Detection of genetically modified organisms by electrochemiluminescence PCR method

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Abstract

With the development of biotechnology, more and more genetically modified organisms (GMOs) have entered commercial market. Because of the safety concerns, detection and characterization of GMOs have attracted much attention recently. In this study, electrochemiluminescence polymerase chain reaction (ECL-PCR) combined with hybridization technique was applied to detect the GMOs in genetically modified (GM) soybeans and papayas for the first time. Whether the soybeans and the papayas contain GM components was discriminated by detecting the Cauliflower mosaic virus 35S (CaMV35S) promoter. The experiment results show that the detection limit for CaMV35S promoter is 100 fmol, and the GM components can be clearly identified in GM soybeans and papayas. The technique may provide a new means in GMOs detection due to its simplicity and high efficiency.
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1. Introduction

A genetically modified organism (GMO) is a living organism with its genome modified by the introduction of an exogenous gene. The gene is able to express an additional protein that confers new characteristics, i.e. herbicide tolerance or resistance to virus, antibiotic and insect (Niederhauser et al., 1996; Droge et al., 1998; Vollenhofer et al., 1999; Hails, 2000; Minunni et al., 2001; Mariotti et al., 2002). The foreign DNA is usually inserted into a gene 'cassette' consisting of an expression promoter (P), a structural gene (encoding region) and an expression terminator (T). Two particular sequences are inserted into most of the available transgenic products: the promoter of the subunit 35S of ribosomal RNA of cauliflower mosaic virus (CaMV35S) and the Nos terminator (Tnos) from *Agrobacterium tumefaciens*. In practice, they are widely used in the commercial production of various transgenic vegetables under brand names such as Roundup Ready for soy, Maigard for maize and the Flaver Savr for tomato (Mannelli et al., 2003).

Many countries have developed laws controlling the marketing of GMOs. At present, in the European Union (EU), labeling is mandatory for food product that contains ingredients derived from genetically modified maize (the Bt-Maize from Novartis) and soybean (RR-Soy from Monsanto) in percentage higher than 1% (Council Regulation (EC), 2000).

Methods for the identification of GMOs can be divided into three categories. The first category includes nucleotide-base amplification methods, such as polymerize chain reaction (PCR), ligase chain reaction (LPR), nucleic acid sequence-based amplification (NASBA), fingerprinting techniques (such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD)), and probe hybridization. The second category includes protein-based methods, such as one-dimensional SDS gel electrophoresis, two-dimensional SDS gel electrophoresis, Western blot analysis and enzyme-linked immunosorbant assay (ELISA). The third category is based on the detection of enzymatic activities. Naturally, every detection method has its own specificities and limitations. The detection using an enzymatic activity method is not recommended for processed foods in which proteins may be denaturized. The methods based on PCR are not suitable for detection of

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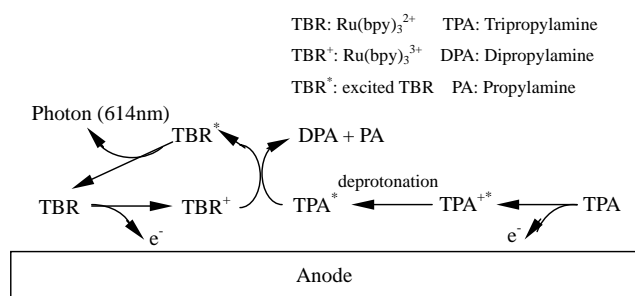*E-mail address:* xingda@scnu.edu.cn (D. Xing).

Fig. 1. Mechanism of ECL excitation. TBR and TPA are oxidized at the anode surface and form TBR$^+$ and TPA^{+*}, respectively. The TPA^{+*} spontaneously loses a proton to form TPA*. The TPA*, a strong reductant, reacts with TBR$^+$, a strong oxidant, to form the excited state of the label, TBR*. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon at 614 nm.
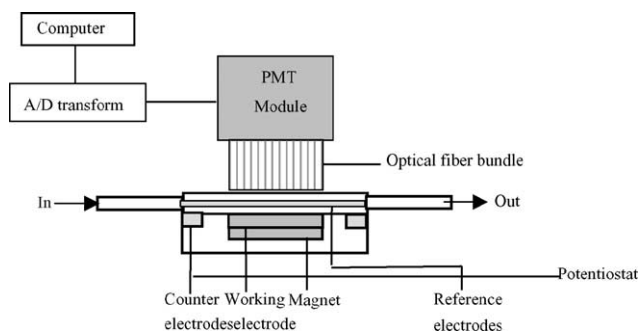

Fig. 2. Schematic of the ECL detection system. The signal from the PMT was amplified and discriminated. The transistor–transistor logic (TTL) pulses were counted with a multi-function acquisition card controlled by Labview software. The voltage applied to the electrodes was controlled with a potentiostat. The signal collection process and data analysis were accomplished with a personal computer.

highly processed foods because DNA fragments in foods could be broken into pieces. Among the three categories, PCR is the most popular method used worldwide (Lin et al., 2001).

Electrochemiluminescence (ECL), where light-emitting species are produced by reactions between electrogenerated intermediates, has become an important and powerful analytical tool in recent years. An ECL reaction using tri-propylamine (TPA) and tris-(2,2'-bipyridyl) ruthenium (TBR) has been demonstrated to be a highly sensitive detection method for quantifying amplified DNA (Leland and Powell, 1990; Blackburn et al., 1991). A previously proposed ECL reaction for TBR + TPA is schematically shown in Fig. 1 (Hsueh et al., 1996; Blackburn et al., 1991; Deaver, 1995).

We employed ECL in GMOs detection because of its high sensitivity. For the first time, ECL, PCR and hybridization were combined to develop a sensitive method to detecting GMOs. In detail, the PCR products of sample (GMOs or non-GMOs) were mixed with a pair of probes designed specially to hybridize with 35S promoter sequence, which is the characteristic of GMOs. After hybridization, the PCR products caught by the probes were collected and then the luminescence signal was detected using the ECL system, and according to the signal we can tell whether the sample was GMOs or not. In this study, we found the ECL signals of GMOs were much higher than those of non-GMOs.

2. Materials and methods

2.1. Materials

2.1.1. Apparatus

A custom-built ECL detection system is described in detail in our previous research (Zhu et al., 2003) (Fig. 2). The instrument is composed of an electrochemical reaction cell, a potentiostat (Sanming Fujian HDV-7C), an ultra high sensitivity single photon counting module (Channel Photomultiplier, Perkin-Elmer MP-962), a multi-function acquisition card (Advantech PCL-836), a computer and labview software. The electrochemical reaction cell contains a working electrode (platinum), a counter electrode (platinum) and a reference electrode (Ag/AgCl$_2$).

2.1.2. Reagents and samples

β-Mercaptoethanol was purchased from AMRESCO, The Netherlands. Taq DNA polymerase, dNTP and 100 bp DNA Ladder were purchased from Shanghai Sangon Biological Engineering & Technology services Co. Ltd. (SSBE), China. The streptavidin MicroBeads were purchased from MACS, Germany. TPA was purchased from Aldrich Chemical Company. GM soybeans (*Brazil soybean No.* 1, *Brazil soybean No.* 2 and *Argentina soybean No.* 1) were from Brazil and Argentina. Non-GM soybeans (*yu soybean No.* 1) were from China. GM papayas (*Huanong-1*) and non-GM papayas (*Suizhonghong*) were gifts from South China Agricultural University.

2.1.3. Primers and probes

PCR primers (Lipp et al., 1999) and probes were all synthesized by SSBE (Table 1). The probes were labeled with biotin by SSBE or ruthenium by our lab, respectively.

2.2. Methods

2.2.1. Principle

The basic principle of the assay was outlined in Fig. 3. PCR amplifications for soybeans and papayas were performed according to the IUPAC method that has been used for GMOs detection (Lipp et al., 1999). Almost all GM soybeans and papayas contain the Cauliflower Mosaic Virus promoter (P-CaMV35S) (Ahmed, 2002; Gachet et al., 1999). We designed a pair of primers to amplify a 195bp fragment in the P-CaMV35S. So, the fragment would be amplified from GMOs instead of non-GMOs through PCR (sometimes, nonspecific amplification would occur). After PCR,

Table 1
Primers and probes used in the study

Name	Sequence (5′–3′)	Product size (bp)	GC content (%)
35S sense primer	gctcctacaaatgccatca	195 (sense primer + antisense primer)	9/19 (47.7%)
35S antisense primer	gatagtgggattgtgcgtca		10/20 (50%)
35S probe 1	cggcagaggcatcttcaacgatggcc-biotin		16/26 (61.5%)
35S probe 2	Ru-tttccacgatgctcctcgtgggtggg		16/26 (61.5%)

the products would hybridize with a pair of oligonucleotide probes. They are designed to hybridize with the 195 bp fragment. Nonspecific amplified products could not hybridize with the probes. One of the probes was labeled by biotin, but another was labeled by ruthenium. The biotin labeled DNA was bound to the surface of streptavidin-coupled beads through the highly selective biotin–streptavidin linkage. The unlinked DNA fragments were washed away. The TBR labeled with the probe would emit light on the anode surface. The light would be recorded as an ECL signal, which reflects the quantity of the hybridized PCR products. Finally, we could confirm whether GM components existed.

2.2.2. DNA extraction

The cetyltrimethyl ammonium bromide (CTAB) method for sample extraction and purification reported by Lipp et al. (1999) was used in this study. The samples with or without GM components were minced with sterile surgical blades and dry samples as flour are moistened with the three-fold amount of water. Then they were extracted with CTAB, precipitated, treated with chloroform, and precipitated with isopropanol to obtain a purified DNA matrix.

2.2.3. PCR amplification

DNA from GM soybeans and papayas and non-GM soybeans and papayas were amplified following the procedure reported by Pietsch et al. (1997). The thermocycler (PTC-100 MJ Research Inc., USA) was programmed with an initial step of denaturation at 94 °C for 3 min. Cycling conditions were: denaturation at 94 °C for 20 s, anneal at 54 °C for 40 s and elongation at 72 °C for 1 min. In total, 40 cycles of above program were performed. The last round of elongation was for 3 min. From the amplification of the DNA regions, fragment of 195 base pairs (bp) was obtained. The control solution (blank) contained all the PCR regents except the DNA template.

2.2.4. Hybridization with a pair of oligonucleotide probes

Hybridizations with biotin labeled probe and TBR labeled probe were performed by adding 20 μl of each to 20 μl of PCR products. The mixture was incubated for 5 min at 95 °C and 10 min at 65 °C in the PCR system (PTC-100 MJ, USA) (Jong et al., 2000).

2.2.5. ECL detection

Twenty μl of hybridization products was added to 20 μl of binding buffer. The solution was incubated at room temperature for 10 min. Then, 10 μl of streptavidin-coated magnetic beads was added. The mixture was then shaken at room temperature for 20 min. After washing and removing the supernatant, the sample was added to the flow ECL detection cell. Then, TPA was added to the reaction cell. A voltage of 1.25 V was applied across the electrodes and the signals of ECL were measured by PMT. At last, computer read the ECL signals by labview software. Each sample was detected 10 times and analyzed with statistical method.

3. Results

3.1. Electrophoresis analysis for PCR products

To verify the feasibility of the method, 2% agarose gel electrophoresis analysis for PCR products was performed in the experiment. As shows in Fig. 4, three bands of 195 bp appear in the lanes of three kinds of GM soybeans PCR products, while no PCR amplification detected in negative control and non-GM soybeans. The results of gel electrophoresis are consistent with the results of ECL detection.

3.2. Capability of ECL detection system

The calibration curve (Fig. 5) was obtained by measuring different quantity of labeled DNA. The minimum detectable quantity was 100 fmol. The curve shows a profile with a linear region from 0.1 to 250 pmol ($R^2 = 0.997$). This wide dynamic range is useful in developing quantification assay.
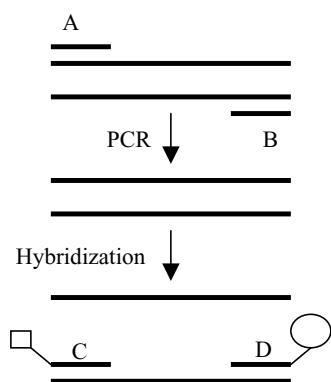

Fig. 3. Basic principle of GMO detection. (A and B) primer; (C) biotin-labeled probe; (D) ruthenium-labeled probe; (□) biotin; (○) ruthenium.
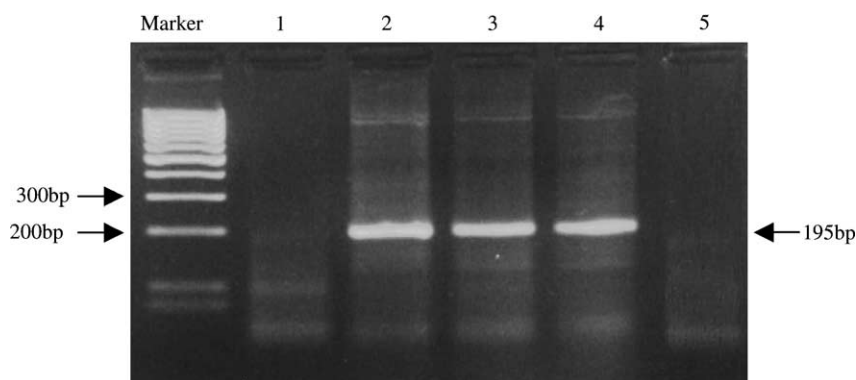
Fig. 4. PCR amplification of genetically modified soybeans DNA. Marker: 100 bp ladder; 1: non-GM soybeans (*yu soybean No.* 1); 2: GM soybeans (*Brazil soybean No.* 1); 3: GM soybeans (*Brazil soybean No.* 2); 4: GM soybeans (*Argentina soybean No.* 1); control: all the PCR regents except the DNA template.

In order to avoid cumulated background signals, the assay started from low quantity to high quantity. The ECL detection cell was cleaned by distilled water after detection.

3.3. ECL detection results

Fig. 6 shows the results of ECL detection for GM soybeans. The signals of blank control are 5.81 ± 0.3 cps (mean $\pm$ standard deviation). And the signals of non-GM soybeans (*yu soybean No.* 1) are 12.8 ± 1.9. According to the data, we set the threshold as 18.5 cps (mean of non-GMOs plus three times SD) to judge the negative. However, the signals of *Brazil soybean No.* 1, *Brazil soybean No.* 2 and *Argentina soybean No.* 1 are 207 ± 10.4 cps, 112 ± 5.6 cps and 83 ± 4.2 cps, respectively. The signal-to-noise ratio of ECL detection was so great (signal-to-noise ratio: ≥ 6.5) that we could confirm whether the samples have GM components by ECL intensity or nor.

Fig. 7 is the results of ECL detection for GM papayas (*Huanong*-1). The signals from control and non-GM papayas (*Suizhonghong*) are 5.3 ± 0.3 cps and 5.8 ± 0.3 cps,
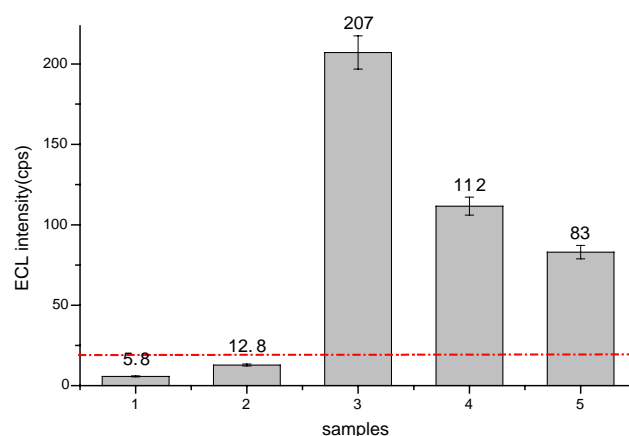

Fig. 6. Detection of genetically modified soybeans. 1: control (TE+TPA); 2: non-GM soybeans (*yu soybean No.* 1); 3: GM soybeans (*Brazil soybean No.* 1); 4: GM soybeans (*Brazil soybean No.* 2); 5: GM soybeans (*Argentina soybean No.* 1).
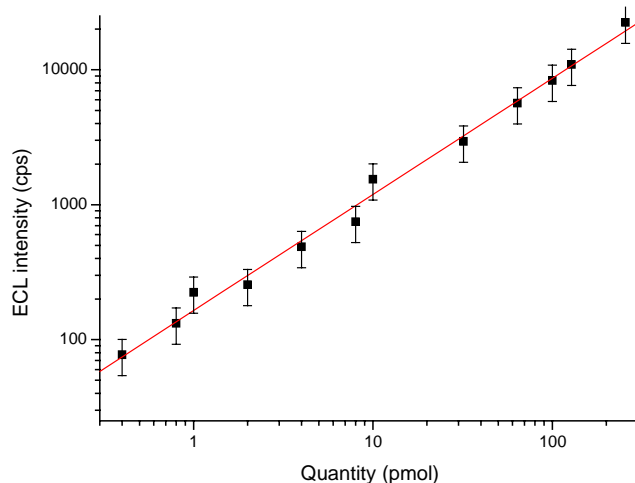

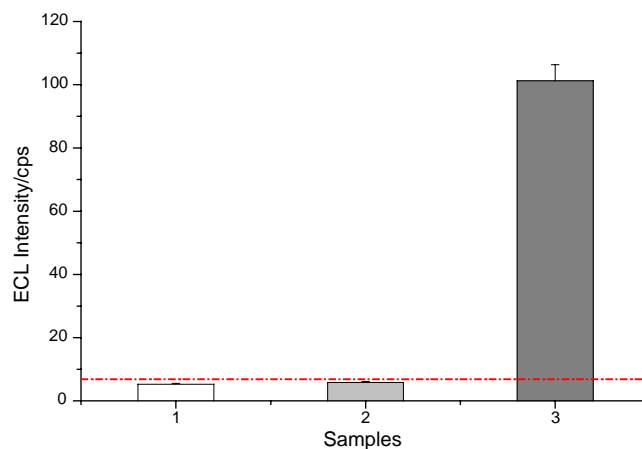Fig. 5. Calibration curve for the ECL system.

Fig. 7. The detection of genetically modified papayas. 1: control (TE + TPA); 2: non-GM papayas (*Suizhonghong*); 3: GM papayas (*Huanong-1*).

respectively. The threshold was set as 6.7 cps. We consider the non-GM papayas were not detectable. The signal of GM papayas is 101.2 ± 5.1 cps. The results strengthen the feasibility of the ECL-PCR detection for GMOs.

Based on the calibration curve, the detection products' quantity of *Brazil soybean No.* 1, *Brazil soybean No.* 2, *Argentina soybean No.* 1 and GM papayas (*Huanong*-1) are 1.27 ± 0.07 pmol, 0.60 ± 0.03 pmol, 0.42 ± 0.02 pmol and, 0.53 ± 0.02 pmol, respectively.

4. Discussion

The amplification products have a double helices structure. The double strands will be separated by thermally denaturing and hybridize with the pair of probes, which was designed for specific selection for CaMV35S promoter in GM soybeans and papayas. Streptavidin-coated magnetic beads could catch the specific PCR products, which has hybridized with the biotin labeled probe, through the biotin–streptavidin conjunction. TBR label will react with TPA at working voltage to emit light for detection. Thus, only the PCR products hybridized with both biotin-labeled and TBR-labeled probes could be detected by ECL assay. The false positive result caused by nonspecific amplification could be avoided, for the probes will not hybridize with the nonspecific amplified products.

In the early 1990s, Kenten and co-workers established the ECL method for nucleic acid analysis. With the rapid development of biotechnology, ECL method was widely used in gene analysis. But, up to now, ECL has not been used to detect GMOs. For the first time, ECL is combined with PCR and hybridization for GMOs detection. The high specificity was realized in our experiment result (Figs. 6 and 7). The results show that the ECL signals of non-GM soybeans and papayas are very low. So we consider they are undetectable. We set the threshold according to the data of known non-GMOs. However, the signals of four kinds of GM samples are far higher than the threshold value. The system has an excellent signal-to-noise ratio. Thus, the ECL-PCR method is feasible for detection for GMOs.

The method does not use any poisonous materials, such as ethidium bromide or isotopes. It provides an extremely sensitive detection at subpicomolar concentration, as well as a very wide dynamic range. Compared with gel electrophoresis analysis, it is no poisonous and easier to operate. In conclusion, the ECL-PCR could be a newly quantitative analysis method for GMOs detection.

5. Conclusion

In this paper, ECL-PCR has been applied to GMOs detection for the first time. The high specificity was realized by hybridization with a pair of probes labeled with biotin and TBR. The method can detect GMOs with high sensitivity, wide dynamic range and rapidness. It could potentially become a rapid and convenient method for daily GMOs detection.

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