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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′-adenyltransferase (*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.[1,2] Several other GM crops/events under field trials were either indigenously developed or imported for research purposes. The National Bureau of Plant Genetic Resources (NBPGR) 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 accessions of transgenic planting material comprising 13 crop species have been processed through NBPGR 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" primers[11] or two "stem" primers[12] 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

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Table 1. Oligonucleotide Primers Designed and Employed in This Study

primer name	sequence (5′−3′)	target	GenBank accession number
F3-p35S	CTCCTCGGATTCCATTGC	*P-35S*	V00141
B3-p35S	TCTACAGGACGGACCATG		
FIP-p35S	ACGATGCTCCTCGTGGGTCATCGTTGAAGATGCCTCT		
BIP-p35S	CGTTCCAACCACGTCTTCAAGTCTTGCGAAGGATAGTGG		
LoopF-p35S	ATCTTTGGGACCACTGTCG		
LoopB-p35S	TGATATCTCCACTGACGTAAGG		
F3-pFMV	AACAATTCTGCACCATTCCT	*P-FMV*	NC_003554.1
B3-pFMV	AATTCTCAGTCCAAAGCCTC		
FIP-pFMV	TGCATCATGGTCAGTAAGTTTCAGATGCTCGATGTTGACAAGATT		
BIP-pFMV	TGTGCTGGAACAGTAGTTTACTTTGAAGGTCAGGGTACAGAGTC		
LoopF-pFMV	AAGACATCCACCGAAGACTTAA		
LoopB-pFMV	AGATTCTTCATTGATCTCCTGTAGC		
F3-nptII	TGATGCTCTTCGTCCAGA	*nptII*	AM887683
B3-nptII	CTCGACGTTGTCACTGAAG		
FIP-nptII	CCATTCGACCACCAAGCGACATCCTGATCGACAAGACC		
BIP-nptII	TAGCCGGATCAAGCGTATGCTCATCTCACCTTGCTCCT		
LoopF-nptII	CGTACTCGGATGGAAGCC		
LoopB-nptII	TTGCATCAGCCATGATGGATA		
F3-aadA	AGCTTTGATCAACGACCTTT	*aadA*	M86913
B3-aadA	GATCCTGTTCAGGAACCG		
FIP-aadA	CAGTTCGCGCTTAGCTGGATGCTGTAGAAGTCACCATTGT		
BIP-aadA	GGTATCTTCGAGCCAGCCACAACGCTATGTTCTCTTGCTT		
LoopF-aadA	AACGCCACGGAATGATGT		
LoopB-aadA	TGATCTGGCTATCTTGCTGAC		
F3-uidA	GTGGACGATATCACCGTG	*uidA*	U12639
B3-uidA	TGGTTAATCAGGAACTGTTGG		
FIP-uidA	CCGCTAGTGCCTTGTCCAGGGTGATGTCAGCGTTGAA		
BIP-uidA	GAATCCGCACCTCTGGCAAATCACACTCTGTCTGGCT		
LoopF-uidA	CCACCTGTTGATCCGCAT		
LoopB-uidA	TCTCTATGAACTGTGCGTCAC		

copy 10^9−10^{10} times during a 15−60 min reaction.[10] LAMP products show a ladderlike pattern on an agarose gel or can be monitored in real time using turbidometry.[13] The amplicons specific for DNA can alternatively be visualized after completion of the LAMP reactions using nucleic acid staining or fluorescent dyes such as SYBR Green I[4,14] and hydroxyl naphthol blue.[14] Real-time LAMP, based on bioluminescence,[15] intercalating fluorescent dyes,[16] and modified fluorescent primers,[17] has been demonstrated. The technique is gaining popularity in diagnostics because of its time efficiency, effectiveness, robustness, and ease of use in human medicine[18] and, more recently, in plant health.[16,19,20]

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.[9] LAMP assays for *T-nos*, *P-35S*, *P-nos*,[21] and the *pat* marker gene[22] have been reported for GMO screening. A bioluminescent real-time reporter (BART) of LAMP has been recently used for GMO screening targeting *P-35S*, *T-nos*, and the *Zea mays* alcohol dehydrogenase (*ADH1*) gene in GM maize event MON810.[8] The LAMP assay for detection of the *cry1Ab* gene in GM rice has also been recently reported,[23] and the results were compared with the qPCR results. Event-specific LAMP assays were developed for two GM soybean events (GTS 40-3-2 and MON88788),[4] seven GM maize events (DAS-59122-7, T25, *Bt*176, TC1507, MON810, *Bt*11, and MON863),[24] and three GM rice events (KMD1, TT51, and KF6).[14]

In this study, LAMP-based visual and real-time assays have been developed to be employed as cost-efficient, rapid, and reliable screening tools for checking the GM status of the sample irrespective of the GM trait or crop species. The developed LAMP assays targeting commonly employed promoters (*P-35S* and *P-FMV*) and marker genes (*aadA*, *nptII*, and *uidA*) are being reported. To evaluate the practicability of LAMP for simple, rapid, and cost-effective GMO screening, two types of chemistries, namely, conventional mix with *Bst* DNA polymerase large fragment and ready-to-use isothermal master mix, and two visualization approaches, namely, visual LAMP by adding SYBR Green I dye after reaction completion and real-time LAMP monitoring, were tested for each assay. The utility of four amplification systems, i.e., conventional heating block, thermal cycler, Light Cycler480 real-time PCR system, and isothermal real-time system (Genei II), for LAMP reactions was compared. The implication for cost-effectiveness and on-site detection of GMOs has also been discussed.

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 *cry1Ac* and *cry2Ab* genes, GFM-*cry1A* with the fused *cry1Ab-Ac* gene, Event1 with the synthetic *cry1Ac* gene, and MLS-9124 with the *cry1Ca* 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 Nath Seeds, Event1 from M/s J. K. Agri Genetics Ltd., and MLS-9124 from M/s Metahelix 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 (Widestrike) of Dow AgroSciences LLC, with *cry1F* and *cry1Ac* genes for insect resistance. These events were imported for the purpose of research through the NBPGR, 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 Widestrike (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 *Pisum 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*, *P-FMV*, *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 *P-FMV*[28] 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*, *P-FMV*, *aadA*, *nptII*, and *uidA*.

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 quality 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 *P-FMV*, 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× ThermoPol Buffer, 0.5−1.0 M betaine, 600 μM to 1.3 mM dNTP, 8−16 units 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 ThermoPol Reaction Buffer was optimized at 2× to obtain satisfactory visualization after SYBR Green I addition. The amplifications were performed in replicates 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× ThermoPol Reaction Buffer [40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 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 Genepro thermal cycler (Bioer Co.) 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 *Bst* 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 10000× SYBR Green I (Sigma Aldrich Co.) to the reaction mixture. Using SYBR Green I dye, a change from an orange color to a green color means that LAMP amplification occurs, whereas no color change depicts the absence of LAMP amplification. The specificity of amplified products was further confirmed by checking the ladderlike 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 (0.32 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 concatemers of a target-specific sequence.[6] The melting temperature (T_m) is the temperature at which the double-stranded DNA product dissociates into single strands. Therefore, the T_m 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 I 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

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

		P-35S		*P-FMV*		*aadA*		*nptII*		*uidA*	
GM event	status in India	S[d]	R[e]	S[d]	R[e]	S[d]	R[e]	S[d]	R[e]	S[d]	R[e]
MON531	C[b]	+	+	−	−	+	+	+	+	−	−
MON15985	C[b]	+	+	−	−	+	+	+	+	+	+
Event1	C[b]	+	+	−	−	−	−	+	+	−	−
GFM-*cry1A*	C[b]	+	+	−	−	−	−	+	+	+	+
MLS-9124	C[b]	+	+	−	−	−	−	+	+	−	−
MON1445	IP[c]	−	−	−	−	+	+	+	+	−	−
Widestrike	IP[c]	−	−	−	−	−	−	−	−	−	−
MON88913	IP[c]	+	+	+	+	−	−	−	−	−	−

[a]Plus and minus signs show the presence and absence of a target in a particular event, respectively. [b]Commercialized. [c]Imported for the purpose of research through NBPGR, New Delhi. [d]Theoretical data based on information available in GM databases, viz., http://www.igmoris.nic.in, http://www.cera-gmc.org, http://www.gmo-compass.org, and http://www.envfor.nic.in/divisions/csurv/geac/geac_home.html. [e]Verified experimentally with real-time PCR assays. Further specificity was checked using LAMP assays.

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 *P35S*, 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-35S*, *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.[3] 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.[30] Screening tests, based on the detection of regulatory sequences commonly used in developing GMOs, such as *P-35S* 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.[31]

Because *P-35S* 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,[1] *P-35S*- 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 *Bst* DNA polymerase large fragment, we used eight GM cotton events (MON531, MON15985, Event1, GFM-*cry1A*, MLS-9124, MON1445, MON88193, 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[4,14] or hydroxyl naphthol blue[14] after end-point reaction. Li and

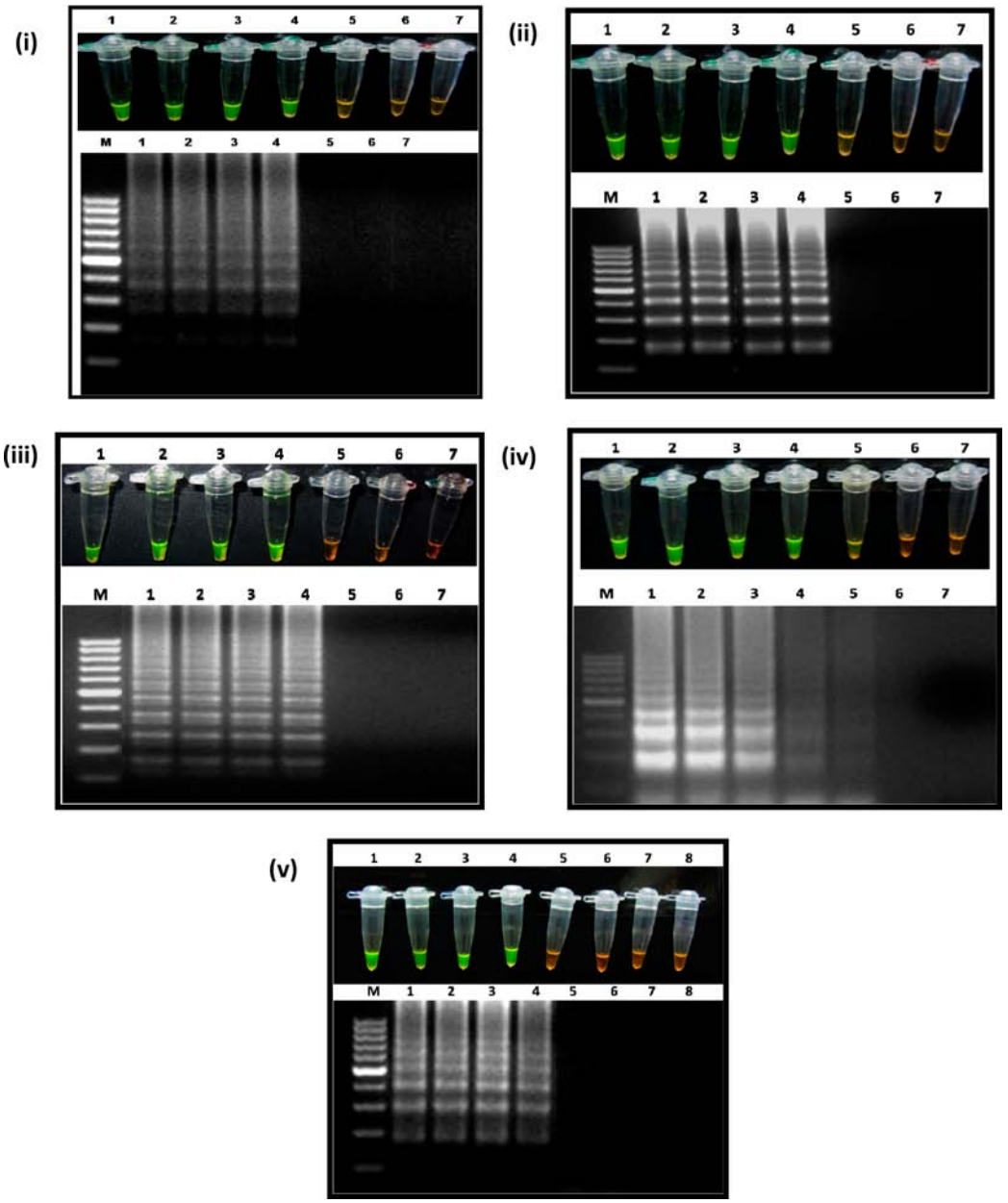

Figure 1. Limits of detection of the LAMP assays using *Bst* polymerase. Tests performed using serial dilutions of the respective GM cotton event with different percentages of GM content for specific transgenic elements: event MON15985 for (i) *P-35S*, (ii) *aadA*, (iii) *nptII*, (iv) and *uidA* and event MON88913 for (v) *P-FMV*. Amplification as observed by visual detection using SYBR Green I dye (top row) and electrophoretic analysis using 2% agarose gels (bottom row). (i–iv) Lane M, 100 bp DNA ladder; lanes 1–6, event MON15985 with 100, 10, 1, 0.1, 0.05, and 0.01% GM content, respectively, corresponding to 40000, 4000, 400, 40, 20, and 10 copies of GMO per reaction mixture, respectively; lane 7, non-GM cotton. (v) Lane M, 100 bp DNA ladder; lanes 1–6, event MON88913 with 100, 10, 1, 0.1, 0.05, and 0.01% GM content, respectively, corresponding to 40000, 4000, 400, 40, 20, and 10 copies of GMO content per reaction mixture, respectively; lane 7, non-GM cotton; lane 8, nontemplate control.

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.[32] 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.[33] 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-35S* 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-35S* may be attributed to the variation in the sequence of *P-35S* incorporated into the GM event, such as *P-35S* with a duplicated enhancer region (doubly enhanced *P-35S*) in events MON531, MON15985, and Event1 and the chimeric *P-35S/ACT8* promoter in event MON88913 (http://www.cera-gmc.org). The mean T_m values for the specific products for *P-35S*, *P-FMV*, *aadA*, *nptII*, and *uidA* were compared and were found to be 87.8 ± 0.7, 84.2 ± 0.1, 88.0 ± 1.0, 90.1 ± 0.3, and 89.3 ± 0.1 °C, respectively (Figure 2). Use of melting curve analysis presents a real advantage for specificity checking as it is faster (first signal detected within approximately 10 min) than gel electrophoresis. It also provides more precise information than gel electrophoresis, in which it is difficult to distinguish a ladder of true positive LAMP products from that of products due to LAMP cross reaction, if the products are similar in size.

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-35S*-specific and 25 min for *nptII*- 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)

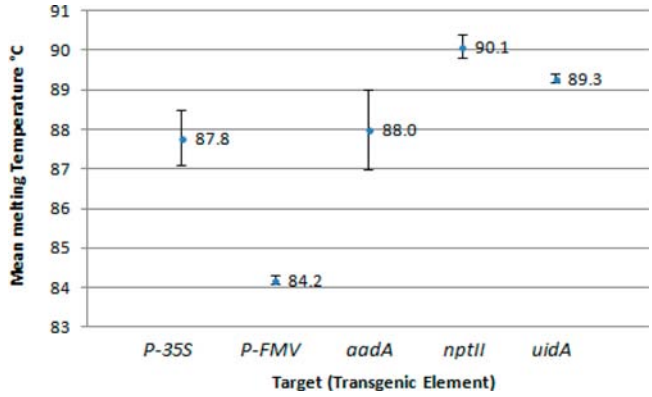

Figure 2. Mean melting temperatures (T_m) of the real-time LAMP products. T_m values for the specific amplicons for *P-35S*, *P-FMV*, *aadA*, *nptII*, and *uidA* targets are indicated. The bars show the standard deviations between target GM events. Data were obtained with the Light Cycler480 system.

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,[34] 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*, *nptII*, and *uidA* amplicons, whereas amplicons for *P-35S* 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

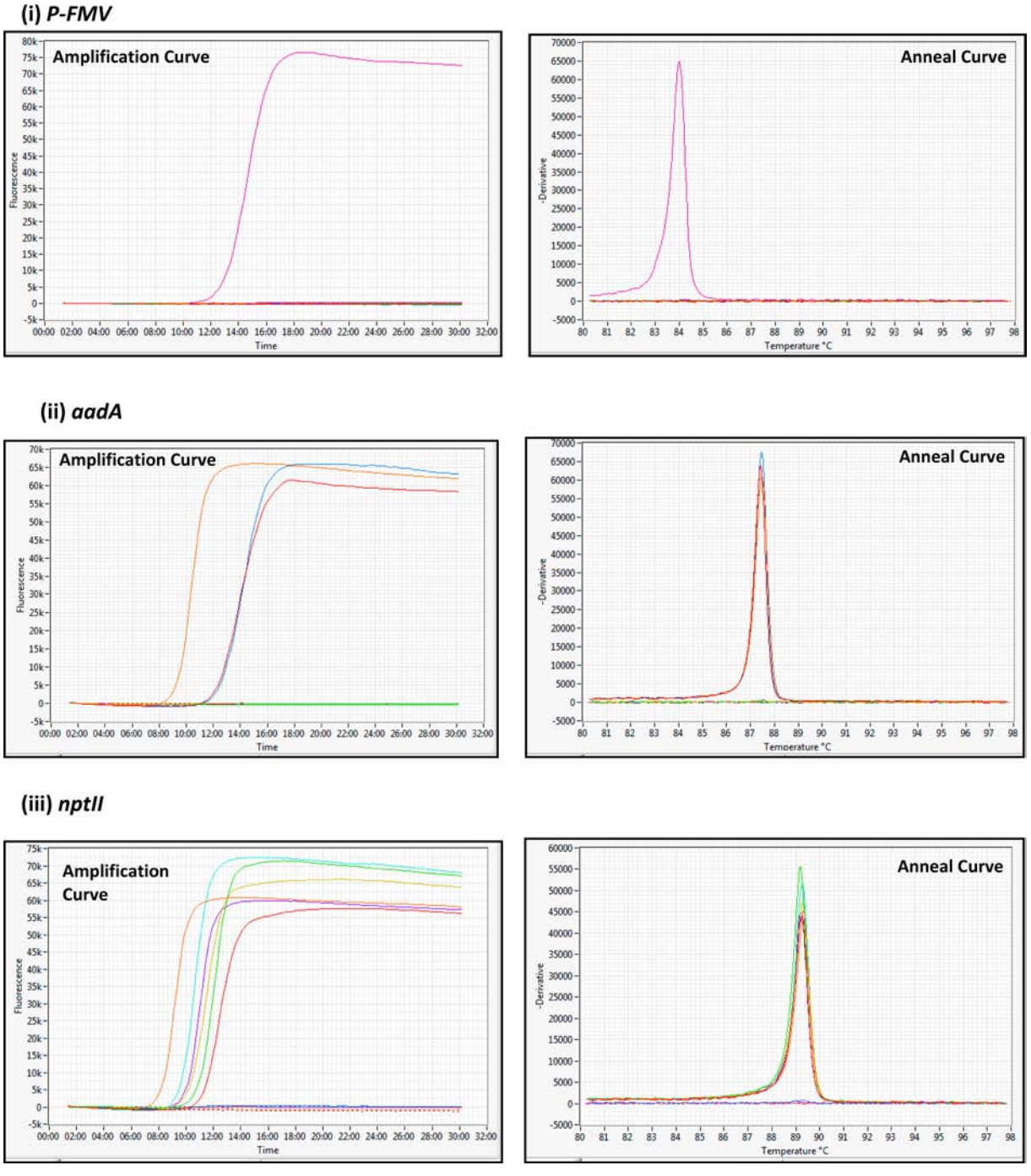

Figure 3. Amplification and annealing curves for LAMP assays for (i) *P-FMV*, (ii) *aadA*, and (iii) *nptII* on the isothermal real-time system (Genie II). Amplification was detected in the respective positive test samples for each target.

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).

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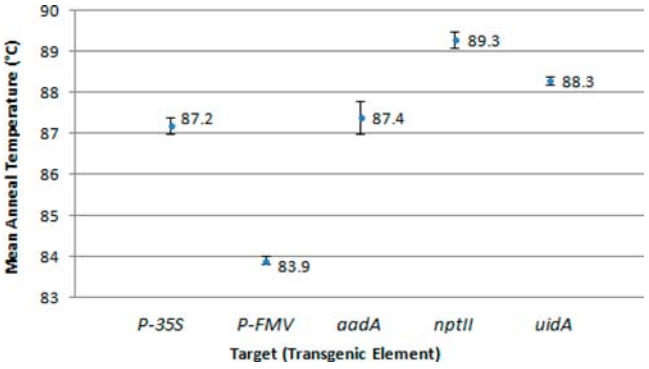

Figure 4. Mean annealing temperatures of the real-time LAMP products obtained using the Genie II system for specific amplicons for *P-35S*, *P-FMV*, *aadA*, *nptII*, and *uidA*. The bars show the standard deviations between target GM events.

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,[8,16,24,35] using either a portable isothermal real-time system or a heating block, which would be further 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-35S*, (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-35S*, (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-35S* 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-35S*, Cauliflower Mosaic Virus 35S 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_p, time of positivity; *uidA*, *β*-glucuronidase

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