Rapid Identification of Transgenic Cotton (*Gossypium hirsutum* L.) Plants by Loop-mediated Isothermal Amplification

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Abstract: In an attempt to speed up the process of screening of transgenic cotton (*G. hirsutum* L.) plants, a visual and rapid loop-mediated isothermal amplification (LAMP) assay was adopted. Genomic DNA was extracted from fresh leaf tissues of T_2 transgenic cotton containing chitinase (*chi*) and *cry1A(b)* genes. Detection of genes of interest was performed by polymerase chain reaction (PCR), LAMP and real-time PCR methods. In LAMP assay the amplification was performed after 30 min at 65°C when loop primers were involved in the reaction. The involvement of loop primers decreased the time needed for amplification. By testing serial tenfold dilutions (10^{-1} to 10^{-8}) of the genes of interest, the detection sensitivity of LAMP was found to be 100-fold higher than that of PCR. The rapid DNA extraction method and LAMP assay can be performed within 30 min and the derived LAMP products can be directly observed as visually detectable based on turbidity in the reaction tube. The accuracy of LAMP method in the screening of transgenes was confirmed by PCR and real-time PCR. The developed method was efficient, rapid and sensitive in the screening of cotton transgenic plants. This method can be applied to any other crops.

Keywords: Bt; chitinase; CryIA(b); detection; loop-mediated isothermal amplification; transgenic cotton

Transformation of crop plants requires transfer of genes to plant cells coupled with labour-intensive selection and regeneration of fertile plants. Equally time-consuming are the molecular and biological investigations of primary regenerants and their offspring. Therefore it is important to identify those plants which carry the transgene as early as possible in the procedure and a great deal of efforts have been directed at shortening the process (Berthomieu & Meyer 1991; Levin & Gilboa 1997; Rogers & Parkes 1999; Lee *et al*. 2009b).

Polymerase chain reaction (PCR) is the method of choice for detection and screening of transgenic offspring, when time and cost may be important factors. One major difficulty in applying PCR is that a large number of samples need to be processed in a short time. In order to process a large number of samples, crude DNA extracts must be used that can be amplified by PCR (Mannerlof & Tenning 1997). Isolation of DNA from cotton and recalcitrant plant can be problematic as the yield of DNA isolated is often low and the quality can be poor (Li *et al*. 2001). Despite their high potential, PCR based methods have some limitations in specific areas of application (Wassenegger 2001; Hernandez *et al*. 2005; Lipp *et al*. 2005; Morisset *et al*. 2008). To alleviate the inconveniences associated with PCR technology, various alternative nucleic-acid target amplification methods have been developed.

Recently, a new technique called loop-mediated isothermal amplification (LAMP) has been developed which can amplify nucleic acids with high

specificity, sensitivity and speed under isothermal conditions (Fu *et al*. 2010). This method depends on auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase, and the amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isothermal conditions between 60°C and 65°C, thereby obviating the need for a thermal cycler (Notomi *et al*. 2000). Moreover, this method can be carried out with simple systems and the LAMP reaction can be monitored in real-time through measurement of turbidity, which is correlated with the production of magnesium pyrophosphate, by means of an inexpensive photometer (Mori *et al*. 2001). Although LAMP was first described using a set of four primers, enhanced sensitivity was reported using an additional pair of loop primers (Nagamine *et al*. 2002).

During the last 10 years, LAMP method and its numerous modifications have been widely used for nucleic acid analysis because of its simplicity, rapidity, high efficiency, and outstanding specificity (Machado *et al*. 2009; Fu *et al*. 2010). Lee *et al*. (2009b) applied the LAMP method to amplify GMO-related DNA sequences. Guan *et al*. (2010) reported an optimized visual LAMP method for the detection of exogenous DNA targets from two genetically modified (GM) soybean events. The limits of detection of these established visual LAMP assays were about four copies of haploid soybean genomic DNA and which were much higher than those reported for conventional PCR assays (Fu *et al*. 2010). To circumvent the need for DNA extraction, Lee *et al*. (2009a) applied the LAMP method to tissue samples disrupted in water. They showed that LAMP is highly sensitive, requiring only a few copies of the target for amplification and detection of GM plants.

Here we report an optimized visual LAMP method for the screening of transgenic cotton events based on the DNA sequence of the recombinant construct inserted into the cotton genome. The cotton lines used contain two transgenes: *cry1A(b)* and *chitinase*. Using an easy, rapid and inexpensive method of DNA extraction, one minute DNA extraction, and optimizing a visual LAMP method we speed up the process of transgenic plant screening. The amplification efficiencies, specificities and sensitivities of these systems will be discussed.

MATERIALS AND METHODS

Plant materials and DNA preparation

The seeds of T_2 transgenic cotton (homozygous lines) containing the chitinase (*chi*) gene (Tohidfar *et al*. 2009) and *Bt* cotton containing the *cry1A(b)* gene (Yazdanpanah *et al*. 2009) along with the DNA sample of constructs were used in this study. The non-transgenic cotton variety Cocker 100 was used as control. The DNA samples of tobacco, barley, pomegranate and olive were provided by Molecular Genetics Laboratory, Zanjan Universiy.

Extraction of genomic DNA

The cell lysate including genomic DNA was extracted from fresh leaf tissue using the method according to Hosaka (Hosaka 2004). In this method, a piece of fresh leaf (5 × 5 mm) was placed in a plastic bag. One ml of extraction buffer (100mM Tris-Cl buffer pH 0, 50mM EDTA pH 8.0, 500mM NaCl, 1.25% SDS and 0.2% 2-mercaptoethanol) was added to each sample. Samples were crushed by pressing a pestle or by rotating a bottle over the plastic bag. Thus, 100 µl of the solution was collected into a 1.5 ml tube containing 32 µl of 5M potassium acetate and mixed by pipetting up and down several times. After spinning the tube at maximum speed for 10 s, 10 µl of the clear solution was collected into a 1.5 ml tube containing 990 µl of sterile water. The sample was used for PCR amplification.

The quality and quantity of DNA were estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analysed by 1% agarose gel electrophoresis.

PCR analysis

A set of six primers containing two inner (FIP and BIP), two outer (F3 and B3) and two loop primers (LF and LB) and a set of four primers containing two inner primers (FIP and BIP) and two outer primers (F3 and B3) (Fermentas Co., St. Leon-Rot, Germany) were designed respectively, for target sequences of *cry1A(b)* and *chi* genes (Figure 1 and Table 1). All of the LAMP primers used in this study were designed using the specific software

of Primer Explorer V4 available on the Eiken Genome site (http://primerexplorer. jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan).

The primers used for PCR were forward: 5'-CTCTAGGTTGGAAGGATTG-3' (F-cry) and reverse: 5'-GAGCATCGGTGTAGATAG-3' (R-cry) for the *cry1A(b)* gene and forward: 5' GCCATAACCGACTCC AAGCA-3' (F-chi) and reverse: 5'-GAG TGG TGT GGA TGC TGT TG-3' (R-chi) (CinnaGen Co., Iran) for the *chi* gene. The primers for real-time PCR were forward: 5'-TTCGCAGTCCAGAACTACCAAG-3'(CRT-F), reverse: 5'TTGTAACGGCTATTGATGGTTGC-3'(CRT-R) (CinnaGen Co., Iran) and the TaqMan probe was 5'-FAM-TCCCCACCTT TGCCCAAACACGCT-BHQ-1 (CRT-P) for the *cry1A(b)* gene. For the *chi* gene the primers were designed as forward: 5'-GAGCCTGCCCAGCCAAAG-3' (CHRT-F) reverse: 5'-CCGTGTCTC CGGTGTTTCC-3' (CHRT-R) and the TaqMan probe was designed as 5'-Fam-TGCCTTCATCGCCGCCGCCAA-BHQ1-3' (CHRT-P) (CinnaGen Co., Karaj, Iran).

Optimization of LAMP reaction conditions

Reaction conditions were optimized to establish fast and efficient parameters for amplifica-

Table 1 Oligonucleotide primers and probe designed for LAMP, PCR and real-time PCR reaction of the genes *cry1A(b)* and *chi*

Primers + probes	Type	Position on gene	Sequence (5'–3')	Length
FIP-cry	forward inner	560–579 and 604–625	GCGTGGTCGGTGTAGTTTCCAATTTTTGCTG-CAACCATCAATAGCC	46 mer
BIP-cry	backward inner	651–671 and 712–731	GAGCGTGTCTGGGGTCCTGATTTTTGTC-CAAAACTGTGAGGGTCA	45 mer
F3-cry	forward outer	542–559	GGCAAAGGTGGGGATTCG	18 mer
B3-cry	backward outer	746–765	TCTGGAGTCATAGTTCGGGA	20 mer
LF-cry	loop forward outer	581–603	CAGCCTAGTAAGGTCGTTGTAAC	23 mer
LB-cry	loop backward outer	683–707	GGATTAGATACAACCAGTTCAGGAG	25 mer
FIP-chi	forward inner	420–441 and 380–397	CGCAATCTCCCTCTTGCGAGTGTTTTC-CAAGGCTTACCCCAGCT	44 mer
BIP-chi	backward inner	443–464 and 499–518	CCTTCTTGGGGCAAACGTCTCATTTT-TATCCCCATGCGTATGGTCC	46 mer
F3-chi	forward outer	346–365	GGCTTCTACACCTACGATGC	20 mer
B3-chi	backward outer	522–539	TTCCGCTCCCTCACGAAG	18 mer
F-cry	forward	295–314	CTCTAGGTTGGAAGGATTG	19 mer
R-cry	reverse	915–933	GAGCATCGGTGTAGATAG	18 mer
CRT-F	forward	446–464	TTCGCAGTCCAGAACTACCAAG	22 mer
CRT-R	reverse	563–586	TTGTAACGGCTATTGATGGTTGC	23 mer
CRT-P	probe	530–554	FAM-TCCCCACCTTTGCCCAAACACGCT-BHQ-1	24 mer
F-chi	forward	85–105	GAGTGGTGTGGATGCTGTTG	20 mer
R-chi	reverse	937–957	GCCATAACCGACTCCAAGCA	20 mer
CHRT-F	forward	328–346	GAGCCTGCCCAGCCAAAG	18 mer
CHRT-R	reverse	399–418	CCGTGTCTCCGGTGTTTCC	19 mer
CHRT-P	probe	937–957	Fam-TGCCTTCATCGCCGCCGCCAA-BHQ1	21 mer

Figure 1. Nucleic acid sequence of *cry1A(b)* gene (a) and *chi* gene (b) used to design inner, outer and loop primers; the nucleic acid sequences used for the primer design and their positions are marked by lines

tion. Different parameters were tested including amplification temperatures (60, 65 and 67°C), periods of time (15, 30, 45, 60, 75 and 90 min) and concentrations of loop primers (0.3, 0.4, 0.5 and 0μM) which have the capacity to accelerate the reaction. The target DNA was added to the reaction and amplification was performed for 10, 20, 25 and 30 ng of target DNA using a heating block (Wise Therm HB-48. Germany).

PCR amplification

In PCR, the reaction was carried out in a 20 μl volume, including 1× PCR buffer, 2.5mM $MgCl_2$, 0.2mM each dNTP, 0.2μM each primer, 1 U Taq DNA polymerase (CinnaGen Co., Karaj, Iran) and 50 ng of template DNA. PCR was performed in a BioRad thermal cycler (DNA Engine Model, New York**,** USA) according to the following program: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min for the *cry1A(b)* gene and initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min extension at 72°C for 1 min and final extension at 72°C for 5 min for the *Chi* gene (Tohidfar *et al*. 2008, 2009). The PCR products were analzsed by 1% agarose gel electrophoresis in 1× tris-acetate-EDTA (TAE) stained with ethidium bromide and visualized on a UV transilluminator (Figure 2c).

Real-time PCR reaction

In order to confirm the accuracy of screening process by the LAMP method, real-time PCR experiments were performed for each sample with a Rotor Gene 3000 (Corbett Research, Sydney, Australia). Each 20 μl of PCR reaction contained 0.2mM d NTP (Genet Bio), 5mM $MgCl_2$, 1× PCR buffer, 100nM probe, 0.125 U *Taq* DNA polymerase (Genet Bio, Cheonan, Koren) All primer concentrations were 200nM. The thermal conditions were as follows: initial denaturation step at 95°C for 2 min, 40 cycles of denaturation at 94°C for 8 s, hybridization at 60°C for 20 s for the *cry1A(b)* gene and initial denaturation step at 95°C for 2 min and 40 cycles of denaturation at 94°C for 10 s, hybridization at 60°C for 30 s for the *chi* gene (Figure 2d).

LAMP reaction

LAMP was carried out in 25 µl reaction mixture in a 0.2 ml tube with 1× ThermoPol Reaction Buffer (20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X100) (pH 8.8), 0.2mM each of F3 and B3, and 0.8mM each of FIP and BIP, 0.4mM each of LF and LB primers (loop primers used just for the *cry1A(b)* gene), 0.1mM dNTPs, 8 units of *Bst* DNA polymerase large fragment (New England Biolabs, Hitchin, UK), 100mM betaine (Sigma, Northbrook, USA), and 2 µl of template DNA. LAMP was performed in a BioRad thermal cycler according to the following program: reactions were incubated at 65°C for 1 h, followed by 82°C for 10 min to inactivate the enzyme.

The LAMP assay was carried out in triplicate for each template DNA, and non-transgenic cotton was used as negative control. LAMP amplification products were detected by a ladder-like appearance on agarose gel electrophoresis or by the naked-eye (Figure 2a, b).

RESULTS

The DNA extracted by so called one-minute DNA extraction method (Hosaka 2004) was successfully

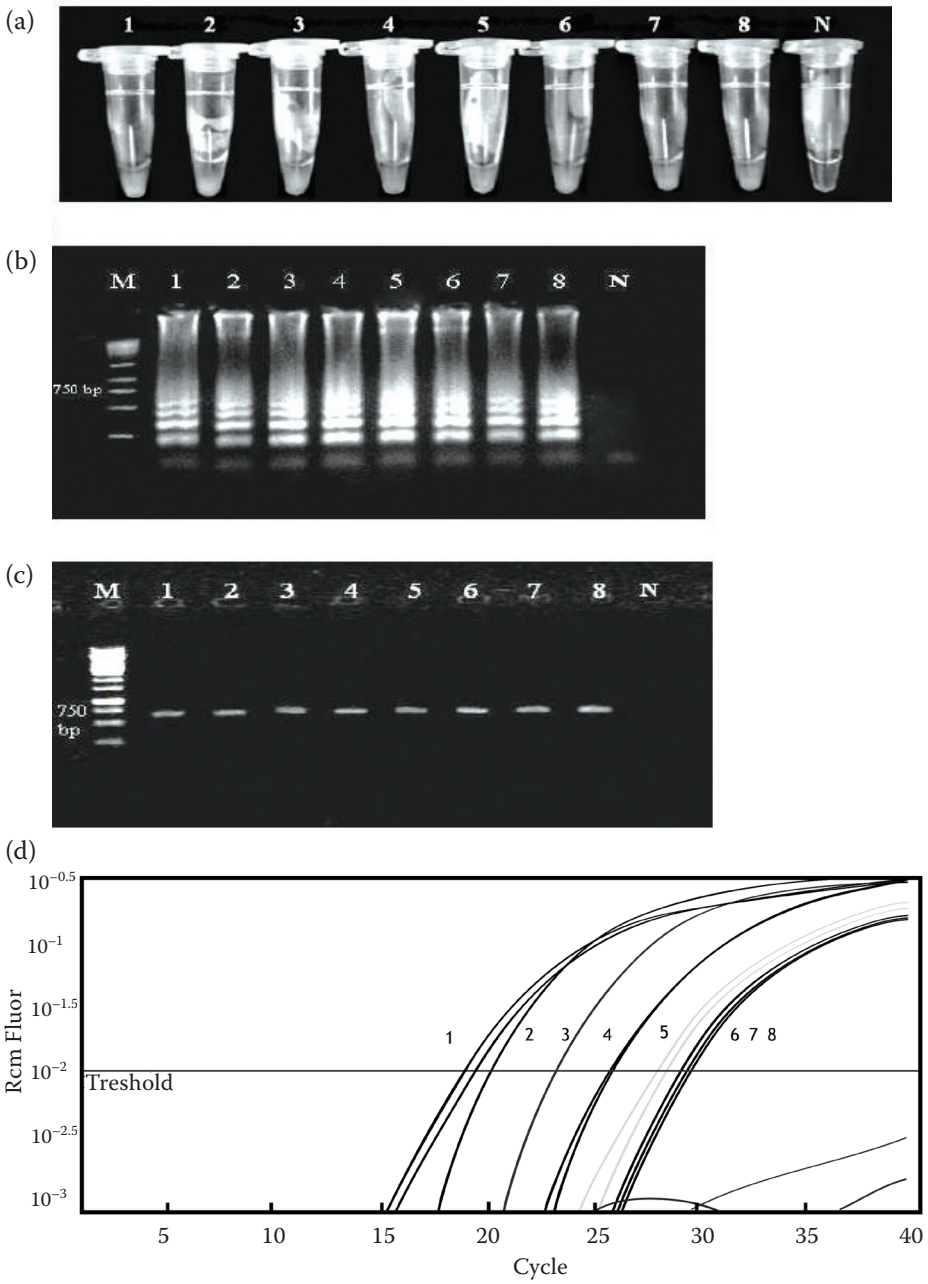

Figure 2. *cry1A(b)* LAMP assay; (a) visual detection of LAMP products by observation of turbidity as white precipitate, N: non-transgenic cotton; (b, c) electrophoresis of LAMP (b) and PCR (c) products amplified from the *cry1A(b)* gene samples; lane M, 1-kb DNA ladder (Fermentase); lane 1, plasmid; lane 2–8, transgenic cotton plants; lane N, non-transgenic cotton; (d) Real-time PCR (d) analysis for the same DNA templates

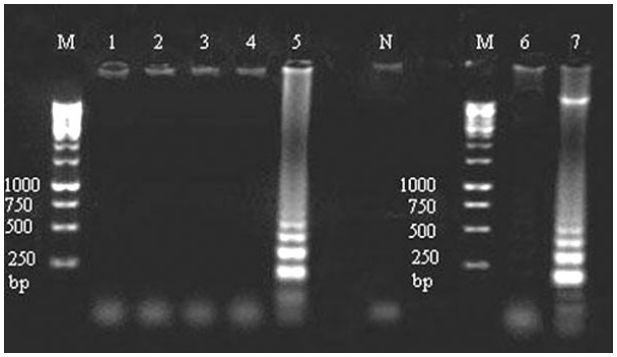

Figure 3. Different amplification times for the *cry1A(b)* gene in LAMP assay using 4 primers (FIP, BIP, F3, and B3) and 6 primers (FIP, BIP, F3, B3, LB, and LF) at 65°C; lane M, 1-kb DNA ladder; lanes 1–5, reactions with 4 primers at 15, 30, 45, 60 and 75 min, respectively; lane 6–7, reactions with 6 primers at 15 and 30 min, respectively; lane N, non-transgenic cotton; the LAMP assay detected the *cry1A(b)* gene at 75 min with 4 primers and at 30 min with 6 primers

used for PCR, LAMP and real-time PCR assay. This method is easy, rapid, inexpensive and feasible for extraction of a large number of LAMP-quality DNA samples without any special equipment.

Optimization of LAMP reaction conditions

The reaction temperature was optimized at 65°C with 20 ng of template DNA and then heated at 82°C for 10 min to terminate the reaction. No amplification was observed at 60 and 67°C. The reaction mixture of the *cry1A(b)* gene LAMP assay with FIP, BIP, F3, B3, LB, and LF primers as well as the reaction mixture with only 4 primers (excluding LB and LF) were incubated for different periods of time (15, 30, 45, 60, 75, 85, and 95 min) to determine the shortest required amplification time at a predetermined temperature (65°C). Successful amplification was achieved after 30 min at 65°C, however, when the

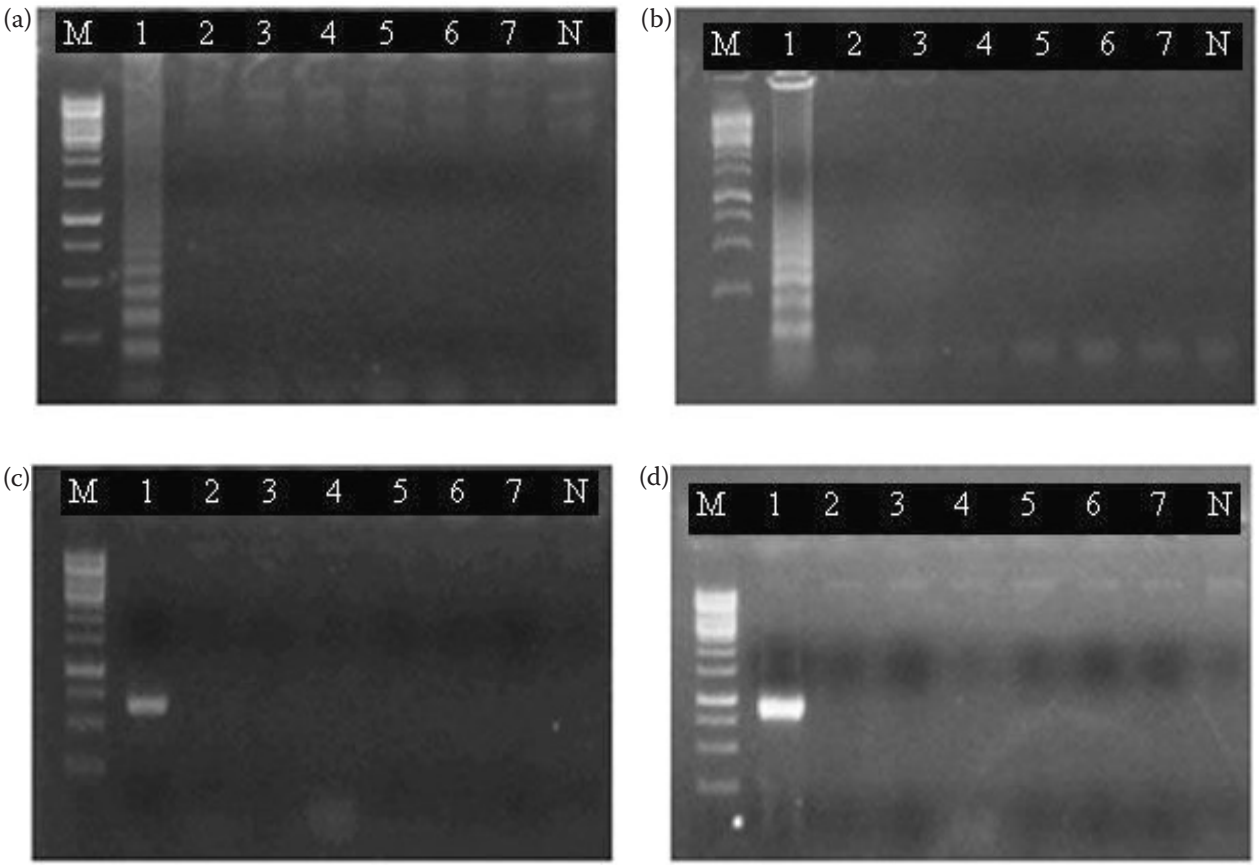

Figure 4. Specificity of LAMP (a, b) and PCR (c, d) assays revealed by gel electrophoresis; (a and c) lane M, 1-kb DNA ladder; lanes 1–7, DNA from *cry1A(b)* gene, *chi* gene, Cocker (non-transgenic cotton), tobacco, barley, pomegranate, olive, respectively; N is the negative control (water); (b), (d) lane M, 1-kb DNA ladder; lanes 1–7, DNA from *chi* gene, *cry1A(b)* gene, Cocker (non-transgenic cotton), tobacco, barley, pomegranate, olive, respectively; lane N, negative control (water)

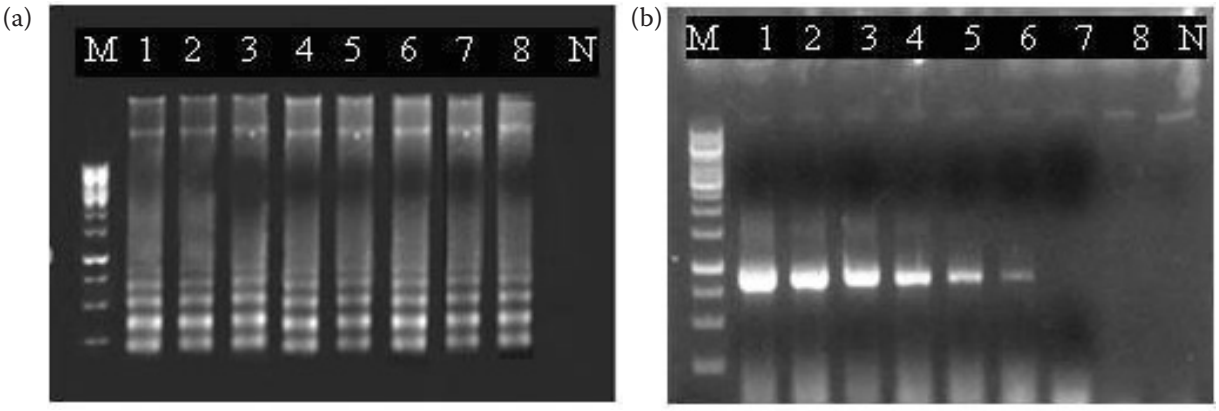

Figure 5. Comparative sensitivities of LAMP (a) and PCR (b) in detection of *chi* gene; Tenfold serial dilutions of *chi* gene were amplified by LAMP; lane M, 1-kb DNA ladder; lanes 1–8, 10^{-1} to 10^{-8} dilutions of *chi* gene; N, negative control

loop primers were not used in the reaction, amplification was observed only after 75 min at the same temperature (Figure 3). Amplified products exhibited a typical ladder-like pattern.

Specificity of the LAMP assay

To evaluate the specificity of the developed LAMP assays several non-transgenic plants of olive, barley, pomegranate, tobacco, non-transgenic cotton (cv. Cocker) and transgenic cotton were used. In the specificity test, 25 ng of transgenic cottos genomic DNA was used as the template in each LAMP assay of *cry1A(b)* and *chi* genes. The typical ladder-like pattern products were observed only in the tests using genomic DNA samples of transgenic cotton as templates. No amplified products were observed in non-transgenic crops (Figure 4). These data confirmed that the developed LAMP assays have high specificity for amplifying the target DNAs.

Sensitivity of the LAMP assay

The sensitivity of the LAMP assay in the detection of *chi* gene was ascertained by testing serial tenfold dilutions (10^{-1} to 10^{-8}) of the target DNA with the detection limit of the PCR and LAMP. The LAMP assay detection limit was10^{-8}. On the other hand, the PCR detection limit was 10^{-6} as shown in Figure 5. Therefore, the detection sensitivity of LAMP was 100-fold higher than that of PCR.

The screening of transgenic and non-transgenic offspring of cotton plants for *cryI A(b)* and *chi* genes using the LAMP method, was confirmed by PCR and real-time PCR analyses (Figure 2). More than 50 samples were tested by all three methods of analysis and it was revealed that the LAMP method is highly sensitive and specific in the identification of transgenic cotton plants.

DISCUSSION

The DNA extracted by so called one-minute DNA extraction method (Hosaka 2004) was successfully used for PCR, LAMP and real-time PCR assay. This method has not been specifically tested for cotton. Our results confirmed its applicability to both PCR and LAMP assays. An important advantage of the isothermal amplification techniques is their tolerance to some inhibitory materials (Kaneko *et al*. 2007) such as secondary compounds in cotton (*G. hirsutum*) plants that can affect the efficiency of PCR. Therefore, using the LAMP technique, the process of DNA purification from samples could be omitted. Using microtitre plates and a multi-channel pipette could shorten the processing time as well.

Before PCR became available, Southern blots were performed to confirm the presence of foreign DNA and its correct integration into the plant genome for this purpose. Large-scale preparations of pure genomic DNA, large amounts of expensive restriction endonucleases, and labelled probes were required. PCR is an efficient alternative to Southern blot analyses and its most convincing advantage is the observation that the integration of foreign DNA can be examined from as little as 200 pg of genomic DNA

(Clark *et al*. 1994; Wassenegger 2001). However, despite their high potential, PCR based methods are also time consuming, expensive and labour demanding, which limits these methods from being widely used for transgenic screening. Our study shows that the LAMP method is even more efficient than PCR in the fast, inexpensive and reliable amplifying of target DNA. Real-time and conventional PCR analyses approved the presence of the transgene in all screened lines by LAMP method. Screening efficiency and sensitivity were enhanced by using an additional pair of loop primers in detection of the *cry1A(b)* gene. Previously, it was reported that the LAMP reaction could be accelerated by using two loop primers (Nagamine *et al*. 2002). However, using only two inner and two outer primers, Guan *et al*. (2010) indicated that their established LAMP assays had high reaction efficiency without loop primers. In our study, the involvement of loop primers decreased the time needed for amplification and increased the production of turbidity which can be observed as a white precipitate

The efficiency of LAMP depends on the size of target DNA. According to Notami *et al.* (2000) the best results could be obtained with 130 to 200 bp of target DNA. Therefore, the size of target DNA should be set to less than 300 bp, including FIP (FIP-cry and FIP-chi) and BIP (BIP-cry and BIP-chi). Most of the LAMP methods used for GMO detection generally focus on the sequence of frequently used genetic elements, such as promoters, terminators (Fukuta et al. 2004; Lee *et al*. 2009a), marker genes (Guan *et al*. 2010), etc. However, LAMP primers can also be easily adapted to other target genes. To be specific to transgene sequence, we designed our primers on the basis of the sequences of *cry1A(b)* and *chitinas*e genes (Tohidfar *et al*. 2009; Yazdanpanah *et al*. 2009). This allowed us to screen our transgenic cotton plants for specific genes. This might be advantageous, as the DNA sequence comprises a sufficient length to provide flexibility for designing primers of choice and as the LAMP method is highly specific to the target sequence, a particular event in the sequence of transgene can be easily detected. Because of the specific features of LAMP primers, target selectivity is expected to be higher than that in PCR (Notami *et al*. 2000). High specificity is assured since target recognition is mediated by four distinct primers targeting six different sequences (Notami *et al*. 2000; Nagamine *et al*. 2002; Fukuta et al. 2004). In some studies, LAMP was even shown to be more selective (Seki *et al*. 2005) and less sensitive to background DNA (Notami *et al*. 2000) than PCR. LAMP has high amplification efficiency and with a lower amount of sample DNA (1–10 ng) (Guan *et al*. 2010, it yields DNA in quantities of more than 500 g/ml (Morriset *et al*. 2008). In our study the sensitivity of the LAMP reaction was more than 100-fold greater than that of the PCR reaction.

Althoug, LAMP assays are complicated by the primer design and by the number of primers required for each assay, in our case by adding loop primers, it could be performed within 35 min without PCR equipment, and the derived LAMP products could be directly visualized by the naked eye. In breeding programs for which hundreds or even thousands of individuals may need to be screened to detect the correct genetic constitution, efforts put into this aspect of experimental design may pay dividends in reduced time and costs of sample preparation, together with the ease of genetic screening.

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