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SHORT COMMUNICATION

Rapid identification of transgenic black pepper using loop-mediated isothermal amplification (LAMP) and real-time LAMP assays

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Abstract A loop-mediated isothermal amplification (LAMP) and real-time LAMP based assays were developed for quick and sensitive detection of transgenic black pepper plants. Primers (six each) were designed based on the nucleotide sequence of two target regions [kanamycin and *Cauliflower mosaic virus* (CaMV) 35S promoter] integrated into the genome of transgenic black pepper. Both assays successfully detected the transgenic plants and no cross-reaction was recorded with non-transgenic plants. The sensitivity of LAMP was up to 10^4 times that of conventional PCR while real-time LAMP was up to 10^3 times that of LAMP and 10^7 times to that of PCR. The addition of 6 mM magnesium sulphate and 0.4 M betaine with 1 h reaction time proved optimal for amplification through LAMP assay. The assays were validated by testing putative transformants of black pepper. The present study clearly established that LAMP and real-time LAMP assays can provide a rapid and simple approach for screening transgenic black pepper and other plants transformed by using the above target gene sequences.

Keywords CaMV 35S promoter · Detection · Kanamycin · Sensitivity · Validation

Abbreviations

CaMV 35S	Cauliflower mosaic virus 35S promoter
LAMP	Loop-mediated isothermal amplification
PCR	Polymerase chain reaction

A number of methods including polymerase chain reaction (PCR), real-time PCR and Southern blot or western blot are available for the identification of transgenic plants (Karami et al. 2011). PCR is the most commonly used method of choice for detection and screening of transgenic plants. However, PCR based method requires thermal cycler, time consuming and not applicable for field tests. Notomi et al. (2000) developed a new technique called loop-mediated isothermal amplification (LAMP), which can amplify nucleic acid with high specificity, sensitivity and speed under isothermal conditions. 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). As the reaction is performed at a single temperature, LAMP assays can be performed very quickly at constant temperature condition without expensive thermal cyclers. LAMP assays are able to recognize six distinct regions flanking target DNA sequences making the technique more specific. LAMP products can be detected by conventional agarose gel electrophoresis that give a typical ladder like pattern, by visual inspection of turbidity/ colour changes/ or in real-time using real-time LAMP instrument (Mori et al. 2001; Iwamoto et al. 2003; Tomlinson et al. 2010). LAMP assay have been used to identify transgenic oilseed rape (Lee et al. 2009), soybean (Guan et al. 2010), cotton (Rostamkhani et al. 2011; Randhawa et al. 2013), rice (Chen et al. 2012) and maize (Huang et al. 2014). In view of its quick, simple to perform and higher sensitivity compared to PCR, we report development of LAMP and real-

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time LAMP assays for the identification of transgenic black pepper plants based on the DNA sequence of the CaMV 35S promoter and kanamycin region present in the recombinant construct inserted into the black pepper genome.

Transgenic plants of black pepper plants produced in our laboratory for development of *Agrobacterium* mediated transformation system using the vector, pBI121 that carry Cauliflower mosaic virus (CaMV) 35S promoter and kanamycin marker gene were used. Non transgenic plants of black pepper were used as negative control. The status of the plants were confirmed by subjecting total DNA isolated from putative transgenic plants by PCR using CaMV 35S promoter and kanamycin marker gene specific primers. Initial standardization of the LAMP and real-time LAMP assays was performed with one plant each of transgenic and non-transgenic black pepper, whereas the validation was done on 20 putative transgenic black pepper plants. The total DNA of the test plants was isolated from 50 mg leaf sample using CTAB procedure as described previously (Hareesh and Bhat 2006).

Primers for LAMP and real-time LAMP assays were designed (Table 1) for the kanamycin marker gene and CaMV 35S promoter region sequences using a software package, namely Primer Explorer version 4 (http://primer explorer.jp/e/). Two external primers, F3 and B3, two internal primers FIP and BIP, and the loop primers (B-loop and F-loop), were designed. Desalted primers were custom synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).

Reaction components for the LAMP assays were optimized in 0.2 ml tubes using total nucleic acids extracted from the transgenic (the positive control) and non- transgenic black pepper plant (the negative control). The third control was the 'water control' (a negative control, in which water was used instead of nucleic acids). The LAMP reaction mixture (25 μl) contained 1 μl (about 60 ng) of the template nucleic acids, 2× thermopol buffer (New England Bio Labs, Ipswich, Massachusetts, USA), 1.4 mM each of dNTPs, 0–14 mM $MgSO_4$ and 0.4–2.4 M betaine (Sigma Chemicals, Bengaluru, India), 0.2 μM each of the external primers F3 and B3, 2 μM each of the internal primers FIP and BIP, 1 μM of the each loop primer (B-loop and F-loop) and 8 U of *Bst* polymerase (New England Bio Labs). The reaction was carried in an incubator (Thermo Scientific, USA) at 65 °C for 60 min followed by incubation at 80 °C for 5 min to inactivate the *Bst* polymerase. In determining the optimum concentration of $MgSO_4$, betaine was maintained at 0.8 M whereas in determining the optimum concentration of betaine, $MgSO_4$ was maintained at 6 mM for both target regions. To find out the optimum duration, the reaction was carried out at 65 °C for different durations (30, 45 and 60 min). LAMP results were analysed through agarose gel electrophoresis. Reaction components for the real-time LAMP assay was optimized in 0.2 ml strips (Optigene, UK) using same nucleic acid templates used in the LAMP assay. The real-time LAMP reaction mixture contained 1 μl of the template nucleic acids, 15 μl isothermal master mix (Optigene) and primers as indicated in the LAMP assay. The reaction was carried out in a real-time LAMP instrument (Genie II, Optigene) held at 65 °C for 60 min. Real-time LAMP results were analysed in terms of Tp values (Tp is the time taken to generate a positive result based on the fluorescence). Annealing/melting temperature analysis from 98 to 80 °C was used to validate the authenticity of the LAMP products. Initially the specificity of the real-time LAMP product was also confirmed through gel electrophoresis.

Table 1 Primers used for loop-mediated isothermal amplification (LAMP) and real-time LAMP assays for the detection of transgenic black pepper plants.

Target region	Primer	Sequence (5′ …….3′)
Kanamycin	F3	CTGTTCGCCAGGCTCAAG
	B3	CGCCAAGCTCTTCAGCAATA
	FIP	GAAAAGCGGCCATTTTCCACCA GCGATGATCTCGTCGTGAC
	BIP	GGATTCATCGACTGTGGCCGG GGTAGCCAACGCTATGTCC
	BL	GTGTGGCGGACCGCTAT
	FL	TTCGGCAAGCGGCATCGC
CaMV 35 S promoter	F3	CTCCTCGGATTCCATTGC
	B3	GTCTTGCGAAGGATAGTGG
	FIP	GGCAGAGGCATCTTCAACGA GGAAGGTGGCTCCTACAA
	BIP	CACGAGGAGCATCGTGGAAA CGTCAGTGGAGATATCACATC
	FL	TTTCCTTTATCGCAATGATGGC
	FL	AGAAGACGTTCCAACCACG

The degree of sensitivity of the LAMP and real-time LAMP assays were tested using serially diluted (10^0 to 10^{-10}) total DNA isolated from transgenic black pepper plant. The PCR was carried out using 1 μl of each of the above dilutions and the respective F3 and B3 primers as described by Jiby and Bhat (2011). In order to validate the LAMP and real-time LAMP assays for the detection of transgenic plants, total DNA isolated from 20 putative transgenic plants were initially amplified through PCR using the specific primer and subsequently subjected to LAMP and real-time LAMP assays along with negative control and positive control using primers for both target regions.

Products of LAMP obtained using two sets of primers corresponding to CaMV 35S promoter region, kanamycin gene and 1 μl of the nucleic acid from the transgenic black pepper plant showed a typical ladder-like pattern in agarose gel electrophoresis while no amplification was observed in the negative controls (Fig. 1a). The optimum concentration of $MgSO_4$ was found to be 6 mM and that of betaine was found to be 0.4 M for both target regions (data not shown). Amplification

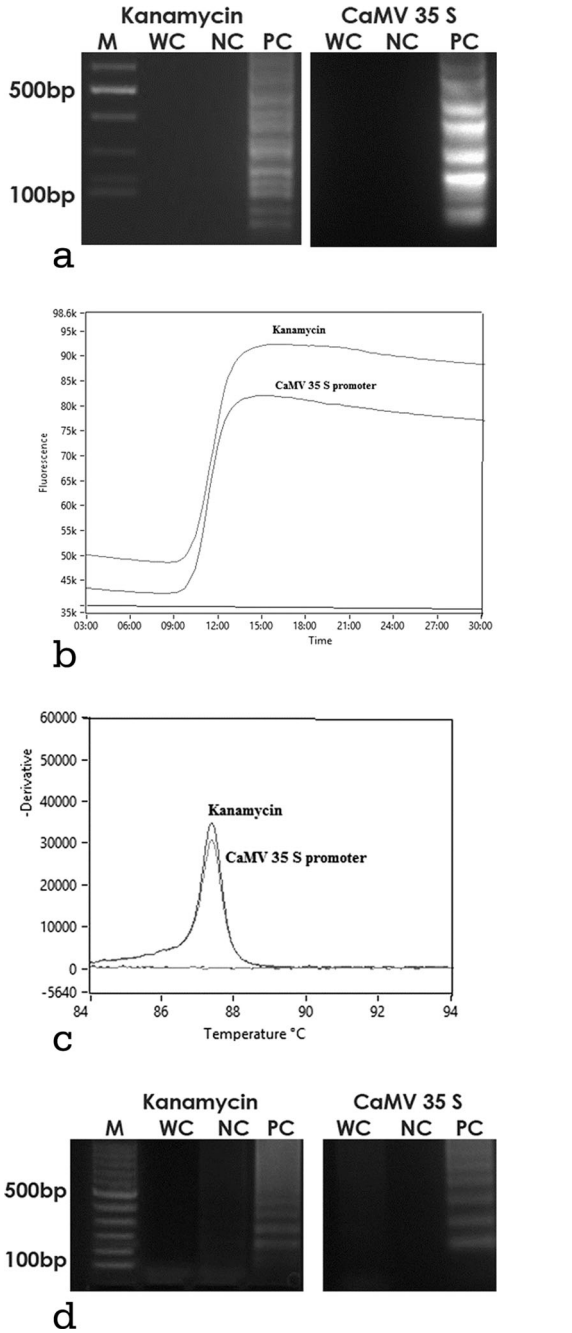

Fig. 1 Standardization of loop-mediated isothermal amplification (LAMP) and real-time LAMP for the identification of transgenic black pepper plants by amplifying kanamycin and Cauliflower mosaic virus 35S promoter (CaMV 35S) specific sequences present in transgenic black pepper plant. Lane M: 100 bp DNA ladder, Lane WC: water control, Lane NC: negative control, PC: positive control. **a** LAMP, **b** real-time LAMP, **c** Anneal curve of real-time LAMP product, and (d) agarose gel electrophoresis of real-time LAMP products

was not optimum at 30 and 45 min but good at 60 min (in terms of number and intensity of bands). Real-time LAMP detected both CaMV 35S promoter and kanamycin target regions only in transgenic plant but not in non-transgenic plant and water control (Fig. 1b). Each assay was specific for its target region, and amplification was observed within 10 min. The specificity of real-time LAMP product was confirmed through anneal curve that showed a single peak at 89 and 88 °C for kanamycin and CaMV 35S promoter region respectively (Fig. 1c). Further, specificity of the product was confirmed by gel electrophoresis that showed typical ladder like bands only in transgenic plant (Fig. 1d).

The detection limits for both kanamycin and CaMV 35S promoter target regions were 10^{-5} using LAMP and 10^{-8} using real-time LAMP while it was only 10^{-1} using PCR (Fig. 2). The 20 putative transgenic black pepper plants were used for validation of the LAMP and real-time LAMP assays. Initially when total DNA from these plants were subjected to PCR using F3 and B3 primers specific for each target regions, 12 plants showed positive reaction indicating that they are true transgenics (Fig. S1a). Non amplification of remaining plants

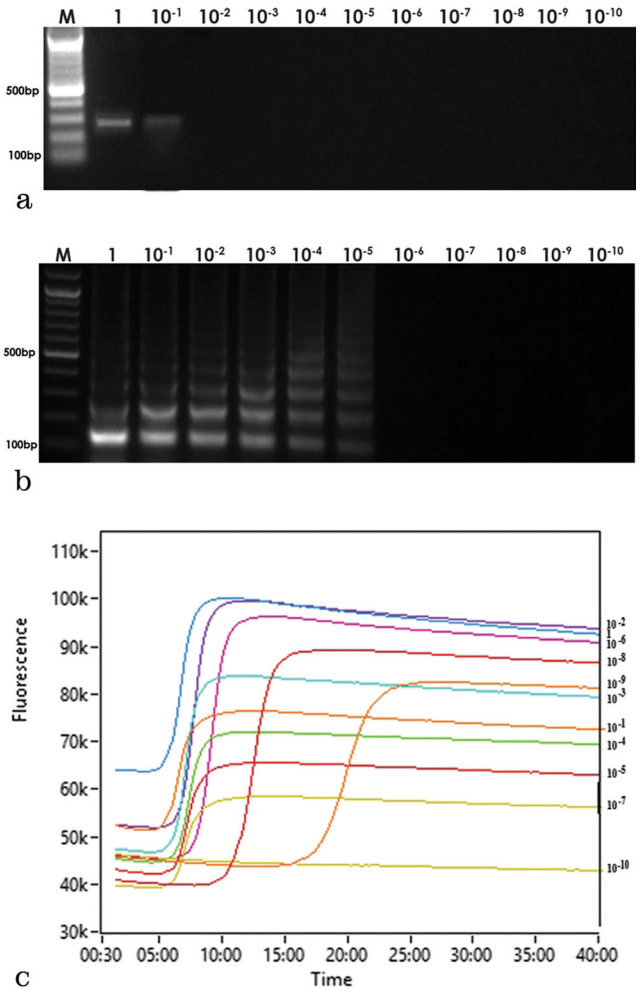

Fig. 2 Comparison of sensitivity of detection of transgenic plants by PCR, loop-mediated isothermal amplification (LAMP) and real-time LAMP by amplifying CaMV 35S promoter gene present in transgenic plant. **a** PCR, **b** LAMP, **c** real-time LAMP. Lanes 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, $10^{-9}$and 10^{-10} show different dilutions of the original extract of total nucleic acids; Lane M shows a 100 bp DNA ladder

indicate that they are non- transgenics. Similar results were obtained when these samples were subjected to LAMP and real-time LAMP assays confirming their accuracy in the detection of transgenic black pepper plants, whereas no amplification was observed in the non-transgenic control (Fig. S1b; S1c). The time taken for the detection in the real-time LAMP assay varied from 6 to 15 min in different samples probably indicating variation in the copy number of the transgene in the plants.

Quick and sensitive detection of transgenic plants early in the selection process is important to track the target traits and currently, PCR-based methods are used for the identification of such plants. The present study was successful in achieving the same result through alternative means: LAMP and real-time LAMP assays which are sensitive and quick to perform. In the present study LAMP and real-time LAMP assays were developed for the identification of transgenic black pepper plants by targeting CaMV 35S promoter and kanamycin gene sequences inserted into the genome of transgenic black pepper. The sensitivity of LAMP and real-time LAMP assays was also compared with that of PCR. To our knowledge, this is the first report of a LAMP and real-time LAMP assay for the detection of transgenic black pepper.

Compared to normal PCR that requires minimum reaction time of 3 h; LAMP is simple, more sensitive and can be performed in laboratories with minimum facilities in a short time (about 1 h) while in real-time LAMP, detection was possible as early as 10 min after start of the reaction. The products of real-time LAMP were confirmed both through anneal curve and agarose gel electrophoresis. The use of real-time LAMP instrument is more practical for quick primary on-site screening (detection within 7–15 min). The anneal curve specifies the accuracy of the reaction and further confirm the results.

With respect to dilution limits, our results are consistent with those obtained by many researchers (Chen et al. 2011; Rostamkhani et al. 2011; Chen et al. 2012). The sensitivity of the LAMP was up to 10^4 times that of PCR while real-time LAMP was up to 10^3 times that of LAMP and 10^7 times that of PCR, suggesting that real-time LAMP and LAMP assays are more sensitive than PCR. The optimized LAMP and the real-time LAMP procedures proved satisfactory even with the screening of putative black pepper transformants further proving their suitability for rapid identification of transgenic black pepper plants. The technique established here can be extended for use in the detection of other transgenic plants carrying the CaMV 35S promoter and / kanamycin gene inserted into their genomes.

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