Reverse transcription loop-mediated isothermal amplification to rapidly detect *Rice ragged stunt virus*

Dingwang Lai a, b, Yuliang Zhang a, Qixing Huang a, Guohua Yin a, c, *, Kayla K. Pennerman c, Zhixin Liu a, Anping Guo a, *

a Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan 571101, China
b College of Agriculture, Hainan University, Haikou, Hainan 570228, China
c Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901, USA

Received 22 October 2015; revised 20 February 2016; accepted 23 February 2016
Available online 02 March 2016

**KEYWORDS**
*Rice ragged stunt virus* (RRSV); Reverse transcription loop-mediated isothermal amplification (RT-LAMP); RT-PCR

**Abstract** *Rice ragged stunt virus* (RRSV) is a very important virus that infects rice and causes serious yield losses in Asian countries and other major rice planting areas. Thus, it is urgent to establish an efficient and practical approach for identification and diagnosis in the field. Our results indicated that reverse transcription loop-mediated isothermal amplification (RT-LAMP) reactions are more efficient and sensitive than RT-PCR for RRSV detection. The optimal LAMP conditions were as follows: 0.4–1.2 μM internal primers, 0.2–0.25 μM external primers, 0.8 μM loop primers, and incubation at 62 °C or 63 °C for 30 min. Furthermore, the RT-LAMP primers specifically targeted RRSV virus and resulted in typical waterfall-like bands by gel electrophoresis and sigmoidal amplification curves. The primers could not be used to amplify other common plant viruses including *Papaya ringspot virus* (PRSV), *Rice yellow stunt virus* (RYSV), *Sorghum mosaic virus* (SrMV), *Cactus virus X* (CVX), *Melon yellow spot virus* (MYSV) and *Southern rice black-streaked dwarf virus* (SRBSDV). Ten-fold serial dilutions of RRSV cDNA indicated that RT-LAMP is much faster and at least ten times more sensitive than RT-PCR in detecting the virus. The waterfall-like product bands could be observed within one hour. In the field study, about 77% samples were identified as RRSV. RT-LAMP has many benefits over RT-PCR such as low cost and high accuracy, sensitivity,
1. Introduction

Rice ragged stunt virus (RRSV), a member of Reoviridae, is mainly transmitted and spread by the brown plant hopper in a persistent manner (Cabauatan et al., 2009). It was first discovered in 1976 in Indonesia and the Philippines (Ling et al., 1978). In China, RRSV occurrence is very low in Hunan, Zhejiang, Fujian, and Guangdong provinces where it appears to spread by mechanical sap inoculation, grafting and vegetative propagation (Li et al., 2014; Wang et al., 2014). Symptoms of RRSV infection included curly and serrate rice leaves, swelled veins and abnormal protruding “tumors” or enations (Fig. 1).

Indicator plants, electron microscopy, enzyme-linked immunosorbent assays (ELISA), and RT-PCR are often used approaches to inspect viruses. However, these methods are slow, require high virus titer and/or sometimes yield false positives. RT-PCR is generally considered sensitive and reliable, but it necessitates a special thermocycler that cannot be practiced in the field. RT-LAMP is a novel approach based on autocycling strand displacement DNA synthesis. It was usually performed with a set of six specially designed primers that recognize the distinct sequences of the target under isothermal conditions. It has been used to detect a wide array of pathogens and transgenes in many different biological systems (Almasi et al., 2013; Aydin-Schmidt et al., 2014; Kil et al., 2015; Kimura et al., 2005; Notomi et al., 2000). It can detect target sequences at a constant temperature from 60 to 65 °C with high sensitivity and specificity. With the addition of a DNA binding dye such as SYBR Green I (Life Technologies, USA), the success of the reactions can be easily evaluated without downstream processing (Almasi et al., 2013; Peng et al., 2012; Shen et al., 2014a,b).

In this study, we aimed to establish an efficient RT-LAMP method to detect RRSV in infected rice. Our method may serve as a basis for seedling or propagule quarantine, and diagnosis of RRSV.

2. Materials and methods

2.1. Plant samples and reagents

Rice infected with RRSV was collected in the Hainan winter-breeding areas in Sanya, Lingshui and Qionghai in Hainan Island, China. Positive controls were provided by the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. E.Z.N.A. RNA Isolation Kit was purchased from Omega Bio-Tek, Inc. (USA); Bst DNA polymerase, betaine solution, dNTPs, fluorescent dyes, and DL2000 DNA marker were purchased from the Guangzhou Gene Deaou Company (China); PrimeScript RT-PCR kit, RNase Inhibitor, and DEPC were purchased from Takara Bio (Japan).

![Figure 1 Typical symptoms on rice infected by RRSV.](image)

2.2. Primers

Using the RRSV genome sequence (NCBI accession No. GQ329711.1), we performed homology analysis with DNA-MAN 7.0 (Lynnon Biosoft) to identify the unique sequences for primer design using Primer3 Input (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Three pairs of specific primers for LAMP and one pair of primers for PCR were made (Table 1). All the primers were synthesized by the Shanghai Sangon Company (China).

2.3. Total RNA extraction, RT-PCR and RT-LAMP

Total RNA was extracted from ~200 mg of fresh rice leaf samples using the E.Z.N.A. RNA Isolation Kit according to the manufacturer’s instructions and was stored at −80 °C for later use. RT-PCR was performed in a mixture containing 1.25 μM of random primers, 1.25 μM/L of oligo(dT)18, 0.25 mM of dNTPs, 1 μL of total RNA (50 ng), and added nuclease-free water to a final volume of 15 μL. First, the mixture was incubated at 65 °C for 5 min and chilled on ice for 2 min. Next, 0.5 μL of MLV reverse transcriptase (100 U), 0.5 μL of RNase Inhibitor (20 U) and 4 μL of 5X M-MLV buffer were added. Then, the mixture was incubated at 42 °C for 1 h followed by inactivation of the reverse transcriptase at 75 °C for 15 min and stored at −80 °C for later use. PCR was performed in a 20 μL reaction system including 1 μL cDNA template, 0.2 μL Pfu DNA polymerase (2.5 U/μL), 0.5 μL of 10 μM RRSV-P1/RRSV-P2 primers, 1 μL dNTPs, and added ddH2O to a final volume of 20 μL. The thermocycler program was performed as follows: pre-heated at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Eight microliters of PCR product was mixed with 1 μL GoldView dye for electrophoresis on a 1.5% agarose gel at 120 V for 15 min.

RT-LAMP was performed in a 25 μL system including 0.2 μM of external primers RRSV-F3 and RRSV-B3, 1.6 μM of internal primers RRSV-FIP and RRSV-BIP, and specificity. This technology meets the requirements for rapid diagnosis of plant virus diseases in the field to best guide management practices for growers.

© 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Table 1  Primers used for RRSV virus detection by LAMP and RT-PCR.

| Primers   | Sequences (5'-3') | Genome position | Purpose                      |
|-----------|-------------------|-----------------|------------------------------|
| RRSV-F3   | GGAAGCAGTTAAGTAAGTTGTG | 541–562         | RT-LAMP external primers    |
| RRSV-B3   | CCACCTACGTTGCTTT | 797–814         |                              |
| RRSV-FIP  | GAGGGGCGCTCATCGAGATGCTGGATGCAAGGTA | 647–667, 593–611 | RT-LAMP internal primers    |
| RRSV-BIP  | ACAACAAATGGCTTTTGCGAGCTTAGATCGAAGGTTGATGGA | 691–712, 746–766 |                              |
| RRSV-FLP  | TCCCTAGGCGGATATGTTGTAAGTC | 622–643         | RT-LAMP loop primers        |
| RRSV-BLP  | GGAATTAGCTAGAGGACAGACACAGG | 715–736         |                              |
| RRSV-P1   | TTCAACCAGCGCTGATCAAGCAG | 197–217         | RT-PCR primers              |
| RRSV-P2   | TACTCTGTGTCCATTAGCTTCGCTCG | 883–905         |                              |

Figure 2  RT-LAMP detection of RRSV under different reaction temperatures. RT-LAMP amplification products were subjected to 1.5% agarose gel electrophoresis (A) and their amplification curves were monitored under different temperatures (B). Lane M: DL2000 DNA Marker; other lanes were RT-LAMP products from different reaction temperatures: 50 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, and 75 °C.
0.8 μM of loop primers RRSV-FLP and RRSV-BLP, 1.4 mM dNTPs, 0.8 M betaine, 6 mM MgSO₄, 2.5 μL 10X ThermoPol II (Mg-free) Reaction Buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100), 8 U Bst DNA polymerase (New England Biolabs, USA), 5 U AMV reverse transcriptase XL (Takara, Japan), 0.5 μL total RNA, and added nuclease-free water to a final volume of 25 μL. The mixture was covered with 20 μL mineral oil and was loaded on a 308 °C thermostat fluorescence detection system (Deaou Biological Technology, China). The reaction was performed at a certain temperature between 50 and 75 °C for at least 30 min, and then heated at 80 °C for 10 min to end the reaction. The amplification curve was checked to determine if there was RRSV virus in the plant sample. The products were inspected on a 1.5% agarose gel after electrophoresis at 120 V for 15 min.

2.4. RT-LAMP optimization

To optimize the RT-LAMP reaction, we selected eight different reaction temperatures (50 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, and 75 °C), four reaction times (30 min, 45 min, 60 min, and 75 min) and four different concentrations of primers (0.4 μM, 0.8 μM, 1.2 μM, and 1.6 μM for internal primers; 0.1 μM, 0.15 μM, 0.2 μM, and 0.25 μM for external primers; 0.1 μM, 0.2 μM, 0.4 μM, and 0.8 μM for loop primers) to compare the amplification effects of different reaction conditions on LAMP.

2.5. Comparison of the specificities and sensitivities of RT-LAMP and RT-PCR

The cDNA from RRSV and six other plant viruses including Rice yellow stunt virus (RYSV), Rice grassy stunt virus (RGSV), Sorghum mosaic virus (SrMV), Southern rice black-streaked dwarf virus (SRBSDV), Cactus virus X (CVX), and Melon yellow spot virus (MYSV) were selected to determine the specificity of LAMP. The cDNA from rice leaves infected with RRSV was 10-fold serially diluted (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸).

2.6. Field diagnosis of rice virus using RT-PCR and RT-LAMP

Twenty-six rice leaves from plants suspected to be infected with RRSV were collected in the field. RT-LAMP and RT-PCR were performed to determine RRSV infection.

3. Results

3.1. Optimization of the LAMP reaction to detect RRSV

The reaction temperature, time, and suitable concentrations of three pairs of primers were optimized using total RNA extracted from plant leaves infected with RRSV. Successful RT-LAMP reactions can produce waterfall-like bands after gel electrophoresis. RT-LAMP was performed under different reaction temperatures from 50 °C to 75 °C. The results showed

Figure 3  RT-LAMP detection of RRSV under different reaction times. RT-LAMP amplification bands were subjected to 1.5% agarose gel electrophoresis (A) and the application curves under different reaction times (B). Lane M: DL2000 DNA Marker; other lanes were LAMP products from different reaction times: 30 min, 45 min, 60 min, and 75 min.
that no bands could be produced at 50 °C and 75 °C, and successful RT-LAMP reactions are from 60 °C to 65 °C. RT-LAMP at 63 °C produced very clear and bright stepwise bands, and a typical sigmoidal amplification curve (Fig. 2). Other optimization results indicated that 30 min was adequate for reaction completion and useful concentrations for the internal, external and loop primers were 0.4–1.2 μM, 0.2–0.25 μM, and 0.8 μM, respectively (Figs. 3 and 4). For field diagnosis, we chose 1.2 μM of internal primers, 0.25 μM of external primer, and 0.8 μM of loop primers to detect RRSV infection.

3.2. Specificity of RT-LAMP to detect RRSV

Total RNA extracted from fresh tissues, each infected with RRSV or one of six other plant viruses (RYSV, RGSV, SrMV, SRBSDV, CVX, and MYSV), was used to evaluate the specificity of RT-LAMP with designed primers. Agarose gel
electrophoresis and RT-LAMP results indicated that only RRSV-infected tissues yielded the characteristic waterfall-like bands and a sigmoidal amplification curve while these were not detected within the other six plant viruses (Fig. 5).

3.3. Sensitivity comparison of RT-PCR and RT-LAMP for RRSV

To compare the sensitivities of RT-PCR and RT-LAMP, the cDNA was serially 10-fold diluted (50–5.0 ng). RT-PCR could detect $10^{-1}$ and $10^{-2}$ diluted cDNA (Fig. 6A) while RT-LAMP could detect $10^{-1}$ to $10^{-3}$ diluted cDNA (Fig. 6B and C). Therefore, RT-LAMP was at least ten times more sensitive than RT-PCR.

3.4. Field diagnostic of RRSV by RT-PCR and RT-LAMP

In order to test the possibility of RT-LAMP technology in practical applications, we collected and analyzed twenty-six samples suspected to be infected with RRSV to perform RT-LAMP. Altogether, 20 samples (77%) were diagnosed as being infected with RRSV (Fig. 7). These results were consistent with the RT-PCR results.

4. Discussion and conclusion

Assessment of rice virus disease incidence is done by visually inspecting the plants for symptoms, which happens when the disease is already prevalent. Consequently, this impacts the efficacy of subsequent control measures. Visual observation is not very reliable as similar symptoms can be due to very different reasons. Current detection methods are expensive and time-consuming, and sometimes unable to recognize low amounts of viruses. Using RT-LAMP to quickly detect RRSV would mean reduced misdiagnosis of rice diseases and timely delivery of pest management systems, translating into reduced costs from expenditure and misuse of pesticides.

In our study, the sensitivity of RT-LAMP technology was at least ten times higher than traditional RT-PCR methods. Moreover, RT-LAMP also indicated high specificity in detection of plant viruses. This is similar to what has been previously observed for other viruses (Chen et al., 2013; Fan et al., 2013; Liu et al., 2010; Wen et al., 2010). False positive rates can be further reduced by careful cleaning of workspaces and avoidance of sample aerosolization to prevent cross-contamination.

We established an efficient RT-LAMP protocol for RRSV detection that can be practically applied in field inspections. Due to its simplicity, rapidness and specificity over RT-PCR, RT-LAMP could be a preferred diagnostic tool for technical and non-technical personnel.
Author contributions

GHY and APG conceived and designed the experiments. DWL and QXH performed the experiments. DWL, YLZ, and GHY analyzed the data. All the authors contributed to the writing of the paper. The authors have declared that no competing interests exist.

Acknowledgments

This work was partially funded by the Special Fund for Agro-scientific Research in the Public Interest of the People’s Republic of China (Grant No. 201403075) and the Major Science and Technology Program of Hainan Province (ZDDXZ2013010). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Almasi, M.A., Erfan Manesh, M., Jafary, H., Dehabadi, S.M., 2013. Visual detection of potato leaf roll virus by loop-mediated isothermal amplification of DNA with the GeneFinder dye. J. Virol. Methods 192, 51–54.

Aydin-Schmidt, B., Xu, W., Gonzalez, I.J., Polley, S.D., Bell, D., Shakely, D., Msellem, M.I., Bjorkman, A., Martenson, A., 2014. Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias. PLoS One 9, e103905.

Cabauatan, P.Q., Cabunagan, R.C., Cho, I.-R., 2009. Rice viruses transmitted by the brown planthopper Nilaparvata lugens Stål. In: Heong, K.L., Hardy, B.D. (Eds.), Planthoppers: New Threats to the Sustainability of Intensive Rice Production Systems in Asia. International Rice Research Institute, Los Baños (Philippines), pp. 357–368.

Chen, X.F., Zhang, J.H., Cui, J.X., Zhang, H.L., Guo, L.X., Yu, S.Q., Fan, X., Dong, Y.F., Zhang, Z.P., Ren, F., Hu, G.J., Zhu, H.J., 2013. Detection of Arabis mosaic virus by RT-PCR. Acta Phytophylacica Sin. 40, 189–190 (in Chinese).

Chen, X.F., Zhang, J.H., Cui, J.X., Zhang, H.L., Guo, L.X., Yu, S.Q., Fan, X., Dong, Y.F., Zhang, Z.P., Ren, F., Hu, G.J., Zhu, H.J., 2013. RT-LAMP assay for detection of Grapevine rupestris stem pitting-associated virus. Acta Phytopathol. Sin. 43, 286–293 [in Chinese].

Kil, E.J., Kim, S., Lee, Y.J., Kang, E.H., Lee, M., Cho, S.H., Kim, M.K., Lee, K.Y., Heo, N.Y., Choi, H.S., Kwon, S.T., Lee, S., 2015. Advanced loop-mediated isothermal amplification method for sensitive and specific detection of Tomato chlorosis virus using a uracil DNA glycosylase to control carry-over contamination. J. Virol. Methods 213, 68–74.

Kimura, H., Ihira, M., Enomoto, Y., Kawada, J., Ito, Y., Morishima, T., Yoshikawa, T., Asano, Y., 2005. Rapid detection of herpes simplex virus DNA in cerebrospinal fluid: comparison between loop-mediated isothermal amplification and real-time PCR. Med. Microbiol. Immunol. 194, 181–185.

Li, S., Wang, H., Zhou, G., 2014. Synergism between Southern rice black-streaked dwarf virus and Rice ragged stunt virus enhances their insect vector acquisition. Phytopathology 104, 794–799.

Ling, K.C., Tiongco, C.E., Aguero, V.M., 1978. Rice ragged stunt, a new virus disease. Plant Dis. Rep. 62, 701–705.

Liu, J., Huang, C.L., Wu, Z.Y., Zhang, X.H., Wang, Y.Q., 2010. Detection of Tomato aspermy virus infecting chrysanthemums by LAMP. Sci. Agric. Sin. 43, 1288–1294 [in Chinese].

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Peng, J., Zhang, J., Xia, Z., Li, Y., Huang, J., Fan, Z., 2012. Rapid and sensitive detection of Banana bunchy top virus by loop-mediated isothermal amplification. J. Virol. Methods 185, 254–258.

Shen, W., Tuo, D., Yan, P., Li, X., Zhou, P., 2014a. Detection of Papaya leaf distortion mosaic virus by reverse-transcription loop-mediated isothermal amplification. J. Virol. Methods 195, 174–179.
Shen, W., Tuo, D., Yan, P., Yang, Y., Li, X., Zhou, P., 2014b. Reverse transcription loop-mediated isothermal amplification assay for rapid detection of *Papaya ringspot virus*. J. Virol. Methods 204, 93–100.

Wang, H., Xu, D., Pu, L., Zhou, G., 2014. *Southern rice black-streaked dwarf virus* alters insect vectors’ host orientation preferences to enhance spread and increase *Rice ragged stunt virus* co-infection. Phytopathology 104, 196–201.

Wen, W.G., Yang, C.Y., Cui, J.X., Zhang, Y., 2010. Detection of *Bean pod mottle virus* by RT-PCR. Plant Prot. 36, 139–141 (in Chinese).