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Electrochemiluminescence polymerase chain reaction detection of genetically modified organisms

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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. Electrochemiluminescence (ECL) method is a chemiluminescent (CL) reaction of species generated electrochemically on an electrode surface. It is a highly efficient and accurate detection method. In this paper, ECL polymerase chain reaction (PCR) combined with two types of nucleic acid probes hybridization was applied to detect GMOs for the first time. Whether the organisms contain GM components was discriminated by detecting the cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase (NOS) terminator. The experiment results show that the detection limit is 100 fmol of PCR products. The promoter and the terminator can be clearly detected in GMOs. The method may provide a new means for the detection of GMOs due to its simplicity and high efficiency.
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Keywords: ECL; GMOs; CaMV35S promoter; NOS terminator

1. Introduction

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 (II) (TBR) has been demonstrated to be a highly sensitive detection method for quantifying amplified DNA [1,2]. A previously proposed ECL reaction for TBR + TPA is schematically shown in Fig. 1. TPA and TBR are oxidized at approximately the same voltage on the anode surface. After deprotonation, TPA chemically reacts with TBR and results in an electron transfer. The resulted TBR molecule relaxes to its ground state by emitting a photon. TPA decomposes to dipropyl amine and is therefore consumed in this reaction. TBR, on the other hand, is recycled. Since both reactants are produced at the anode, luminescence occurs there [3]. Compared with other detection techniques, the ECL has some advantages: no radioisotopes are used; detection limits are extremely low; the dynamic range for quantification extends over six orders of magnitude; the labels are extremely stable compared with those of most other chemiluminescence (CL) systems; and the measurement is simple and rapid, requiring only a few seconds [2,4].

Genetically modified organisms (GMOs) are referred to as living organisms that genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics [5–10]. 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 (T-NOS) from *Agrobacterium tumefaciens*. In practice, they are widely

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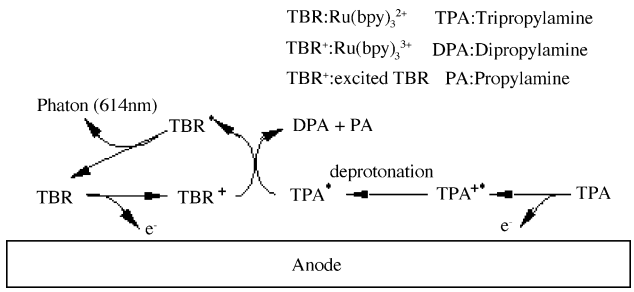

Fig. 1. Mechanism of ECL excitation. TBR and TPA are oxidized on the anode surface and form TBR^+ and TPA^{+*}, respectively. The TPA^{+*} spontaneously loses a proton to form TPA^*. The TPA^*, a strong reductant, reacts with TBR^+, a strong oxidant, to form the excited state of the label, TBR^*. The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

used in the production of commercialised various transgenic vegetables under brand names such as Roundup Ready for soy, Maigard for maize and the Flaver Savr for tomato [11].

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% [12].

Methods for the identification of GMOs can be divided into three categories. The first category includes nucleotide-based 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 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 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. Among the three aforementioned categories, PCR is the most popular method used worldwide [13].

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 detect GM capsicums, tomatoes and *Arabidopsis thalianas*. In detail, the PCR products of sample (GMOs or non-GMOs) were mixed with two pairs of probes designed specifically to hybridize with 35S promoter sequence and NOS terminator sequence, which are the characteristics of GMOs. After hybridization, the PCR products caught by the probes were collected and then the luminescence signal was detected by 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 [14,15] (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, Perkinelmer MP-952), 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, 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. Non-GM and GM capsicums were gifted from Vegetable Research Institute Science, Guang-Dong Agricultural Sciences. Non-GM and GM tomatoes were from Huazhong Agricultural Unversity. Non-GM and GM *Arabidopsis thalian*as were gifts from Sun Yat-Sen University.

2.1.3. Primers and probes

PCR primers [16] and probes were all synthesized by SSBE (Table 1). The TBR–NHS ester, synthesized in our laboratory according to Terpetschnig's paper [17], was intro-

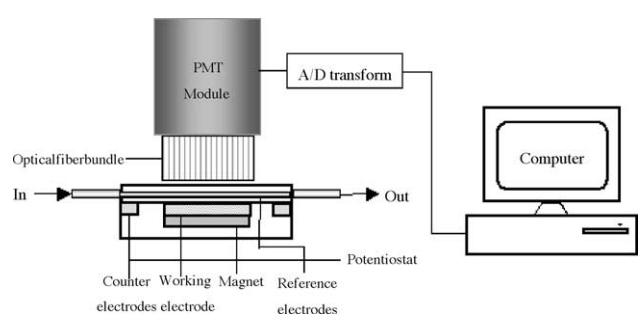

Fig. 2. Schematic diagram of the ECL detection system. The signal from the PMT was amplified and discriminated. The transistor–transistor logic (TTL) pulses were counted with a multifunction acquisition card controlled by labview software. The voltage applied to the electrodes was controlled with a potentiostat.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

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%)
NOS sense primer	gaatcctgttgccggtcttg	180 (sense primer + antisense primer)	11/20 (55%)
NOS antisense primer	ttatcctagtttgcgcgcta		9/20 (45%)
35S probe 1	cggcagaggcatcttcaacgatggcc-biotin		16/26 (61.5%)
35S probe 2	Ru-tttccacgatgctcctcgtgggtggg		16/26 (61.5%)
NOS probe 1	ccatctaaataacgtcatgcat-biotin		8/22 (36.4%)
NOS probe 2	Ru-cgcgtattaaatgtataattgcg		8/23 (48.2%)

duced into forward primer as described by Kenten et al. [18]. The biotin was introduced into reverse primer by SSBE.

2.2. Methods

2.2.1. Principle

PCR amplifications for capsicums, tomatoes and *Arabidopsis thalianas* were performed according to the IUPAC method that had been used for GMOs detection [16]. Almost all GM capsicums, tomatoes and *Arabidopsis thalianas* contain the cauliflower mosaic virus promoter (P-CaMV35S) and nopaline synthase terminator (T-NOS) [19,20]. We designed two pairs of primers to amplify a 195 bp fragment in the P-CaMV35S and an 180 bp fragment in the T-NOS. So, the fragments would be amplified from GMOs instead of non-GMOs through PCR. After the PCR amplifications, the products would hybridize with a pair of oligonucleotide probes. They are designed to hybridize with the 35S or NOS-PCR products. Non-specific amplified products could not hybridize with the probes. One of the probes was labeled by biotin, but another was labeled by TBR. The biotin-labeled DNA was linked on to the surface of streptavidin-coupled beads through the highly selective biotin–streptavidin linkage. The unlinked DNA fragments were washed away. The TBR-labeled 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. [16] was used in this study. The samples with or without GM components were minced with sterile surgical blades and dry samples as flour were moistened with the three-fold amounts 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 and non-GM capsicums, tomatoes and *Arabidopsis thalianas* was amplified following the procedure reported by Pietsch et al. [21]. 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 the above program were performed. The last round of elongation lasted for 3 min. From the amplification of the DNA regions, fragment of 195 or 180 base pairs (bp) was obtained. The control solution (blank) contained all the PCR reagents except the DNA template.

2.2.4. Hybridization with a pair of oligonucleotide probes

Hybridizations with biotin- and TBR-labeled probes 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) [22].

2.2.5. ECL detection

Twenty microliters 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, the computer read the ECL signals by labview software. Each sample was detected 10 times. The averages and the standard deviations were calculated by using Microsoft Excel spread sheet function.

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 shown in Fig. 3, the bands of 195 bp or 180 bp appear in the lanes of GM capsicums, tomatoes and *Arabidopsis thalianas* PCR products, while no PCR amplification was detected in negative control and non-GMOs. The results of gel electrophoresis are consistent with the results of ECL detection.

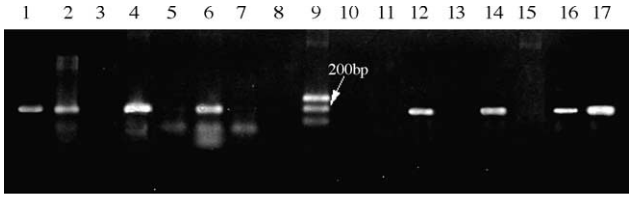

Fig. 3. PCR amplification of genetically modified organisms DNA. 1, positive (NOS); 2, GM capsicums (NOS); 3, non-GM capsicums (NOS); 4, GM tomatoes (NOS); 5, non-GM tomatoes (NOS); 6, GM *Arabidopsis thalianas* (NOS); 7, non-GM *Arabidopsis thalianas* (NOS); 8, control (NOS, all the PCR regents except the DNA template); 9, marker (100 bp ladder); 10, control (35S); 11, non-GM *Arabidopsis thalianas* (35S); 12, GM *Arabidopsis thalianas* (35S); 13, non-GM tomatoes (35S); 14, GM tomatoes (35S); 15, non-GM capsicums (35S); 16, GM capsicums (35S); 17, positive (35S).

3.2. Capability of ECL detection system

The calibration for detection sample was $\lg I = 2.21526 + 0.86062 \lg Q$ (I is the ECL intensity (cps), Q the quantity (pmol), $n = 16$, $r = 0.997$). The linear range was 0.1–250 pmol. The experimental quantitation limit, defined as the lowest quantity of sample which gives rise to a signal-to-noise ratio of 7.3, was found to be 0.1 pmol. This wide dynamic range is useful in developing quantification assay. In order to avoid cumulated background signals, the assay started from the low quantity to the high quantity. The ECL detection cell was cleaned by distilled water after the detection.

3.3. ECL detection results

Hybridization products separated by magnetic particles gave significant ECL signals, when injected in the flow cell. Fig. 4 shows the ECL-PCR detection results of non-GMOs of 20 categories were under the threshold. The false positive rate of ECL detection system was very low. The results strengthen the feasibility of the ECL-PCR detection for GMOs. Fig. 5 shows the results of ECL detection for GMOs. The signals of blank control are 19.64 ± 0.98 cps for 35S and 18.55 ± 1.30 cps for NOS (mean ± S.D., cps). According to the data, we set the threshold as 22.58 cps (mean of blank control plus three times S.D.) to judge the negative [23,24]. The result shows that the signals of non-GMOs are under the threshold value. And the signals of GMOs are much higher than the threshold value. The signal-to-noise ratio of ECL detection for GMOs was higher than 7.3. We could confirm whether the samples have GM components by ECL intensity clearly. The results strengthen the feasibility of the ECL-PCR detection for GMOs.

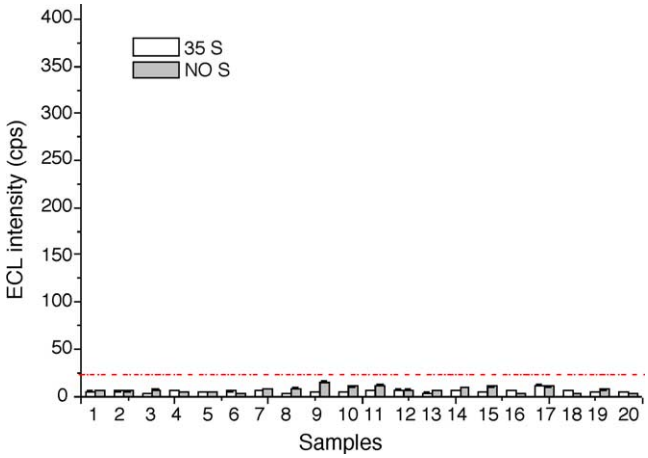

Fig. 4. Detection of negative samples by ECL system. 1, azalea; 2, pinaster; 3, sweet potato; 4, peanut; 5, cajuput; 6, cushaw; 7, towel gourd; 8, ceiba; 9, lettuce; 10, hyacinth; 11, pawpaw; 12, lidded cleistocalyx; 13, grassiness; 14, canna; 15, magnolia; 16, papaya; 17, aloe; 18, shallot; 19, leek; 20, tung.

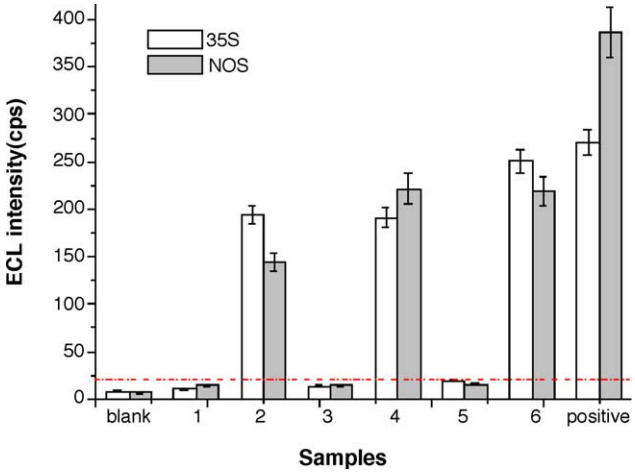

Fig. 5. Detection of genetically modified organisms by ECL system. Blank, TE + TPA; 1, non-GM *Arabidopsis thalianas*; 2, GM *Arabidopsis thalianas*; 3, non-GM tomatoes; 4, GM tomatoes; 5, non-GM capsicums; 6, GM capsicums; Positive.

4. Discussion

The amplification products have a double helices structure. The double strands will be separated by thermally denaturing and hybridize with two pairs of probes, which were designed for specific selection for 35S promoter and NOS terminator in GM capsicums, tomatoes and *Arabidopsis thalianas*. Streptavidin-coated magnetic beads could catch the specific PCR products, which had hybridized with the biotin-labeled probe, through the biotin–streptavidin conjunction. TBR label would react with TPA at working voltage to emit light for detection. Thus, only the PCR products hybridized with both biotin- and TBR-labeled probes could be detected by ECL assay. The false positive result caused by non-specific amplification could be avoided, for the probes would not hybridize with the non-specific amplified products. Both the 35S promoter and the NOS terminator are detected at the same time. So, the sample can be confirmed as GMO if the 35S or the NOS were detectable by using ECL-PCR method.

In the early 1990s, Kenten et al. established the ECL method for nucleic acid analysis. With the rapid development

of biotechnology, ECL method has been 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 GM capsicums, tomatoes and *Arabidopsis thalianas* detection. The high specificity was realized in our experiment (Fig. 4). The results show that the ECL signals of non-GM capsicums, tomatoes and *Arabidopsis thalianas* 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 three 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 the detection of GMOs.

The method does not involve any poisonous materials, such as ethidium bromide or isotopes. It provides an extremely sensitive detection at subpicomolar concentration, as well as in a very wide dynamic range. Compared with gel electrophoresis analysis, it is not poisonous and easy 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.

Acknowledgements

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