Reverse Transcription Loop-mediated Isothermal Amplificationを用いたRaspberry ringspot virusの検出

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| 著者 | 森本, 圭  
筒井, 康貴  
前川, 晃演  
藤原, 裕治  
小原, 達二 |
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Detection of *Raspberry ringspot virus* Using Reverse Transcription Loop-mediated Isothermal Amplification

Kei MORIMOTO, Yasutaka TSUTSUI¹, Akinobu MAEKAWA², Yuji FUJIWARA³ and Tatsuji OHARA⁴

Kansai Airport Substation, Kobe Plant Protection Station
¹I. Senshukukounaka, Tajiricho, Sennangun, Osaka, 549-0011 Japan
²Kinki Regional Agricultural Administration Office, Kobe Plant Protection Station
³Research Division, Yokohama Plant Protection Station
⁴Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries

**Abstract:** *Raspberry ringspot virus* (RpRSV), which has not occurred in Japan, infects many plants such as raspberry, gooseberry, cherry, grapevine, and strawberry. Biological assay, enzyme-linked immunosorbent assay (ELISA), and reverse transcription polymerase chain reaction (RT-PCR) have been introduced for the detection of RpRSV in postentry quarantine in Japan. We studied reverse transcription loop-mediated isothermal amplification (RT-LAMP) for RpRSV detection. As a result, the detection limit in RT-LAMP that employed primers specific for RpRSV was more than 10 times as high as that in the RT-PCR. Furthermore, simple and rapid sample preparation methods were evaluated, and the virus detection time was able to be reduced compared to modified SDS-Potassium acetate method.

**Key words:** *Raspberry ringspot virus*, RT-LAMP, simple, rapid, nucleic acid extraction

**Introduction**

*Raspberry ringspot virus* (RpRSV) has not occurred in Japan. The virus was first discovered in Scotland on *Rubus idaeus* (CADMAN et al., 1956) and then was reported in Turkey, Kazakhstan, and other European countries. The virus belongs to the genus *Nepovirus*, and it was isolated from a variety of fruit trees such as raspberry, gooseberry, cherry, grapevine, and herbaceous plants such as strawberry, narcissus, anemone, chickweed, and various wild plants. In particular, enormous damage caused by infection with this virus was reported on raspberry and strawberry. Symptoms such as leaf curl, chlorotic spot, and dwarfism were observed, and these plants were sometimes seriously infected and then withered and died (NISIO et al., 1981; CROP PROTECTION COMPENDIUM 2007).

Biological assay, enzyme-linked immunosorbent assay (ELISA), and reverse transcription polymerase chain reaction (RT-PCR) have been introduced for the detection of the virus in postentry quarantine in Japan. In this study, loop-mediated isothermal amplification (RT-LAMP) (NOTOMI et al., 2000), which is a method for rapid detection and has an excellent detection limit, was evaluated as a detection tool for RpRSV.

In addition, simple and rapid sample preparation procedures for RT-LAMP are required at the sites of plant quarantine. Therefore, simple and rapid two-sample preparation proceedings for RT-LAMP were tested.

**Materials and Methods**

1. **Virus isolate**

We detected an RpRSV infected anemone (*Anemone japonica*) that had been imported from the United Kingdom at postentry quarantine in 1997. This infected plant has been maintained under the special import permit issued by the Minister of Agriculture, Forestry and Fisheries. RpRSV was mechanically transmitted from the RpRSV infected anemone plant to *Chenopodium quinoa* by the sap inoculation method, and an isolate used in this study was obtained by the single lesion isolation method.
2. Design of RT-LAMP primers for RpRSV detection

The LAMP primer set was designed using Primer Explorer V3 (Eiken Chemical Co. Ltd. and Fujitsu Ltd.) from sequence data in the coat protein-coding region of seven isolates of RpRSV (GenBank accession No. AF110796 [Lloyd George], AF116189 [Orr], AF116190 [Shepherd], AF116191 [Tarvit], S46011 [Scottish], AF111114 [MX], AF110476 [Himalaya El]), which were multiple-aligned using ClustalW (DDBJ).

3. Extraction of total nucleic acid

The SDS–Potassium acetate method described by DELLAPORTA et al. (1983) was modified, and total nucleic acid was extracted. This method is as follows. Plant tissue (0.05 g) was ground in 1.0 ml extraction buffer (1% 2-mercaptoethanol, 0.1 M Tris-HCl pH 8.0, 0.05 M EDTA pH 8.0, 0.5 M NaCl). Seven hundred μl of suspension was transferred to a new tube, and 50 μl of 20% SDS solution was added. Mixture after incubation for 10 min at 65°C was added to 250 μl of 5 M potassium acetate, and put on ice for 20 min. After centrifugation for 10 min at 13,000 × g at 4°C, 450 μl of supernatant was transferred to a new tube and the total RNA was precipitated by adding 270 μl of 2-propanol and put on ice for 10 min. After centrifugation at 20,000 × g for 5 min at 4°C, the pellet was dried and resuspended in 25 μl of sterilized water. This suspension was used as the template RNA for the RT-LAMP reaction.

4. RT-LAMP reaction

The Loopamp RNA amplification reagent kit (Eiken Chemical Co.) was used for RT-LAMP reaction as follows. Reaction mixture consisted of 25 μl of the mixture containing 0.2 μM each of F3 and B3 primers, 1.6 μM each of FIP and BIP primers, 0.8 μM of LB primer, 1 μl of Enzyme mix, and 2 μl of the total nucleic acid. The target gene amplification was measured by a real-time turbidity meter (LA-200, Teramecs Co.). Reactions were carried out at 60°C for 60 min, and 0.5 μl of the RT-LAMP amplification products was analyzed by electrophoresis in 2% agarose gel in order to confirm characteristic ladder bands as appropriate.

5. Specificity of the designed primers

Arabis mosaic virus (ArMV), Grapesvine fanleaf virus (GFLV), and Tomato ringspot virus (ToRSV) belong to the genus Nepovirus and were used in this test. The total nucleic acid of each virus was extracted by the modified SDS–Potassium acetate method from infected C. quinoa, and reaction specific of RT-LAMP was tested for each.

6. Comparison of RT-LAMP and RT-PCR detection limits

Detection limits of RT-LAMP and RT-PCR were compared by 10-fold serial dilution samples (10⁻¹ to 10⁰). In RT-PCR, the RT reaction was conducted using a mixture containing Random Hexamer (TaKaRa) and AMV reverse transcriptase (TaKaRa). After RT reaction at 42°C for 30 min, PCR was conducted using AmpliTaq Gold mixture (Applied Biosystems) with primer set Rp F1 (5’-CTATGAGGTAGATCCATTAC-3’) and Rp R1 (5’-GGGAGATGATATCCCATTC-3’), which was designed for this study based on the coat protein coding region of RpRSV. The PCR cycles were as follows; one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min. PCR products were analyzed by electrophoresis in 2% agarose gel, and the limit of detection was compared between RT-LAMP and RT-PCR.

7. Evaluation of simple and rapid preparation methods of samples for RT-LAMP

(1) Toothpick method and crude sap method

Two sample preparation methods, the toothpick method and the crude sap method (FUKUTA et al., 2005; TAKEUCHI et al., 2006), were evaluated in terms of simple and rapid preparation methods. Leaves of RpRSV-infected anemone and C. quinoa were used as samples, 20 samples were prepared by each method, and processing time was measured for each.

(a) Toothpick method

The leaf was picked with a toothpick, and this handling was repeated five times. The tip of the toothpick was soaked in RT-LAMP reaction mixture. The samples were assayed by RT-LAMP.

(b) Crude sap method

A sample of 100 mg of a leaf was ground with 400 μl of 0.1 M Tris-HCl pH 8.0, and 5 μl of the sap was diluted with...
Table 1. RT-LAMP primer sequences for RpRSV detection

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<tr>
<th>Primer</th>
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<tr>
<td>RpRSV F3</td>
<td>GCATCAATAACATTGCGGA</td>
</tr>
<tr>
<td>RpRSV F3</td>
<td>TGCCGTAAGTACAGAT</td>
</tr>
<tr>
<td>RpRSV FIP</td>
<td>ACATCATATTCCCACGCGCTGGTGAACCAGGTA</td>
</tr>
<tr>
<td>RpRSV BIP</td>
<td>GTTTTCAGTTACCGATGGCCTTTGACTCAACAAGA</td>
</tr>
<tr>
<td>RpRSV LB</td>
<td>ACGTCTCCATATTACGGTGCC</td>
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Fig. 1. Turbidity of RT-LAMP reaction using designed primer set. RpRSV positive samples (●: C. quinoa and ■: anemone) and negative samples (○: healthy C. quinoa, □: healthy anemone, and △: distilled water).

195 μl of the same buffer; 2 μl of the diluted sap was assayed by RT-LAMP.

(2) Simultaneous processing of a large number of samples

The toothpick method was modified and tested as a simultaneous processing method for treatment of a large number of samples. An infected leaf was picked with a toothpick five times, and the tip of the toothpick was soaked in 250 μl of 0.1 M Tris-HCl pH 8.0; this procedure was repeated the prescribed number of times. The simultaneous processing consisted of samples of 10, 20, 30, 40, and 50 anemone leaves, each including one infected anemone leaf; 2 μl of suspension was tested by RT-LAMP reaction.

Results

1. Detection of RpRSV by RT-LAMP using the designed primer set

Table 1 shows each primer sequence of the designed primers set, and figure 1 shows RT-LAMP reaction using the primer set. Turbidity of infected C. quinoa and anemone samples increased characteristically 25 to 30 min after the reaction started, and ladder bands peculiar to LAMP amplification products were observed by electrophoresis with positive control (Fig. 2). However, the rise of turbidity was not observed on negative controls (healthy C. quinoa and anemone, and water) and ArMV, GFLV, and ToRSV for 90 min.

Figure 3 shows the result of the comparative test of detection limits between RT-LAMP and RT-PCR. RpRSV could be detected by RT-LAMP from the extracted total nucleic acid that was diluted to $1 \times 10^{-5}$, and the RpRSV detection limit by RT-PCR was $1 \times 10^{-4}$ dilution.
Fig. 2. Agarose gel electrophoresis of the specific amplification products by RT-LAMP for RpRSV detection. Lane M: 100 bp DNA ladder marker; lane 1: infected anemone; lane 2: healthy anemone; lane 3: distilled water.

Fig. 3. Comparison on the detection limit of RpRSV between RT-LAMP (A) and RT-PCR (B). (A) Turbidity of the RT-LAMP reaction with RpRSV-infected C. quinoa after serial 10-fold dilution from $10^{-6}$ to $10^{-5}$ (○: $10^{-5}$, ▲: $10^{-4}$, ■: $10^{-3}$, ○: $10^{-2}$, □: $10^{-1}$). (B) Agarose gel electrophoresis of RT-PCR from total RNA extracted from infected C. quinoa. Lane M: DNA size marker (100 bp ladder). Lane 1: $1 \times 10^{-5}$; Lane 2: $1 \times 10^{-4}$; Lane 3: $1 \times 10^{-3}$; Lane 4: $1 \times 10^{-2}$; Lane 5: $1 \times 10^{-1}$; Lane 6: $1 \times 10^{-3}$; Lane 7: $1 \times 10^{-6}$.

2. Evaluation of simple and rapid preparation methods of samples for RT-LAMP

(1) Toothpick method and crude sap method

The virus could be detected from RpRSV-infected anemone and C. quinoa leaves using the toothpick method. Twenty samples were able to be prepared in approximately 15 min using this method.

Also, the virus could be detected from crude sap prepared by grinding RpRSV-infected anemone and C. quinoa with 0.1 M Tris-HCl pH 8.0. Twenty samples were able to be prepared in approximately 30 min. On the other hand, the modified SDS-Potassium acetate method required approximately 150 min for preparation of 20 samples.

(2) Simultaneous processing for a large number of samples

RpRSV could be detected from any simultaneously processed samples used in this study (Fig. 4). RT-LAMP could
even detect the virus in the suspension that was prepared by the toothpick method from a sample of 50 anemone leaves that included one infected leaf.

**Discussion**

The RT-LAMP primer set for RpRSV detection was designed in a conserved region of seven RpRSV strain sequences. The primer set could constantly detect RpRSV within 60 min. Turbidity of positive controls increased in 25 to 30 min after the reaction started, and it was not observed in healthy plants. In addition, ArMV, GFLV, and ToRSV used in this study as closed virus species, which belong to the genus Nepovirus, were not detected by the RT-LAMP test. This result showed that this RT-LAMP primer set was RpRSV-specific and has useful characteristics for RpRSV-specific detection. The detection limits of RpRSV using RT-LAMP and RT-PCR were compared using 10-fold serial dilution samples. The limit of detection of RpRSV using RT-LAMP was 10 times as high as that using RT-PCR. Moreover, RT-LAMP could detect the virus more rapidly compared to RT-PCR because the RT-LAMP reaction was carried out under isothermal conditions, and verification by gel electrophoresis was not necessary.

RT-LAMP reaction requires approximately 60 min, but nucleic acid extraction from a lot of samples using the modified SDS-Potassium acetate method requires a few hours. So, the two simple and rapid sample preparation methods, the toothpick method and the crude sap method, were reported by FUKUTA et al. (2005) and TAKEUCHI et al. (2006), respectively, and were examined as simple nucleic acid extraction methods. In the toothpick method, 20 samples could be prepared in 15 min, whereas they could be prepared in 30 min in the crude sap method. The results showed that the two simple and rapid sample preparation methods are meaningful. In addition, the toothpick method could reduce the risk of contamination because used toothpicks could be disposed of. Therefore, we concluded that detection of RpRSV using the toothpick method is more useful than the crude sap method as a plant quarantine technique. Furthermore, screening assay is necessary in the preparation of many samples because accurate and rapid inspection that doesn’t obstruct logistics is required at plant quarantine inspection sites. Thus, simultaneous processing was tested, and as a result, RpRSV could even be detected from a sample of 50 anemone leaves that included one infected leaf. This result suggested that simultaneous processing was an efficient sample preparation method for the detection of RpRSV from many samples. Hence, the detection procedures for RpRSV that we concluded are as follows. The toothpick method is performed when small and medium-scale samples must be tested. On the other hand, when a large number of samples are processed, the method combining the toothpick method with simultaneous processing should be used. It was considered that these simple and rapid sample preparation methods would be useful at plant quarantine inspection sites.

We evaluated RpRSV detection methods from herbaceous plants in this study. RpRSV detection methods for fruit trees will require further evaluation.

**REFERENCES**


Reverse Transcription Loop-mediated Isothermal Amplification
を用いたRaspberry ringspot virusの検出

Raspberry ringspot virus（RpRSV）は、我が国未発生のウイルスで、諸外国ではキイチゴ、スギリ、オウトウ、ブドウなどの果樹類や、オランダチゴ、スイセン、アネモネ、ハコベ等多くの植物から分離されている。中でもキイチゴ、オランダチゴは被害が大きいとされる。植物防除では、輸入関連検疫において、これまで生物検査、ELISA検査及びRT-PCR法を用いてウイルスの検出を行ってきた。今回、簡易・迅速かつ高感度に遺伝子診断が行えるRT-LAMP法を用によるRpRSVの検出について検討した。RpRSV検出用RT-LAMPプライマーセットを設計し、アネモネ感染葉からの抽出核酸を模板としてRT-LAMPを行ったところ、反応開始後60分以内に特異的にRpRSVを検出することができた。RT-PCR法との検出度を比較した結果、RT-LAMP法の検出感度が10倍高かった。また、核酸抽出を行わず、つまようじを用いる方法と磨碎粗榨液を用いる方法で反応に用いる抽出型調製の簡略化を試みたところ、両法とも検出可能であり、さらに多検体を同時処理することで大量検定にも適応できると考えられ、今回設計したプライマーを用いたRT-LAMP法によるRpRSVの検出は十分な検出感度を有し、また簡易的な抽出型調製法を組み合わせることで、RpRSVを簡易かつ迅速に検出する有効な手法であった。