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# Reverse transcription loop-mediated isothermal amplification assay for rapid and sensitive detection of *Banana bract mosaic virus* in cardamom (*Elettaria cardamomum*)

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**Abstract** Cardamom being a perennial and propagated vegetatively, *Banana bract mosaic virus* (BBrMV) in cardamom spreads mainly through infected material. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for easy and quick detection of the virus. The following conditions proved optimal for amplification: 4 mM of magnesium sulphate, 1.2 M of betaine, 65 °C, and 1 h of reaction time. The results were assessed visually by turbidity and green fluorescence (induced by adding manganese chloride and calcein) in the reaction tube and also by gel electrophoresis. The assay successfully detected the virus in infected plants whereas no cross-reaction was recorded with healthy plants. The detection limit for RT-LAMP was up to 100 times that for conventional RT-PCR and on a par with that for real-time RT-PCR. The assay was validated by testing field samples of cardamom plants from different cardamom-growing tracts in Kerala, India.

**Keywords** RT-LAMP · Sensitivity · RT-PCR · Real-time RT-PCR · Validation

Cardamom (*Elettaria cardamomum* Maton), family Zingiberaceae, is a herbaceous rhizomatous perennial native to the evergreen forests of the Western Ghats of southern India (Ravindran 2002). One of the most important and highly valued spices, next only to saffron, cardamom is also grown in Guatemala, Papua New Guinea, Sri Lanka and Tanzania. In India, cardamom is cultivated in the states of Kerala, Karnataka, and Tamil Nadu at elevations of 800–1,300 m. In recent years, a virus, namely the *Banana bract mosaic virus* (BBrMV, an ssRNA virus in the genus *Potyvirus*, family *Potyviridae*) has been found infecting the crop. Until recently, BBrMV was reported to infect only banana, causing bract mosaic disease in India, Philippines, Sri Lanka, Thailand, Vietnam and Western Samoa, and lowering yields by up to 40 % (Rodoni et al. 1999). The virus is known to spread vertically by vegetative means and horizontally through aphids in a non-persistent manner. The first natural infection of cardamom with BBrMV causing chlorotic streak disease was reported in 2012 (Siljo et al. 2012). The disease is characterized by continuous or discontinuous spindle-shaped yellow or light green intravenous streaks along the veins and the midrib. The streaks later coalesce, and the veins also turn yellow or light green. In severe cases of symptoms tillering in infected plants was suppressed. The disease was found to be more prevalent in those plantations in which banana were either the preceding crop or grown nearby. The virus spreads primarily through tillers, whereas the secondary spread, from banana to cardamom, might be through aphids (Siljo et al. 2012).

Cardamom is mainly propagated vegetatively through tillers, which makes the use of virus-free plants a crucial element in checking the spread of the

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virus, especially to new areas. Because the symptoms vary with age, the degree of resistance or susceptibility, and also the season, infected plants cannot be identified reliably by visual examination. So far, RT-PCR has been the only method to detect BBrMV in cardamom (Siljo et al. 2012), a method both expensive and time-consuming for large-scale use in the field. Loop-mediated isothermal amplification (LAMP), on the other hand, is not only sensitive but can also be carried at a constant temperature of 60–65 °C in a water bath. LAMP assays use specific sets of four to six primers and a DNA polymerase with strand-displacing activity to generate amplification products from DNA templates (Notomi et al. 2000; Nagamine et al. 2002). The assays can be also be performed with RNA templates by adding reverse transcriptase enzyme—hence often referred to as reverse transcription loop-mediated isothermal amplification (RT-LAMP)—and take about as long as LAMP assays to perform (Fukuta et al. 2003). The products of LAMP and RT-LAMP can be viewed in several ways, such as by noting the degree of turbidity or colour changes or by agarose gel electrophoresis (Mori et al. 2001; Nagamine et al. 2002; Tomita et al. 2008). RT-LAMP has been successfully used for detecting many RNA viruses infecting plants (Fukuta et al. 2003, 2004, 2005; Nie 2005; Varga and James 2006; Liu et al. 2010; Peng et al. 2012), and the present paper describes the design and optimization of a RT-LAMP assay to detect the BBrMV in infected cardamom plants.

For standardizing the assay, a cardamom plant infected with the BBrMV and a healthy cardamom plant were used. Total RNA isolated from these plants was subjected to RT-PCR for confirming the presence or absence of the virus (Siljo et al. 2012). The same two plants were used for determining the detection limits of the assay and for comparing the results with those obtained by using conventional and real-time RT-PCR. For validating the RT-LAMP assay, samples from 20 cardamom plants (9 with and 11 without the characteristic symptoms) collected from different cardamom-growing tracts of the Indian state of Kerala were used.

Primers for the RT-LAMP assay were designed from the conserved region in the coat protein gene identified by multiple sequence alignment of coat protein gene sequences of BBrMV available in GenBank (Benson et al. 1999). The primers were designed using the LAMP primer design software Primer Explorer V4 (http://primerexplorer.jp/e/) and synthesized at Integrated DNA Technologies, Coralville, Iowa, USA. The software generated four primers (F3, B3, FIP and BIP) and only one kind of loop primer (BL) for the target sequence used. Thus five RT-LAMP primers were synthesized for the virus, namely the external primers F3 (5′- GAGATATATG CCCAGGTGG-3′) and B3 (5′- CCTCTCTGTATTCT CTTCACC-3′), the internal primers FIP (5′-GCCCTG TTTGTAGTTTTTGATGTGAGGATTGAATGACA TAAGCTTAGC -3′) and BIP (5′- GAGAAGCACA CACGCAGATGAAGGTCCAAGATTTCCATCC AA-3′), and the loop primer (B-loop, 5′- GCTGCAGC TATTCGTGGGTCA-3′).

The components of the RT-LAMP assay were optimized by using total RNA extracted from an infected plant (the positive control) and from a healthy plant (the negative control); the third control was the water control (a negative control using water instead of RNA). The RT-LAMP mixture (25 μl) contained 1 μl (about 100 ng) of the template RNA, 2× thermopol buffer (New England Biolabs, Ipswich, Massachusetts, USA), 1.4 mM each of dNTPs, 0–12 mM $MgSO_4$, and 0–1.6 M betaine (Sigma Chemicals, Bangalore, India), 200 nM each of the external primers F3 and B3, 2 μM each of the internal primers FIP and BIP, 1 μM of the loop primer (B-loop), 1 mM $MnCl_2$ and 50 μM calcein (Sigma Chemicals), 8 U of *Bst* DNA polymerase (New England Biolabs), and 1.5 U of thermoscript reverse transcriptase (Invitrogen, Carlsbad, California, USA). For determining the conditions optimal for the reaction (concentration of $MgSO_4$ and of betaine), it was carried out at 65 °C for 75 min followed by incubation at 80 °C for 10 min to inactivate the *Bst* polymerase. While determining the optimum concentration of $MgSO_4$, betaine was maintained at 1.2 M and in determining the optimum concentration of betaine, $MgSO_4$ was maintained at 4 mM. To find out the optimum temperature, the reaction was carried out for 75 min at different temperatures (56 °C, 59 °C, 62 °C, 65 °C, and 68 °C); to find out the optimum duration, the reaction was carried out at 65ºC for different durations (15 min, 30 min, 45 min, 60 min, and 75 min). Initially, the RT-LAMP reaction was optimized using a thermo cycler (Master cycler gradient, Eppendorf, Germany) for maintaining a constant reaction temperature. Later on, three other options were evaluated to check their suitability for the purpose: a heat block (Henry Troemner, Thorofare, New Jersey, USA), a water bath

(Plastocrafts, Mumbai, India), and an incubator (Thermo Scientific, Waltham, Massachusetts, USA).

DNA is synthesized in large amounts during a RT-LAMP assay. The process yields large amounts of magnesium pyrophosphate as a by-product, which, being insoluble, makes the contents of the reaction tube turbid. The extent of turbidity can be gauged by different methods, and we used three: (1) visual examination in normal daylight for the presence or absence of turbidity, (2) visual examination under UV light for green fluorescence induced by adding 1 mM $MnCl_2$ and 50 μM calcein to the reaction mixture, and (3) visualization by gel electrophoresis on 2 % agarose.

To compare the detection limits of different assays, serial dilutions of the total RNA extract from the virus-affected plant were subjected to RT-LAMP, RT-PCR, and SYBR Green based real-time RT-PCR. The following dilutions were made using total RNA extracted from the healthy plant as a diluent ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$). All the dilutions were subjected to RT-LAMP, conventional RT-PCR, and SYBR Green real-time RT-PCR. The RT-PCR was carried out using 1 μl of each of the above dilutions and the F3 and B3 primers as described by Siljo et al. (2012). Real-time RT-PCR was carried out using the Rotor Gene Q system (Qiagen, Hilden, Germany) in 25 μl of reaction mixture that contained 12.5 μl of 2× QuantiFast™ SYBR Green PCR Master mix, 1.0 μl (1 μM/μl) each of the forward and reverse primers, 50 U of Revert Aid reverse transcriptase, and 1 μl of the template. The thermocycling steps were as follows: initial cDNA synthesis at 42 °C for 45 min, denaturation at 95 °C for 5 min, and 35 cycles of 95 °C for 15 s and 60 °C for 45 s.

To validate the detection of BBrMV by RT-LAMP assay in field samples of cardamom collected from different locations in Kerala, India, the samples were subjected first to a direct antigen coated (DAC) ELISA (using BBrMV-specific polyclonal antiserum at $10^{-3}$ dilution) (Clark et al. 1986) and SYBR Green real-time RT-PCR and then to RT-LAMP along with the positive and negative controls.

Products of the RT-LAMP assay obtained using 1 μl of the total RNA from the infected cardamom plant showed turbidity, green fluorescence under UV light, and the typical ladder-like pattern in agarose gel electrophoresis characteristic of RT-LAMP, whereas none of these (turbidity, fluorescence, and ladder-like patterns) was observed either in the negative control (the healthy plant) or in the 'water control' (Fig. 1). The optimum concentrations of $MgSO_4$ and betaine were found to be 4 mM and 1.2 M respectively. When the optimized reaction components were subjected to RT-LAMP at different temperatures for 75 min, no amplification was seen either at 56 °C or at 68 °C but was evident at 59 °C, 62 °C, and 65 °C (data not shown). There was little difference in the intensity of bands obtained at 62 °C and 65 °C. Similarly, when temperature was held constant at 65 °C, amplification was very slight at 45 min but satisfactory at 60 min and at 75 min (data not shown). There was little difference in the intensity of bands obtained in 60 min and 75 min. Thus, the optimum temperature and duration for RT-LAMP were 65 °C and 60 min. The amplification was successful with all the three devices (incubator, heating block, and water bath). The optimal RT-LAMP reaction components and conditions are given in Table 1.

The detection limits for BBrMV were $10^{-4}$ dilution of total RNA in both RT-LAMP and SYBR-Green-based real-time RT-PCR but $10^{-2}$ in RT-PCR (Fig. 2). The results were confirmed by repeating the experiment a total of three times. The RT-LAMP assay was 100 times as sensitive as conventional RT-PCR and

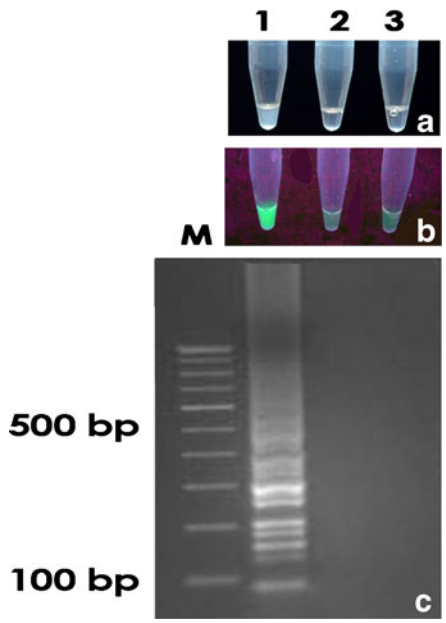


**Fig. 1** Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for the detection of *Banana bract mosaic virus* in cardamom. Visualization: **a** normal light, **b** UV light, **c** after gel electrophoresis; *lane M*: 100 bp DNA ladder; *lane 1*: infected cardamom; *lane 2*: healthy cardamom; *lane 3*: water control

**Table 1** Components of the optimized reverse transcription loop-mediated isothermal amplification (RT-LAMP) for the detection of *Banana bract mosaic virus* (BBrMV) in cardamom

| Component | Stock conc. | Required conc. | Required volume per reaction (μl) |
|---|---|---|---|
| Thermopol reaction buffer | 10× | 2× | 5.0 |
| $MgSO_4$ | 50 mM | 4 mM | 2.0 |
| dNTPs | 10 mM | 1.4 mM | 3.5 |
| F3 primer | 10 μM | 0.2 μM | 0.5 |
| B3 primer | 10 μM | 0.2 μM | 0.5 |
| FIP | 100 μM | 2 μM | 0.5 |
| BIP | 100 μM | 2 μM | 0.5 |
| BL primer | 100 μM | 1 μM | 0.25 |
| Betaine | 5 M | 1.2 M | 6.0 |
| *Bst* polymerase | 8 U/μl | 8 U | 1.0 |
| Thermoscript RT | 1.5 U/μl | 1.5 U | 1.0 |
| $MnCl_2$ | 20 mM | 1 mM | 1.25 |
| Calcein | 1 mM | 50 μM | 1.25 |
| Sterile water | – | | 0.75 |
| Template RNA | 100 ng/μl | | 1.0 |
| Total reaction volume | – | | 25.0 |

Reaction mixture incubated at 65 °C for 60 min followed by 80 °C for 10 min in an incubator

as sensitive as real-time RT-PCR. Each of the three methods of examining the products of RT-LAMP visually (turbidity, green fluorescence under UV light, and electrophoresis) gave similar results in terms of detection limits although using turbidity was somewhat subjective, especially in borderline cases (Fig. 2).

To validate the RT-LAMP assay, samples from nine symptomatic and 11 non-symptomatic cardamom plants collected from different locations were used. In DAC-ELISA, all except three non-symptomatic cardamom plants tested positive. When subjected to real-time RT-PCR using 1 μl of template RNA isolated from these samples along with the positive and negative controls, all the samples tested positive except the negative control and the water control. These samples were then subjected to RT-LAMP using 1 μl of template RNA along with the positive and negative controls. Results of RT-LAMP also showed a positive reaction in all samples except the two controls, confirming successful detection of BBrMV in the infected plants.

Chlorotic streak disease is a newly emerging threat to cardamom production. The disease is of recent origin and was mainly observed in plantations where banana was either the previous crop or grown nearby. In banana, BBrMV is known to be transmitted through suckers and horizontally through many aphid species including *Pentalonia caladii* (Magnaye and Espino 1990; Suresh et al. 2005). The aphid *P. caladii* is known to feed and breed in both banana and cardamom plantations (Venugopal 2002) and might be responsible for horizontal transmission of BBrMV from banana to cardamom. Once infected, vertical transmission of BBrMV might take place through infected tillers, cardamom being propagated vegetatively. Hence, early diagnosis of the virus at the incubation stage itself and selection of healthy mother plants are important to check the spread of the disease. Currently, assays based on RT-PCR are being used for detection of the virus (Siljo et al. 2012).

Compared to the conventional RT-PCR, RT-LAMP is less expensive in terms of equipment required, more sensitive, and has been used for detecting many pathogens including viruses. Developing a RT-LAMP assay for each virus requires appropriately designed primers, standardization of the reaction components (especially the concentration of $MgSO_4$ and of betaine), and optimal temperature and duration. In the present study, all these parameters for detecting BBrMV were standardized and the RT-LAMP assay was carried out successfully using three different devices, namely an incubator, a heating block, and a water bath, confirming that any of them can maintain a set temperature accurately enough for RT-LAMP amplification. As several researchers (Tomita et al. 2008; Ravindran et al. 2012) have done in the past, we too found that it is possible to detect the products of RT-LAMP through direct observation by looking for turbidity or for green fluorescence (by adding manganese chloride and calcein to the reaction tube). All the three visual methods (turbidity, green fluorescence, and gel electrophoresis) gave similar results in terms of detection limits, although turbidity was to some extent subjective, especially in borderline cases. However, the presence or absence of green florescence serves as a simple and objective proof. Our results show that such simple indications as turbidity or green fluorescence are adequate for performing RT-LAMP in laboratories with minimum facilities. As reported by many workers (Fukuta et al. 2003; Varga and James 2006; Boubourakas et al. 2009; Liu et al. 2010), the results of the present study also showed that RT-LAMP was up to 100 times as sensitive as conventional RT-PCR and as sensitive as SYBR-Green-based real-time RT-PCR. That

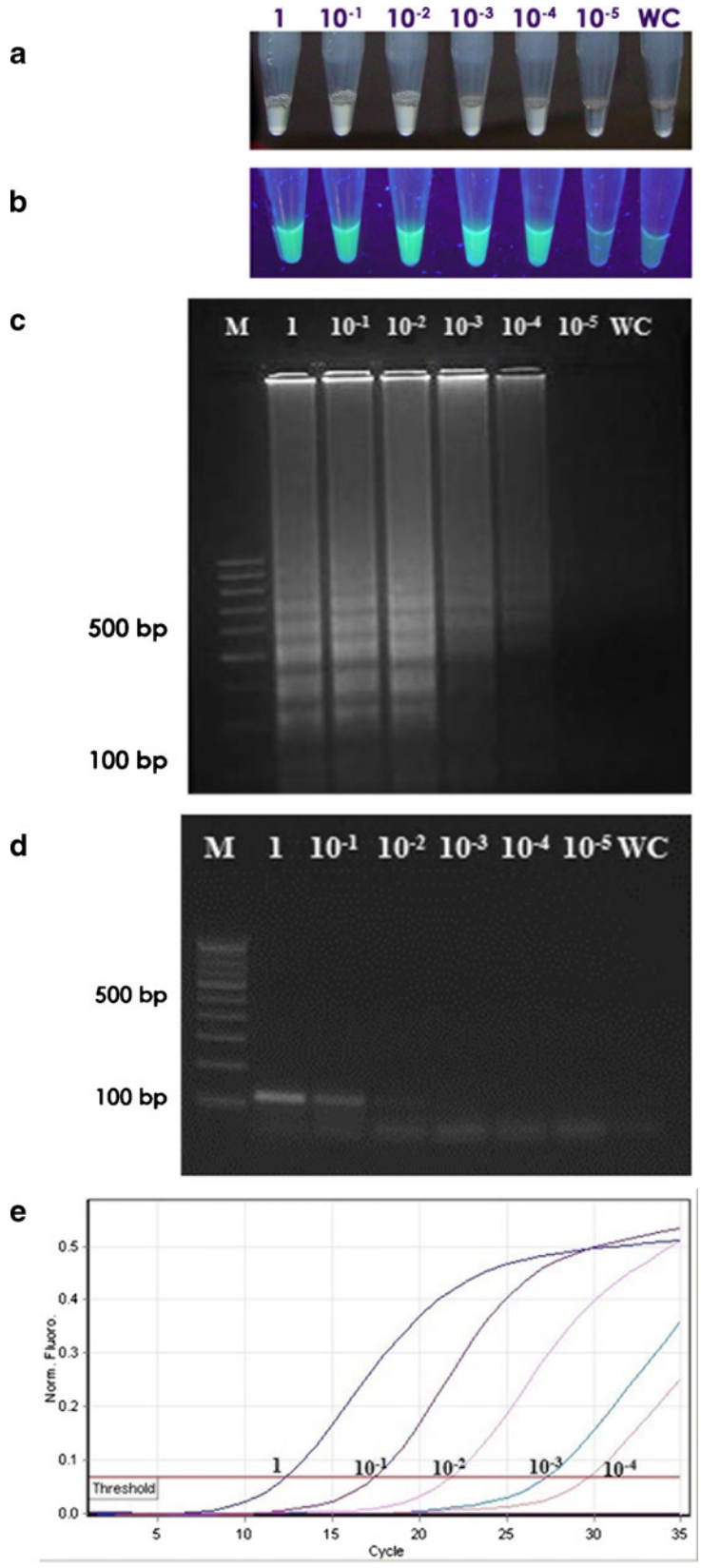


**Fig. 2** Comparison of sensitivity of detection of *Banana bract mosaic virus* (BBrMV) by reverse transcription loop-mediated isothermal amplification (RT-LAMP) (**a**–**c**), RT-PCR (**d**), and real-time RT-PCR (**e**). *Lanes 1*, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ show different dilutions of the original extract of total RNA; *lane M* shows a 100 bp DNA ladder; and *lane WC* shows water control. Visualization: **a** under normal light, **b** under UV light, **c** gel electrophoresis, **d** agarose gel electrophoresis of RT-PCR product, **e** amplification curve obtained with real-time RT-PCR carried out using different dilutions of the original extract of total RNA

the optimized RT-LAMP assay worked well with field samples collected from different regions shows that the assay is suitable for rapid detection of BBrMV in cardamom. Because RT-LAMP detected the virus in a larger number of non-symptomatic plants than DAC-ELISA did, RT-LAMP is the more reliable method of detecting the virus and screening the mother plants.

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