Anal Bioanal Chem
DOI 10.1007/s00216-014-7791-y

RESEARCH PAPER

Potential of cross-priming amplification and DNA-based lateral-flow strip biosensor for rapid on-site GMO screening

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Received: 28 November 2013 / Revised: 17 March 2014 / Accepted: 24 March 2014

Abstract The requirement to monitor the presence of genetically modified organisms (GMO) in a variety of marked products has generated an increasing demand for reliable, rapid, and time and cost-effective analytical methods. Here we report an on-site method for rapid detection of cauliflower mosaic virus promoter (CaMV 35S), a common element present in most GMO, using cross-priming amplification (CPA) technology. Detection was achieved using a DNA-based contamination-proof strip biosensor. The limit of detection was 30 copies for the pBI121 plasmid containing the CaMV 35S gene. The certified reference sample of GM maize line MON810 was detectable even at the low relative mass concentration of 0.05 %. The developed CPA method had high specificity for the CaMV 35S gene, as compared with other GM lines not containing this gene and non-GM products. The method was further validated using nine real-world samples, and the results were confirmed by real-time PCR analysis. Because of its simplicity, rapidity, and high sensitivity, this method of detecting the CaMV 35S gene has great commercial prospects for rapid GMO screening of high-consumption food and agriculture products.

Keywords Genetically modified organism screening · Cross-priming amplification · on-site detection · Cauliflower mosaic virus promoter

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-7791-y) contains supplementary material, which is available to authorized users.

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Introduction

Genetically modified (GM) crops have attracted much attention because of their potential advantages in terms of raising agricultural productivity and reducing the need for environmentally harmful pesticides [1, 2]. GM crops covered 170.3 million hectares in 2012, a 100-fold increase in hectarage from 1996 to 2012. However, the long-term effects of GM products on human beings and the global ecology remain uncertain. These modified foods have not gained worldwide acceptance because of consumer mistrust resulting from environmental and public health safety concerns [3]. In response to this, many countries and the EU have enacted legislation to regulate the presence of genetically modified organisms (GMO) in crops, foods, and ingredients [4]. The ability to detect GMO is essential for enabling consumers to choose whether to consume food containing GMO. It is therefore necessary to develop reliable, sensitive, and rapid methods for GMO detection [5, 6].

Currently, the most commonly used detection methods for GM materials include conventional polymerase chain reaction (PCR), real-time PCR, and enzyme-linked immunosorbent assay (ELISA) [7–9]. However, most of these methods are time consuming, use expensive equipment, and require that well-trained personnel be available to perform field tests [10]. Immunoassay-based lateral-flow tests have been developed for on-site detection of several GM crops [11–13]. These use specific monoclonal and polyclonal antibodies in an immunochromatographic format incorporating antibody-coated particles. However, the immunoassay-based strip test is not useful for testing foods that have been highly processed, because the protein will probably have been destroyed.

DNA is more resistant than protein to processing, and can be extracted from even highly processed foods. However, there are only a few nucleic-acid lateral-flow strip tests developed for detecting DNA amplification products, because of the need for specialized instruments and potential risk of cross

Published online: 16 April 2014

contamination [14, 15]. Isothermal amplification is more convenient than the traditional PCR method, because all reactions can be conducted at one defined temperature, without complex instrument-based thermal cycle steps. A variety of isothermal-amplification methods have been developed, for example strand-displacement amplification [16], nucleic-acid-sequence-based amplification [17], rolling circle amplification [18], loop-mediated amplification [19], and helicase-dependent amplification [20]. However, these methods are still relatively complex procedures requiring the use of multiple enzymes and/or special reagents.

Cross-priming amplification (CPA) is a novel isothermal-amplification technology that uses a strand-displacement enzyme and a set of ingeniously designed primers [21]. The primer design usually includes: a pair of displacement primers that detach single-strand sequences at their 3 ends, one or more cross primer that introduces an additional priming site during each round of amplification, a biotinylated primer, and a probe labeled with FITC [22–24]. CPA products dual-labeled with biotin and FITC can be easily read out on a lateral-flow strip, where target sequences are immobilized by fixed FITC antibody and visualized by streptavidin-coated latex particles [25]. Screening of food by amplification of genetic elements frequently introduced into genetically modified plants is an effective method for rapidly detecting GM products. The cauliflower mosaic virus 35S promoter (CaMV 35S) is one of the most commonly used promoters, and the ability to detect this gene will enable the detection of most GMO plant products. Thus, the rapid and sensitive screening of the 35S gene is of much interest to consumers. We developed a nucleic-acid lateral-flow strip biosensor, based on CPA, for high-sensitivity on-site detection of CaMV 35S promoter, and proved it to be a simple, rapid, specific, and cost-effective field-based method of GMO screening.

Experimental

Materials and reagents

Bst DNA polymerase large fragment (BstDNApol) and 10× ThermoPol buffer were purchased from New England Biolabs Inc. Betaine Solution was purchased from Sigma-Aldrich Inc. dNTP and $MgSO_4$ solution were purchased from Sangon Biotech Inc. 10 % GM maize lines MON810, Bt176, MON863, MIR604, and GA21 standard, GM soybean line Roundup Ready standard, GM canola line RT73, and GM cotton line GHB614 were purchased from JRC-IRMM (Geel, Belgium). The 35S plasmid of pBI121, GM rice line Bt63, and non-GM maize were provided by the Chinese Academy of Inspection and Quarantine (CAIQ). The nine real-world samples were supplied by National Detection and Monitoring Technology Research Center of Import and Export Genetically Modified Organisms (NDMTRCIEGMOs). Primers and probes (Table 1) were synthesized by Takara.

DNA template and pretreatment

A serial dilution of plasmid pBI121 was prepared by stepwise dilution with double-distilled water. The concentrations of the solution ranged from one to 1×10^6 copies of plasmid per μL.

The milled non-GM maize and 10 % Mon810 were mixed to 1.0 % (mass per mass) content. The 1.0 % test sample material was then further mixed with ground non-GM rice to obtain MON810 contents of 0.5 %, 0.1 %, 0.05 %, and 0.01 %.

DNA was extracted from the GM and non-GM samples using the Plant Genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's procedure. The extracted DNA was quantified by use of the NanoDrop 2000 Spectrophotometer, and stored at −20 °C until use.

Primer design for CPA

A set of five primers, including two displacements, one cross, and two detector primers, which recognized six distinct regions on the sequence of the 35S gene, was designed by primer 5.0 software, as shown in Fig. 1. The 5≠end of the single cross-primer (Primer CPR) was not complementary to the template, enabling it to be displaced when DNA polymerase extended the upstream displacement primer. The two detector primers were designated as detector forward sense (Probe F, 5≠end labeled with fluorescein isothiocyanate (FITC)), and detector reverse anti-sense (Probe B, 5≠end labeled with biotin). Details of the primers are given in Table 1.

Conditions of CPA reaction

To optimize the primer concentration, the CPA reaction was performed in a total volume of 10 μL reaction mixture containing one of four different primer combinations, shown in Table S1 (Electronic Supplementary Material). The

Table 1 DNA sequences of CPA primers

Name	Sequence (5'-3')
Primer DPF	CGACAGTGGTCCCAAAGA
Primer DPR	CCAATGAAATGAACTTCC
Primer CPR	TATCTCCACTGACGTAAG-AGGAAGGGTCTTGCGAAG
Probe F	FITC-ATATCTCCACTGACGTAAGGG
Probe B	BIOTIN-ACGCACAATCCCACTATC

F, forward primer; R, reverse primer; DPF, displacement forward primer; DPR, displacement reverse primer; CPR, crossing primer; Probe F, primer labeled with FITC; Probe B, primer labeled with biotin

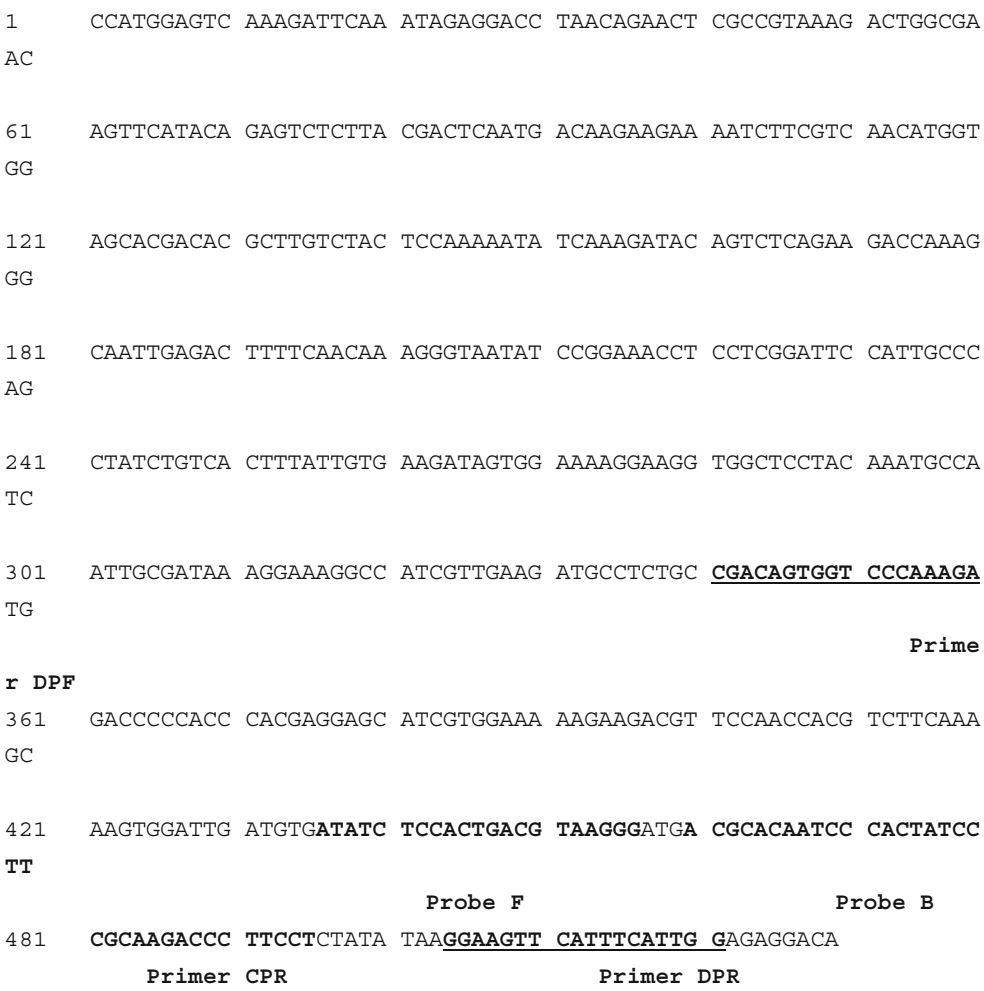

Fig. 1 The DNA sequences of the CaMV 35S gene and locations of CPA primers

concentration of other CPA components was: 8.0 U BstDNApol, 1 mol L^{-1} betaine solution, 0.8 mmol L^{-1} dNTP, 6 mmol L^{-1} $MgSO_4$ solution, and the specified amounts of target DNA. The reaction was performed at 63 °C for 50 min, and the mixture was then stored at 4 °C. The optimum primer concentration was then determined, and used during optimization of the remaining components of the CPA assay. Details are given in the section "Optimization of CPA reaction for CaMV 35S promoter detection", below.

Detection of CPA products

In the optimization reaction, the amplification products of different CPA conditions were identified by electrophoresis analysis using a 1.0 % agarose gel containing SYBR Green I. A digital picture was taken using the Gel Doc imaging system (Bio-Rad Laboratories, Hercules, CA, USA) under UV light. When detecting pBI121 plasmid and GM real samples under the optimized conditions, the reaction was repeated three times and the amplification products were identified by inserting the reaction tubes into the cross-contamination-proof disposable detection device, in which amplified GM-specific products can be visualized on nucleic-acid lateral-flow strips. The principle of the DNA strip is similar to that of the immunological lateral-flow strip. PrimerF and PrimerB are the primers labeled at their 5≠ends with FITC and biotin, respectively. These primers produce FITC–biotin dual-labeled CPA products, which are captured by FITC antibody fixed to the T line of the strip. Immobilized CPA products can be readily visualized by streptavidin-coated red latex particles, which combine with the biotin label to reveal the positive result. Only specific amplification products link the two haptens together, and these are detected by the immuno strip with a high degree of specificity. As a control, biotins were fixed to the C line of the strip, which can be clearly visualized by streptavidin-coated latex particles. The result was determined by observing the presence (positive result) or absence (negative result) of the test line on the lateral-flow DNA test strip.

CPA detection of the GMO real samples

The insect-resistant GM maize line MON810 was used as the template for validation of the CPA method. DNA extracted from MON810 samples with different mass fractions was amplified

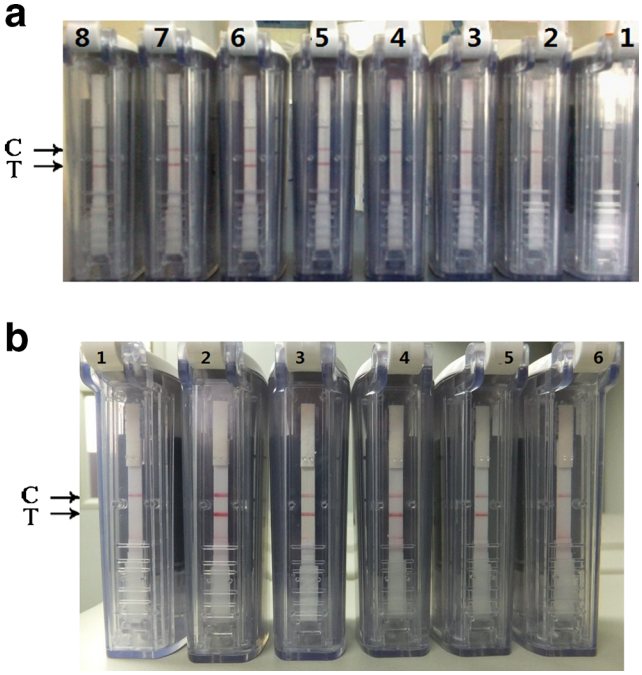

Fig. 2 (**a**) Amplification of the plasmid at different concentrations. *1*, blank control, the template was ddH_2O; *2*, three copies; *3*, 30 copies; *4*, 3×10^2 copies; *5*, 3×10^3 copies; *6*, 3×10^4 copies; *7*, 3×10^5 copies; *8*, positive control; *C*, control line; *T*, test line (**b**) Detection result from different concentrations of plasmid, using the modified procedure: *1*, blank control; *2*, 3×10^4 copies; *3*, 3×10^3 copies; *4*, 3×10^2 copies; *5*, 30 copies; *6*, 3 copies; *C*, control line; *T*, test line

using an optimized CPA assay and method. Each reaction was repeated three times. The products were detected using the cross-contamination-proof lateral-flow DNA strip device.

Results and discussion

Optimization of CPA reaction for CaMV 35S promoter detection

The reaction system and procedure were optimized to improve the sensitivity of the CPA method. To determine the optimum concentration of the five primers, the reaction was performed using four combinations of primer concentrations, as shown in Table S1 (Electronic Supplementary Material). Distinct amplified products were observed on the agarose gel at the optimum combination of 0.4 μmol L^{-1} Primer DPF and Primer DPR, 1.0 μmol L^{-1} Primer CPR, 0.6 μmol L^{-1} Probe F, and Probe B (Fig. S1, Electronic Supplementary Material). The concentration of Primer CPR is much higher than that of the other primers because it introduces an additional priming site during each round of amplification and forms a stem-loop structure of the target sequence, whose concentration is directly related to the amount of amplification products. Increasing the concentration of Primer CPR results a higher number of visible products on the agarose gel (Fig. S1, Electronic Supplementary Material). The optimization of other CPA components was performed using the primer combination described above with different concentrations of dNTP, betaine, BstDNApol, or Mg^{2+}. The reaction results are shown in Fig. S2–5 (Electronic Supplementary Material); distinct amplified products were observed on the agarose gel at the optimum conditions of 6.4 U BstDNApol, 0.5 mol L^{-1} betaine solution, 0.8 mmol L^{-1} dNTP, and 12 mmol L^{-1} $MgSO_4$ solution.

To determine the optimum reaction temperature, the reaction was performed at different temperatures in the range 60–66 °C using 4×10^4 copies of the pBI121 plasmid. As shown in Fig. S6 (Electronic Supplementary Material) the temperature had little effect on the sensitivity of the CPA method. We chose 62 °C as the optimum temperature.

Sensitivity of the CPA reaction for the CaMV 35S promoter

The optimum reaction conditions were used to amplify the serially diluted CaMV 35S plasmid to determine the minimum level of target DNA needed for the CPA assay. Each reaction was repeated three times. As shown in Fig. 2a, amplicons were observed when 3×10^3 copies of DNA were present. We surmised that at the low reaction temperatures, primers were not able to sufficiently bind to the double-stranded DNA (dsDNA). Heat denaturation of dsDNA can increase PCR amplification efficiency. Therefore, to further improve the sensitivity of the CPA, the procedure was modified by adding an initial denaturation step before adding the BstDNApol. The result (Fig. 2b) clearly showed that this improved the sensitivity of the assay, with a resulting limit of detection of 30 copies using the disposable nucleic-acid detection device.

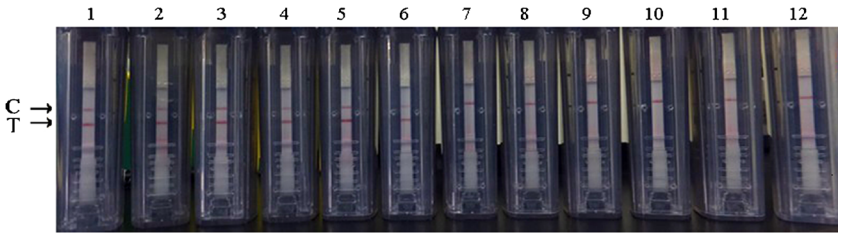

Fig. 3 Detection results for CaMV 35S promoter in different GMO crops. *1*, pBI121 plasmid; *2*, GM maize line MON810; *3*, GM maize line Bt176; *4*, GM maize line Bt11; *5*, GM maize line MON863; *6*, GM soybean line Roundup Ready; *7*, GM maize line MIR604; *8*, GM canola line RT73; *9*, GM maize line GA21; *10*, GM rice line Bt63; *11*, GM cotton line GHB614; *12*, non-GM maize; *C*, control line; *T*, test line

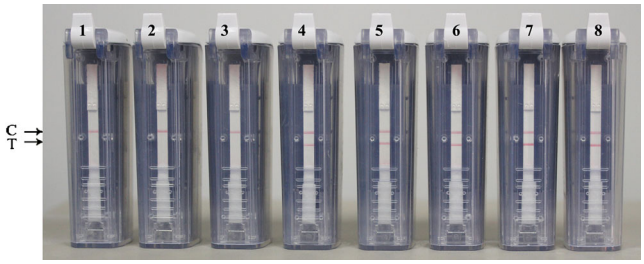

Fig. 4 Detection results for the MON810 samples with different mass fractions, using the modified procedure: *1*, blank control; *2*, non-GM maize; *3*, 0.01 % MON810; *4*, 0.05 % MON810; *5*, 0.1 % MON810; *6*, 0.5 % MON810; *7*, 1 % MON810; *8*, pBI121 plasmid; *C*, control line; *T*, test line

The previously reported loop-mediated isothermal amplification (LAMP) assay had a detection limit of four copies of GM genomic DNA [26]. However, LAMP is subject to non-specific amplification, especially when the target DNA is absent or there are low amounts of DNA present in the reaction [27]. LAMP relies on the detection of by-products of DNA synthesis [28, 29], e.g. magnesium pyrophosphate precipitation or use of SYBR Green dye, meaning it may not be able to distinguish between real and false positives. Consequently, the CPA detection method for CaMV 35S promoter would be expected to be a more reliable rapid diagnostic tool for GMO detection, and is particularly convenient for fast screening.

Specificity of the CPA assay

The specificity of the CPA primers was evaluated by CPA amplification of the genomic DNA from five GM lines containing 35S promoter gene, five GM lines not containing the 35S promoter gene, and one non-GM maize. The CPA amplification products of all GM lines and non-GM maize were determined by use of the nucleic-acid lateral-flow strip, as described above. The results reveal that two red lines (test line and control line) on the DNA strip could be seen for the GM maize lines MON810, Bt176, Bt11, and MON863, and the GM soybean line Roundup Ready, all of which contain the 35S gene. In the reaction of non-GM maize, GM maize lines MIR604 and GA21, GM rice line Bt63, GM canola line RT73, and GM cotton line GHB614, only the control line in the test strip was produced (Fig. 3). These results reveal that the CPA primers were highly specific to the CaMV 35S promoter gene. The genome-amplification method always has the possibility of false positives caused by cross-contamination [30, 31]. In our study, the test strips are housed in leak-proof plastic devices and the specific amplified products are detected without opening the caps of the amplification tubes, preventing the possibility of amplicon cross-contamination and reducing the risk of false-positive results. The positive result would be observed only when both detector probes were detected by the immuno strip, resulting in a high degree of specificity.

Practical application of the CPA method for GMO samples

To verify the reliability of CPA for GMO screening, we detected the extracted DNA of insect-resistant GM maize line MON810 samples with different mass fractions, using the optimized conditions and procedure. The results (Fig. 4) reveal that with this technique we could detect maize flour with 0.05 % GM maize MON810 content. The results had excellent repeatability in the three repeats of the CPA reactions, even at the lowest GM content of 0.05 %, indicating that CPA is a reliable assay for detecting commercially available certified reference samples.

The CPA method was also validated by analyzing real-world samples. Nine samples, derived from crackers (one sample), biscuits (one sample), soybean flour (one sample), maize flour (two samples), soybean meal (two samples), animal feed (two samples), and 0.05 % GM maize MON810 (positive control), were analyzed both by the proposed method and by real-time PCR, a method of choice for GMO analysis. As shown in Fig. 5, both test line and control line were observed for samples 2, 4, 5, and 9 and for the positive control, indicating that these samples contain genetically modified ingredients with the CaMV 35S gene. For samples 1, 3, 6, 7, and 8, only control lines in the test strip were produced, and the CaMV 35S gene was undetectable in these samples.

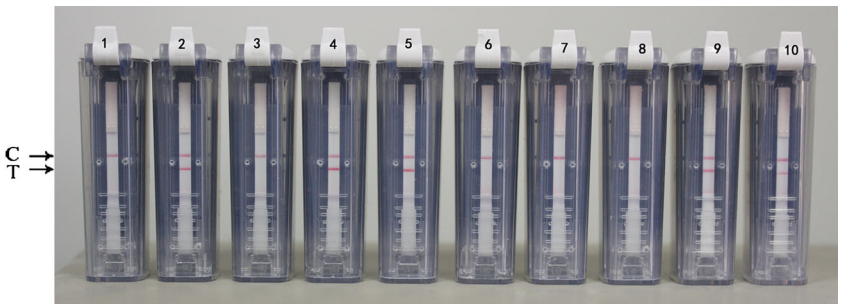

Fig. 5 Detection results for real-world samples using the developed CPA method: *1*, sea-salt crackers; *2*, paprika biscuit; *3*, soybean flour; *4*, maize flour-BJ; *5*, maize flour-QD; *6*, soybean meal-BJ; *7*, soybean meal-YD; *8*, distillers dried grains with soluble; *9*, oil cake; *10*, positive control, 0.05 % MON810; *C*, control line; *T*, test line

The real-time PCR analysis was performed on these samples independently, by NDMTRCIEGMOs [8], and the results (shown in Fig. S7 Electronic Supplementary Material) confirmed those of the CPA method. The results obtained by the two methods were in good agreement, demonstrating the reliability of the CPA method for real sample detection.

The real-time PCR system is widely used as a crucial technology for quantifying GM in samples, and is also used for qualitative detection of GMO [32–34]. Real-time PCR methods for MON810 detection have been reported, and the detection limit is 0.5 % [35, 36], which is much higher than that of the CPA-based strip method in our study. However, the real-time PCR system requires difficult and time-consuming procedures and expensive instruments and reagents. Protein-based strip tests for GMO detection are simple, cheap, and fast, and do not require any laboratory facilities or technical expertise. Some methods have been reported that use antigen–antibody interaction for detection of GMO including GM line MON810, Bt11, GTS-40-3-2, and genetically engineered proteins CP4-EPSPS, CryIAb, Cry9c, PAT/pat, and PAT/bar [37, 38]. All results revealed the detection sensitivity to be only ~0.1–0.8 %. Furthermore, the protein-based methods are trait specific, thus lacking the characteristics of a broad screening test, and the exogenous proteins expressed are unstable in most processed GM products. Protein-based detection methods are unable to distinguish between different biotechnology-derived products that express the same protein. Furthermore, because proteins are often denatured by processing, protein-based detection methods are most suitable for use on non or minimally processed materials (e.g. grain, flour). However, assays for use on specific processed materials have occasionally been developed. The developed nucleic-acid strip test for CaMV 35S, a common element present in most GMO, is reliable, highly sensitive, convenient, and practical for monitoring the presence of GM materials.

Conclusion

In this report, a novel CPA-based nucleic-acid-strip detection method for the CaMV 35S gene was developed and demonstrated. The detection limit is 30 copies for plasmid DNA and 0.05 % for real-world GM samples, which is more sensitive than that of DNA-based PCR and of the protein-based strip test. The CPA method had high specificity for the CaMV 35S gene, as compared with other GM lines not containing this gene and non-GM products. The detection can be performed without specialist equipment, and is suitable for on-site use. Use of the DNA-based lateral-flow strip device greatly reduces the chance of sample contamination, and means results can be read by the naked eye . The CaMV 35S screening test is of utmost importance in providing an initial insight into the possibility of accidental GMO contamination. Because of its simplicity, rapidity, and high sensitivity, this detection method for the CaMV 35S gene has great commercial potential for the fast GMO screening of high-consumption food and agriculture products.

Acknowledgments We are grateful for the support of the Important National Science and Technology Specific Projects (2013ZX08012-001) and AQSIQ Non-profit Industry Research Projects (20141004).

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