Loop-Mediated Isothermal Amplification: Rapid Visual and Real-Time Methods for Detection of Genetically Modified Crops

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Supporting Information

ABSTRACT: A rapid, reliable, and sensitive loop-mediated isothermal amplification (LAMP) system was developed for screening of genetically modified organisms (GMOs). The optimized LAMP assays using designed primers target commonly employed promoters, i.e., Cauliflower Mosaic Virus 35S (P-35S) and Figwort Mosaic Virus promoter (P-FMV), and marker genes, i.e., aminoglycoside 3’-adenytransferase (aadA), neomycin phosphotransferase II (nptII), and β-glucuronidase (uidA). The specificity and performance of the end-point and real-time LAMP assays were confirmed using eight genetically modified (GM) cotton events on four detection systems, employing two chemistries. LAMP assays on the isothermal real-time system were found to be most sensitive, detecting up to four target copies, within 35 min. The LAMP assays herein presented using alternate detection systems can be effectively utilized for rapid and cost-effective screening of the GM status of a sample, irrespective of the crop species or GM trait. These assays coupled with a fast and simple DNA extraction method may further facilitate on-site GMO screening.

KEYWORDS: loop-mediated isothermal amplification (LAMP), genetically modified organism (GMO), screening elements, real-time, visual detection

INTRODUCTION

The level of commercialization of genetically modified (GM) crops is increasing rapidly worldwide, with respect to the acreage of cultivated area as well as the event/trait diversification. By 2012, 25 GM crops comprising 319 GM events had been commercialized worldwide in 59 countries.1 In India, only GM cotton has been commercially cultivated, with six Bt cotton events, namely, MON531 (Bollgard I), MON15985 (Bollgard II), GFM-cry1A, Event1, BNLA-601, and MLS-9124, covering an area of more than 10.8 million ha.12 Several other GM crops/events under field trials were either indigenously developed or imported for research purposes. The National Bureau of Plant Genetic Resources (NBPR) is the nodal agency under the Indian Council of Agricultural Research, New Delhi, for issuance of import permits and quarantine processing of imported transgenic planting material. So far, 172 imports with 5115 accesses of transgenic planting material comprising 13 crop species have been processed through NBPR for the purpose of research. With the increase in the number and complexity of GM events, the development of commensurate, reliable, and cost-effective GM diagnostics for identification and quantification has become a real challenge.

Polymerase chain reaction (PCR)3 or real-time PCR (qPCR), targeting engineered DNA, is the most direct and widely applied analytical approach. PCR, being a reliable, robust, and sensitive technique, has broad application in GM detection. However, high-precision equipment and procedures associated with PCR analysis are some of the constraints, which limit their use for on-site detection.4 Moreover, the qPCR technique is often sensitive to inhibitors present in plant extracts.5

Loop-mediated isothermal amplification (LAMP),6 an isothermal nucleic acid amplification technique, is less sensitive to inhibitors,7,8 does not require sophisticated equipment, and has the potential to be deployed on site. LAMP is attracting attention because of its sensitivity and specificity, being superior to PCR, and usually comparable to qPCR. In addition, LAMP provides results on site in a significantly shorter time than conventional PCR-based techniques.

LAMP is characterized by the use of four different primers, specifically designed to recognize six distinct regions on the target DNA template. An inner primer containing sequences of sense and antisense strands of the target DNA initiates LAMP reaction, which proceeds at a constant temperature, followed by strand displacement DNA synthesis primed by an outer primer set.6,9,10 The addition of two so-called “loop” primers11 or two “stem” primers12 further increases the speed of amplification of the LAMP assay. Amplification and detection of target genes can be completed in a single step at a constant temperature, by incubating the DNA template, primers, and a strand displacement DNA polymerase. The significant advantage of LAMP is that it can amplify DNA isothermally (60–65 °C) with a simple isothermal instrument, based on strand displacement synthesis of DNA by Bst DNA polymerase. It provides a high amplification efficiency, with replication of the original template.
copy $10^9-10^{10}$ times during a 15–60 min reaction. LAMP products show a ladderlike pattern on an agarose gel or can be monitored in real time using turbidometry. The amplicon products show a ladderlike pattern on an agarose gel or can be resolved on a sequencing gel, and, more recently, in plant health. 

In 2004, a GMO screening method using LAMP targeting P-35S was first reported by Fukuta et al., in which turbidometry was used for real-time monitoring. LAMP assays for T-nos, P-35S, and the pat marker gene have been reported for GMO screening. A bioluminescent real-time reporter (BART) of LAMP has been recently used for real-time monitoring. 

<table>
<thead>
<tr>
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<th>target</th>
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MATERIALS AND METHODS

Test Samples. Seed samples of eight GM cotton events were used for this study. Five of these events, i.e., MON531 with the cry1Ac gene, MON15985 with the cry2Ab gene, and MON15985 with the cry1A gene, are for insect resistance, which have already been commercialized in India. The seed samples of these commercialized Bt cotton events were procured from authorized sources, specifically the developers of the respective events: MON531 and MON15985 from M/s Maharashtra Hybrid Seeds Co. Ltd., GFM-cry1A from M/s Neth Seeds, Event1 from M/s J. K. Agri Genetics Ltd., and MLS-9124 from M/s Metaphlic Life Sciences Private Ltd.
The three other events include MON1445 and MON88913 events of Monsanto Co., both with CP4-EPSPS gene for glyphosate herbicide tolerance, and the stacked event 281-24-236 × 3006-210-23 (Widestripe) of Dow AgroSciences LLC, with cry1F and cry1Ac genes for insect resistance. These events were imported for the purpose of research through the NBPCR, New Delhi. Prior to the LAMP experiments, all the test samples were checked for specific events and/or traits using respective qPCR assays. Event-specific TaqMan qPCR assays were performed using published protocols for GM events MON531, MON15985, MON1445, and Widestripe (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). Construct-specific qPCR,25 targeting the 120 bp junction region between the E9 terminator gene derived from Pismum sativum and the T-DNA gene derived from the Agrobacterium tumefaciens Ti plasmid, was performed for GM event MON88913. Transgene-specific qPCR assays were conducted for the rest of the events, i.e., cry1Ac in GFM-cry1A and Event1 and cry1C in MLS-9124, using protocols with designed primers and the TaqMan probe (not published).

All test samples were further checked for transgenic elements in this study, namely, P-35S, F-PMV, aadA, nptII, and uidA, using qPCR. TaqMan qPCR was used for P-35S,26 nptII, and uidA.27 SYBR Green qPCR using optimized conditions was performed for F-PMV28 and aadA (using designed primers). These samples amplified the specific products, as expected. Hence, these eight GM cotton events were used as respective positive and negative controls to test the specificity of the LAMP assays for the detection of transgenic screening elements, namely, P-35S, F-PMV, aadA, nptII, and uidA, and event MON88913 for P-FMV, for preparation of test samples. For the LAMP assay using Bt DNA polymerase large fragment, test samples with 10, 1.0, 0.1, 0.05, and 0.01% (copy/copy ratio, cp/cp) GM content representing 4000, 400, 40, 20, and 4 copies of GMO per reaction mixture, respectively, were prepared by serially diluting DNA of Bt cotton event MON15985 with the non-GM counterpart.

DNA Extraction. Seed samples were ground to a fine powder using an electric grinder. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The quantity and purity of purified DNA were measured and evaluated using the Q5000 UV/vis spectrophotometer (Quawell). Also, the quality of DNA was analyzed by 0.8% (w/v) agarose gel electrophoresis in 1× TAE stained with ethidium bromide.

Design of LAMP Primers. Sequences of the commonly used promoters, i.e., P-35S and F-PMV, and marker genes aadA, nptII, and uidA were employed. The primers for each element were designed on the basis of the strategy described by Notomi et al.6 and using LAMP Designer (Premier Biosoft, Palo Alto, CA). Primers were synthesized by Pivotal Marketing. The specificity of designed primers was further confirmed using the BLAST algorithm (standard nucleotide BLAST available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). Details of the sequences and targets of the designed primers are listed in Table 1.

Optimization of LAMP Reactions. A series of reactions were performed with the primer sets for each target to optimize visual LAMP reaction conditions with varying concentrations of buffer, dNTPs, primers, and DNA. The reaction mixture consisted of 1×–2× ThermolPol Buffer, 0.5–1.0 M betaine, 600 μM to 1.3 mM dNTP, 8–16 μl of Bst DNA polymerase large fragment, forward (FIP) and backward (BIP) inner primers (0.6–1.2 μM each), LoopF and LoopB primers (0.4–0.8 μM each), forward (F3) and backward (B3) primers (1.0–2.4 μM each), and 100–125 ng of DNA. The reactions were performed at three temperatures, i.e., 60, 62, and 65 °C. The optimal temperature for isothermal amplification of all LAMP systems was found to be 65 °C, based on the results of preliminary specificity tests. The concentration of ThermolPol Reaction Buffer was optimized at 2× to obtain satisfactory visualization after SYBR Green 1 addition. The amplifications were performed in triplicates on the thermal cycler and conventional heating block. For real-time LAMP assays, varying concentrations of isothermal master mix ranging from 12.5 to 15.0 μL were used. Primer concentrations used were 0.04–0.1 μM for F3 and B3 primers, 0.16–0.4 μM for LoopF and LoopB primers, and 0.24–0.6 μM for FIP and BIP primers.

LAMP-Based Visual Detection Assay. The LAMP assay was performed in a 25 μl total reaction mixture containing 2× ThermolPol Reaction Buffer [40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM (NH₄)₂SO₄, 2 mM MgSO₄, and 0.2% Triton X-100], 1.0 M betaine (Sigma Aldrich Co.), 2.4 μM F3, 2.4 μM B3, 1.2 μM FIP, 1.2 μM BIP, 0.8 μM LoopF, 0.8 μM LoopB, and 1.3 mM dNTP mix (MBI Fermentas Inc., Hanover, MD). After the addition of 5.0 μL of 25 ng/μL DNA as a template, the mixture was incubated at 95 °C for 5 min and cooled on ice, and then 16 units of Bst DNA polymerase large fragment (New England Biolabs, Inc.) was added. The mixture was incubated at 65 °C for 75 min in a conventional heating block (Neolab, Mumbai, India) and then cooled to 4 °C by being kept on ice. The same set of reactions was also performed on a GeneXpro thermal cycler (Bior Cie) for comparing the results obtained on the heating block.

For the determination of specificity and sensitivity, the reactions were conducted in triplicate for each template DNA along with a nontemplate control. Approximately 40000 copies of genomic DNA, as calculated according to the work of Arumuganathan and Earle,29 were used as a template in the LAMP reactions. For specificity tests, 100% genomic DNA was used. For sensitivity experiments, event MON15985 was selected, as it is positive for four transgenic elements, namely, P-35S, aadA, nptII, and uidA, and event MON88913 for P-FMV, for preparation of test samples. For the LAMP assay using Bt DNA polymerase large fragment, test samples with 10, 1.0, 0.1, 0.05, and 0.01% (copy/copy ratio, cp/cp) GM content representing 4000, 400, 40, 20, and 4 copies of GMO per reaction mixture, respectively, were prepared by serially diluting DNA of Bt cotton event MON15985 with the non-GM counterpart.

LAMP-amplified products were directly observed by the naked eye by adding 0.2 μL of 10000X SYBR Green 1 (Sigma Aldrich Co.) to the reaction mixture. Using SYBR Green 1 dye, a change from an orange color to a green color means that LAMP amplification occurred, whereas no color change depicts the absence of LAMP amplification. The specificity of amplified products was further confirmed by checking the ladder-like profile using gel electrophoresis on 2% agarose (Lonza, Rockland, ME) in 1× TAE stained with ethidium bromide.

Real-Time LAMP Assay on the Light Cycler480 System. LAMP reactions were performed in triplicates on a Light Cycler480 system (Roche Applied Science, Mannheim, Germany) in 96-well real-time PCR plates. The total reaction volume of 25 μL contained 5.0 μL of template DNA, 12.5 μL of isothermal master mix (OptiGene Ltd., Horsham, U.K.), and LAMP primers at the following final concentrations: 0.04 μM for F3 and B3, 0.16 μM for LoopF and LoopB, and 0.24 μM for FIP and BIP for P-FMV, aadA, nptII, and uidA. For P-35S, F3 and B3 (0.08 μM each), LoopF and LoopB (0.52 μM each), and FIP and BIP primers (0.48 μM each) were used.

Cycling conditions were set as follows: one cycle of uracil-N-glycosylase (UNG) activation at 62 °C for 1 s, denaturation at 62 °C for 1 s followed by 45 cycles of amplification at 62 °C for 2 s and 62 °C for 59 s (with a single acquisition mode) followed by one cycle of melting at 98 °C in a continuous mode.

LAMP products are concatamers of a target-specific sequence.6 The melting temperature (Tm) is the temperature at which the double-stranded DNA product dissociates into single strands. Therefore, the Tm of a given LAMP amplicon is specific under given reaction conditions and differs between amplicons of test samples with their nucleotide composition. Hence, in addition to monitoring the increase in fluorescence, we also performed melting curve analysis to further verify the positive samples for the specific product obtained with real-time LAMP assays.

Further, real-time LAMP products were subjected to visual detection using SYBR Green 1 dye and electrophoretic analysis on 2% agarose gels (as described above for visual LAMP), for checking the consistency in different detection systems.

For the determination of sensitivity, test samples were prepared by mixing DNA of Bt cotton event MON15985 (for P-35S, aadA, nptII, and uidA) and event MON88913 (for P-FMV) with the non-GM counterpart to obtain 400 (1.0% cp/cp), 200 (0.5%), 40 (0.1%), 20 (0.05%), 10 (0.025%), and 4 (0.01%) copies of GMO per reaction mixture. A sample of 100% (cp/cp) Bt cotton (40000 copies) was used as a positive amplification control, whereas non-GM cotton sample and nontemplate control were used as negative controls.

Real-Time LAMP Assays on the Isothermal System (Genie II). LAMP reactions were performed in triplicate in Genie tubes on the Genie II system (OptiGene Ltd.) using the same test samples of GM
cotton described in the previous section (Real-Time LAMP Assay on the Light Cycler480 System). Reactions were conducted in 25 μL volumes using 5.0 μL of template DNA, 15.0 μL of isothermal master mix (OptiGene Ltd.), and LAMP primers at final concentrations of 0.1 μM for F3 and B3, 0.2 μM for LoopF and LoopB, and 0.4 μM for FIP and BIP.

Amplification conditions were set as follows: amplification at 62 °C for 30 min, followed by annealing from 98 to 80 °C with a ramping rate of 0.1 °C/min. For P3SS, the amplification time was increased to 35 min. Fluorescence data were acquired during the amplification phase, while fluorescence derivative data were acquired during the annealing phase.

For the determination of sensitivity, samples were prepared by mixing DNA of GM events MON15985 (for P-3SS, aadA, nptII, and uidA) and MON88913 (for P-FMV) with their non-GM counterparts to obtain 400 (1.0% cp/cp), 200 (0.5%), 40 (0.1%), 20 (0.05%), 10 (0.025%), 4 (0.01%), and 2 (0.005%) copies of GMO per reaction mixture (as described in the previous section).

## RESULTS AND DISCUSSION

With the number and complexity of GM events increasing globally at a faster pace, testing for the presence or absence of every GM trait in each crop is becoming extremely time-intensive and cost-intensive. Initial screening for the presence or absence of transgenic elements common to multiple GM events can allow rapid and cost-efficient discrimination of GM and GM-free samples. A hexaplex PCR approach targeting commonly employed marker genes, i.e., aadA, bar, hpt, nptII, pat, and uidA, for screening of GM crops has been previously reported by our laboratory. Screening tests, based on the detection of regulatory sequences commonly used in developing GMOs, such as P-3SS and T-nos, are usually applied initially to detect the presence of a GMO, irrespective of the expressed GM trait. From a practical viewpoint, screening methods are useful for rapid and reliable reduction of test samples by direct identification of negative samples, which do not need to be further analyzed.

Because P-3SS and P-FMV are the most commonly used promoters in the Bt cotton events commercially cultivated in India and among most of the GM events commercialized worldwide, P-3SS- and P-FMV-specific LAMP assays were developed in this study. In addition to promoter-specific LAMP assays allowing screening of a wide range of GM crops and events, LAMP assays were developed for the detection of commonly used marker genes, namely, aadA, nptII, and uidA. After optimization of the reaction, the same setup (concentration and amplification temperature) was used for all LAMP reactions. This uniformity of reaction condition is a real advantage as it allows standardization and simplification of the experiments, especially in cases in which assays need to be performed on site with simple equipment such as a conventional heating block.

### Specificity and Sensitivity of the Visual LAMP Assay.

To evaluate the specificity of the developed visual LAMP assays using Bt DNA polymerase large fragment, we used eight GM cotton events (MON531, MON15985, Event1, GFM-cryIA, MLS-9124, MON1445, MON88913, and Widestrike) as positive and negative control samples for the respective targets as shown in Table 2.

Color change, an indicator of LAMP amplification, was only observed in reaction mixtures containing GM cotton events with the target sequences. On agarose gels, the specificity of the LAMP assays was further confirmed by the presence of the typical ladderlike pattern of the products resulting solely from reactions with the DNA containing the target sequences (Figure S1 of the Supporting Information).

The specificity test results obtained from gel electrophoresis and visual observation were consistent in all three replicates. Moreover, the pattern of these results is in line with the theoretical data for the presence or absence of tested elements in individual GMOs as shown in Table 2. The data confirmed that the developed LAMP assays show high specificity for amplifying the target DNAs.

Because the efficiency of the LAMP reactions was consistent in both the heating block and the thermal cycler (data not shown), the heating block in combination with a fast DNA extraction method may be preferred for cost-effective and on-site detection of GMOs. This consistency demonstrates the ability of the LAMP assays to be used on site with very simple and affordable equipment, making the technology available also to developing countries for rapid, cost-effective, and on-site screening of the GM status of samples.

The sensitivity was assessed as the limit of detection (LOD) for each LAMP assay. The LOD determined as the lowest concentration at which all replicates resulted in a positive LAMP reaction signal was 40 target copies for all the assays. Along with visual detection, the LOD was also confirmed on agarose gels (Figure 1). However, as product analysis is mostly conducted by gel electrophoresis, their application is limited to laboratories, and the advantage of rapid LAMP amplification may not be efficiently exploited. The concept of "visual" LAMP has already been proposed via the addition of high concentrations of fluorescent dyes such as SYBR Green I or hydroxyl naphthol blue after end-point reaction. Li and

### Table 2. GM Cotton Events Used To Check the Specificity of LAMP Assays for Their Respective Targets

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collaborators developed a LAMP assay for the rapid detection of the cry1Ab gene in GM rice by formation of a white precipitate or measuring the fluorescence intensity under ultraviolet irradiation, both visible to the naked eye.23 So far, LAMP-based assays have been developed for the detection of few screening elements, namely, P-35S, T-nos, pat, and cry1Ab.21−23 The visual detection of products using SYBR Green I can be useful in screening the GM events in a quick and cost-effective manner and, if combined with a fast and simple DNA extraction method, could also be applied for on-site inspection in the fields or on the ports of entry. In addition to P-35S, LAMP assays targeting screening elements, namely, P-FMV, aadA, nptII, and uidA, have been reported in this study.

By combining several carefully chosen LAMP screening assays, one could obtain a fairly good idea about the GM or non-GM status of the sample. With the combination of five LAMP assays presented in this study, 62% of the globally known GM events and 91% of the events authorized, tolerated, or in the pipeline for authorization in the European Union that are gathered in the GMOseek project matrix (Debode et al., personal communication) can be detected. Using the GMOseek algorithm, P-35S has been identified as the element with the best potential to screen the Bt cotton events.
commercially cultivated in India. The developed LAMP assays have potential to screen all the GM events authorized for commercial cultivation in India and also more than 70% of the GM events approved for field trials over the past five years (2006–12). Moreover, using a matrix approach, the analyst could already infer the identity of the GM events potentially present in the sample. The visual LAMP approach offers the advantage of having a very simple setup that can be performed on a heating block. Once the visual LAMP-based screening results are obtained on site (or in a laboratory), the LAMP products showing the potential presence of GM event(s) can be further analyzed by gel electrophoresis or even sequenced for further confirmation.

Specificity and Sensitivity Tests for the Real-Time LAMP Assay. Light Cycler480 System. The analytical specificity of the real-time LAMP assays was tested on a real-time PCR system (Light Cycler480 system) using the same samples that were used for the visual LAMP assays. All real-time LAMP assays showed the desired specificity of amplification for their respective targets, giving no signal when tested on respective negative control(s). Similarly, in non-GM cotton samples and nontemplate controls (negative control using water instead of DNA), amplification signals were not detected. The amplification results were as expected and in accordance with the details given in Table 2.

To confirm the specificity of the real-time LAMP amplification product, and to distinguish between true and false positive reactions, we performed melting curve analysis after amplification reactions. Under constant reaction conditions, $T_m$ values for the different amplicons are relatively constant, P-3SS showing the largest variability from 87.4 °C in event MON15985 to 88.3 °C in event Mls-9124. For other amplicons, the variability of $T_m$ was negligible or null. The variability in $T_m$ values observed in the case of P-3SS may be attributed to the variation in the sequence of P-3SS incorporated into the GM event, such as P-3SS with a duplicated enhancer region (doubly enhanced compared to the use of more sophisticated equipment but also to

![Figure 2. Mean melting temperatures ($T_m$) of the real-time LAMP products.](image)

The positive nature of a reaction is expressed by its time of positivity ($t_p$) value, i.e., amplification time at which the fluorescence second derivative reaches its peak above the baseline value. The $t_p$ values of different assays tested on the MON15985 DNA dilution series differed depending on the targeted sequence. The first signal was observed within 13 min with reaction mixtures containing 400 copies of aadA or uidA, followed by 15 min for P-3SS-specific and 25 min for nptIII and P-FMV-specific LAMP assays (Figure S2 of the Supporting Information).

Analysis using real-time LAMP took only 45 min to detect 10 copies of the target and can be much quicker at higher target concentrations, whereas the conventional LAMP required 75 min for the reaction to reach completion (visual LAMP) followed by 120 min for gel electrophoresis, if specificity needs to be verified. Real-time LAMP products were also subjected to visual detection using SYBR Green I dye and gel electrophoresis analysis, after amplification. Upon addition of SYBR Green I dye, the products showing specific positive results in real-time LAMP turned green, whereas the nonamplified real-time LAMP products remained orange. Similarly, a typical ladderlike pattern was detected only for positive products (Figure S2 of the Supporting Information).

For all the tested LAMP assays on the real-time system, the LOD was up to 10 target copies. No amplification was detected in test samples with fewer target copies per reaction mixture or in the negative control reaction mixture containing non-GM cotton DNA or in the nontemplate control. The sensitivity of the developed real-time LAMP assays (up to 10 copies or 0.025%) was found to be superior to that of the visual LAMP (up to 40 copies or 0.1%). This higher sensitivity may be due not only to the use of more sophisticated equipment but also to the use of more efficient chemistry and/or enzymes in the reaction mix: when products of real-time LAMP assays were further observed visually after addition of SYBR Green I dye or after gel electrophoresis, a LOD of 10 copies could be observed (data not shown).

The squared correlation coefficient ($R^2$) was also calculated as the correlation coefficient of the standard curve obtained by line regression analysis. As per method acceptance criteria and method performance requirements of the Codex Alimentarius Commission, the average value of $R^2$ has been considered to be suitable when it is less than 0.98. The $R^2$ values for the plots generated for the test samples with 400, 200, 40, 20, and 10 copies of GMO were also found to be above 0.98 for all real-time LAMP assays (Figure S3 of the Supporting Information).

Isothermal Real-Time Genie II System. The real-time LAMP assays described above were also evaluated on an isothermal Genie II system (OptiGene Ltd.) using the same test samples of GM cotton events. LAMP assays showed the expected specificity for each transgenic element, as also shown in Table 2. The first signal was detected within approximately 10 min for the aadA, nptIII, and uidA amplicons, whereas amplicons for P-3SS and P-FMV were observed after amplification for 16 min (Figure 3 and Figure S4 of the Supporting Information). The real-time LAMP assays performed in this system were more time-efficient than those performed on the real-time PCR.
system (where the first signals appeared between 16 and 25 min, depending on the amplicons tested). Real-time LAMP assays were faster than LAMP assays developed for conventional visual or gel electrophoresis analysis (where 75 or 120 min is required, respectively, for detection of amplicons).

The mean annealing temperature for the P-35S amplicons (87.2 ± 0.2 °C) was found to be 87.0 °C in GFM-cry1A, 87.1 °C in MLS-9124 and MON88913, and 87.2 °C in MON531, MON15985, and Event1. In other LAMP assays performed on the isothermal real-time system, the mean annealing temperature was 83.9 ± 0.1 °C for P-FMV, 87.4 ± 0.4 °C for aadA, 89.3 ± 0.2 °C for nptII, and 88.3 ± 0.1 °C for uidA (Figure 4).

The amplification profiles and anneal curves of the real-time LAMP assays obtained using the isothermal real-time detection system are shown in Figure 3 and Figure S4 of the Supporting Information.

The sensitivity experiments showed that the LOD of LAMP assays on the isothermal real-time system was up to 4 copies of GMO per reaction mixture, which was slightly higher than those determined on the real-time PCR system (where the LOD was up to 10 target copies per reaction mixture). Real-time LAMP assays were also found to be more sensitive than the conventional LAMP assays (where the LOD was up to 40 target copies per reaction mixture).
In conclusion, a rapid, cost-effective, and sensitive LAMP-based detection system has been developed to detect five screening elements commonly being employed in several GM crops. The developed assays can be used in the screening phase of GM testing to determine the GM status of samples. Two types of chemistries, namely, Bst DNA polymerase large fragment and ready-to-use OptiGene isothermal master mix, were employed for LAMP amplification on four detection systems, i.e., conventional heating block, thermal cycler, real-time PCR system, and isothermal real-time system. Both chemistries performed equally well in terms of the sensitivity and specificity of the assays. However, assays based on the isothermal master mix, performed on the isothermal real-time system, are faster and more sensitive and offer flexibility, as these assays can also be performed on real-time PCR systems with similar efficiencies. Real-time LAMP analysis involves simple interpretation of the amplification results, obtained by observing the amplification and melting curves, in comparison to previously used LAMP-based GMO detection strategies employing turbidometry, visual checkup, and/or gel electrophoretic analysis.

The reported conventional LAMP assays can also be employed on a simple heating block or a thermal cycler, if the suitable equipment for real-time LAMP is not available. In that case, the LAMP amplification is visually verified by adding SYBR Green I after the reaction; for further confirmation, gel electrophoresis analysis can also be performed.

The flexibility of the reported LAMP assays can facilitate its applicability for reliable GMO detection in the laboratory and also on site, if it is combined with simple and fast DNA extraction methods like those recently applied for the other LAMP assays, using either a portable isothermal real-time system or a heating block, which would be more useful for GMO screening by customs authorities to check the unauthorized imports at ports of entry or by the field inspectors or farmers in the fields.

ASSOCIATED CONTENT

Supporting Information

Specificity test of the visual LAMP assays in the selected GM cotton events for transgenic elements (i) P-3SS, (ii) P-FMV, (iii) aadA, (iv) nptII, and (v) uidA (Figure S1); specificity of the real-time LAMP assays as obtained using the real-time system via (a) amplification curves, (b) melting peaks, (c) visual detection using SYBR Green I, and (d) electrophoretic analysis of real-time LAMP products to check the specificity of designed LAMP primers for (i) P-3SS, (ii) P-FMV, (iii) aadA, (iv) nptII, and (v) uidA (Figure S2); linearity of the real-time LAMP assays as obtained on the real-time PCR system (Figure S3); and amplification and annealing curves for LAMP assays for (i) P-3SS and (ii) uidA on the isothermal real-time system (Genie II) (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

aadA, aminoglycoside 3′-adenytransferase; BLAST, Basic Local Alignment Search Tool; F3 and B3, forward and backward primers, respectively; FIP and BIP, forward and backward inner primers, respectively; LAMP, loop-mediated isothermal amplification; LOD, limit of detection; nptII, neomycin phosphotransferase II; P-3SS, Cauliflower Mosaic Virus 3SS promoter; PCR, polymerase chain reaction; P-FMV, Figwort Mosaic Virus promoter; GM, genetically modified; GMO, genetically modified organism; qPCR, real-time polymerase chain reaction; t_{fp}, time of positivity; uidA, β-glucuronidase

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