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ORIGINAL ARTICLE

Development of reverse transcriptase-recombinase polymerase amplification (RT-RPA) assay for rapid detection of large cardamom chirke virus

P. Malavika[1] · A. I. Bhat[1] · M. Greeshma[1]

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Abstract
Large cardamom chirke virus (LCCV) causing *chirke* disease of large cardamom is a major production constraint of this crop. Rapid and accurate detection of LCCV is important for managing the disease. In the present study an isothermal assay namely, reverse transcriptase-recombinase polymerase amplification (RT-RPA) was developed for the detection of LCCV. Total RNA isolated by two different methods and crude extracts isolated using five different methods as templates were assessed for their ability to detect LCCV. Of these, only the total RNA isolated by both methods gave consistent and repeatable results while all the crude extracts used as templates gave non-specific amplification. RT-RPA was up to 1000 times more sensitive than conventional RT-PCR for the detection of LCCV. The detection limit of RPA was 10 fg when recombinant plasmid was used as the template. The RT-RPA assay was validated using field samples and found suitable for large-scale screening of large cardamom plants against LCCV for the selection of virus-free plants.

Keywords RT-PCR · RT-RPA · Diagnosis · Sensitivity

Introduction

Large cardamom (*Amomum subulatum*) belongs to the family Zingiberaceae and is a perennial spice crop grown in moist shady areas along hilly slopes at an elevation of 2500–5500 ft in the north-eastern states of India, Nepal, and Bhutan. The economic part of this plant is the dried capsules which are used as a spice for flavouring food and in medicine. India is the largest producer of large cardamom. Of the two viral diseases infecting large cardamom, *chirke* is serious with incidence ranging from 19 to 35% causing yield loss of up to 85% [13]. The disease is characterized by mosaic streaks on the leaves which gradually coalesce and eventually turn brownish and dry up. The flowering in the diseased plant is extremely reduced and only 1–5 flowers develop in one inflorescence as against 16–20 flowers borne by healthy plants [19]. Besides vegetative means, LCCV is transmitted through sap and also by aphids, *Rhopalosiphum maidis* as well as *Myzus persicae* in a non-persistent manner. The disease is caused by the large cardamom chirke virus (LCCV) (Genus: *Macluravirus*; Family: *Potyviridae*) and is prevalent in all large cardamom growing regions [13, 20]. LCCV is monopartite with flexuous filamentous particles of about 625–650 nm in length and 12.2 nm wide containing a linear, single-stranded, positive sense RNA of about 8 kb in size [22]. In addition to large cardamom, LCCV infection on chilli has been reported recently [21]. Currently, polyclonal antiserum-based assays such as enzyme-linked immunosorbent assay (ELISA), dot-immunobinding assay (DIBA), and lateral flow immunoassay (LFIA) have been reported for the detection of LCCV [25]. Serological assays are less sensitive compared to nucleic acid-based assays such as PCR and real-time PCR assays. However, these assays require expensive equipment, and more time to perform the tests, thus limiting the usage of these assays for large-scale screening of plants.

In contrast, isothermal assays such as recombinase polymerase amplification assay (RPA) can be performed without the need for a sophisticated laboratory in a short time [5]. RPA is performed using two primers at a constant temperature of ~ 39 °C for 15–30 min using primers, recombinase, single-stranded DNA binding proteins, and strand-displacing polymerase [18]. RNA viruses can be easily

✉ A. I. Bhat
Ishwarabhat.a@icar.gov.in

[1] ICAR-Indian Institute of Spices Research, Kozhikode, Kerala 673012, India

detected through reverse transcriptase RPA (RT-RPA) and the products can be visualized through gel electrophoresis. RT-RPA has been successfully used for the detection of a large number of viruses infecting different crops [5], such as barley yellow dwarf virus [10], chilli veinal mottle virus [9], cucumber green mottle mosaic virus [8], ginger chlorotic fleck-associated virus 1 & 2 [17], maize chlorotic mottle virus [7], potato virus Y [26] and rose rosette virus [1]. Multiplex RT-RPA that can be used to simultaneously detect more than one plant virus has also been developed for the detection of cucurbit leaf crumple virus, cucurbit yellow stunting disorder virus, and cucurbit chlorotic yellows virus [6]. In the present study, we report the development and validation of RT-RPA assay for the detection of LCCV and its comparison with RT-PCR assay.

Materials and methods

Virus isolates

For the initial standardization of RT-RPA assay, RT-PCR tested LCCV-infected (positive control) (also confirmed through sequencing of the RT-PCR product) and LCCV-free (negative control) large cardamom plants collected from Sikkim, India was used. The RT-RPA assay was validated using field samples of large cardamom with and without symptoms of the disease. For determining the specificity of RT-RPA, a large cardamom sample infected with cardamom bushy dwarf virus (CBDV) confirmed through PCR using specific primers was used.

Primer design and synthesis

Based on the coat protein gene nucleotide sequence of the LCCV (GenBank accession number JN 257715), primers were designed for the RT-RPA assay. The RT-RPA primers were designed as per the guidelines of the RPA reaction kit manufacturer (TwistDx, United Kingdom) (https://www.twistdx.co.uk). Primers for RT-PCR were based on the coat protein gene sequence of the LCCV reported earlier [13]. All the designed primers were synthesized at Eurofins, Bangalore, India [Table 1].

Template preparation

Seven methods of template isolation from *chirke* disease-affected large cardamom plants were attempted. (i) Total RNA was isolated using a commercial kit (RNeasy Plant Mini kit, Qiagen, Germany) according to the manufacturer's instructions. (ii) Total RNA was extracted by LiCl (lithium chloride) method as described previously [4], by grinding 150 mg of leaf tissue in 2 ml freshly prepared CTAB buffer [2% cetyltrimethylammonium bromide (CTAB), 1% polyvinyl pyrrolidone (PVP), 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA]. One ml of the homogenate was incubated at 65 °C for 15 min followed by the addition of chloroform: isoamyl alcohol (24:1). The resulting mixture was centrifuged at 11,000 g for 10 min, aqueous layer was added with an equal volume of 4 M LiCl and incubated overnight at 4 °C. On the next day, the contents were centrifuged at 13,000 g for 25 min, and the pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µl of nuclease-free water. (iii) Crude extract prepared as described earlier [24] in a laminar airflow cabinet by grinding the leaf tissue in 500 vol of 100 mM Tris–HCl (pH 8.0), diluted 10^{-1} to 10^{-3} in the same buffer and used as a template. (iv) Crude sap extracted by placing the leaf in a sterile plastic bag and pressing using a sterile micro pestle in a laminar airflow cabinet as described [11]. The extracted sap was then collected with a micropipette and diluted 10^{-1} to 10^{-3} with sterile distilled water and used as a template. (v) Crude extract was prepared by grinding 50 mg of leaf tissue in 1 ml TE buffer (50 mM Tris, 10 mM EDTA), centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was used as the template [14]. (vi) Tissue was ground in alkaline-PEG buffer (6% PEG 200 and 20 mM NaOH), vortexed, and used as a template [23]. (vii) Touch-and-Go method [2] involved puncturing the leaf sample using a 20 µl pipette tip and mixing it into the reaction mixture. In the first five methods, the template was denatured by heating at 80 °C for 10 min followed by snap cooling in ice for 10 min, and 1 µl of this was used as a template for RT-RPA and RT-PCR, while in the other two methods, the plant extract was directly used as the template.

Table 1 List of primers used for the detection of large cardamom chirke virus by RT-RPA and RT-PCR

Assay	Primer	Sequence	Amplicon (bp)
RT-RPA	Forward	5′– AACAACAGCACAATTTGAGAGATGGGTGGAGG –3′	175
	Reverse	5′– ATATTGGCCACTAGAATGTATTTCCATGTTTTGG –3′	
RT-PCR	Forward	5′– CAGCAAATGATGAGGATGTT –3′	648
	Reverse	5′– TGCTACCTTGAATCTTTCCA –3′	

Development and optimization of RT-RPA

To set the RT-RPA reaction, the supplied freeze-dried reaction pellet of the Twist Amp® basic kit was dissolved in 29.5 µl of rehydration buffer, 2.4 µl (480 nM) each of forward and reverse primer, 1.5 U of RevertAid reverse transcriptase (0.5 µl), 0.25 µl of RNase inhibitor and 7.45 µl nuclease-free water. The resultant mixture was aliquoted equally (8.5 µl each) into five PCR tubes, and a 1 µl template was added to the tubes. For initiating the RT-RPA reaction, 0.5 µl of 280 mM magnesium acetate solution (final concentration of 14 mM) was added and the tube was incubated for 40 min at 39 °C with moderate mixing and spinning briefly after 4 min. The RT-RPA reaction was stopped by keeping the tube at 65 ºC for 10 min. Initially, to check the specificity of designed primers, three RT-RPA reactions were set up using positive control, negative control, and water control (without a template). A 10 µl of RT-RPA product was loaded and run on 2% agarose gel and visualized under the GelDoc system. The specificity of the RT-RPA product was confirmed by cloning the gel-purified DNA band to TA vector, PTZ57R/T (Fermentas, USA) and subjecting it to Sanger sequencing from both directions at the automated sequencing facility at the Eurofins Genomic India Pvt Ltd., Bengaluru, India. The RT-RPA reaction conditions were optimized using different concentrations of magnesium acetate (12–20 mM), temperature (37–41 °C), and different duration of incubation time (5–30 min) and 10 µl of the products from each reaction were analysed through agarose gel electrophoresis.

RT-PCR

Conventional RT-PCR performed as described [3] consisted of 1 µl template, 1 × *Taq* polymerase buffer that contained 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 400 µM dNTP mix, 10 pM each of forward and reverse primers, 1 U of RNase inhibitor, 1.25 U of RevertAid reverse transcriptase, 0.75 U of *Taq* polymerase and RNase free water to make 50 µl. The temperature profile in the thermocycler involved cDNA synthesis at 42 ℃ for 45 min followed by 35 cycles at 94 ℃ for 30 s, 52 ℃ for 30 s and 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. Products of RT-PCR were run on 1% agarose gel electrophoresis and documented using a GelDoc system.

Determination of detection limits and comparison of sensitivity

To determine the sensitivity of RT-RPA and RT-PCR, the total RNA isolated from an LCCV-infected large cardamom plant was subjected to serial dilution (from 10^0 to 10^{-5}) with nuclease-free water, and each dilution was subjected to RT-RPA and RT-PCR. Products were run on an agarose gel to compare the sensitivity of the assays. The experiment was repeated three times to confirm the results. Similarly, 10 ng of the recombinant plasmid DNA harbouring LCCV insert was serially diluted from 10 ng to 1 fg and subjected to RPA reaction to determine the detection limit.

Validation of RT-RPA

For validating the optimized RT-RPA assay, the total RNA isolated from field samples of large cardamom was tested by RT-RPA assay along with RT-PCR assay.

Results

Development and optimization of RT-RPA assay

RT-RPA using total RNA isolated by a commercial kit and LiCl methods was specific for the detection of LCCV as only the positive control showed amplification (Fig. 1). No amplification was seen with healthy and water controls used in the assay. The specificity of the RT-RPA products obtained from both methods was confirmed by sequencing that showed 99% identity to the LCCV. Both these templates were also suitable for the detection of LCCV by RT-PCR assay. RT-RPA performed using total RNA isolated from CBDV-infected large cardamom plant did not show amplification indicating that the primers are specific for the detection of LCCV (Supplementary Fig. S1). RT-RPA carried out using crude extract prepared by Tris-HCl buffer, squeezed sap, TE buffer and alkaline-PEG buffer methods showed amplification in healthy control also, while the Touch-and-Go method showed amplification only in infected samples (Supplementary Table S1). However, sequencing the RT-RPA product obtained from these methods showed no identity with the LCCV sequence. Hence, only the total RNA was used as a template in all further experiments. A magnesium acetate concentration of 14 mM, temperature of 39 °C, and an incubation time of 15 min was found optimum for the detection of LCCV through RT-RPA (Fig. 2).

Determination of detection limits of the virus

The sensitivity of RT-RPA for the detection of LCCV when using total RNA isolated by the kit method was 10^{-4} (Fig. 3a) while it was 10^{-3} for the detection by RT-PCR (Fig. 3c) indicating that RT-RPA is 10 times more sensitive than RT-PCR. The detection limit of RT-RPA using total RNA isolated by LiCl method was 10^{-4} (Fig. 3b) while it was 10^{-1} in RT-PCR (Fig. 3d) indicating that RT-RPA is 1000 times more sensitive than RT-PCR. The detection limit

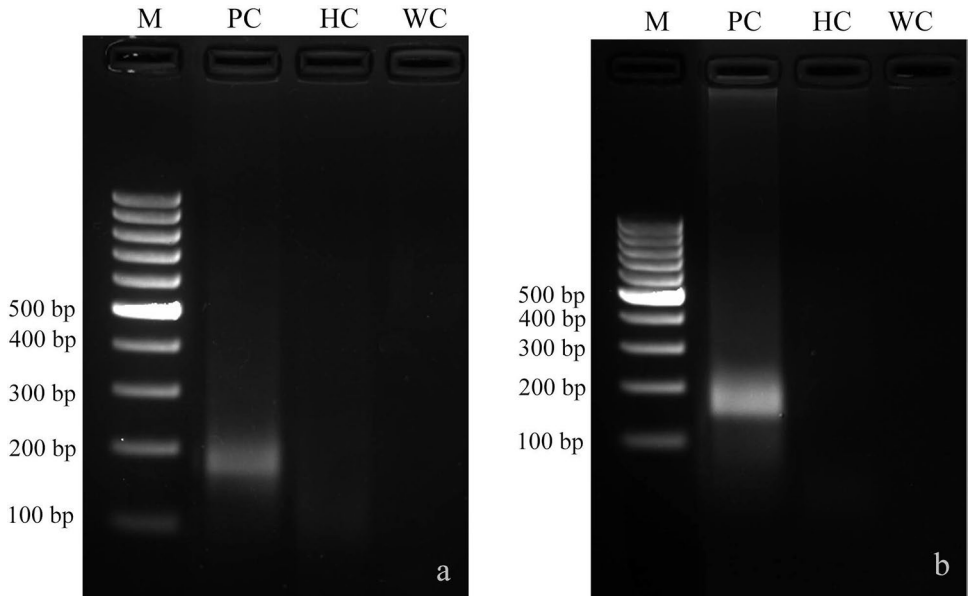

Fig. 1 Detection of large cardamom chirke virus (LCCV) by reverse transcriptase-recombinase polymerase amplification (RT-RPA) using total RNA isolated by **a** a commercial kit and **b** LiCl method. Lane M: Molecular weight markers; Lane PC: Known LCCV-infected large cardamom plant; Lane HC: Known virus-free large cardamom sample and Lane WC: water control

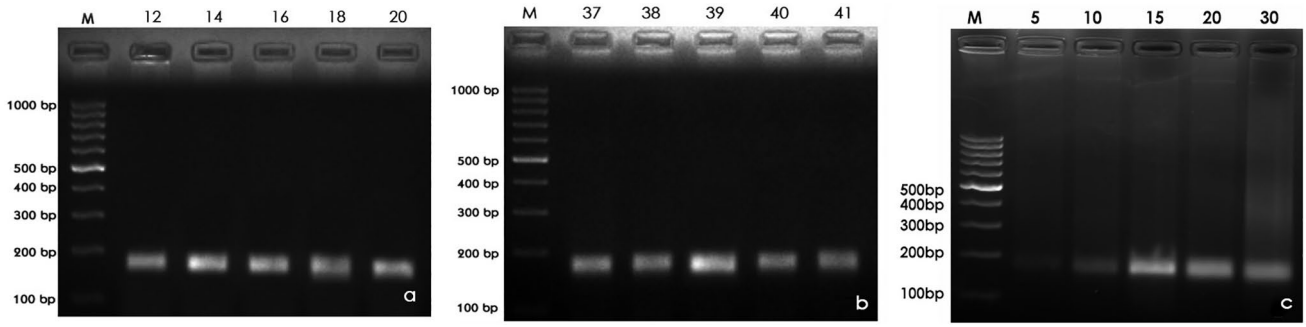

Fig. 2 Optimization of different parameters for the detection of large cardamom chirke virus (LCCV) by reverse transcriptase-recombinase polymerase amplification (RT-RPA). **a** Magnesium acetate. Lanes 12, 14, 16, 18, and 20 are loaded with products of RT-RPA carried out using different concentrations of magnesium acetate in mM. **b** Temperature. Lanes 37–41 are loaded with products of RT-RPA carried out at different temperatures in °C. **c** Time. Lanes 5, 10, 15, 20, and 30 are loaded with products of RT-RPA carried out at different durations in min. Lane M shows molecular weight markers

of the RPA assay was 10 fg when recombinant plasmid DNA was used as the template (Supplementary Fig. S2).

Validation of RT-RPA assay

Validation of RT-RPA assay was carried out with field samples of large cardamom plants collected from Sikkim and Darjeeling areas of northeast regions of India. When total RNA isolated by a commercial kit and LiCl methods from 14 plants (2 symptomatic and 12 asymptomatic) were tested, 9 plants (including two symptomatic) were positive in RT-RPA while the remaining 5 plants (all non-symptomatic) showed a negative reaction to LCCV (Fig. 4a, b). When the templates isolated by LiCl method were subjected to RT-PCR, only 6 plants showed a positive reaction to LCCV, while the remaining 8 plants were negative (Fig. 4c).

Discussion

The currently available diagnostic assays for LCCV detection are time-consuming and require expensive equipment. In the current study, we developed, optimized, and validated RT-RPA assay for the detection of LCCV in large cardamom. The total RNA isolation using a commercial kit and LiCl methods was successful as templates in the detection of LCCV. The specificity of the RT-RPA product was assessed by sequencing. Further, the RT-RPA assay developed in the current study was specific for the detection of LCCV as it did not cross-react with CBDV infecting large cardamom. The methods of crude extracts reported earlier such as the Tris–HCl method used in RT-LAMP to detect chrysanthemum stem necrosis virus in chrysanthemum

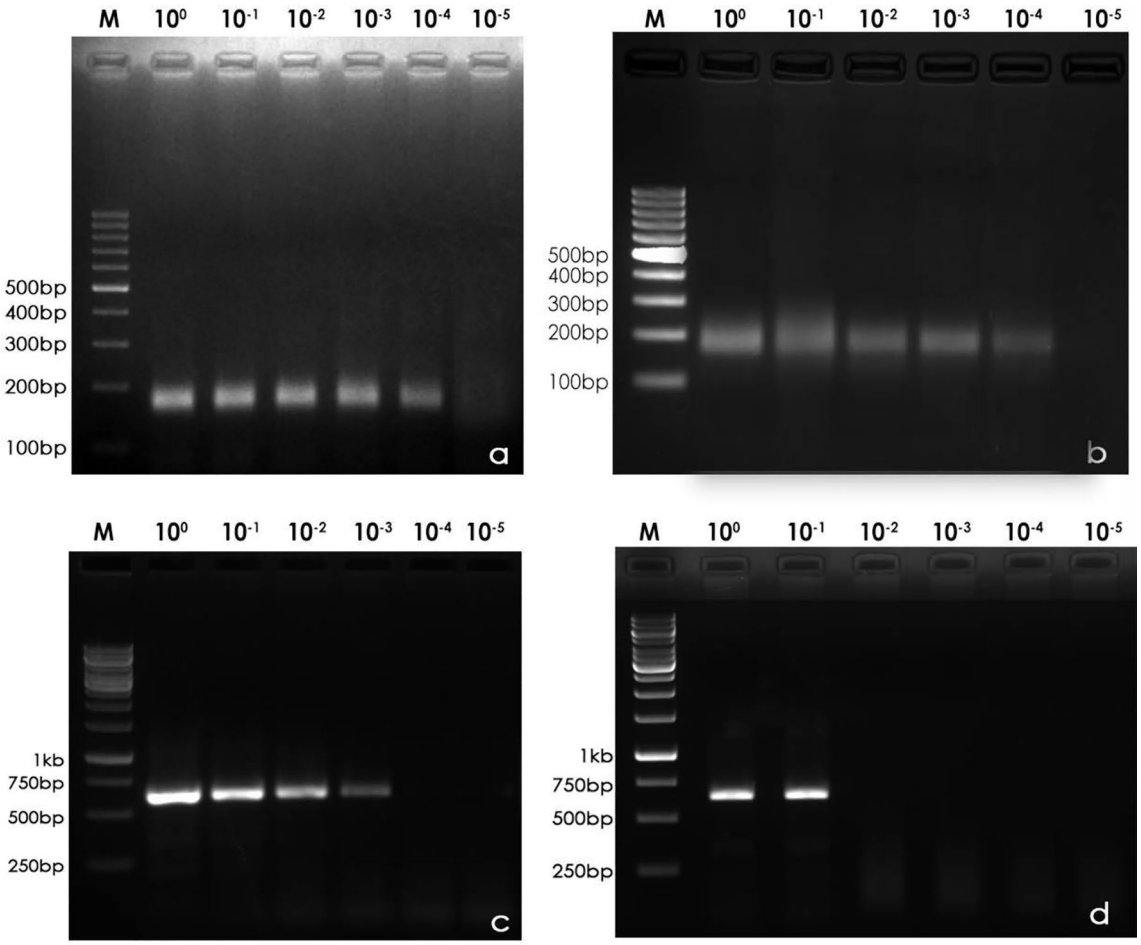

Fig. 3 Comparison of the sensitivity of reverse transcriptase-recombinase polymerase amplification (RT-RPA) (**a**,**b**) and reverse transcriptase-polymerase chain reaction (RT-PCR) (**c**,**d**) assays for detection of large cardamom chirke virus (LCCV) using total RNA isolated by kit method (**a**, **c**) and LiCl method (**b**, **d**). Lanes 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} show different dilutions of the total RNA; lane M shows molecular weight markers

and tomato [24], squeezed sap method used in RT-PCR to detect dendrobium viroid in *Dendrobium* plants [11], TE buffer method used in RT-PCR to detect apple stem grooving virus in apple trees [14], alkaline-PEG buffer method used in RT-RPA to detect yam mosaic virus and yam mild mosaic virus in yam [23] and Touch-and-Go method used to isolate template from *Arabidopsis thaliana* for RT-PCR [2] failed in the present study for the detection of LCCV in large cardamom by RT-RPA. Sequencing of the RT-RPA products obtained when crude extracts from the infected and healthy plants were used as templates revealed no identity with LCCV sequence thus confirming non-specific amplification in both virus-infected and virus-free plants. The failure of crude extracts as templates in the detection of LCCV by RT-RPA might be due to the presence of contaminants or inhibitors specific for large cardamom in the crude extracts.

In the present study, the sensitivity of RT-RPA was 10 times greater than that of RT-PCR for the detection of LCCV when total RNA isolated by the kit method was the template, while it was 1000 times more sensitive than that of RT-PCR when the template isolated by LiCl method was used. Thus, the relative sensitivity of RT-RPA when compared to RT-PCR differed according to the RNA isolation method, while the absolute sensitivity of RT-RPA remained same using both methods. Similar results of higher sensitivity in RT-RPA using total RNA were reported for the detection of barley yellow dwarf virus in oats [10], chilli veinal mottle virus in tobacco [9], citrus concave gum-associated virus in apples [12] and cucumber green mottle mosaic virus in watermelon [8]. The RT-RPA assay developed in the current study was validated for the detection of LCCV using field plants of large cardamom including asymptomatic plants. Three plants that tested

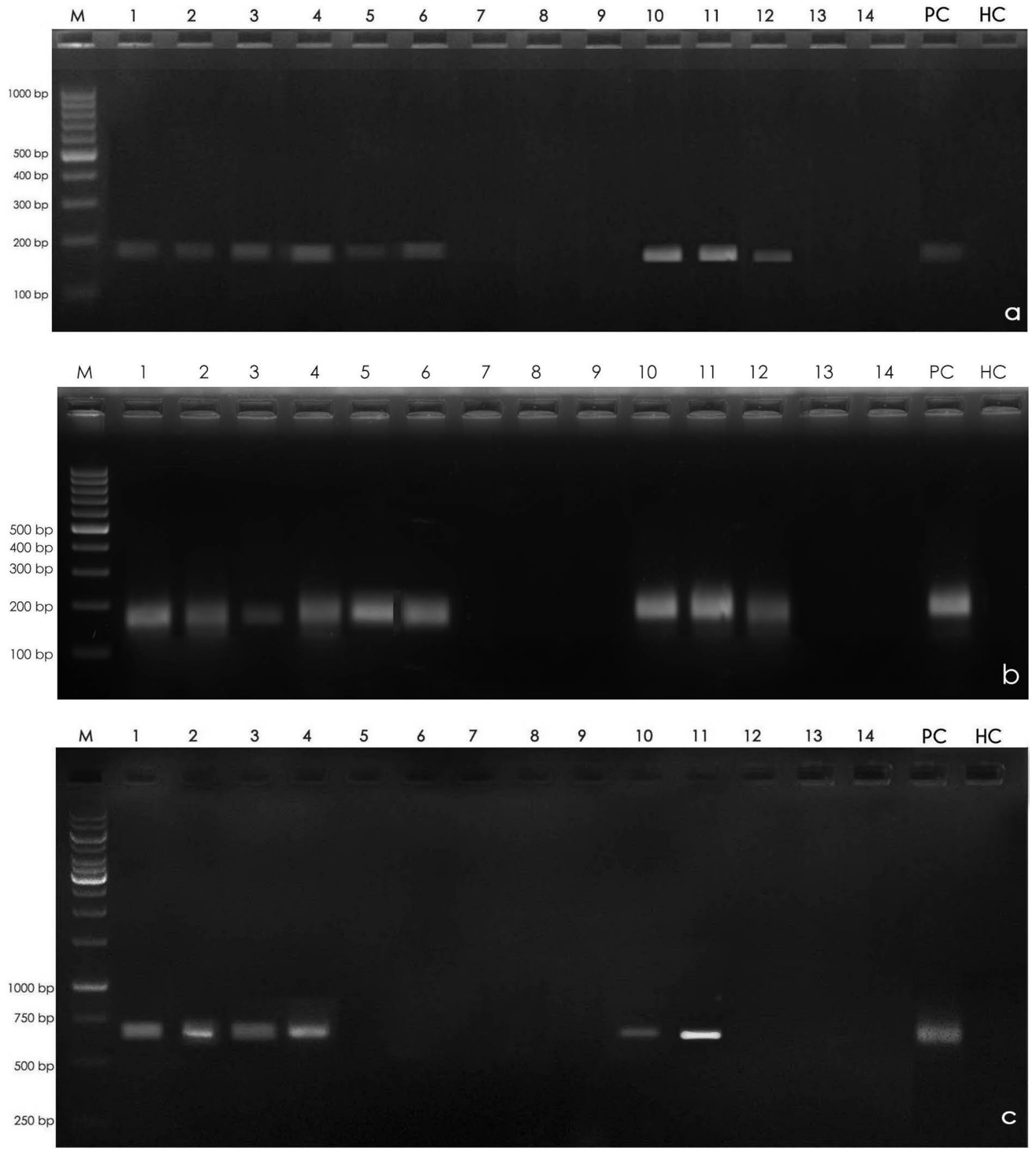

Fig. 4 Validation of reverse transcriptase-recombinase polymerase amplification (RT-RPA) for the detection of large cardamom chirke virus (LCCV) in field samples of large cardamom using **a** total RNA isolated by a commercial kit and **b** LiCl method **c** Detection of LCCV in the same plants through reverse transcriptase-polymerase chain reaction assay (RT-PCR) using total RNA isolated by LiCl method as template. Lane M shows molecular weight markers; lanes 1–14 show different field samples, where 1–9 and 12–13 were asymptomatic while 10 and 11 were symptomatic plants; lane PC indicates positive control, and lane HC indicates healthy control

positive in RT-RPA showed negative reactions when subjected to RT-PCR (Fig. 4). The low sensitivity of RT-PCR to detect LCCV may be due to the PCR inhibitors present in the extract [15].

Assays such as RT-PCR need more time and are expensive in terms of equipment required to perform the tests which limits the wide usage of these assays for routine detection. An alternate technique like RT-RPA is rapid and does not require expensive equipment like a thermal cycler and hence can be performed in resource-poor laboratories [5]. Thus RT-RPA technique is a more practical method to screen a large field population of large cardamom for LCCV as it needs only an incubator for setting up the reaction. Furthermore, it is a very simple assay that requires a lower incubation temperature (about 37–42 °C) and lesser incubation time (about 15–30 min) [16]. In conclusion, in the present study molecular isothermal assay, namely RT-RPA was optimized for the specific and sensitive detection of LCCV infecting large cardamom using total RNA as a template. We believe that the assay will enable the identification of LCCV-free plants that can be used as mother plants for the propagation of virus-free large cardamom plants. Besides, diagnostics can also be used to detect LCCV in potential insect vectors of the virus and screen large cardamom germplasm to identify sources of resistance and in epidemiology studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13337-024-00861-2.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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