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ORIGINAL PAPER

Establishment of a loop-mediated isothermal amplification (LAMP) detection method for genetically modified maize MON88017

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Abstract In this study, we developed a visual and rapid assay for the detection of MON88017 maize using the LAMP method. The LAMP method was specific for MON88017 event and takes only 40 min and the LAMP assay sensitivity is about 40 copies, which is the same level as that of conventional PCR method. LAMP amplicons can directly be detected by naked-eye inspection after adding SYBR Green I. In summary, the LAMP method is visual, faster, and more sensitive and does not need special equipment compared to the traditional PCR technique, which makes it a very higher efficiency approach for field tests and fast screening of GMO crops, especially for on-site, large-scale testing purposes in the field.

Keywords GMO · LAMP · MON88017 · Detection

Introduction

Modern plant breeding techniques have revolutionized agro-food industry by introducing genetically modified organisms (GMOs) with the aim to improve the agricultural practices and nutritional quality [5]. A large number of GM crops with new beneficial traits have been developed by integration of foreign genes into the plant genomes. Since 1996, the planting area and events of GM crops have been continuously increased. In 2014, a record 181.5 million hectares of biotech crops were grown globally, an increase of more than six million hectares from 2013 [14], and global hectarage has increased more than 100-fold since the first plantings of biotech crops according to a report released by the International Service for the Acquisition of Agri-Biotech Applications (ISAAA). GM maize is also grown on a big scale in the USA, Brazil, Argentina and so on. There are two important traits in GM maize production, one is the resistance to insect damage such as MON88017, 59122, Bt11, MON810 and Bt176, and the other is particular tolerance to herbicide such as MON88017 and NK603. Therefore, MON88017 is more likely to be widely cultivated in the future.

Nowadays, it is a growing controversy: Should GMO foods always be labeled so consumers are aware that the product contains genetically modified ingredients? Increasingly, consumers are interested in GMO monitoring and labeling. To ensure the consumer's choice of freedom, many countries have established regulatory systems and labeling regulations for GM food [34]. To protect the "right to know" of consumers for the GMO products, in several groups and countries, GMO labeling policies have been instituted and stated that the GMO products must be labeled when they are above the threshold between 0 and 5 % [10]. Therefore, to guarantee the GMO traceability, the stricter laws must rely on more accurate and efficient methods for detecting GMOs in raw materials as well as in highly processed food and feed. Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification test that can be used in fields with resource-limited settings. Since LAMP method was first reported in 2000 [24], the number of studies performed using LAMP to detect the GMO ingredient is increasing every year. The direct evidence on the LAMP robustness is its application for the detection of GMO crops [2, 9, 13, 18, 20, 22, 35, 39]. However, to date, only one conventional multiplex qualitative PCR method [25] and

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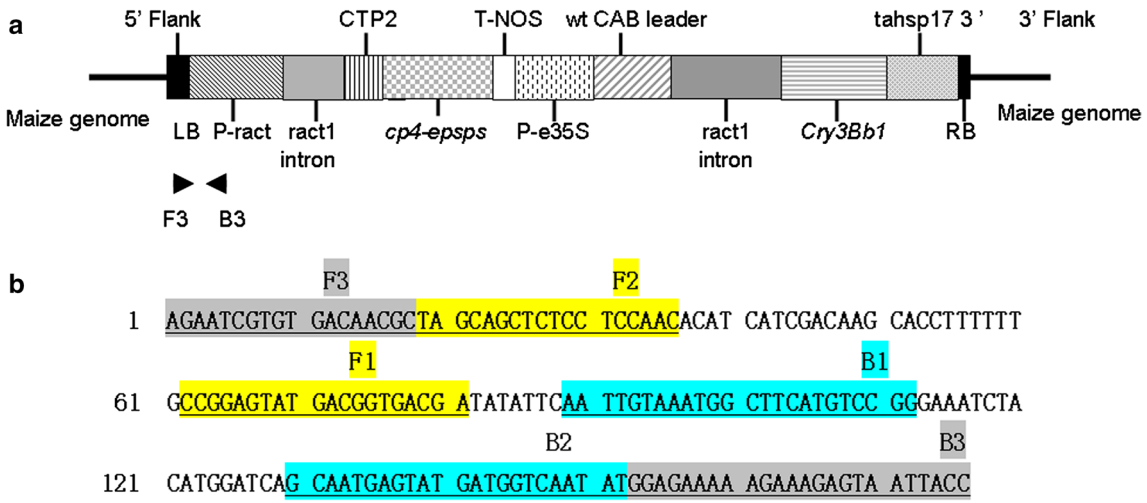

Fig. 1 Nucleotide sequences used as the event-specific LAMP primers for MON88017. **a** Schematic representation of LAMP primer design, **b** nucleotide sequences used for this LAMP assay are indicated by the *lines* and *highlights*

one quantification PCR method [6] have been developed for MON88017 and no report of LAMP detection method for it. In this study, we aimed to establish a sensitive, specific and rapid LAMP method to detect the flanking sequence of exogenous gene in GM maize MON88017.

Materials and methods

Plant materials

The 10 % (w/w) GM maize MON88017 was purchased from CRM/CM Information center of China, and the other 1.0 % (w/w) GM crop samples: GM maize (MON88017, MON863 and NK603), GM soybean (GTS 40-3-2 and MON89788), GM rice (TT51-1 and Kefeng 6), GM cotton (MON1445 and MON88913), GM canola (GT73) and GM sugar beet (H7-1) were supplied by the Center of Science and Technology Development, Ministry of Agriculture of China. Non-GM maize was purchased from the local market in Harbin, China.

Primer design

The primers were designed according to the 5′ border sequence in MON88017 corn obtained from the GMDD database (http://gmdd.shgmo.org/event/view/85). The LAMP primer set was designed by the online software http://primerexplorer.jp/e, including two outer primers (F3 and B3), a forward inner primer FIP (F1c + F2) and a backward inner primer BIP (B1c + B2). The detailed locations of LAMP primers in the target genome DNA sequence are shown in Fig. 1.

DNA extraction and purification

The plant genomic DNAs were isolated and purified from 100 mg sample of above compounds using Plant Genomic DNA Extraction Kit (Tiangen, China) according to the manufacturer's instructions. The quantity and quality of DNA were initially determined by measuring the absorption at 260 nm and the absorption ratio of 260/280 nm with a Nano-100 spectrometer (Allsheng, China). And the concentrations of all samples have been accurately determined as 100 ng/μL by dilutions of template with ddH_2O.

LAMP reaction assay

The LAMP reaction was carried out in a total of 25 μL solution containing 1× ThermoPol buffer, 0.2 μM each outer primer F3 (5′-AGAATCGTGTGACAACGC-3′) and B3 (5′-GGTAATTACTCTTTCTTTTTCTCC-3′), 1.2 μM each inner primer FIP (5′-TCGTCACCGTCATACTCCGGTAGCAGCTCTCCTCCAAC-3′) and BIP (5′-AATTGTAAATGGCTTCATGTCCGGATATTGACCATCATACTCATTGC-3′), 0.4 mM dNTPs (TaKaRa, China), 1.0 M betaine (Sigma, St. Louis, MO, USA), 2 mM $MgSO_4$, 8 U/μL *Bst* DNA polymerase large fragment (New England Biolabs, USA) and 1 μL (100 ng) target DNA. The mixture was incubated at 63 °C for 35 min and then heated at 80 °C for 5 min to terminate the reaction. The LAMP assay was carried out in triplicate, and the no template control (NTC) contained ddH_2O instead of the template.

Detection of LAMP products

The LAMP amplified products were directly watched by the naked eye through adding 2 μL of 1/10-diluted

original SYBR Green I (10,000×) (Sigma, USA) into the reaction tube to observe the change in color. The mixtures were visually inspected and photographed under the natural light. In addition, a portion of each product was analyzed by 2 % agarose gel electrophoresis stained with ethidium bromide (EB) and photographed using a Multi-Image (Alpha, USA). The variation in the color was visually observed in the positive, negative and NTC reaction products.

Conventional PCR assay

Conventional PCR was also established to compare the specificity and sensitivity with the developed LAMP method. The primers employed for conventional PCR were MON88017 LF/LR. The expected product size was 199 bp. The 20 μL volume PCR mixture contained 1× *rTaq* buffer (TaKaRa, China), 0.25 mM each dNTP, 0.25 μM each primer (MON88017-LF: TTGTCCTGAACCC-CTAAAATCC and MON88017-LR: CCCGGACATGAA GCCATTTA), 1 U *rTaq* DNA polymerase (TaKaRa, China) and 1 μL template DNA (100 ng). Each reaction was initially denatured at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min using a LabCycler SensoQuest (SENSO, Germany). PCR products were electrophoresed in a 2 % agarose gel and photographed.

Specificity of LAMP system

In the specificity test, 100 ng total GM maize MON88017 (1.0 %, w/w), non-GM maize and other ten GM crops (including MON863, NK603, GTS 40-3-2, MON89788, TT51-1, Kefeng 6, MON1445, MON88913, GT73 and H7-1) genomic DNAs (1.0 %, w/w) were used as templates in each LAMP assay under the optimized conditions. In addition, non-GM maize template was used as negative control, and template DNA was replaced by ddH_2O as NTC.

Limit of detection (LOD) of LAMP system

To test the LOD of the established LAMP assay, eight different folds dilutions of MON88017 genomic DNA template (4000, 2000, 400, 200, 40, 20, 4 and 2 copies/μL) and 1 μL of each concentration were used as template for the LAMP reactions. According to the mean molecular weight and size of the maize genome (*Zea mays*, 2.73 pg/genome or copy) [29], 1 ng maize DNA equals approximately 366 copies of DNA. In the DNA template, the concentration of the "first DNA mixture" maize haploid genomic DNA was 4000 copies/μL. The "first DNA mixture" was serially diluted in ddH_2O to final concentrations of 2000, 400, 200, 40, 20, 4 and 2 copies/μL of total maize haploid genomic DNA, respectively. Each sample was amplified in three parallel reactions, and the entire experiment was repeated three times.

Application of the LAMP system for MON88017 maize in practical samples

To evaluate the LAMP system method for GM maize MON88017, DNAs of seven maize-derived samples (corn kernel, maize juice, corn flakes, maize meal, maize noodle, popcorn and maize starch) and one artificially mixed sample were prepared and exogenous ingredient of MON88017 was detected by this LAMP system. In each reaction, a total of 100 ng genomic DNA was amplified, and three replicates were carried out per sample.

Results and discussion

Optimization of this LAMP system

In this LAMP assay, auto-cycling strand displacement DNA synthesis is carried out by a *Bst* DNA polymerase with high strand displacement activity, and a set of four special primers (F3, B3, FIP, BIP) can recognize a total of six distinct regions on each of the target DNA sequences of MON88017. The final products are known to be cauliflower-like structures with multiple overhangs and loops, which can be observed during agarose gel separation of LAMP products [33]. So, the LAMP amplicons detected

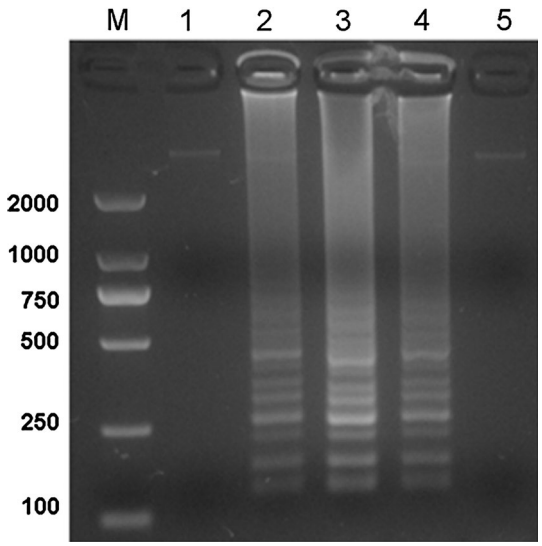

Fig. 2 Optimization of the LAMP reaction temperature. *Lanes 1–5* temperature of 59, 61, 63, 65, 67 °C, respectively. *Lane M* DL2000 DNA marker

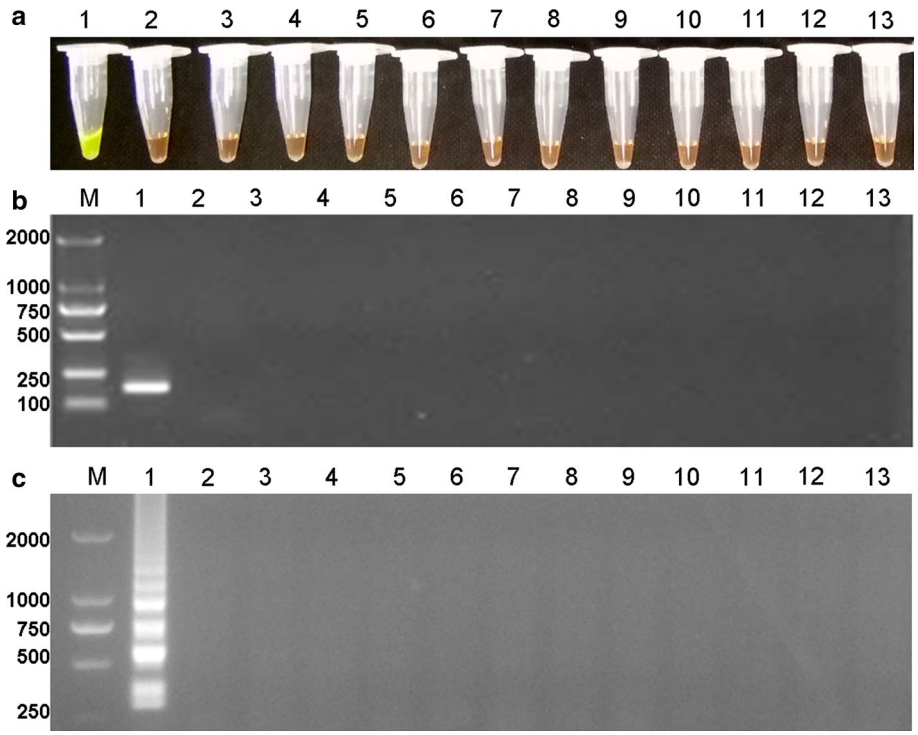

Fig. 3 Specificity test of MON88017 LAMP system. **a** Direct visual inspection with fluorescent dye of SYBR Green I, **b** 2 % agarose gel electrophoresis for MON88017 traditional PCR assay and **c** 2 % agarose gel electrophoresis for MON88017 LAMP assay. *Tube* and *lane 1–11*, amplification of MON88017, MON863, NK603, GTS 40-3-2, MON89788, TT51-1, Kefeng 6, MON1445, MON88913, GT73 and H7-1, respectively; *lane 12* non-GM maize; *lane 13* NTC; *lane M* DL2000 DNA marker

by gel electrophoresis exhibited ladder-like multiple bands. In this study, when the reaction temperature was between 61 and 65 °C, agarose gel electrophoresis showed visible ladder-like DNA fragments (Fig. 2). However, there were more intense ladder-like bands at the temperature of 63 °C. The above result demonstrated that 63 °C should be the optimized LAMP reaction temperature of MON88017. After adding SYBR Green I to the reaction tubes, the positive LAMP reaction was also observed by visualization of a color change (from orange to green) following the addition of diluted SYBR Green I dye (Fig. 3a).

Specificity analysis of LAMP system

The LAMP assays have been used to identify GMO events, such as soybean [31], maize [1, 35, 36, 38], rice [19], wheat [3], cotton [17, 20] and transgenic target DNA [7, 15, 26]. To assess the event specificity of this MON88017 LAMP detection system, different GM crops, including MON88017, MON863, NK603, GTS 40-3-2, MON89788, TT51-1, Kefeng 6, MON1445, MON88913, GT73 and H7-1, were used to perform LAMP assays. All DNA templates except MON88017, especially the other six GM event DNAs (MON1445, NK603, GTS 40-3-2, MON89788, GT73 and H7-1) carrying the *Cp4-epsps* gene, failed to amplify the target fragment and observed changed color by this LAMP assay (Fig. 3a), which has proved to be event-specific in the detection of MON88017 maize. This result indicates that the LAMP detection system could specifically amplify the MON88017 event-specific target fragment. To make sure that the expected target was amplified, a traditional PCR was performed using primer set of MON88017-LF and MON88017-LR–MON88017 DNA. Then, a 199-bp amplicon was successful obtained (Fig. 3b). So, the result of the conventional PCR was consistent with that of the LAMP assay (Fig. 3a–c).

Sensitivity analysis of LAMP system

That LAMP is able to detect very small amounts of target and does that even in high amounts of background DNA makes it ideal for GMO detection [17]. To evaluate the relative sensitivity of the assay, LAMP and PCR were carried out using a series of dilutions of MON88017 DNA ranging from 4000 to 2 copies. Positive LAMP amplifications were observed up to dilutions to 40 copies per reaction, as detected by both gel electrophoresis and SYBR Green I staining (Fig. 4a, b). Conventional PCR provided positive results up to dilutions to 40 copies per reaction, as indicated by the presence of an expected amplicon (199 bp) after gel electrophoresis (Fig. 4c). Sensitivity assay revealed that 40 copies of the MON88017 genome could be detected by this method at genome DNA level, which had the same sensitivity as conventional PCR (40 copies) for the detection of MON88017 and similar to nested PCR [37]. The detection limitation was consistent with other reported LAMP assays [13, 20] and close to the sensitivity of real-time PCR for the detection of some other GM crops [18, 30].

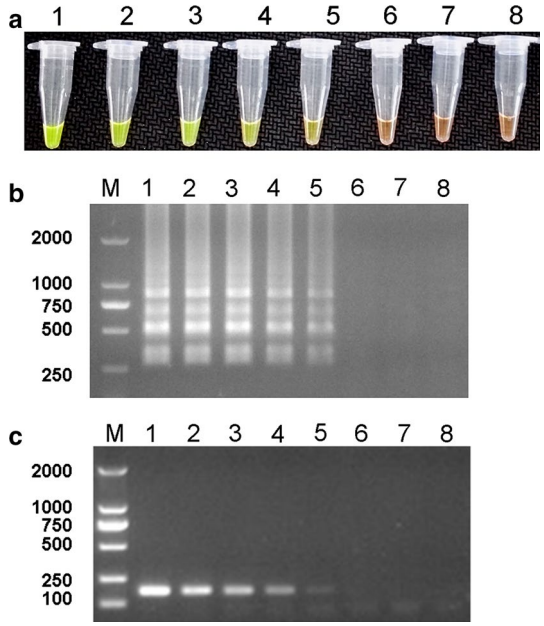

Fig. 4 LOD test of MON88017 LAMP and PCR system. **a** Direct visual inspection with fluorescent dye of SYBR Green I, **b** 2 % agarose gel electrophoresis for MON88017 LAMP assay and **c** 2 % agarose gel electrophoresis for MON88017 traditional PCR assay. *Tube* and *lane 1–8* LAMP products were amplified from MON88017 maize DNAs with different concentrations of 4000, 2000, 400, 200, 40, 20, 4 and 2 copies, respectively; *lane M* DL2000 DNA marker

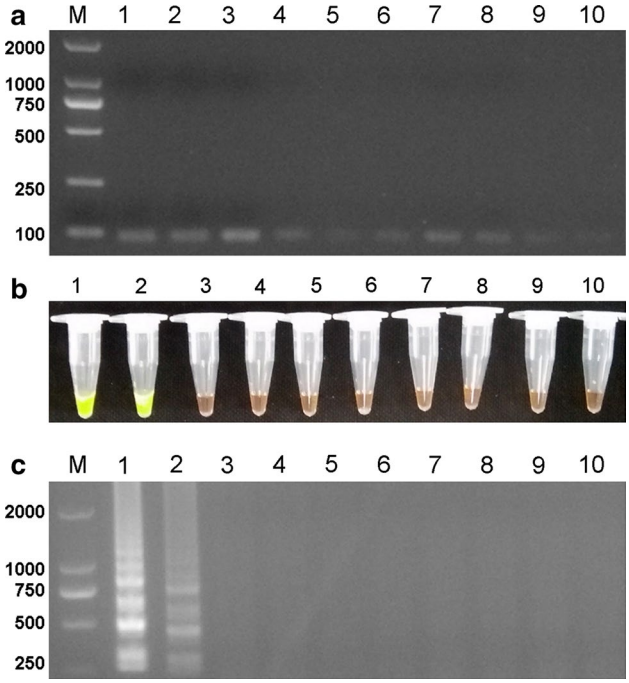

Fig. 5 Application of LAMP system to detect maize processed food samples. **a** 2 % agarose gel electrophoresis for *zSSIIb* maize-specific reference gene assay, **b** direct visual inspection with fluorescent dye of SYBR Green I and **c** 2 % agarose gel electrophoresis for MON88017 LAMP assay. *Tube 1–10* MON88017 maize, artificially mixed sample, non-GM maize, corn kernel, maize juice, corn flakes, maize meal, maize noodle, popcorn and maize starch, respectively; *lane M* DL2000 DNA marker

Analyses of mixed samples using this MON88017 LAMP system

Seven maize processed food samples and one artificially mixed sample, which was made by spiking MON88017 maize powder into non-GM maize powder and heating up the mixture, were subjected to this LAMP assay. Our results showed that all ten samples contained *zSSIIb* gene (Fig. 5a), which confirmed that all samples tested contained maize ingredient. Seven maize processed product samples were negative for MON88017 maize, whereas artificially mixed sample was positive. Similar results were obtained by the addition of SYBR Green I dye (Fig. 5b, c). Therefore, this inexpensive, rapid and visible LAMP protocol for MON88017 is significant for GMO detection. Accordingly, this LAMP method can be used as a powerful GMO detection tool for MON88017 maize and its derivates, due to its high specificity, sensitivity and practicability under isothermal condition.

Conclusion

At present, to guarantee the GMO traceability, a number of new approaches for GMO detection have been developed, such as PCR capillary gel electrophoresis (CGE) [11, 12], microarrays technology [28], Luminex technology [4, 8], digital PCR [16, 23] and next-generation sequencing [21, 32], PCR-ELISA [27]. The cost efficiency and high-throughput could also be achieved by the combinatory SYBR Green qPCR and matrix-based approach. Therefore, LAMP techniques have been applied for qualitative detection and identification GMOs [36, 38]. In this study, a rapid LAMP method was successfully developed for the detection of event-specific sequence in GM maize MON88017. Compared to the conventional PCR method, the LAMP system established in this study could be performed within 40 min. Furthermore, the LOD of this LAMP system for identifying MON88017 is as low as 40 copies, indicating that the LAMP system had the same sensitivity as the general PCR approach. In addition, the LAMP reaction results could be determined by the naked-eye observation without any time-consuming gel electrophoresis.

In conclusion, the MON88017 LAMP detection method established in this study is prompt, visual, specific, accurate, low cost, identical sensitive and does not need special equipment compared to conventional PCR technique, which is very necessary for field tests and fast screening of GM maize. Since the assays are performed at one

temperature, the LAMP reactions of GMO products can used as a convenient tool for GM maize detection without electrophoretic analysis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

Informed consent Not applicable.

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