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Detection of *Potato Spindle Tuber Viroid* by Reverse Transcription Loop-mediated Isothermal Amplification

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Abstract: *Potato spindle tuber viroid* (PSTVd) is one of the most important pathogens of potatoes and tomatoes, and is a pathogen cautioned about for invasion to Japan. It is known to be transmitted mechanically and through true seeds of potatoes and tomatoes. RT-PCR is reported as one of the detection method for PSTVd. However, the RT-PCR method takes much time to inspect and its procedures are complex. Therefore, Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP), a novel gene amplification method, was evaluated for detection of PSTVd instead of RT-PCR. RT-LAMP is efficient, rapid, and has high specificity. PSTVd was specifically detected within about 60 minutes by the RT-LAMP method using whole nucleic acid extracted from potato (leaves, tubers, and true seeds) and tomato (leaves and seeds). Sensitivity of the RT-LAMP method was about ten times higher than that of the RT-PCR method.

Key words: RT-LAMP, *Potato spindle tuber viroid*, detection, potato, tomato

Introduction

Potato spindle tuber viroid (PSTVd) is a species of the genus *Pospiviroid* in the family *Pospiviroidae* that occurs in North America (DIENER and RAYMER, 1971), China (HE *et al*., 1987), Europe, and so on. PSTVd infects potatoes (*Solanum tuberosum*), tomatoes (*Lycopersicon esculentum*), and other *Solanum* spp. (SINGH, 1973). PSTVd causes stunting, smaller and elongated tubers, and yield losses of potatoes (PFANNENSTIEL and SLACK, 1980). The occurrence of PSTVd has not been recorded in Japan, except a temporary occurrence in a greenhouse in Fukushima Prefecture in 2008. As a result of investigation of its cause, it could be thought to originate from imported tomato seeds. And it was concluded that the temporary occurrence has been eradicated in July, 2009 (MAFF, 2009). PSTVd is mainly transmitted through potato tubers and also is known to be transmitted mechanically (DIENER *et al*., 1979) and through true seeds of potatoes and tomatoes (LOEBENSTEIN *et al*., 2001; HADIDI *et al*., 2003). In order to prevent injurious diseases of potato such as PSTVd, imported potato plants such as tubers and tissue cultures are subjected to post-entry quarantine, and imported potato true seeds and tomato plants, which has shown characteristic symptoms, are inspected by RT-PCR for infection of PSTVd at entry points in Japanese import quarantine. However, the RT-PCR method used in the import inspection takes much time to inspect and the inspection procedures are complex.

RT-LAMP, a novel gene amplification method, is efficient, rapid, and has high specificity (NOTOMI *et al*., 2000; USHIKUBO, 2004). In this study, the RT-LAMP method was developed and evaluated for detection of PSTVd from potatoes and tomatoes instead of the RT-PCR method.

Materials and Methods

1. PSTVd infected potatoes and tomatoes

PSTVd severe strain (PSTVd-S) and mild strain (PSTVd-M) supplied by Dr. R. P. Singh in 1969 from Canada under special permit issued by the Ministry of Agriculture, Forestry and Fisheries were used in this study as PSTVd inoculum. The total nucleic acid extracted from the PSTVd-S and PSTVd-M infected potato leaves (cv. Norin No. 1) were

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Table 1. The RT-LAMP Primers for detection of PSTVd

Name	Size	Sequence 5′ to 3′	Position*
PSTVd-F3	19	AAACTCGTGGTTCCTGTGG	8–26
PSTVd-B3	19	GAGGAAGGACACCCGAAGA	192–210
PSTVd-FIP	39	ATCCCTGAAGCGCTCCTCCGTTCACACCTGACCTCCTGA	27–45, 71–90
PSTVd-BIP	42	ACTGGCAAAAAAGGACGGTGGGGGAAGGGTGAAAACCCTGTT	112–133, 170–189
PSTVd-LF	18	GCCGCCTTCTTTTTTCTT	52–69
PSTVd-LB	17	CCGACAGGAGTAATTCC	147–163

*Primers design position on GenBank Accession No. AY937179.

mechanically inoculated to healthy potato seedlings (cv. Norin No. 1) and healthy tomato seedlings (cv. Rutgers), respectively. The inoculated seedlings were grown in a containment greenhouse at 25–30°C and used as a positive control and for seed production. PSTVd-M infected tomato seeds could be produced, but PSTVd-S infected tomato seeds couldn't be obtained from PSTVd-S infected tomato plants, because stunting of the plants were severe and consequently tomato fruits could not be grown. PSTVd-S and PSTVd-M infected potato true seeds also couldn't be obtained respectively because of a poor fruition of potato in the greenhouse. The PSTVd infection of the inoculated plants and seeds produced was confirmed by RT-PCR (NAKAHARA *et al.*, 1998). After reverse transcription of extracted total RNA using Random primer (TAKARA Co.), PCR using primers PPSTV-1P (5′-CGCGCCCGCAGGACCAC-3′) and PPSTV-1M (5′-TGTCGGCCGCTGGGCACT-3′) (NAKAHARA *et al.*, 1998) was performed for 40 cycles under the following conditions: 0.5 min. at 94°C, 2 min. at 68°C, and 8 min. at 72°C, with an initial denaturation step of 4.5 min. at 94°C. Amplified products were electrophoresed on 2% agarose and confirmed specific amplified products approximately 300 bp.

Tomato chlorotic dwarf viroid (TCDVd), which is closely related taxonomically to PSTVd (SINGH *et al.*, 1999), was also used as a control to confirm the specificity of newly designed RT-LAMP primers.

2. Design of RT-LAMP primers for PSTVd detection

RT-LAMP primers for PSTVd detection were designed based on the reported PSTVd sequences (OWENS and THOMPSON, 2005, GenBank Accession No. AY937179) by LAMP primer design software Primer Explorer version 3 (Eiken Chemical Co., Fujitsu Ltd.) (Table 1).

3. Extraction of total nucleic acid from potato leaf, potato tuber, and tomato leaf

The total nucleic acid of infected sample was extracted by the SDS-Potassium acetate method (DELLAPORTA *et al.*, 1983). Fifty milligrams of sample were ground in 2 ml of extract buffer solution (0.1 M Tris-HCl [pH 8.0], 0.5 M NaCl, 1% 2-mercaptoethanol, 0.05 M EDTA [pH 8.0], 1.25% SDS, 3.3% PVP, and 1.25 M potassium acetate) (40v/w) and stirred thoroughly. The solution was centrifuged for 10 minutes at 15,000×*g*, and the supernatant was obtained. A half volume of 2-propanol was added to the supernatant, the solution was mixed, it was then centrifuged for 10 minutes at 15,000×*g*, and the supernatant was obtained. Equal amounts of 99.5% ethanol and 1/10 volume of 3 M sodium acetate were added to the supernatant, mixed, and the total nucleic acid was precipitated. The total nucleic acid was dissolved in 50 μl of 0.1 TE (pH 8.0), and 1 μl of it was used as a template for the RT-LAMP reaction.

4. RT-LAMP reaction

RT-LAMP reaction was carried out with a Loopamp RNA amplification reagent kit (Eiken chemical Co.) in 25 μl of the mixture containing 1×reaction mix (40 mM Tris-HCl [pH 8.8], 20 mM KCl, 16 mM $MgSO_4$, 20 mM $[NH_4]_2SO_4$, 0.2% Tween 20, 1.6 M Betain, 2.8 mM each dNTPs), 0.2 μM each of F3 and B3 primers, 1.6 μM each of FIP and BIP primers, 0.8 μM each of LF and LB primers, 1 μl Enzyme mix (AMV reverse transcriptase and *Bst* DNA Polymerase), and 1 μl of the total nucleic acid. Turbidity that indicated target gene amplification was measured by a real-time turbidity meter (LA-200, Teramecs Co.). Reactions were carried out at 65°C for 80 minutes. If the turbidity increased characteristically within 60 minutes, it was considered positive.

5. Sensitivity comparison of RT-LAMP and RT-PCR

Total nucleic acid extracted from the PSTVd-S infected potato leaf was suspended in sterilized distilled water and

serially diluted to 10^{0-4} times with sterilized distilled water. RT-LAMP and RT-PCR were carried out using these templates as described above, and detection sensitivities of RT-LAMP and RT-PCR were compared.

6. Detection of PSTVd from potato seeds and tomato seeds

A model test of PSTVd detection from a sample of 400 potato seeds, including only one PSTVd-M infected tomato seed instead of infected potato seed, was performed because potato true seed infected with PSTVd couldn't be obtained. In order to confirm the infection of tomato seed, One quarter of a tomato seed infected with PSTVd-M, equal to one potato seed weight, was mixed with 399 healthy potato seeds (cv. Norin No. 1, about 0.3 g), and two halves of tomato seeds infected with PSTVd-M were mixed with 399 healthy tomato seeds (cv. Rutgers, about 1.4 g) (HOSHINO *et al.*, 2006). Four hundred healthy seeds were used as a negative control. Extractions of total nucleic acid from seed samples were carried out as described above.

7. Evaluation of simplified sample preparation methods for RT-LAMP

RT-LAMP for PSTVd detection from potato and tomato was carried out by two simplified LAMP methods reported by FUKUTA *et al.* (2005a, 2005b), using clarified crude sap and toothpicks. In our study, an insect pin was used in place of a toothpick.

(1) RT-LAMP using clarified crude sap

Fifty milligrams each of potato and tomato leaves and 400 seed samples were ground with 0.1 M Tris-HCl (pH 8.0) solution (200 v/w). They were centrifuged for one minute at 10,000×*g*. Two microliters of clarified crude sap were used in the RT-LAMP reaction as template (FUKUTA *et al.*, 2005b).

(2) RT-LAMP using insect pins

Like toothpicks method in LAMP to detect Tomato yellow leaf curl virus (TYLCV) (FUKUTA *et al.*, 2005a) and Melon yellow spot virus (MYSV) (TAKEUCHI *et al.*, 2006), potato leaves and tomato leaves infected with PSTVd-M were stuck with insect pins (No. 5) several times, and then the pins were dipped into 25 μl of RT-LAMP reaction mixture. In order to evaluate the influence of the times of sticking, PSTVd-M infected samples were stuck with insect pin 3, 5, and 10 times.

Results

1. Detection of PSTVd using the primers designed

Detection of PSTVd by RT-LAMP was carried out using the designed primers (PSTVd-F3, -B3, -FIP, -BIP, -LF, and -LB) and total nucleic acid extracted from the PSTVd infected potato and the tomato leaves. An increase of turbidity (a white precipitate in RT-LAMP reaction mixture) began to be observed in about 30 minutes and reached its highest in about 45–60 minutes. An increase of turbidity did not appear in the TCDVd infected sample. No reaction was observed on the total nucleic acid of healthy potato and healthy tomato leaf samples after 80 minutes. It is known that RT-LAMP reaction produces many bands of different sizes (NOTOMI *et al.*, 2000). Many bands of different sizes were observed from the total nucleic acid of the tomato leaves infected with PSTVd, but not from tomato leaf infected with TCDVd and the healthy tomato leaf. These specific amplifications of the target RNA of PSTVd were confirmed by agarose gel electrophoresis (Fig. 1).

Detection sensitivity of the RT-LAMP method compared with that of the RT-PCR method is shown in Fig. 2. It was possible to detect PSTVd from the extracted total nucleic acid diluted to 10^{-3} times in the RT-LAMP method and diluted to 10^{-2} times in the RT-PCR method. It was suggested that the detection sensitivity of the RT-LAMP method was about ten times higher than that of the RT-PCR method.

2. Detection of PSTVd from potato tuber, potato seeds, and tomato seeds

Detection of PSTVd by the RT-LAMP method was carried out using total nucleic acid extracted from a potato tuber, 400 potato seeds, and 400 tomato seeds. As a result, it was recognized that PSTVd is able to be detected stably from all the infected samples (Fig. 3).

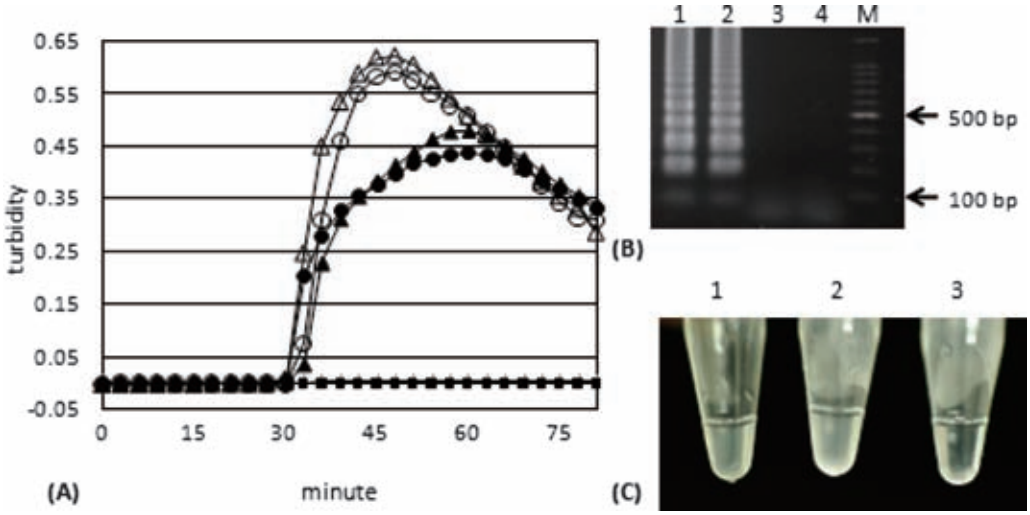

Fig. 1. Detection of PSTVd with the designed RT-LAMP primer. (A) Measurement result of turbidity by real-time turbidity meter (LA200, Teramecs). Turbidity of the RT-LAMP reaction at 65°C from the total RNA extracted from the potato leaves (●: PSTVd-S, ▲: PSTVd-M, and ■: healthy potato) and the tomato leaves (○: PSTVd-S, △: PSTVd-M, ◇: TCDVd, and □: healthy tomato). (B) Agarose gel electrophoresis of the specific amplification products from the total RNA extracted from the tomato leaves by RT-LAMP. Lane 1: PSTVd-S, 2: PSTVd-M, 3: TCDVd, and 4: healthy tomato. M: DNA size marker (100 bp ladder). (C) Confirmation of the amplification by the white precipitate in the RT-LAMP reaction mixtures. 1: potato leaf infected PSTVd-S, 2: potato leaf infected PSTVd-M, and 3: healthy potato.

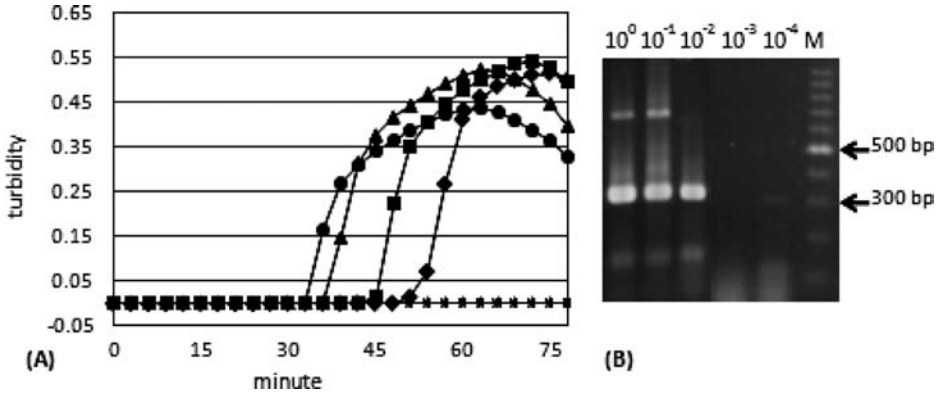

Fig. 2. Sensitivity comparison of RT-LAMP (A) and RT-PCR (B) for detection of PSTVd. (A) Turbidity of the RT-LAMP reaction from the total RNA extracted from the potato leaves from PSTVd-S after serial 10-fold dilution from 10° to 10^{-4} (●, ▲, ■, ◆, ×) and healthy potato leaf (✱). (B) Agarose gel electrophoresis of RT-PCR from the total RNA extracted from the potato leaves infected with PSTVd-S after serial 10-fold dilutions from 10° to 10^{-4}. Lane M: DNA size marker (100 bp ladder).

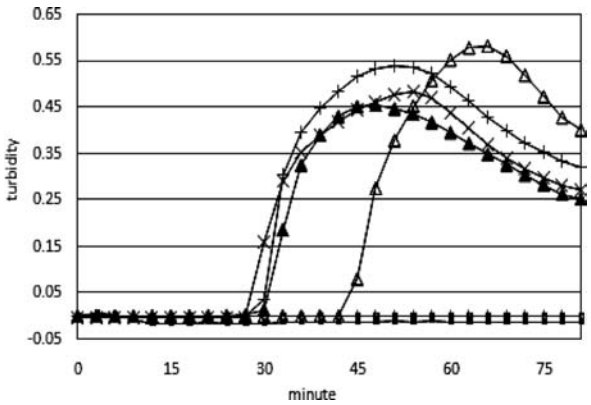

Fig. 3. Turbidity of the RT-LAMP reaction at 65℃ from the total RNA extracted from potato tubers (×: PSTVd-S, +: PSTVd-M, and ○: healthy potato), 400 potato seeds (▲: PSTVd-M and ■: healthy potato), and 400 tomato seeds (△: PSTVd-M and □: healthy tomato)

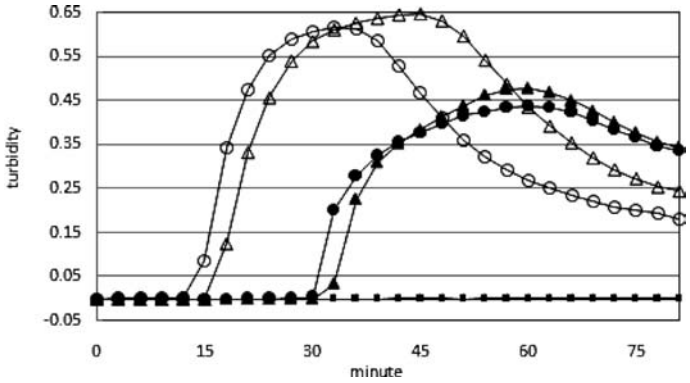

Fig. 4. Turbidity of the RT-LAMP reaction at 65℃ from the sap obtained from potato leaves (●: PSTVd-S, ▲: PSTVd-M, and ■: healthy potato) and tomato leaves (○: PSTVd-S, △: PSTVd-M and □: healthy tomato)

Table 2. Evaluation of times of pin sticking on detection of PSTVd-M by RT-LAMP using insect pins.

Plant part	Potato Leaf			Tomato Leaf
Times of sticking	3	5	10	3
Detection rate	4/5	2/5	4/5	4/4
Time to have required turbidity ＞0.1	41:06	40:12	59:36	45:36
	26:42	29:36	32:24	34:24
	50:06	–	36:00	38:36
	43:06	–	31:12	33:36
	–	–	–	

3. Evaluation of simplified sample preparation methods for RT-LAMP

(1) The simple method using clarified crude sap

The simple method using clarified crude sap was able to detect PSTVd from PSTVd infected potato leaves and tomato leaves within 60 minutes (Fig. 4). But an increase of turbidity did not appear in 400 seed samples of potato and tomato including infected seed, respectively. On the other hand, it was able to detect PSTVd from one infected tomato seed by this method (data not shown).

(2) The simple method using insect pins

Regardless of times of sticking samples by insect pin, PSTVd-M could be detected from infected potato leaves. But the detection rate (percentages of positive reaction) was not stable, and the time needed for detection (time to beginning of increased turbidity) varied widely. On the other hand, PSTVd-M was able to be detected stably from the infected tomato leaf when the leaf was stuck three times with the pin (Table 2).

Discussion

Both PSTVd-S and PSTVd-M strains could be detected stably by the RT-LAMP method using the designed primers (PSTVd-F3, -B3, -FIP, -BIP, -LF, and -LB) and total nucleic acid extracted from potato leaf, potato tuber, and tomato leaf within 60 minutes, and PSTVd-M could also be detected from 400 seeds of both potato and tomato including only one infected seed, respectively. An increase of turbidity appeared in about 30 minutes in infected plants and not in healthy plants, so it was recognized that the primers work specifically and effectively. Detection sensitivity of the RT-LAMP method using the designed primers set and total nucleic acid extracted from infected potato leaves as a template was compared with that of the RT-PCR method described previously (NAKAHARA *et al.*, 1998). The sensitivity of the RT-LAMP method was about ten times higher than that of the RT-PCR method. Also the time needed for detection of PSTVd in the RT-LAMP method is about one-third that of the RT-PCR method because PSTVd could be detected by the RT-LAMP method under isothermal conditions and without gel electrophoresis. As a result, it was obvious that the

RT-LAMP method could detect PSTVd rapidly and with high sensitivity compared with the RT-PCR method.

On the other hand, in the preparation of templates for LAMP, FUKUTA *et al.* (2005a, 2005b) reported two simplified LAMP methods using clarified crude sap and toothpicks. So, in our study, similar simple methods for PSTVd detection were evaluated. PSTVd could be detected stably by RT-LAMP using the crude sap from infected potato leaves and tomato leaves within an hour, but not from 400 potato seeds and 400 tomato seeds including one infected seed respectively. It was suggested that the RT-LAMP reaction might be inhibited by the amplification-inhibiting substances in crude sap of seeds while PSTVd could be detected from one infected tomato seed by this simple method. So, it was also suggested that the extraction of total nucleic acid should be indispensable to detect PSTVd from 400 seeds of potato and tomato by the RT-LAMP method. PSTVd could be detected stably from infected tomato leaves by another simple method using insect pins. But the detection rate was not stable and the time needed for detection varied widely from infected potato leaves. It was considered that PSTVd might distribute unevenly in the infected potato leaves and/or that the RT-LAMP reaction might be inhibited by the amplification-inhibiting substances in the infected potato leaves. These simple methods without extraction of total nucleic acid were rapid and convenient. The crude sap method seems to be superior to the insect pins method for stable detection of PSTVd from infected potato leaves but it is considered that the insect pins method is more convenient than the crude sap method for detection of PSTVd from infected tomato leaves.

In this study, it is concluded that the RT-LAMP method was a very useful method for detection of PSTVd using the total nucleic acid extracted from potato and tomato. Moreover, it is considered that an introduction of the crude sap method and the insect pins method for detection of PSTVd from potato leaves and tomato leaves would be very effective at plant quarantine inspection sites.

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和 文 摘 要

RT-LAMP法による*Potato spindle tuber viroid*（PSTVd）の検出

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Potato spindle tuber viroid（PSTVd）はジャガイモ、トマトに甚大な被害を及ぼす我が国が侵入を警戒している重要病原体である。本ウイロイドは汁液伝染の他、種子伝染することが知られている。植物検疫においてはこれまで、RT-PCRによって本ウイロイドの検出を行ってきた。しかし、RT-PCRは操作が煩雑であり検出まで時間がかかる等の問題がある。そこで、簡易、迅速、高感度の遺伝子増幅法であるRT-LAMP法による、PSTVdの検出について検討した。抽出核酸を鋳型としてRT-LAMPを行ったところ、ジャガイモ葉、塊茎、種子及びトマト葉、種子から約60分以内に特異的にPSTVdを検出することができた。RT-PCR法と検出感度を比較したところ、RT-LAMP法の方が約10倍感度が高かった。また、ジャガイモ葉及びトマト葉からは、核酸抽出を行わず磨砕汁液を鋳型としても、さらにトマト葉では、葉を昆虫針で刺し、針先に付着した汁液を鋳型としても検出可能であった。

* 現在、消費・安全局植物防疫課