Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 582 (2007) 275–280
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

An improved electrochemiluminescence polymerase chain reaction method for highly sensitive detection of plant viruses

Ya-bing Tang, Da Xing *, De-bin Zhu, Jin-feng Liu

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631,China

Received 31 May 2006; received in revised form 27 August 2006; accepted 13 September 2006
Available online 16 September 2006

Abstract

Recently, we have reported an electrochemiluminescence polymerase chain reaction (ECL-PCR) method for detection of genetically modified organisms. The ECL-PCR method was further improved in the current study by introducing a multi-purpose nucleic acid sequence that was specific to the tris(bipyridine) ruthenium (TBR) labeled probe, into the 5′ terminal of the primers. The method was applied to detect plant viruses. Conserved sequence of the plant viruses was amplified by PCR. The product was hybridized with a biotin labeled probe and a TBR labeled probe. The hybridization product was separated by streptavidin-coated magnetic beads, and detected by measuring the ECL signals of the TBR labeled. Under the optimized conditions, the experiment results show that the detection limit is 50 fmol of PCR products, and the signal-to-noise ratio is in excess of 14.6. The method was used to detect banana streak virus, banana bunchy top virus, and papaya leaf curl virus. The experiment results show that this method could reliably identity viruses infected plant samples. The improved ECL-PCR approach has higher sensitivity and lower cost than previous approach. It can effectively detect the plant viruses with simplicity, stability, and high sensitivity.

Keywords: Electrochemiluminescence; Polymerase chain reaction; Plant viruses; Tris(bipyridine) ruthenium-probe; Biotin-probe

1. Introduction

Electrochemiluminescence (ECL) is a chemiluminescent reaction of species generated electrochemically at an electrode surface. Initially, $Ru(bpy)_3^{2+}$ (TBR) and tri-propylamine (TPA) are oxidized at the surface of an anode. $TPA^{\bullet+}$ immediately loses a proton and becomes a powerful reducer. When $TPA^{\bullet}$ and $Ru(bpy)_3^{3+}$ react, the latter enters an excited state by a high energy electron transfer from the electron carrier, $TPA^{\bullet}$. Relaxation of $Ru(bpy)_3^{2+*}$ to the ground state results in a light emission, at 620 nm [1,2]. Noticeable, $Ru(bpy)_3^{2+}$ is not consumed during the reaction and may be oxidized and excited repeatedly, if there is excessive TPA in the buffer. Since Kenten et al. [3] first used ECL to DNA probe, this method has been widely used in DNA analysis.

Plant viruses can have a considerable impact on the general public with respect to environment, food supply, and also safety, quality and diversity of food available. There are no up-to-date estimates of world losses attributable to plant viruses, the ever increasing world population requires higher productivity on a constantly declining area of agricultural land, a effective viruses control measure, will play a vital role in providing the world with an adequate and varied supply of safe, high quality food [4].

The variety of techniques that have been developed for the detection of plant viruses includes: electron microscopy coupled with serological techniques as in immunoelectron microscopy [5]; direct observation of viral double stranded RNAs following gel electrophoresis [6]; use of serological techniques (ELISA) [7]; detection of the viral genome using molecular hybridization technology [8]; detection of the viral genome using polymerase chain reaction (PCR) technology [9]. Naturally, every method has its own specificities and limitations. But, a common shortcoming of these methods is that they are usually tedious, multi-stage, low-sensitivity, high cost.

Electrochemiluminescence PCR method is a highly sensitive nucleic acid analytical method, which was described in detail in our previous publications [1,2,10]. In this paper, we improved electrochemiluminescence PCR method and used it to detect plant viruses for the first time. Specific nucleic acid sequences (20 bp) were added to 5′ terminal all of the primers.

* Corresponding author. Tel.: +86 20 85210089; fax: +86 20 85216052.
E-mail address: xingda@scnu.edu.cn (D. Xing).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.09.021

The TBR-probe sequence was complementary to the special nucleic acid sequence. Electrochemiluminescence assay was coupled with nucleic acid probes hybridization to detect PCR products. Whether the plants infected by viruses was discriminated by detecting the PCR products of viruses. The PCR products hybridized with a pair of probes that designed specially can specifically select the target for detection, thus can avoid pseudo-positive result. After hybridization, the PCR products caught by the probes were collected and then the luminescence signal was detected using an ECL system. The ECL signals of samples infected by viruses would be much higher than those of healthy samples. Therefore, according to the threshold value of ECL signal we can discriminate whether the sample was infected. This is a highly effective method to detect plant viruses with stability, high sensitivity, simple operation procedures, and accurate result.

2. Experimental

2.1. Materials

2.1.1. Reagents and samples

Taq DNA polymerase, dNTP and 600 bp DNA Ladder were purchased from Shanghai Sangong Biological Engineering & Technology Services Co. Ltd. (SSBE), China. β-Mercaptoethanol and cetyltrimethyl ammonium bromide (CTAB) were purchased from AMRESCO, Netherlands. The streptavidin micro-beads were purchased from MACS, Germany. TPA was purchased from Aldrich Chemical Company, the $Ru(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR-NHS ester) was purchased from Sigma, USA. Healthy and virus-infected (BSV: banana streak virus; BBTV: banana bunchytop virus; PaLCV: papaya leaf curl virus) leaves were obtained from Institute of Plant Viruses, South China Normal University (Guangzhou, China).

2.1.2. Primers and probes

PCR primers and probes [10] were all synthesized by SSBE (Table 1). The probes were labeled with biotin by SSBE or TBR by our lab, respectively.

2.2. Apparatus

An ECL detection system has been custom-built [1,11]. 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 ($Ag/AgCl_2$).

2.3. Assay procedures

2.3.1. Principle

Fig. 1 shows the basic principle of the improved ECL-PCR. DNA was extracted from the healthy and virus-infected leaves, repetitively, and then amplified by viruses primers, only the DNA of virus-infected leaves can be amplified. After PCR, the products would hybridize with a pair of oligonucleotide probes. The TBR-probe is designed to hybridize with the specific nucleic fragment [12]. Nonspecific 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 bound to the surface of streptavidin-coupled beads through the highly selective biotin–streptavidin linkage [13,14]. The unlinked DNA fragments were washed away. The TBR labeled with the probe would emit light on the anode surface [15]. The light would be recorded as an ECL signal, which reflects the quantity of the hybridized PCR products. Finally, we could confirm whether infected viruses components existed.

Compared with ECL-PCR method, the improved method has introduced a a specific nucleic acid sequences (20 bp) to the 5′ terminal of the primers for hybridization with TBR-probe. The sequences, which were added to forward primer and reverse primer are complementary. TBR-probe sequence was complementary to the special nucleic acid sequence, which was added to reverse primers, and was the same as the special nucleic acid sequences which was added to forward primers. After PCR, one PCR product can hybridize with two TBR-probes, which

Table 1
Primers and probes used in the study

Name		Sequence (5′–3′)	Accession number
Papaya leaf curl virus (PaLCV)	Sense primer	5′gatgcaaggtcgcatatgagtggttgaatcggatttgt3′	AY650283 (1316–1723)
	Anti-sense primer	5′ctcatatgcgaccttgcatcttcccactatcttcctctgc3′	
	Biotin-probe	5′Biotin-gatgcggtgctggactttgattgg3′	
Banana bunchytop virus (BBTV)	Sense primer	5′gatgcaaggtcgcatatgagggcaggaggaagtatgga3′	U97526 (43–322)
	Anti-sense primer	5′ctcatatgcgaccttgcatcgatggctatgttcaggtttt3′	
	Biotin-probe	5′Biotin-agcaaggcggcaacaagccacgac3′	
Banana streak virus (BSV)	Sense primer	5′gatgcaaggtcatatatgaggaatccaagaacataaaatcaagac3′	DQ115591(1–443)
	Anti-sense primer	5′ctcatatgcgaccttgcatcggtacgcagaccactctttacat3′	
	Biotin-probe	5′Biotin-cagcaatgacgatcaatgggcaagg3′	
	TBR-probe	5′TBR-gatgcaaggtcgcatatgag-3′	

Underscore indicates the region of anti-sense primers is complementary to TBR-probe sequences, and sense primer is in the same of the TBR-probe sequences. Accession number: Genebank accession number of DNA and genomic sequences, available at http://www.ncbi.nlm.nih.gov.

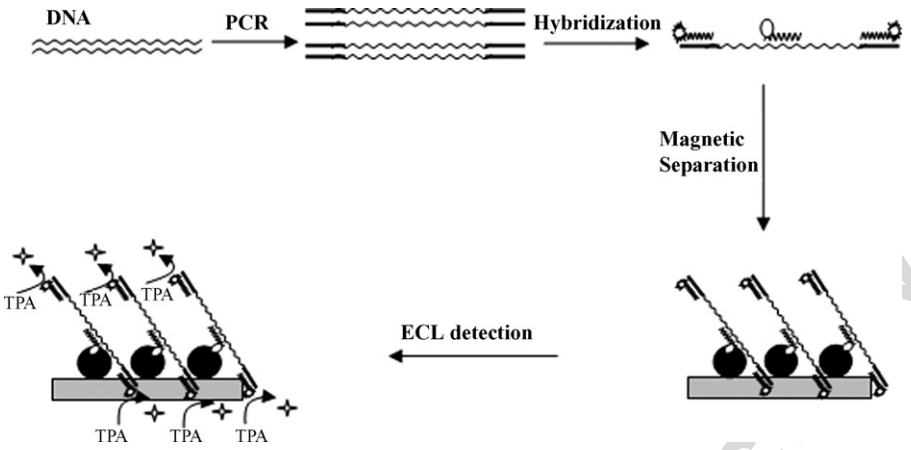

Fig. 1. The basic principle of the improved ECL-PCR method for detection of plant viruses: TBR-probe (); biotin-probe (); magnetic beads (●); photon ().

improved the sensitivity of ECL detection, and the same TBR-probe can be used for different virus detection, the universal ECL probe can be developed. In a word, that's an effective method for improving the sensitivity of ECL detection and reducing ECL detection cost.

2.3.2. 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 leaves infected or without infected viruses 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.

2.3.3. PCR amplification

We chose the conserved sequence of viruses to amplification; The PCR reaction was carried out in 25 μL mixtures containing 1 μL of sample DNA, 2.5 μL 10× Taq polymerase buffer, 0.5 μL dNTP, 5pmol sense and anti-sense primers, 1U Taq polymerase. The amplification reaction was performed on thermal circler. (PTC-100, MJ Research Inc., USA).

2.3.4. 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 55 °C in the PCR system.

2.3.5. ECL detection

Twenty microliters of hybridization products was added to 20 μL binding buffer. The solution was incubated at room temperature for 10 min. Then, 10 μL of streptavidin coated magnetic beads was added [17,18]. 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 ECL signals were read and recorded using labview software. Each sample was detected 10 times and analyzed with statistical method.

3. Result and discussion

3.1. Capability of ECL detection system

The calibration curve (Fig. 2) was obtained by measuring different quantity of labeled DNA. The minimum detectable quantity was 50 fm. The curve shows a profile with a linear region from 0.1 to 250 pmol ($R^2 = 0.997$). The wide dynamic range is useful in developing quantification assay, in order to avoid cumulated background signals, the assay started from low quantity to high quantity.

3.2. The improved ECL-PCR conditions optimization

Several factors that influence on ECL signals were investigated, including the condition of hybridization and ECL detec-

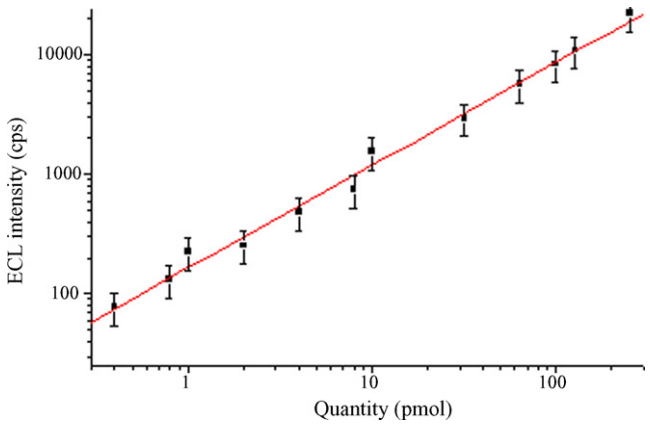

Fig. 2. Calibration curve for the ECL system: the calibration curve was obtained by measuring different quantity of labeled DNA. The minimum detectable quantity was 100 fmol. The curve shows a profile with a linear region from 0.1 to 250 pmol ($R^2 = 0.997$). This wide dynamic range is useful in developing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

tion conditions. We study the pH value and probe concentrations of hybridization running solution, the detection temperature and magnetic beads incubated time of ECL solution [19].

3.2.1. Effect of probes hybridization conditions in ECL detection

It is a very important factors that hybridization efficiency for the ECL detection [20]. The optimal concentration of probe and the pH value hybridization buffer are necessary to detect the specific PCR product was determined. Fig. 3(a) shows that obvious concentration-dependent differences were observed in this range of probe concentrations. The probe concentration is 1–15 times primer concentration, ECL intensity has a wide range increased. But when the rate exceeds 15 times, the ECL intensity has not improved. This is because the concentration of probe has reached saturation point. So, the optimal concentration of probe is 15 times primer's concentration. The pH value is a very important factor for nucleic acid hybridization. Suitable pH value can improve hybridization efficiency. As shown in Fig. 3(b), we studied the variety of ECL intensity on the pH range of 6.0–9.0. The ECL intensity increased with increasing pH 6.0–7.0, and decreased from 7.0 to 9.0. Therefore, we selected pH 7.0 as the optimal pH value.

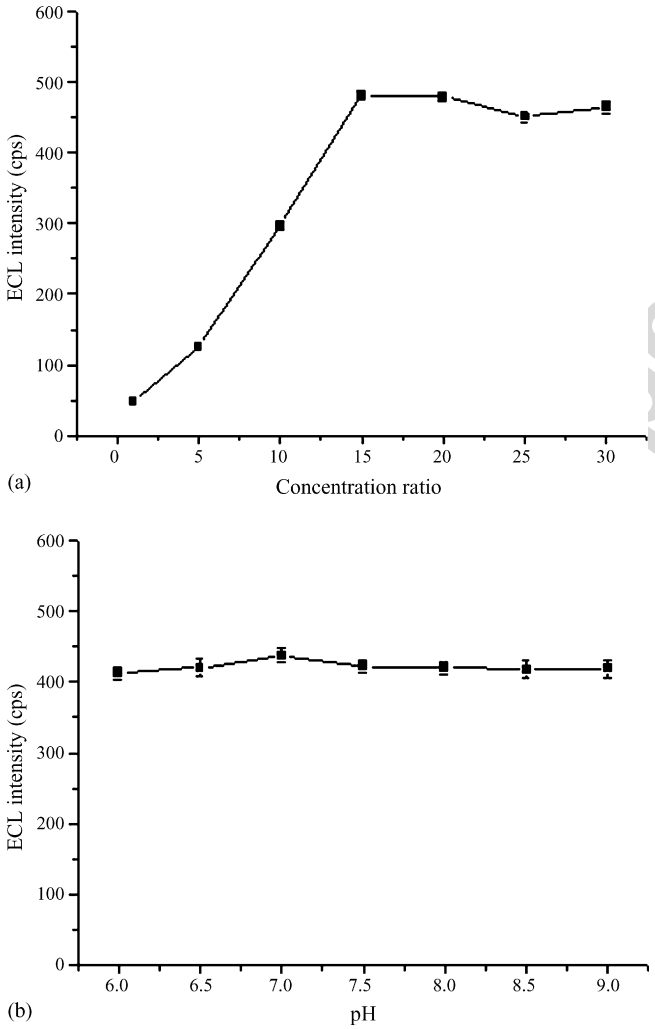

Fig. 3. Hybridization solution pH value (a) and probe concentration (b) optimization. (a) The pH (6–9, in 0.5 increment) of hybridization running solution was added 10 μL papaya leaf curl virus PCR product and 1 μL biotin-probe and 1 μL TBR-probe. After detection of ECL signals, the ECL intensity was plotted vs. pH. (b) The probes were added to hybridization system at concentrations of 1, 5, 10, 15, 20, 25, 30 times primer concentration (20 pmol).

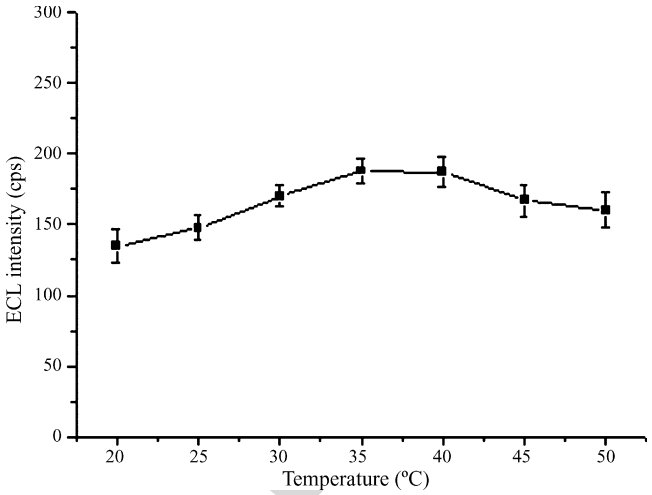

Fig. 4. The effect of the running solution temperature on the ECL intensity. Twenty microliters of hybridization products was added to 20 μL binding buffer. The solution was incubated at room temperature for 10 min. Then, 10 μL of streptavidin coated magnetic beads was added. The mixture was then shaken at room temperature for 20 min. After washing and removing the supernatant, the sample was added to the flow ECL detection cell the running solution temperature was changed from 20 to 50 °C.

3.2.2. Effect of ECL detection conditions

The temperature of ECL detection is an important factor that influences the ECL signals according to the mechanism of ECL reaction. The comfortable temperature can improve the stability of ECL signals [10,13], and strengthen ECL signals intensity. Fig. 4 shows that the highest ECL intensity signals were obtained when the temperature was from 35 to 40 °C. If the temperature of running solution was beyond 40 °C, with the increasing of the temperature, the ECL signals are decrease. So, we chose 37 °C was the optimum temperature.

The quantity of magnetic beads is also of vital importance for the ECL detection [21], the biotin-labeled DNA was linked on to the surface of streptavidin-coupled beads though the highly selective biotin–streptavidin linkage. The unlinked DNA fragments were washed away. The appropriate amount of beads can capture the entire special PCR product, thus improving the sensitivity. But, excessive beads would be absorbed on the surface of electrode, and influence the reaction of TPA and $Ru(bpy)_3^{2+}$ on the surface of electrode. Therefore, 10 μL bead was added to each 20 μL hybridization product is the optimum quantity by study repeatedly.

3.3. ECL detection results

In order to verify the feasibility of this method and the importance of optimization experiment conditions, the determination of papaya leaf curl virus was performed. Fig. 5(a) shows the

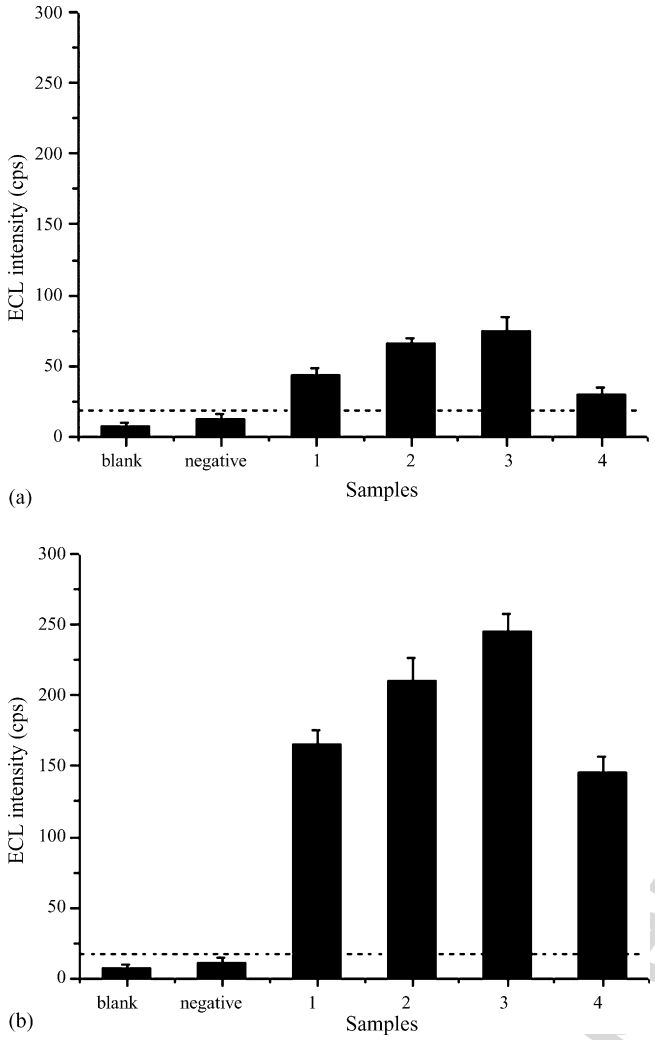

Fig. 5. Improved ECL-PCR detection result of papaya leaf curl virus (PaLCV). Blank: TPA; negative: healthy China papaya leaf; 1–4 positive: infected viruses China papaya leaves (nos. 1–4). (a) Before optimization: hybridization buffer pH 8.0; the concentration ratio of probe and primer 10; ECL detection temperature 25 °C; beads 20 μL. (b) After optimization: hybridization buffer pH 7.0; the concentration ratio of probe and primer 15; ECL detection temperature 37 °C; beads 10 μL. Other condition (a) was the same as (b).

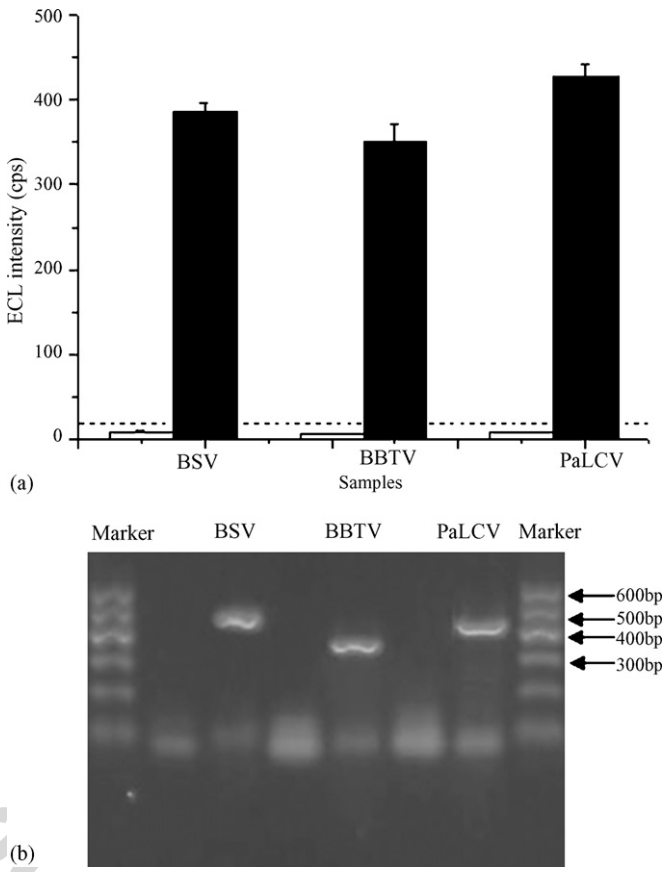

Fig. 6. Improved ECL-PCR detection result of suspect samples. (a) BSV: banana streak virus; BBTV: banana bunchytop virus; PaLCV: papaya leaf curl virus. (b) Agarose gel electrophoresis analysis the PCR product of these viruses.

results of ECL detection for the positive of papaya leaf curl virus samples before optimization above-mentioned experiment conditions. The signals of blank control are 7.0 ± 2.8. And the signals of negative are 12.1 ± 3.5. The threshold value is defined as that, when ECL value measured is above it, the sample is considered to be positive. The threshold value can be derived as the mean three times the standard deviation of the background signal [22,23]. According to the data, we set the threshold as 22.6 cps. The result shows that the signals of controls are under the threshold value. And the signals of samples are higher than threshold value. But, the signals-to-noise ratio of ECL detection for infected samples is very low (≥ 2.43). Fig. 5(b) shows the results of the same samples of papaya leaf curl virus after optimization above-mentioned conditions. The signals of blank control are 7.2 ± 2.1. And the signals of negative are 11.3 ± 4.1. According to the data, we set the threshold as 23.6 cps. The signals-to-noise ratio of ECL detection for infected samples is so great (≥ 14.6). Compared with Fig. 5(a) and (b), the result of optimization above-mentioned experiment condition is much better than without optimization. So, we thought this method can clearly detect whether the papaya leaf curl virus exist or not. But, the optimum experiment condition is necessary for the improved ECL-PCR detention plant viruses.

Fig. 6(a) shows that the results of ECL detection for three kinds of suspect plant viruses' samples, the signals of negative (9 ± 1.2, 6 ± 0.9, 9 ± 1.3 cps). According to the data, we set the threshold as 12.6 cps (mean of normal samples plus three times S.D.) to judge the negative. The signals of BSV, BBTV, PaLCV are 385 ± 12.3, 350 ± 22.3, 428 ± 14.5, respectively. The signal-to-noise ratio of ECL detection was so great (signal-to-noise ratio > 38.8) that we could confirm whether the samples have infected viruses by ECL intensity or not. In order to verity the feasibility of the method, 1% agarose gel electrophoresis analysis for PCR products was performed in the experiment. Fig. 6(b) shows the lane of BSV (483 bp) have a band between 400 and 500 bp; the lane of BBTV (320 bp) have a band between 300 and 400 bp; the lane of PaLCV(448 bp) have a band between 400 and 500 bp. The results of gel eleectrophoresis are consistent with the results of ECL detection. According to the calibration curve (Fig. 2), the detection limit of the improved method is 50 fmol PCR products, which is lower than that of ECL-PCR method

(100 fmol). The signal-to-noise ratio of the improved method is two times higher than that of the ECL-PCR method [10]. This is because that the improved ECL-PCR method introduced the specific nucleic acid sequences into both of the forward and the reverse primers, so that one PCR product can hybridized with two TBR-probes, thus improved the sensitivity of the method. On the other hand, the introduction of the specific nucleic acid sequences make one kind of TBR-probe can be used to detect different plant viruses, thus a universal ECL probe was developed, which can greatly reduce the cost.

4. Conclusion

In summary, an improved ECL-PCR method we described here for the detection of plant viruses. Compared to ECL-PCR, the improved ECL-PCR has higher sensitivity and lower detection cost. This method is a safe, low background noise, high sensitive, specific and cost-effective detection technique, and is suitable for fast, convenient detecting plant and animal viruses, bacteria, fungi, and other special nucleic acid sequence.

Acknowledgements

We are grateful to Dr. Guohui Zhou (South China Agricultural University, Guangzhou, China) for kindly providing BSV, BBTV, PaLCV. The authors thank Prof. Ja Li for critical reading of the manuscript and helpful suggestions. This research is supported by the National Natural Science Foundation of China (60378043; 30470494), and the Natural Science Foundation of Guangdong Province (015012; 04010394; 2004B10401011).

References

[1] J.F. Liu, D. Xing, X.Y. Shen, D.B. Zhu, Biosens. Bioelectron. 20 (2004) 436.
[2] D.B. Zhu, D. Xing, X.Y. Shen, J.F. Liu, Biochem. Biophys. Res. Commun. 324 (2004) 964.
[3] J.H. Kenten, J. Casadei, J. Link, et al., Clin. Chem. 37 (1991) 1626.
[4] D.W. Parry, Plant Pathology in Agriculture, Cambridge University Press, 1990, p. 3–4.
[5] K.S. Derrick, Virology 56 (1973) 6521.
[6] T.J. Morris, J.A. Dodds, Phytopathology 69 (1979) 854.
[7] Collins, M. Sedgley, J. Virol. Meth. 114 (2003) 65.
[8] W.W. Hu, S.M. Wong, J. Virol. Meth. 70 (1998) 193.
[9] P. Srifah, S. Loprasert, N. Rungroj, Gene 179 (1996) 105.
[10] S. Song-hua, S.K. Lung, A. Richard, et al., Biochem. Biophys. Res. Commun. 30 (2003) 377.
[11] J. Liu, D. Xing, X.-Y. Shen, D.-B. Zhu, Anal. Chim. Acta 537 (2005) 119.
[12] D.B. Zhu, D. Xing, X.Y. Shen, et al., Biosens. Bioelectron. 20 (2004) 448.
[13] G.F. Blackburn, H.P. Shah, J.H. Kenten, et al., Clin. Chem. 37 (1991) 1534.
[14] M. Lipp, P. Brodman, K. Pietsch, J. Pauwels, E. Anklam, J. AOAC Int. 82 (1999) 923.
[15] G.H. Yan, D. Xing, S.C. Tan, J. Immunol. Meth. 288 (2004) 47.
[16] T.E. Schutzblank, J. Smith, J. Clin. Microbiol. 33 (1995) 2036.
[17] D. Menno, D.E. Jone, F.L. Jan, T. Schuurman, M.E. Pauline, V. Wertheim, R. Boom, J. Clin. Microbiol. (2000) 2568.
[18] A. Richard, Collins, L.S. Ko, Y.F. King, L.T. Lau, J. Xing, C.H. Albert, Biochem. Biophys. Res. Commun. 297 (2002) 267.
[19] H.J. Stern, R.D. Carlos, T.E. Schutzbank, et al., Clin. Biochem. 28 (1995) 470.
[20] A.M. Siddiqi, V.M. Jennings, M.R. Kdd, et al., J. Clin. Lab. Anal. 10 (1996) 423.
[21] S. Gudibande, J.H. Kenten, J. Link, et al., Mel. Cell Probes. 6 (1992) 495.
[22] J. DiCesare, B. Grossman, E. Katz, E. Picozza, R. Ragusa, T. Woudenberg, Biotechniques 15 (1993) 1152.
[23] K. Motmans, J. Raus, C. Vandevyver, J. Immunol. Meth. 190 (1996) 1107.