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Visual and Rapid Detection of Two Genetically Modified Soybean Events Using Loop-mediated Isothermal Amplification Method

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Abstract To develop effective alternatives for detecting genetically modified organisms (GMOs), we reported one optimized visual loop-mediated isothermal amplification (LAMP) method for the detection of exogenous DNA targets from two GM soybean events in this study. This isothermal amplification can be performed within 40 min without polymerase chain reaction (PCR) equipment and the derived LAMP products can be directly observed by naked eye employing SybrGreen I dye instead of conventional gel electrophoresis analysis. The limits of detection of these established visual LAMP assays were about 4 copies of haploid soybean genomic DNA, and which were much higher than those of reported conventional PCR assays. Furthermore, the high specificity of LAMP assays was determined. All the results demonstrated that the developed visual LAMP assays are convenient, cost-efficient, and rapid for on spot detection of GMOs.

Keywords Loop-mediated Isothermal Amplification · Visual Detection · Genetically Modified Soybean

Introduction

In the past two decades, the recombinant DNA technique has been widely applied in modern agriculture and around 150 kinds of genetically modified (GM) crops with new traits have been produced and approved for commercialization in global area Andersen et al. (2006). By the end of 2008, the global area of GM crops being planted has reached 125 million hectares. GM soybean is by far the world's most cultivated GM crop, and its planted area was 65.8 million hectares in 2008, occupying 53% of GM crops worldwide (James 2008). However, since the consumers concern GM food safety, series of laws have been issued for GMOs regulation and labeling in China, European Union (EU), Brazil, Korea, Japan, and Australia, etc.

Currently, various molecular methodologies based on protein or nucleic acid analysis have been developed and used for GMOs detection (Hernández et al. 2005; Rodríguez-Lázaro et al. 2007). In particular, conventional and real-time quantitative polymerase chain reaction (PCR) methods have been widely accepted as standards for identifying and determining the GM contents in food and feeds because of their high efficiency, sensitivity, and stability (Bellocchi et al. 2008; Holst-Jensen et al. 2003; Singh et al. 2008; Marmiroli et al. 2008; Zel et al. 2008). Several conventional and quantitative real-time PCR methods for GM soybean GTS 40-3-2 have been developed and validated (Yang et al. 2008; Taverniers et al. 2005; Berdal

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and Holst-Jensen 2001; Huang and Pan 2005). However, the requirements of either a high-precision instrument for PCR amplification or complicated procedures for PCR analysis are their main disadvantages, which limit these methods being widely used on spot detection.

Loop mediated isothermal amplification (LAMP) is one isothermal nucleic acids amplification technique developed by Notomi et al. (Notomi et al. 2000). The LAMP assay is performed using *Bst* DNA polymerase large fragment (New England Biolabs) with strand displacement activity under isothermal conditions ranging from 60 to 65°C within shorter time. In LAMP assay, a set of four specially designed primers, termed inner primers (FIP and BIP) and outer primers (F3 and B3) were used, which can recognize at least six independent target DNA sequences, providing the exceptionally higher specificity of amplification than conventional PCR method. The LAMP products are typical ladder-like pattern and could be judged by agarose gel electrophoresis or real-time monitored according to the turbidity using turbidimeter (Mori et al. 2001). Alternatively, the LAMP reaction is determined visually by adding SybrGreen I dye to the reaction mixture: the color of the solution would change to green in the presence of LAMP amplicons, while it remains orange for the mixture with no amplification (Iwamoto et al. 2003). LAMP technique has been used successfully to detect bacterial microorganisms (Enosawa et al. 2003; Misawa et al. 2007), pathogenic virus (Poon et al. 2005; Imai et al. 2007), and parasites (Han et al. 2007), etc. However, there are few reports on the plant genomic DNA amplification using the LAMP method because of larger and more complex genomic DNA. Also, few applications using LAMP technique have been reported on GMOs detection (David et al. 2009).

In this study, the optimized visual LAMP methods for the detection of GM soybean events, GTS 40-3-2 and MON89788, have been established. The amplified efficiencies, limits of detection (LODs) and specificities of these systems indicated that the developed LAMP assays are more specific, effective, and sensitive than conventional PCR assays, and which can be used for GMOs detection on spot.

Materials and Methods

Plant Materials

The GM plant events (GTS 40-3-2 and MON89788 soybeans, MON810 maize, GT73 canola, and MON531 cotton) were developed by Monsanto Company, and the certified reference materials of these GM events were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China) or the American Oil Chemists' Society. The seeds of GM Huafan No. 1 tomato were kindly supplied by Huazhong Agricultural University, China. Non-transgenic seeds of soybean, sweet pepper, wheat, and rice were purchased from local market in Shanghai, China.

DNA Extraction and Purification

The plant genomic DNA was extracted and purified using the Plant DNA Mini-Prep kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The quantity and quality of purified DNA samples were measured and evaluated using the NanoDrop 1000 UV/Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Also, the quality of DNA was analyzed by 1% (*w*/*v*) agarose gel electrophoresis in 0.5× TBE with GelRed staining.

DNA Oligonucleotides

The scheme of the LAMP primers design for GM soybeans was shown in Fig. 1a, and a set of four primers containing two inner primers (FIP and BIP) and two outer primers (F3 and B3) were designed for each target sequence. FIP contains the F1c (complementary to F1) and the F2 sequence. BIP contains the B1c sequence (complementary to B1) and the B2 sequence. All of the LAMP primers used in this work were newly designed using the specific software of Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). The conventional PCR primers (89788-F/R) were used according to the previous report (Liu et al. 2009). All primers used in this study were listed in Table 1 and synthesized by Invitrogen Co. Ltd. (Shanghai, China). The detailed locations of LAMP primers in target DNA sequence were shown in Fig. 1b.

Conventional PCR Assay

In conventional PCR, the reaction was carried out in a 30 μL volume, including 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 1.5 U *Taq* DNA polymerase (TaKaRa biotechnology Co., Ltd, Dalian, China) and 5 μL template DNA. The conventional PCR was performed in PTC-100 Thermalcycler (MJ Research, Watertown, MA, USA) according to the following program: an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. The PCR products were analyzed by 2% (*w*/*v*) agarose electrophoresis in 0.5×TBE with GelRed staining.

LAMP Assay

LAMP assay was performed in a 25 μL total reaction mixture containing 20 mM Tris-HCl (pH8.8), 10 mM KCl,

Fig. 1 Primers design for LAMP assays. **a** Schematic diagram for LAMP primer design. **b** Nucleotide sequences used for designing the primers. Primers used for LAMP assay are indicated by *arrows*

10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, 0.5 M betaine (Sigma), 1.2 μM each FIP and BIP, 0.2 μM each F3 and B3, 400 μM each dNTP. After adding 5 μL template DNA, the mixture was incubated for 5 min at 95 °C and cooled on ice, and then 8 U *Bst* DNA polymerase large fragment (New England Biolabs) were added. The mixture was incubated at 63 °C for 60 min in a conventional heating block. LAMP assay was carried out in triplicate for each template DNA, and the no template control (NTC) contained water instead of the template.

Analysis of LAMP Products

In general, 10 μL amplified products were analyzed by 2% agarose gel electrophoresis in 0.5×TBE with GelRed staining after the LAMP amplification. Alternatively, the LAMP amplified products were directly observed by the naked eye through adding 2 μL 1,000×SybrGreen I (Generay Biotech Co., Ltd., Shanghai) into the reaction mixture. The color of reaction mixture will turn green in the presence of LAMP-amplified products, while it remains

Table 1 Oligonucleotide primers used in this study

Primers name	Sequence (5′–3′)	Target	Reference
GTS-F3	AGGAGTAGTACACTCACCA	3′ junction of GTS 40-3-2	This work
GTS-B3	AAGAGAACTACCTTCTCACC		
GTS-FIP	CAACGAGAAGCTATATGTAGATGCTGTGACCCTAATAGGCAACA		
GTS-BIP	ATTTGGGATCGGAGAAGAACTGTCGAGCTTCTTCACGAACT		
89788-F3	TTGTTTATTGCTTTCGCCTAT	3′ junction of MON89788	This work
89788-B3	CTATCACATATGGTTGAAGTCG		
89788-FIP	AGTCACTAGAGTCTGAGCGTTATTAGACGGATCGTAATTTGTCG		
89788-BIP	ACCTTCACTCTCCTCAAGCAGAACGTAAGGGATAATTTGGT		
Lectin-F3	ACAACTTTTGAAAAGTACCCAAT	Lectin gene	This work
Lectin-B3	GAACTTGTTCCAGCTGAAAG		
Lectin-FIP	CATTGCTTTGCTTCAGCTAAATTGCTGCTAGTATAAATAGGGGCATG		
Lectin-BIP	AACCCAGAATGTGGTTGTATCTCTCAGTTTCCGCTGAGTTTGC		
89788-F	ACGTAGACACGTCGAAATAAAGATT	3′ junction of MON 89788	Mori et al. (2001)
89788-R	GGAAGAGGCTGAAATGCTTG		

orange in reaction mixture with no amplification. The sensitivities of gel electrophoresis analysis and visual observation by addition of SybrGreen I were compared using different LAMP products amplified from serially diluted soybean genomic DNA samples.

Results and Discussion

Design of the LAMP Systems for Detecting GM Soybeans

One ideal LAMP assay relies on the concurrence of the *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers capable of recognizing a total of six distinct regions on the target DNA (Notomi et al. 2000). LAMP primers of GTS 40-3-2 event were designed based on the event-specific sequence of 3′ end of exogenous integration (EMBL accession code AJ308515), recognizing a 214 bp target sequence (Windels et al. 2001). The MON89788 LAMP primers were designed based on our revealed event-specific sequence of 3′ end of exogenous integration to recognize a 203 bp target sequence (Liu et al. 2009). LAMP primers for the soybean endogenous reference gene were targeted at the *Lectin* sequence (EMBL accession code. K00821), targeting to a 217 bp target sequence. The detailed sequences of all LAMP primers used in this study are listed in Table 1.

Optimization of the LAMP Assays

In the present study, LAMP assays were performed employing heat-denatured soybean genomic DNA as the template with two inner primers and two outer primers. Previous study reported that LAMP assay could work well using non-denatured DNA samples as templates (Nagamine et al. 2001). However, we found that the amplified efficiency of LAMP assay using non-denatured soybean DNA as template was obviously lower than that using denatured soybean DNA (data not shown). Accordingly, the concentration of base destabilizer, betaine, was decreased to 0.5 M in each reaction. The optimal reaction temperature was determined by comparing the LAMP-amplified efficiencies under various temperatures ranging from 60 °C to 65 °C. In addition, different primer concentrations and the ratios between inner primers (FIP and BIP) and outer primers (F3 and B3) were also optimized. The LAMP assays for soybean *Lectin* endogenous reference gene, GTS 40-3-2 and MON89788 soybean events were established based on the optimized reaction conditions described in "Materials and Method" section.

Evaluation of the Reaction Efficiency of LAMP Assays

Previously, it has been reported that the LAMP reaction can be accelerated by using two loop primers (Nagamine et al. 2002). However, in the present study, only two inner primers (FIP and BIP) and two outer primers (F3 and B3) were used for amplification. In order to test the reaction efficiency of the developed LAMP assays, the different reaction times (10, 20, 30, 40, 50, and 60 min) were selected in GTS 40-3-2 and MON89788 LAMP assays. As shown in Fig. 2, the GTS 40-3-2 event specific LAMP products could be observed after 30-min amplification with 10 ng DNA template or 40-min amplification with 1 ng DNA template. Also, similar results were obtained in MON89788 event-specific LAMP assay (data not shown). These results indicated that the established LAMP assays have high reaction efficiency without the loop primers,

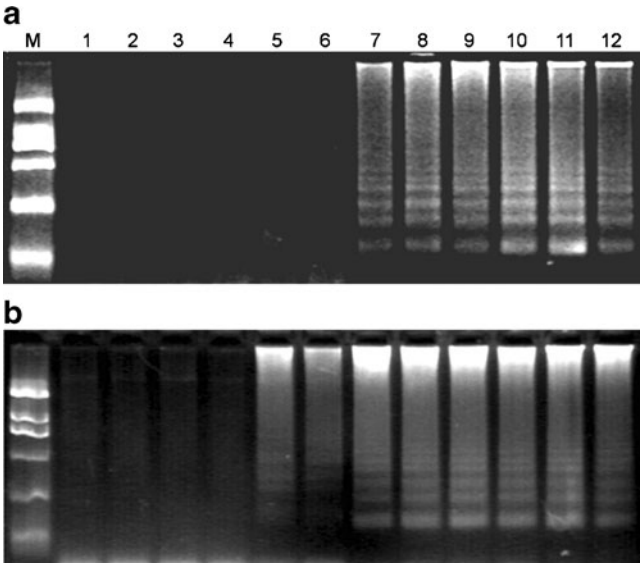

Fig. 2 Determination of the amplified efficiency of LAMP assays. LAMP products were detected on 2% agarose gel after 10 min (*lanes 1 and 2*), 20 min (*lanes 3 and 4*), 30 min (*lanes 5 and 6*), 40 min (*lanes 7 and 8*), 50 min (*lanes 9 and 10*), 60 min (*lanes 11 and 12*); *lane M* DL2000 marker. **a** The products were detectable after 40 min when the total template was 1 ng. **b** The products were detectable after 30 min when the total template was 10 ng

suggesting that these assays are sufficient for rapid amplification of target DNAs.

Specificity Test

To evaluate the specificity of the developed LAMP assays (GTS 40-3-2, MON89788 and *Lectin*), several GM events (GTS 40-3-2 and MON89788 soybean, MON810 maize, Huafan No. 1 tomato, GT73 canola, and MON531 cotton) and non-GM plants (pepper, soybean, potato, wheat, and rice) were used. In the specificity test, 10 ng total corresponding plant genomic DNA was used as the template in each LAMP assay. As expected, in the *Lectin* assay, the typical ladder-like pattern products and green color of the reactions were only obtained in the tests using soybean genomic DNA samples as templates, no amplified products and orange color were observed in other GM and non-GM crops as well as the NTC (Fig. 3a–b). In the GTS 40-3-2 assay, the typical ladder-like pattern products (Fig. 4a) and green color (data not shown) were obtained only in the reaction using GTS 40-3-2 soybean genomic DNA as template. In the MON89788 LAMP assay, the similar result was observed (Fig. 5a). In addition, we found that all the results obtained from gel electrophoresis and visual observation by addition of SybrGreen I was consistent in the three LAMP assays. These data confirmed that the developed LAMP assays have high specificity for amplifying the target DNAs.

Limit of Detection

For GMOs detection, the high sensitivity is important and necessary because the degradation of low quantity DNA derived from GMOs often occurs in practical detection. To test the LOD of *Lectin* LAMP assay, non-GM soybean genomic DNA was serially diluted with 0.1×TE buffer to final concentrations of 20, 2, 0.2, 0.02, 0.002, 0.001, 0.0002, and 0.0001 ng/μL. Five microliter diluted DNA sample was used as template in each reaction. As shown in Fig. 3c–d, the amplified LAMP products and green color were observed in all the dilutions except for the levels of 0.0002 and 0.0001 ng/μL. Thus, the LOD of *Lectin* assay was as low as 5 pg, corresponding to about four copies

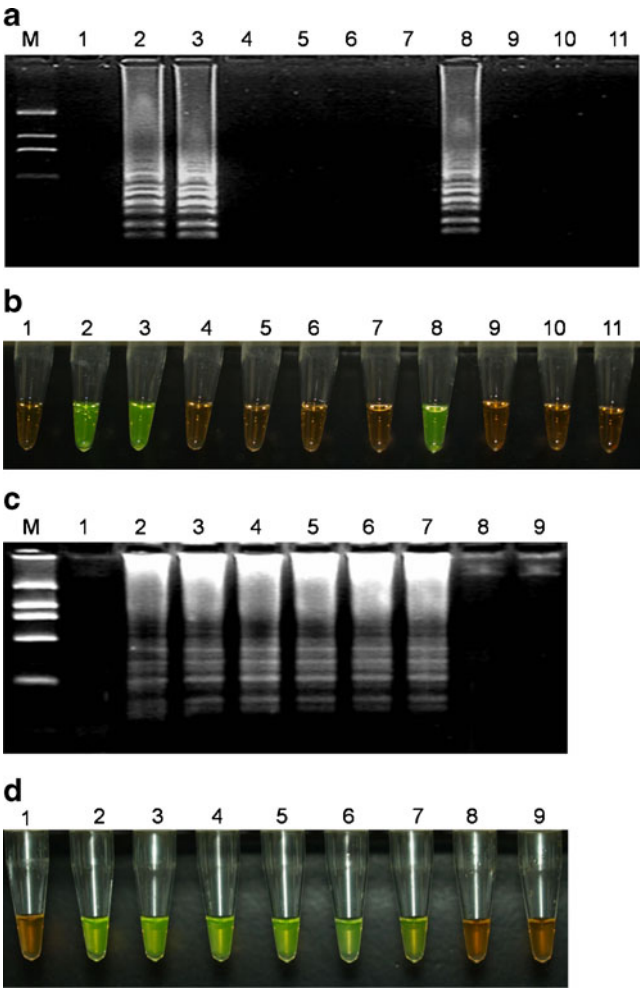

Fig. 3 Lectin LAMP assay. **a**, **b** Specificity test of LAMP assay by agarose gel electrophoresis analysis and visual observation using SybrGreen I. *Lane 1* no template control (NTC); *lanes 2–11* GTS 40-3-2, MON89788, MON810, Huafan No. 1, GT73, MON531, non-GM soybean, pepper, wheat, and rice; *lane M* DL2000 marker. **c**, **d** Sensitivity test of LAMP products by agarose gel electrophoresis analysis and visual observation using SybrGreen I. *Lane 1* NTC; *lanes 2–9* correspond to 100, 10, 1, 0.1, 0.01, 0.005, 0.001, and 0.0005 ng; *lane M* DL2000 marker

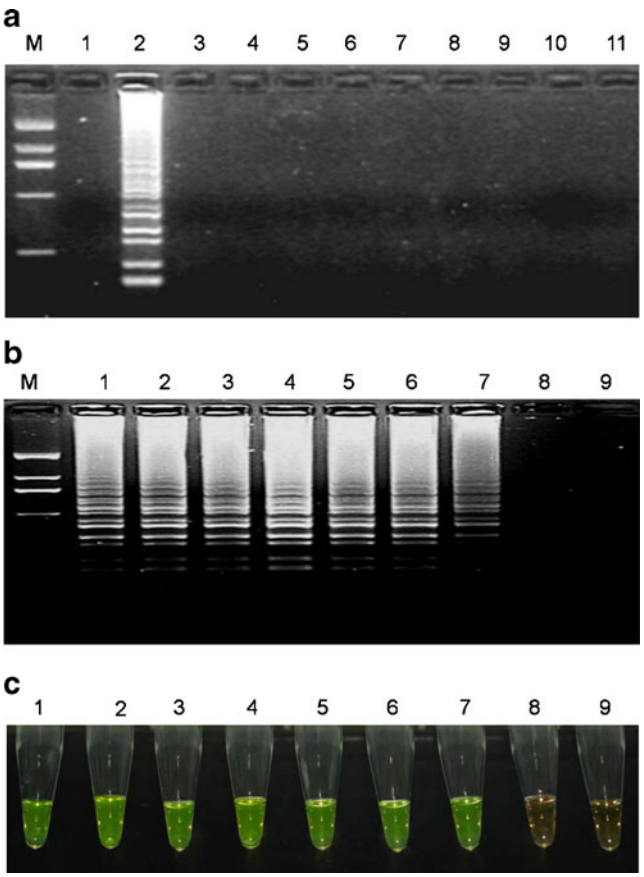

Fig. 4 GTS 40-3-2 LAMP assay. **a** Specificity test of the LAMP assay. *Lane 1* NTC; *lanes 2–11* GTS 40-3-2, MON89788, MON810, Huafan No. 1, GT73, MON531, non-GM soybean, pepper, wheat, and rice; *lane M* DL2000 marker. **b**, **c** Sensitivity test of LAMP products by agarose gel electrophoresis analysis and visual observation using SybrGreen I. *Lanes 1–8* mixed GM GTS 40-3-2 soybean samples with GM contents of 10%, 5%, 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0%; *lane 9* NTC; *lane M* DL2000 marker

soybean haploid genomic DNA according to the soybean genome size (Andersen et al. 2006).

For testing the LODs of GTS 40-3-2 and MON89788 LAMP assays, the tested samples were prepared by mixing the relevant GM soybean event with non-GM soybean at various levels, such as 10%, 5%, 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0% (*w*/*w*). In each reaction, a total of 100 ng soybean genomic DNA was amplified. As shown in Figs. 4b–c and 5b–c, the amplified DNA fragments and the green colors were observed from all the tested levels except for the 0% level in both GTS 40-3-2 and MON89788 LAMP assays. These results indicated that the LODs of both GTS 40-3-2 and MON89788 LAMP assays were 0.005%, corresponding to about 4 copies of haploid soybean genomic DNA. In addition, we also found that the analyzed results from visual observation by addition of SybrGreen I and gel electrophoresis analysis were quite consistent, and there were no more difference between the visual observation and gel electrophoresis analysis. This indicated that the visual observation could be used for LAMP analysis instead of the general agarose gel electrophoresis.

In conventional PCRs, the LODs of *Lectin* and GTS 40-3-2 were reported with the value of 40 copies (Rott et al. 2004), and the LOD of MON89788 was tested as low as eight copies of haploid soybean genomic DNA

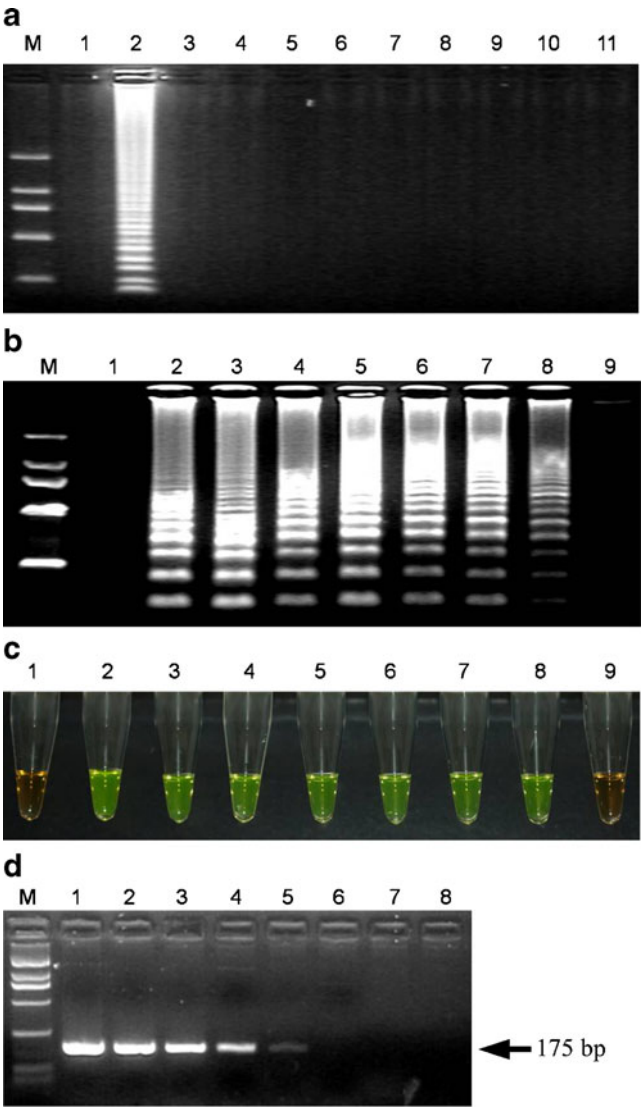

Fig. 5 MON89788 LAMP assay. **a** Specificity test of the LAMP assay. *Lane 1* NTC; *lanes 2–11* MON89788, GTS 40-3-2, MON810, Huafan No. 1, GT73, MON531, non-GM soybean, pepper, wheat, and rice; *lane M* DL2000 marker. **b**, **c** Sensitivity test of LAMP products by agarose gel electrophoresis and visual observation using SybrGreen I. *Lane 1* NTC; *lanes 2–9* mixed GM MON89788 soybean samples with GM contents of 10%, 5%, 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0%; *lane M* DL2000 marker. **d** Sensitivity test of MON89788 conventional PCR. PCR products were amplified from MON89788 soybean DNAs with different concentrations. *Lanes 1–7*, 5%, 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0%; *lane 8* NTC; *lane M* DL2000 marker

(Fig. 5d), respectively. Compared with the LODs of conventional PCR assays, we believed that the developed LAMP assay is more sensitive and could be feasibly used for detection of GTS 40-3-2 and MON89788 soybeans.

Conclusion

In this study, we developed the visual and rapid detection methods for GM soybean MON89788 and GTS 40-3-2 events using the optimized LAMP technique. Compared to the classical PCR analysis, the LAMP assay has the advantages such time saving and low cost or simple procedures for result analysis. LAMP assay can be performed under isothermal conditions (60-65 °C) without PCR thermal cyclers within shorter time (40 min in this study). In combination with SybrGreen I dye, the amplified products can be directly detected by naked eye instead of the conventional gel electrophoresis analysis or fluorescent detection...In addition, the LODs of developed MON89788 and GTS 40-3-2 LAMP assays with the values of four copies are more sensitive than conventional PCR methods. High specificity and sensitivity, cost-effectiveness, and low requirement of equipment of the developed visual LAMP assays allow this method to be useful in GM soybean samples analysis, especially on spot detection combined with the fast DNA extraction method.

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