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Development of the Visual Loop-Mediated Isothermal Amplification Assays for Seven Genetically Modified Maize Events and Their Application in Practical Samples Analysis

Lili Chen,[†,‡,§] Jinchao Guo,[‡,§] Qidi Wang,[‡] Guoyin Kai,[†] and Litao Yang*[,‡]

[†]Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, 100 Guilin Road, Shanghai 200234, People's Republic of China

[‡]National Center for Molecular Characterization of Genetically Modified Organisms, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China

ABSTRACT: As more and more genetically modified (GM) crops are approved for commercialization and planting, the development of quick and on-spot methods for GM crops and their derivates is required. Herein, we established the polymerase chain reaction and agarose gel electrophoresis-free system for the identification of seven GM maize events (DAS-59122-7, T25, BT176, TC1507, MON810, BT11, and MON863) employing a loop-mediated isothermal amplification (LAMP) technique. The LAMP assay was performed using a set of four specific primers at 60−65 °C in less than 40 min, and the results were observed by direct visual observation. In these developed assays, the specificity targeted at each GM maize event based on the event-specific sequence was well confirmed, and the limits of detection were as low as four copies of maize haploid genomic DNA with an exception of 40 copies for MON810 assay. Furthermore, these developed assays were successfully used to test six practical samples with different GM maize events and contents (ranged from 0.0 to 2.0%). All of the results indicated that the established event-specific visual LAMP assays are more convenient, rapid, and low-cost for GM maize routine analysis.

KEYWORDS: Genetically modified maize, loop-mediated isothermal amplification, event-specific

INTRODUCTION

The acreage of commercial cultivation of genetically modified (GM) crops has increased dramatically from 1.7 million hectares to 134 million hectares in past two decades, and the number of countries planting GM crops has reached 25.[1] However, because of the ever-increasing global controversial issues on the food safety, environment risk, and ethical concerns of genetically modified organisms (GMOs), legislations requiring labeling of products containing GMOs with certain threshold are currently issued in more than 50 countries and areas. Accordingly, accurate, quick, and high effectual methodologies for identifying or quantifying the presence of GMOs in food/feed are very important and necessary.

Maize (*Zea mays L.*) is the third important crop under global development, which has been mainly used for human food and animal feed. Up to date, more than 49 GM maize events have been approved for planting worldwide. Nucleic acid-based approaches are mainly used for GMO analysis as compared with the methods on the basis of protein analysis because of the advantages of high sensitivity, low costs, applicability of complex and processed samples, etc. Several alternatives were applied for GMO detection, for example, ligation-dependent probe amplification,[2] nucleic acid sequence-based amplification using transcription techniques (NASBA) in combination with microarray detection,[3] padlock probe ligation in combination with microarray detection,[4] SNPlex technology,[5] optical thin-film biosensor chips,[6] polymerase chain reaction (PCR),[7−9] etc. Also, some qualitative and quantitative PCR approaches have been developed for the detection of GM maize events, for example, MON810 maize,[10,11] MON863 maize,[12] 3272 maize,[13] 59122 maize,[14] and MIR604 maize,[15] etc. Although these approaches showed high specificity and sensitivity, the requirements of either a high-precision instrument for PCR amplification or complicated procedures for PCR analysis may partially account for the limited application of PCR-based approaches.

Loop-mediated isothermal amplification (LAMP), one PCR-free strategy for isothermal nucleic acid amplification, has been developed and widely used for the molecular detection of microorganisms and viruses.[16−21] The LAMP reaction depends on four specific primers (two inner and two outer primers) with six distinct regions of the target DNA, showing higher specificity as compared with typical PCR. The significant advantage of LAMP is that it can amplify DNA isothermally (60−65 °C) with a simple isothermal instrument based on the autocycling strand displacement synthesis of DNA by *Bst* DNA polymerase. Also, it is time-saving for DNA amplification (within 60 min) with high efficiency. Moreover, the presence or absence of LAMP products can be visually judged with naked eyes by adding a high-concentration fluorescent dye, such as SYBR Green I.[22,23] Recently, we reported the successful use of LAMP coupled with SYBR Green I to visually analyze the GM soybean GTS-40-3-2 and MON89788.[23] In this study, we developed seven LAMP assays for seven GM maize events, DAS-59122-7, T25, BT176, TC1507, MON810, BT11, and MON863, respectively, and successfully used them to test practical samples.

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MATERIALS AND METHODS

Plant Materials. The GM maize events (MON810 and MON863) were developed by Monsanto Co. GM maize events BT11 and BT176 were developed by Syngenta Seeds. GM maize event T25 was developed by Aventis CropScience Co. GM maize TC1507 maize was developed by Mycogen. GM maize DAS-59122-7 was developed by Dow Agro Sciences LLC and Pioneer Hi-Bred International. The certified reference materials (CRMs) of MON810, BT11, BT176, and MON863 were purchased from Fluka Co., and other GM maize samples (T25, TC1507, and DAS-59122-7) were supplied by the developers. Nontransgenic seeds of maize, rice, soybean, and cotton were purchased from the local market in Shanghai, China, and checked without GM contents in our lab.

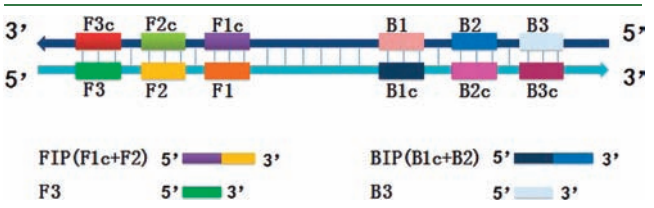

Figure 1. Schematic diagram for LAMP primer design.

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

DNA Oligonucleotides. In LAMP assays, the outer primers included one forward outer primer (F3) and backward outer primer (B3) are the same as those of regular PCR primers. The inner primers included one forward inner primer (FIP) and one backward inner primer (BIP). The FIP consists of the F1c (complementary to F1) and a sense sequence F2, and the BIP consists of the B1c (complementary to B1) and a sense sequence B2. The schematic diagram of LAMP primers is shown in Figure 1. All primers were designed using the specific online software of Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html) and synthesized by Invitrogen Co. Ltd. (Shanghai, China). The primers (F3, B3, FIP, and BIP) of each GM maize event (DAS-59122-7, T25, BT176, TC1507, MON810, BT11, and MON863) were designed based on the event-specific sequences[24] (http://gmdd.shgmo.org/), and the primers of the maize endogenous reference gene were designed based on the sequence of invertase (*Ivr1*) gene (Genbank Acc. No. U16123). The detailed primer sequences are listed in Table 1.

Table 1. Oligonucleotide Primers Used for Maize-LAMP Assays in This Study

target name	primer name	sequence (5′−3′)	target
59122	59122-F3	GCACCTGTGATTGGCTCAT	5′ junction of 59122
	59122-B3	GCTGAGTGGCTCCTTCAAC	
	59122-FIP	TGTCGTTTCCCGCCTTCAGTTT-GAGGGACGGAAGAAAGAGTG	
	59122-BIP	AGGGAGTCACGTTATGACCCCC-GCGGTTCTGTCAGTTCCAA	
T25	T25-F3	TCCCGTCAAGCTCCAGATG	5′ junction of T25
	T25-B3	GCTCCACCATGTTGACGAAG	
	T25-FIP	TGCCGTCGAGGGACATGAACT-AGTCTCCAGCAACCTCTCC	
	T25-BIP	CCGTGCGACAGCGACAATGG-TTGCCCTTTGGTCTTCTGAG	
MON810	MON810-F3	AGACAGCCAAGACCTCGA	3′ junction of MON810
	MON810-B3	CTGCTCGCAAGCAAATTCG	
	MON810-FIP	GCGGCCAGAGGGAACCAGTA-CCTGATCCGCTACAACGC	
	MON810-BIP	ACCACAGCCACCACTTCTCCT-GGCTACCGAAAGTCCTCGT	
MON863	MON863-F3	GTTGGTGAGCCTAGTGAT	5′ junction of MON863
	MON863-B3	CCTTTTCTACTGTCCTTTTGATG	
	MON863-FIP	GGCCGTAACATTTAGCAAAAAACTA-GGAGACTATCTAGCTTGGTTC	
	MON863-BIP	AATGCTGAACTATTGACCCTACTTG-AAGTGACAGGTAGGATCGG	
BT11	BT11-F3	GGTTTCTTAGACGTCAGGT	3′ junction of BT11
	BT11-B3	CAACCCCCATTTTTGATGA	
	BT11-FIP	TCCATGAGCGGATACATATTTGAAT-GGCACTTTTCGGGGAAAT	
	BT11-BIP	TTGGTGGAGACCATTTCTTGGT-AAATAGCCATGAGCGACC	
TC1507	TC1507-F3	TGGACCGAGCCTTGACTT	5′ junction of TC1507
	TC1507-B3	AGAAAGGTCGGTCCCCTTG	
	TC1507-FIP	TAGGGGTACCCCCAAGACTCC-GCCTTTGCAGCTTTGTGC	
	TC1507-BIP	AGTAGCCCCCGAGCCTCAAA-AAAGTGCGACAAAAGCCTCC	
BT176	BT176-F3	TTCAAGCACGGGAACTGG	3′ junction of BT176
	BT176-B3	GGAGAGGGAGAGAGAGGGA	
	BT176-FIP	GATCTCGGTGACGGGCAGGA-GTTTCTGGCAGCTGGACTT	
	BT176-BIP	TCTCCTCCATTGATGCACGCC-GGGGAAGGGAGAAACGGT	
invertase	IVR-F3	GCTCTGTACAAGCGTGCA	*invertase* gene
	IVR-B3	CCCGTTTCCTAGCTCATTGT	
	IVR-FIP	GCGCAAAGTGTTGTGCTTGGAC-TTTCCGTCTACTCGAGCCTA	
	IVR-BIP	GCGACAGAGATGTATGGCGCC-ACAGTCTCTGACTACGGTGT	

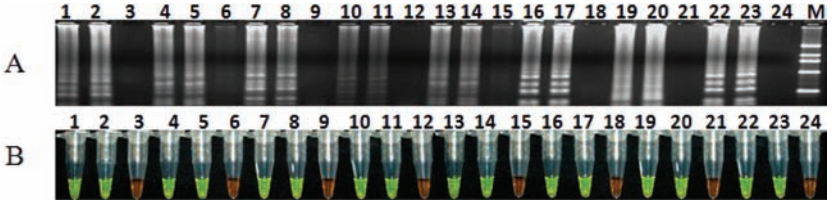

Figure 2. LAMP assays of seven GM maize events. (A) 2% agarose gel electrophoresis and (B) direct visual inspection with fluorescent dye of SYBR Green I. Lanes 1 and 2, DAS-59122-7; lanes 4 and 5, T25; lanes 7 and 8, BT176; lanes 10 and 11, MON810; lanes 13 and 14, TC1507; lanes 16 and 17, MON863; lanes 19 and 20, BT11; lanes 22 and 23, non-GM maize; lanes 3, 6, 9, 12, 15, 18, 21, and 24, NTC; and lane M, DL-2000 marker.

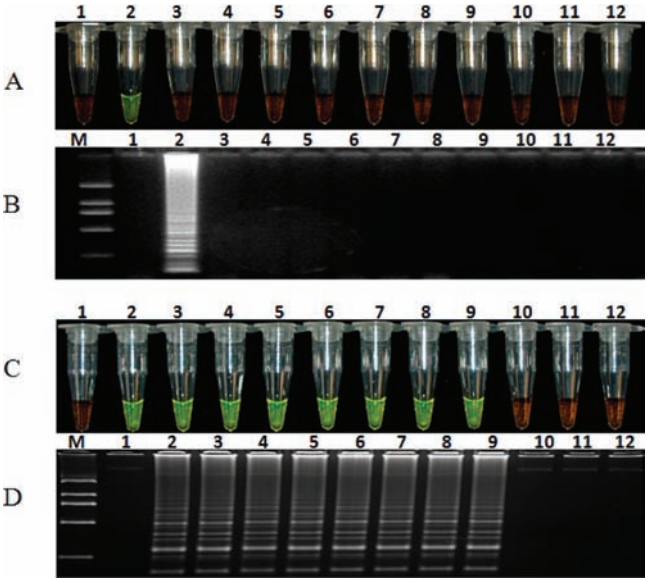

Figure 3. Specificity test of DAS-59122-7 LAMP assay. Panels A and B showed the results of the DAS-59122-7 event LAMP assay, and panels C and D showed the result of the *Ivr1* LAMP assay. Lane 1, NTC; lanes 2−12, DAS-59122-7, T25, BT176, TC1507, MON810, BT11, MON863, non-GM maize, non-GM rice, non-GM soybean, and non-GM cotton; and lane M, DL-2000 marker.

LAMP Reaction. The LAMP reaction was carried out in a 25 μL reaction mixture containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, 0.5 M betaine (Sigma), 1.2 μM concentration each of FIP and BIP primer, 0.2 μM concentration each of F3 and B3 primer, 400 μM concentration each of dNTP, and 5 μL of template DNAs. After the denaturation step at 95 °C for 5 min, the mixture was cooled in ice immediately. After 8 U of *Bst* DNA polymerase large fragment (New England Biolabs) was added, the reaction was incubated at 61 °C for 40 min using S1000 thermal cycler (Bio-Rad Laboratories, Inc., United States) or a thermostatic water bath. The LAMP assay was carried out in triplicate for each template DNA, and the no template control (NTC) contained water instead of the template.

Detection of LAMP Products. LAMP-amplified products could be observed through visual inspection and agarose gel electrophoresis (AGE) analysis. For the visual observation, the color of the LAMP products with target DNA amplification might change from the original orange to green after adding the additional fluorescent dye, 2 μL of 1000× SYBR Green I (Generay Biotech Co., Ltd. Shanghai, China). Otherwise, it remains orange in the reaction mixture without target DNA amplification. For comparing with visual inspection, 10 μL of corresponding LAMP reaction product was loaded on 2% agarose gel in 0.5× TBE with GelRed staining, and the gels were assessed photographically under UV light.

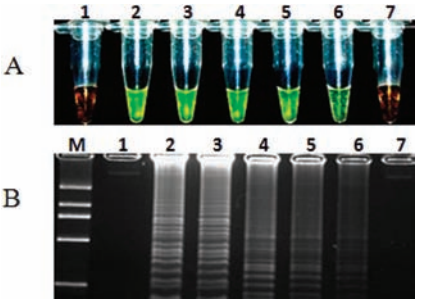

Figure 4. Sensitivity test of DAS-59122-7 LAMP assay. Lane M, DL-2000 marker; lane 1, NTC; and lanes 2−7, 4×10^4 copies, 4×10^3 copies, 4×10^2 copies, 4×10 copies, four copies, and one copy maize haploid genomic DNA, respectively.

RESULTS AND DISCUSSION

Seven Maize Event-Specific LAMP Assays. The aim of this work was to develop the LAMP assays for the approved GM maize events (DAS-59122-7, T25, BT176, TC1507, MON810, BT11, MIR604, MON88017, NK603, GA21, and MON863) in China. However, because of the exceptional lower or higher GC% content of the event-specific sequences of GM maize MIR604, MON88017, NK603, and GA21 events, a total of seven sets of LAMP primers for GM maize events (DAS-59122-7, T25, BT176, TC1507, MON810, BT11, and MON863) were successfully designed. The maize endogenous reference gene *Ivr1* LAMP assay was used for the verification of absence of PCR inhibitors. The optimized conditions of the seven LAMP assays were described above. In each LAMP reaction, a total of 10 ng of corresponding plant genomic DNA was used as the template. In these seven LAMP assays, the typical ladderlike pattern products were obtained from the reaction with positive DNA control using AGE analysis, and the color change from orange to green was also observed from the reaction with positive DNA control by adding the fluorescent dye of SYBR Green I with naked eyes (Figure 2). In addition, the developed LAMP assays were performed in a Bio-Rad S1000 thermal cycler, and the identical results were also observed when in thermostatic water bath (data not shown).

Specificity of the LAMP Assays. Generally, the LAMP assay employed four primers that recognize six different sequences on a target DNA sequence, and the specificity is much higher than that of conventional PCR. Herein, the specificity of each assay was individually evaluated employing the genomic DNAs from GM Maize events (DAS-59122-7, T25, BT176, TC1507, MON-810, BT11, and MON863) and non-GM crops (maize, rice, soybean, and cotton).

In the specificity test of DAS-59122-7 assay, the typical ladderlike pattern products and green color change were only obtained from its positive sample. No color change and ladderlike pattern

Table 2. Analyzed Results of the Practice Samples from U.S. Department of Agriculture/GIPSA Proficiency Program in April 2010 Using Developed LAMP Assays[a]

	T25		MON810		BT11		59122		MON863		TC1507		BT176	
	official values (%)	LAMP results	official values (%)	LAMP results	official values (%)	LAMP results	official values (%)	LAMP results	official values (%)	LAMP results	official values (%)	LAMP results	official values (%)	LAMP results
C4.1	0.8	+	0.1	+	0.0	−	0.1	+	0.4	+	0.8	+	0.1	+
C4.2	0.0	−	0.0	−	0.0	−	0.0	−	0.0	−	0.0	−	0.0	−
C4.3	2.0	+	2.0	+	0.1	+	0.0	−	0.0	−	0.0	−	1.5	+
C4.4	0.5	+	0.1	+	2.0	+	0.1	+	0.8	+	1.5	+	0.1	+
C4.5	0.0	−	0.5	+	1.0	+	0.0	−	0.0	−	0.0	−	0.5	+
C4.6	1.0	+	1.0	+	0.0	−	2.0	+	0.0	−	0.1	+	0.1	+

[a] Key: −, the event was not detected using LAMP assay; and +, the event was detected using LAMP assay.

products were observed in other GM maize events and non- GM maize as well as the NTC (Figure 3). Similarly, in the other six LAMP assays of GM maize events T25, BT176, TC1507, MON810, BT11, and MON863, the expected positive results were only obtained from the corresponding positive sample. All results showed that these LAMP assays have high specificity, suggesting that it was suitable for the identification of the corresponding GM maize events.

Sensitivity of the LAMP Assays. The limit of detection (LOD) is the lowest amount or concentration of analyte that can be reliably detected with an acceptance criterion. To investigate the LODs of the developed LAMP assays, genomic DNA isolated from powdered 100% GM maize events and a non-GM maize were serially diluted to final concentrations equivalent to 8000, 800, 80, 8, 0.8, and 0.2 copies haploid genome/μL. In each reaction, 5 μL of diluted DNA solutions was used as the template. In the DAS-59122-7 LAMP assay, the color change from orange to green was observed in the reactions of all of tested levels except for the level of $0.2 \times 10^{\circ}$ maize genome copies/μL (Figure 4A), and the identical results were also found in AGE analysis (Figure 4B). The results indicated that the absolute LOD of DAS-59122-7 assay was about four copies of haploid maize genomic DNA. Meanwhile, the same LOD values were obtained in the other GM maize LAMP assays (T25, BT176, TC1507, BT11, and MON863). The slightly low LOD of 40 copies were confirmed in the MON810 assay. In the MON810 LAMP assay, although the LOD of 40 copies is slightly lower than those six LAMP assays, the MON810 assay is still suitable for the MON810 maize identification. The slightly low LOD was mainly related to the design of the LAMP primers and the structural of the target DNA sequence. All of the results indicated that the sensitivities of these seven assays were high enough as compared with that of conventional PCR. Also, the visual observation can be used for LAMP analysis with higher sensitivity, and it was convenient and operable on spot detection application instead of the agarose gel electrophoresis.

Visual LAMP as One Novel Analytical Approach in the U.S. Department of Agriculture/GIPSA Proficiency Test. In U.S. Department of Agriculture/GIPSA proficiency test of April 2010, six maize samples named with C4.1, C4.2, C4.3, C4.4, C4.5, and C4.6 were mixed and prepared by commercially available transgenic events (T25, CBH351, Mon810, GA21, E176, Bt11, NK603, TC1507, Mon863, DAS-59122-7, MIR604, and 3272), and the proportion of each event ranged from 0 to 2.0% was predetermined by gravimetric methods.

In this proficiency test, the developed LAMP assays were used to qualitatively analyze the six practical samples (C4.1, C4.2, C4.3, C4.4, C4.5, and C4.6) in our lab, and the results are listed in Table 2. In the test of sample C4.1, the color change was observed in T25, MON810, BT11, DAS-59122-7, MON863, TC1507, and BT176 LAMP assays, indicating that the sample C4.1 was consisted of GM maize events of T25, MON810, BT11, DAS-59122-7, MON863, TC1507, and BT176. In C4.3, GM maize events of T25, MON810, BT11, and BT176 were detected. In C4.4, GM maize events of T25, MON810, BT11, DAS-59122-7, MON863, TC1507, and BT176 were detected. In C4.5, the contents of GM maize MON810, BT11, and BT176 were observed. In C4.6, the contents of GM maize T25, MON810, DAS-59122-7, TC1507, and BT176 were observed. In the C4.2, no color changes were observed in the seven GM maize LAMP assays, which meant the absence of these seven GM maize events. As compared with the official result report (http://archive.gipsa.usda.gov/biotech/quarterly_reports/april_2010_final_report.pdf), we found that the LAMP results were identical with the official results, indicating that the developed LAMP assays of seven GM maize events were well suitable for the practical maize samples analysis.

All of above results indicated the advantages of LAMP assay, such as the high specificity, high sensitivity, low costs without special expensive equipment, and visual inspection with AGE. We believed that the LAMP technology will be widely used for GMO analysis. In particular, if the LAMP assay combines with the fast DNA extraction method/equipment, it can be used for the visual and on-spot detection for GMOs with high throughput. However, the developed visual assays are not perfect for practical GM samples analysis. For instance, (i) the LAMP assay works with the nucleic acids, and the DNA extraction of processed GM samples is still the main limitation of the LAMP application; (ii) as compared with conventional PCR, the design of LAMP primers is more complex and difficult, especially for designing the primers for one target with high GC % contents and limited size; and (iii) it is very difficult to develop the multiplex LAMP assay for simultaneous identification of multiple targets because of the amplified products with a typical ladderlike pattern in positive results.

CONCLUSION

In this study, we established the visual and rapid LAMP assays of PCR-free DNA target amplification for seven GM maize events. Furthermore, the developed assays were successfully used in a U.S. Department of Agriculture/GIPSA proficiency test in April, 2010, with ideal results. The developed LAMP assays have good potential to dramatically simplify the GMO

routine qualitative analysis with the advantages of low cost, simple instrumentation, fast time, and cheap labor.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86 21 34207174. Fax: +86 21 34204869. E-mail: yylltt@sjtu.edu.cn.

Author Contributions

§These two authors contributed equally to this work.

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