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Enhancing *Columnea latent viroid* detection using reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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Abstract *Columnea latent viroid* (CLVd) is an important quarantine plant pathogen, it is of regulatory interest because of its high rate of seed transmission in tomato (*Solanum lycopersicum*). The detection of CLVd from leaves and seeds of solanaceous and cucurbitaceae species were investigated. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was developed for detection of CLVd within 1 h. The method is highly sensitive, being 2,000 times and 100 times more sensitive than conventional RT-PCR in detecting CLVd from leaves and seeds, respectively. The results showed that this detection method was species specific to CLVd with no cross reactivity to other viroids in the same *Pospiviroid* genus. It was successfully applied to detect CLVd from field samples. This appears to be the first application of RT-LAMP for detecting CLVd.

Keywords: Detection, RT-LAMP, viroids, leaves, seeds

Introduction

Viroids are the smallest plant pathogens and consist only of non-encapsidated, single-stranded circular RNA of 250-400 nucleotides (Bussie *et al*., 1999; Gozmanova, 2003; Tsagris *et al*., 2008). Viroids autonomously replicate in susceptible cells and are divided into two families, *Pospiviroidae* and *Avsunviroidae*. The *Columnea latent viroid* (CLVd) is a member of the genus *Pospiviroid* in the *Pospiviroidae* family, has a rod-like genome structure, an asymmetric circle replication mechanism in the nucleus and a wide host range. It was first reported from asymptomatic *Columnea erythrophae* in the

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USA, its genome was characterized and it was reported as transmissible to purple velvet plant (*Gynura aurantiaca*), cucumber (*Cucumis sativus*) tomato (*Solanum lycopersicum*) (Hammond *et al*., 1989), *Nemathanthus wettsteini* (Singh *et al*., 1992) and Gloxinia species (Nielsen and Nicolaisen, 2010). Severe visible symptoms in tomato are difficult to differentiate from those symptoms expressed by other pospiviroids and a universal primer set was not able to distinguish CLVd from other viroids (Bostan *et al*., 2004). Its host range in horticultural plants was further studied and numerous other species may also be hosts for CLVd because most of the currently identified host plants are symptomless (Matsushita and Tsuda, 2015). Seed transmission in tomato and *Petunia* × *hybrida* (Matsushita and Tsuda, 2016) was reported.

In Thailand, CLVd causes damage to tomato seed production and has been reported as having a low percentage of plant infection (Tangkanchanapas, 2005). It caused severe symptoms of necrosis on leaf veins, plant stunting and yield reduction on 'Rutgers' tomato when tested in greenhouse conditions (Tangkanchanapas, 2013). A CLVd isolate was inoculated onto 'Seedathip3' and 'Cherry' tomato plants and they showed severe symptoms of leaf epinasty, plant stunting and severe necrosis on stems and petioles (unpublished). The Department of Agriculture of Thailand has reported that CLVd has damaged a number of tomato cultivars and Bolomaka (*Solanum stramonifolium* Jacq.), and the risk has been included on the Thai plant pest list (Tangkanchanapas, 2013).

An essential component of viroid disease control is the development and use of sensitive diagnostic procedures that help to prevent crop infection and to eliminate infected materials. Many diagnostic techniques have been developed, such as nucleotide sequencing (Hammond *et al*., 1989; Owens *et al*., 2003), molecular hybridization (Stark-Lorenzen *et al*. 1997; Lin *et al*., 2011; Tiberini and Barba, 2012; Torchetti *et al*., 2012) and conventional RT-PCR using specific primers (Bostan *et al*., 2004; Rizza *et al*., 2009; Matsushita *et al*., 2010; Monger *et al*., 2010; Botermans *et al*., 2013; Olivier *et al*., 2014; Papayiannis, 2014). However, RNA extraction from plant tissue is required (Stark-Lorenzen *et al*., 1997) and gel electrophoresis and UV detection are time consuming. To decrease the time needed for viroid detection, reverse transcription loop-mediated isothermal amplification (RT-LAMP) was developed. The LAMP technique amplifies cDNA with high specificity, efficiency and rapidity under isothermal conditions. The main components of the reaction include a DNA polymerase, a set of four specific primers of six distinct sequences on the target DNA, which consist of inner primers containing sequences of the sense and antisense strands of the target DNA (Notomi *et al*., 2000). The efficiency of many RT-LAMP protocols for specific targets has been confirmed (Lan *et al*. 2009; Hassan *et al*., 2013; Mohammad and Dehabadi, 2013; Suzuki *et al*.,

2016). RT-LAMP was developed and reported for some viroids such as *Peach latent mosaic viroid* (PLMVd) (Boubourakas *et al*., 2009), *Potato spindle tuber viroid* (PSTVd) (Tsutsumi *et al*., 2010; Lenarcic *et al*., 2013), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Park *et al*., 2013) and *Coconut cadang-cadang viroid* (CCCVd) (Thanarajoo *et al*., 2014).

Objectives: This research aimed to develop a specific, highly sensitive and efficient RT-LAMP procedure for CLVd detection in both leaves and seeds of vegetable crops. It was also hoped that the technique developed in the current research would be useful for routine viroid detection in a range of other economic plant species.

Materials and methods

Viroid sources, plant materials and RNA preparation

The viroid isolates were obtained from a collection at the Department of Plant Pathology, Kasetsart University, Thailand; CLVd isolate NK-KUKPS1, accession no. KY235369; *Pepper chat fruit viroid* (PCFVd) isolate PCFVd-Thai, accession no. JF446893 (Reanwarakorn *et al*., 2011); and *Chrysanthemum stunt viroid* (CSVd) isolate RCS, accession no. MF803029; and were propagated using mechanical inoculation of 'Rutgers' tomato. The CLVd isolate NK-KUKPS1 was used as the primary viroid inoculum source. The cultivars used in the study were: tomato (*S. lycopersicum* cv. Seedathip 4), eggplant (*S. melongena* cv. Farmer Long), pepper (*Capsicum annuum* cv. PBC365), cucumber (*Cucumis sativus* cv. Bingo) and melon (*C. melo* L. cv. Pot Orange). The third leaf of the solanaceous plants and first true leaf of the cucurbitaceae species were inoculated mechanically using crude sap of CLVd-infected tomato leaves in 0.1M phosphate buffer pH 9.0 at 1 g: 5 mL, mixed with carborundum dust. All CLVd and mock inoculated (buffer) plants were maintained in an insect-proof nursery for 8 weeks after inoculation. Viroid infection was confirmed by conventional RT-PCR.

Total RNA was extracted from 100 mg of leaves or seeds from each sample following a modified CTAB method (Reanwarakorn *et al*., 2011) using the TLES extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0 M, 100 mM LiCl, 1% (v/v) SDS and 2% (w/v) Na2SO3) instead of the CTAB extraction buffer.

Conventional RT-PCR for CLVd detection

The conventional RT-PCR in this study used the CLVd specific CL-P2 primer set (cCL-P2 and hCL-P2), for which the target sequence was the whole

CLVd genome (370 nts). An internal control, amplification of the mitochondrial NADH dehydrogenase gene, based on previously reported primers (Lee and Chang, 2006) was used to monitor RNA extraction. The primers that were used are listed in Table 1. The PCR reaction mixture consisted of 1X of the Green PCR Master Mix (Thermo ScientificTM) as per manufacturer's recommendations, 0.1 µM forward and reverse primers, 2 µL of cDNA template, adjusted to a final volume of 20 µL with RNase-free water (Thermo ScientificTM). The PCR program included 5 min at 96 ℃, followed by 40 cycles of 40 s at 94 ℃, 40 s at 54 ℃ and 40 s at 72 ℃ and a final extension of 7 min at 72 ℃ for the CL-P2 primers. To monitor RNA extraction, plant *nad5* mRNA was determined using the Nad primers followed by the RT-PCR reaction using the CL-P2 primers. The PCR products were analyzed by 2% agarose gel electrophoresis with the RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology) and visualized under UV light.

Design of primer sets and optimization of the two-step RT-LAMP method

The RT-LAMP primers were designed using the CLVd isolate MC-M-11 accession no. AM698095 sequence using PrimerExplorer version 4 software from Eiken Chemical (https://primerexplorer.jp/elamp4.0.0) to provide four primer sets that covered the whole genome of CLVd after multiple sequence alignment of DNA using ClustalW alignment tools (http://www.ebi.ac.uk/Tools/msa/clustalo/) of the GenBank database (data not shown). The best sets of outer primers (F3 and B3), inner primers (FIP and BIP) and gene specific primers are shown in Table 1.

The cDNA was synthesized using the B3 primer and followed the same procedure as the RT reaction using the CL-P2 primers. The 25 µL LAMP reaction mixture contained 6 mM MgSO4, 1X Thermo polymerase buffer (Biolab), 1.4 mM dNTPs mixture, 1.6 µM of FIP and BIP, 0.2 µM F3 and B3, 1M Betaine, 8 units Bst DNA Polymerase Large Fragment (New England Biolabs), 2.0 µL of cDNA and 3.4 µL of RNase-free dH2O. The LAMP cycle was performed at 61 ℃, 63 ℃ and 65 ℃ for 60 min followed by heat inactivation at 80 ℃ for 10 min to terminate the reaction (Boubourakas *et al*., 2009). After the reaction, the products were analyzed by adding 1 µL of 1,000X SYBR green I to the reaction mix to observe the color change from orange to green, and under a blue light transilluminator (LED wavelength: 468 nm) to observe fluorescence for positive samples, and then confirmed by 1.2 % agarose gel electrophoresis using gel staining by the RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology) and visualization on a UV-transilluminator. Each sample was analyzed at least three times.

Table 1. The primer names, sequences and position of each primer set used in this study.

Target	Primer name	Primer	Sequence(5'- 3')	Genome positions[3/]
CLVd	SK-175[1/]	F3	CAGGGTTTTCACCCTTCCTT	175-194 (sense)
		B3	CGGTTCCAAGGGCTAAACAC	337-356 (antisense)
		FIP	CAGAACCTGCGCTGGTCAAGA-TTCTGGTTTCCTTCCTCTGC	237-257 (antisense), 197-216 (sense)
		BIP	CCGGTGGCATCACCGAGTTT-CCCGGAGACCAAGCTAGA	264-283 (antisense), 314-331 (sense)
CLVd	CL-P2[2/]	cCL-P2	CTGCAGCCATGCAAAGA	23-39 (antisense)
		hCL-P2	GGTCAGGTGTGAACCAC	40-56 (sense)

[1]/ RT-LAMP primers obtained from GenBank accession no. AM698095
[2]/ CL-P2 primers - target size at 370 bp used in this study
[3]/ The position of the RT-LAMP primers obtained from GenBank accession no. AM698095

Specificity and sensitivity of the RT-LAMP method to detect CLVd from leaves and seeds

The specificity and sensitivity of CLVd detection by the two-step RT-LAMP method was compared with conventional two-step RT-PCR. The specificity of both methods for the detection of CLVd, PCFVd and CSVd from infected tomato leaves was also compared. (PSTVd was not included in this study as it is a quarantine pest and has never reported in Thailand).

Two groups of sensitivity tests for CLVd detection were studied. With the first group, sensitivity was determined using a 10-fold serial dilution of the total RNAs from CLVd infected leaves with RNAs from healthy leaves of tomato, eggplant, pepper, cucumber and melon. The total RNA was measured and adjusted to 200 ng μL^{-1} using a nanodrop spectrophotometer, and the 10-fold serial dilutions were prepared at 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng μL^{-1}.

With the second group, total RNA from single seeds of viroid-infected and viroid-free seeds of tomato and eggplant was extracted and adjusted at 150 ng μL^{-1}. The serial dilution of total RNAs from viroid infected tomato and eggplant seeds and from viroid-free seeds was performed at: 1:100, 1:200, 1:400, 1:1,000, 1:2,000, 1:10,000, 1:20,000, 1:40,000 and 1:100,000 (volume: volume). The RT-LAMP reactions were conducted as previously described.

Sequencing

RT-LAMP products were cloned into the pGemT-easy vector following the manufacturer's protocol and sent to SolGent Co. Ltd. (Korea) for sequencing. The similarity of the sequences was determined by blast with the

GenBank database by using Blastn tools (http://www.ncbi.nlm.nih.gov/BLAST).

Application of the RT-LAMP method for a field survey for CLVd

To confirm the usefulness of the RT-LAMP method for CLVd detection, 15 field tomato plants from a previous viroid monitoring project in Thailand, that was conducted in 2015 to 2016, were used for assay with the RT-LAMP. These field samples included five PCFVd and 10 CLVd positive samples as determined using the conventional RT-PCR method. Total RNAs of leaves were used to detect the presence of CLVd using the conventional RT-PCR and RT-LAMP methods as previously described.

Results

Optimization of the two-step RT-LAMP procedure

Investigation of the optimal conditions for the RT-LAMP procedure was conducted using total RNA from CLVd infected leaves. The four primer sets were generated and tested at 61 ℃, 63 ℃ and 65 ℃ for 60 min. The results showed that primer set "SK-175" amplified the CLVd amplicons most efficiently at 65 ℃ (data not shown).

Specificity and sensitivity of the RT–LAMP method for detection of CLVd from leaves and seeds

The specificity of RT-LAMP using the SK-175 primer was tested to determine the cross reactivity between viroid species in the same Pospiviroid genus: CLVd, PCFVd and CSVd from infected tomato leaves. Total RNAs were used for the comparison. The results showed that CLVd was detected by RT-LAMP, but neither PCFVd nor CSVd was detected, indicating that the RT-LAMP primers are specific to CLVd (Figure 1).

Two groups were used to the sensitivity for CLVd detection by conventional RT-PCR and RT-LAMP. The detection of CLVd in the first group was determined using a 10-fold serial dilution of total RNA from infected and healthy leaves of tomato, eggplant, pepper, cucumber and melon. The results showed that conventional RT-PCR could detect CLVd from leaves in a range of 100 to 200 ng μL^{-1} from all samples and RT-LAMP could detect the viroid in the range of 0.001 to 200 ng μL^{-1}. The detection of the viroid using the cCL-P2 primers in the conventional RT-PCR, therefore, would require 700 ng (200 ng

μL^{-1}) of total RNA for the cDNA synthesis step while the outer primer (B3) of the SK-175 primer needs an initial RNA concentration of at least 0.35 ng (0.1 ng μL^{-1}). These investigations showed that the RT-LAMP was 2,000 times more sensitive than the conventional RT-PCR for CLVd detection from leaf samples (Table 2).

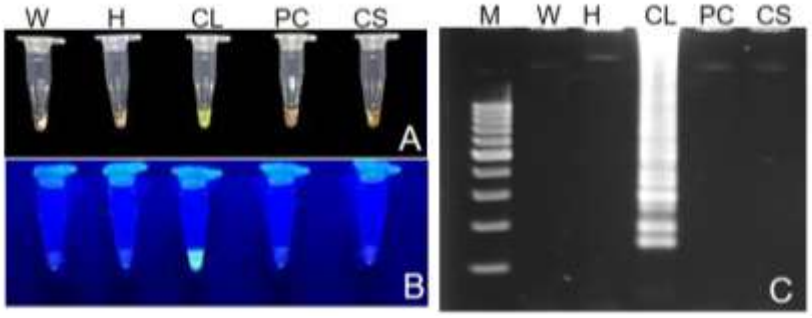

Figure 1. The specificity of RT-LAMP using the SK-175 primer for CLVd detection at 65˚C, (A) visual detection with SYBR Green I stain - the color changed from orange to light green color under normal light in the CLVd positive samples, (B) visual detection using SYBR Green I stain under the Blue Light Transilluminator showed fluorescence in only the CLVd positive sample, (C) agarose gel showing the DNA ladder of the CLVd amplicons. M = 100 bp molecular weight markers; W = dH_2O; H= healthy tomato; CL = CLVd infected tomato; PC = PCFVd infected tomato; CS = CSVd infected tomato

Table 2. Comparison of the sensitivity of conventional RT-PCR and RT-LAMP to detect CLVd from leaves.

Method	**Total RNA concentration (ng μL^{-1})**										
	Plant sp.	dH_2O	H[1/]	200	100	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Conventional RT-PCR	Tomato	-[2/]	-	+[3/]	+	-	-	-	-	-	NT[4/]
	Eggplant	-	-	+	+	-	-	-	-	-	NT
	Pepper	-	-	+	+	-	-	-	-	-	NT
	Cucumber	-	-	+	-	-	-	-	-	-	NT
	Melon	-	-	+	-	-	-	-	-	-	NT
RT-LAMP (SK-175)	Tomato	-	-	+	+	+	+	+	+	-	NT
	Eggplant	-	-	+	+	+	+	+	-	-	-
	Pepper	-	-	+	+	+	+	+	+	+	-
	Cucumber	-	-	+	+	+	+	+	+	-	NT
	Melon	-	-	+	+	+	+	+	-	-	NT

[1]/ H = Healthy plant RNA
[2]/ (-) = negative result
[3]/ (+) = positive result
[4]/ NT= no test

Sensitivity of detection of CLVd in the second group was determined using a serial dilution of total RNA from CLVd infected tomato and eggplant seeds in comparison with viroid-free seeds. The conventional RT-PCR could detect CLVd in seed at a minimum ratio of 1:100 from tomato and of 1:1,000 from eggplant, while RT-LAMP could detect CLVd from seed at ratios of 1:10,000 and 1:40,000 from infected tomato and eggplant seed, respectively. The technique was, therefore, 100 times more sensitive than conventional RT-PCR for CLVd detection from seed samples (Table 3).

Sequencing assay

To confirm the RT-LAMP products obtained when using the SK-175 primer, the sequences of the RT-LAMP products were blasted with the GenBank database using Blastn tools from the website (http://www.ncbi.nlm.nih.gov/BLAST). These results confirmed that the RT-LAMP primer was specific to CLVd due to the high similarity (96-98% identity; data not shown) of the product sequences to those of the CLVd isolate NK-KUKPS1.

Table 3. Comparison of the sensitivity of conventional RT-PCR and RT-LAMP to detect CLVd from seeds.

Method	**Plant sp.**	**Total RNA ratio (infected : healthy)**												
		dH$_2$O	H [1/]	1	1:100	1:200	1:400	1:10	1:2x10^3	1:1$0^4$	1:2x10^4	1:4x10^4	1:1$0^5$	1:2x10^5
Conventional RT-PCR	Tomato	-[2/]	-	+[3/]	+	-	-	NT[4/]	NT	NT	NT	NT	NT	NT
	Eggplant	-	-	+	+	+	+	+	-	-	-	NT	NT	NT
RT-LAMP (SK-175)	Tomato	-	-	+	+	+	+	+	+	+	-	-	NT	NT
	Eggplant	-	-	+	+	+	+	+	+	+	+	+	-	-

[1]/ H = Healthy plant RNA
[2]/ (-) = negative result
[3]/ (+) = positive result
[4]/ NT= no test

Application of the RT-LAMP method for field survey for CLVd

Conventional RT-PCR and RT-LAMP methods were able to detect CLVd in infected samples (Figure 2). However, the conventional RT-PCR method detected CLVd from most but not all of the CLVd infected samples; sample tube no. 12 (Figure 2A), while RT-LAMP detected all of the CLVd infected samples and a PCFVd infected sample, which may have been a mixed infection with CLVd; sample tube no. 3 (Figure 2B).

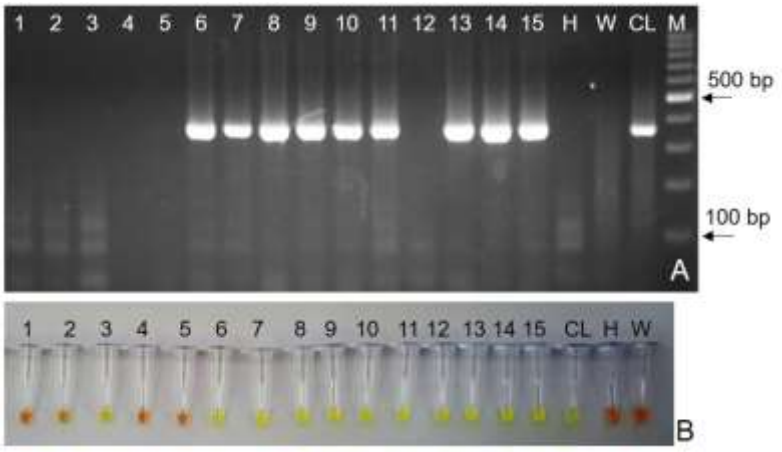

Figure 2. Application of conventional RT-PCR and RT- LAMP for detection of CLVd from field collected samples. (A) DNA products at 370 bp of CLVd using CL-P2 primers, (B) visual detection LAMP products with SYBR Green I stain; the color changed from orange to light green color under normal light in the CLVd positive samples. M=100 bp molecular weight markers; W= dH_2O; H= healthy tomato; CL= CLVd positive control; 1-5= PCFVd infected tomatoes; 6-15= CLVd infected tomatoes

Discussion

This study found that one set of primers could detect CLVd and it showed that the position of the viroid sequences was one critical factor for RT-LAMP primer development. The optimal conditions for viroid detection has previously been shown to depend on the viroid species and their genome sequence. Previous validations have included 62.5 ˚C for 32 min for *Peach Latent mosaic viroid* (PLMVd) (Boubourakas *et al*., 2009), 65˚C for 15-25 min for PSTVd detection (Lenarcic *et al*., 2013), 65˚C for 90 min for *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Park *et al*., 2013) and 60 ˚C for 60 min for *Coconut cadang-cadang viroid* (CCCVd) (Thanarajoo *et al*., 2014). Although four

primer sets for CLVd were designed by the program as specific to certain segments of the viroid genome, the software does not indicate the possibility of primer specificity and the efficiency of detection, so the evaluation of each primer set was compared under different conditions to select the best one.

This investigation proved that the CLVd accumulation in leaf and seed tissue varied with species; it accumulated to a higher level in solanaceous than in cucurbitaceae species and in eggplant seed more than in tomato seed. The results showed that the assay was highly sensitive for detection of CLVd from both leaves and seeds. This RT-LAMP assay provided results in 1 h instead of a few hours when compared with conventional RT-PCR. Similarly, the RT-LAMP for PLMVd was shown to be a sensitive detection method that has been applied to peach, plum, apricot, pear and quince samples with 100 times more sensitivity than the two-step RT-PCR method (Boubourakas *et al*., 2009). Likewise, because PSTVd detection using RT-PCR takes more time and is more complicated than RT-LAMP. This streamlined method was developed for specific PSTVd detection which could be completed in 1 h using the RNA from various parts of potato (leaves, tubers and true seeds) and tomato (leaves and seeds), and was shown to be 10 times more sensitive than the RT-PCR method (Tsutsumi *et al*., 2010). This method could be performed in the field using portable instruments, making it more cost effective (Lenarcic *et al*., 2013). The RT-LAMP method is very sensitive and contamination, due to carry-over of the LAMP product from one assay to another, is of concern because the product is increased three-fold every half cycle (Notomi *et al*., 2000) False positives can, therefore, be a problem (Zhao *et al*., 2018). In this study the false positives were minimized by separating the pipette set and working area from other work, sterilizing the pipette between samples and thoroughly cleaning the working area.

The four primers tested, which recognized the six distinct LAMP set sequences, ensure that there is a high specificity to the target amplification as described previously by Natomi et.al. (2000). This result confirmed that the primers used in this study were specific for CLVd detection from leaves and seeds. RT-LAMP has also been developed for detection of CCCVd from infected oil palm leaves; the sequencing assay proved that the primers are specific for CCCVd and did not detect other viroids such as CEVd and PSTVd (Thanarajoo *et al.*, 2014). Another RT-LAMP was developed for CChMVd, which causes serious diseases in chrysanthemum in Korea. It is a rapid detection method as its primers are specific to the CChMVd genome and have the ability to detect non-symptomatic and symptomatic CChMVd isolates (Park *et al.*, 2013). Other assays using RT-LAMP detection for viruses have been developed as effective genotype-specific techniques, which avoid lengthy

sequencing, for *Pepino mosaic virus* (PepMV) (Ling *et al.*, 2013), *Potato Virus Y* (PVY) (Mohammad and Dehabadi, 2013) and *Grapevine leafroll-associated virus* type 3 (GLRaV-3) (Walsh and Pietersen, 2013). A specific detection method for *Tomato necrotic stunt virus* (ToNStV) was also reported that had no cross-reactivity to other potyviruses or other common tomato viruses (Li and Ling, 2014).

Therefore, a CLVd detection procedure using RT-LAMP was developed. The results showed that the assay was highly sensitive for detection of CLVd from leaves and seeds. It was also more sensitive than conventional RT-PCR when detecting CLVd from leaf and seed samples from the various vegetable species in this study. This RT-LAMP assay gave results within an hour of sampling instead of a few hours when compared with conventional RT-PCR. This supported the objective of decreasing the detection time. The validation of this assay will lead to enhanced efficiency and sensitivity of CLVd detection from leaf and seed samples in both the laboratory and the field.

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References

Bostan, H., Nie, X. and Singh, R. P. (2004). An RT-PCR primer pair for the detection of Pospiviroid and its application in surveying ornamental plants for viroids. Journal of Virological Methods. 116:189-193.

Botermans, M., van de Vossenberg, B. T. L. H., Verhoeven, J. T. J., Roenhorst, J. W., Hooftman, M., Dekter, R. and Meekes, E. T. M. (2013). Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. Journal of Virological Methods. 187:43-50.

Boubourakas, I. N., Fukuta, S. and Kyriakopoulou, P. E. (2009). Sensitive and rapid detection of *Peach latent mosaic viroid* by the reverse transcription loop-mediated isothermal amplification. Journal of Virological Methods. 160:63-68.

Bussie, F., Lehoux, J., Thompson, D. A., Skrzeczkowski, L. J. and Perreault, J. P. (1999). Subcellular localization and rolling circle replication of *Peach latent mosaic viroid*: hallmarks of group a viroids. Journal of Virology. 73:6353-6360.

Gozmanova, M. (2003). Characterization of the RNA motif responsible for the specific interaction of *Potato spindle tuber viroid* RNA (PSTVd) and the tomato protein *Virp1*. Nucleic Acids Research. 31:5534-5543.

Hammond, R., Smith, D. R. and Diener, T. O. (1989). Nucleotide sequence and proposed secondary structure of *Columnea latent viroid*: a natural mosaic of viroid sequences. Nucleic Acids Research. 17:10083-10094.

Hassan, A., Ali, H. B. and Kamel, A. A. E. (2013). One-hour loop-mediated isothermal amplification assay for the detection of quarantinable toxigenic *Fusarium garmanirum*. African Journal of Microbiology Research. 7:1179-1183.

Lan, X., Yang, B., Li, B. Y., Yin, X. P., Li, X. R. and Liu, J. X. (2009). Reverse transcription-loop-mediated isothermal amplification assay for rapid detection of hepatitis E virus. Journal of Clinical Microbiology. 47:2304-2306.

Lee, S. C. and Chang, Y. C. (2006). Multiplex RT-PCR detection of two orchid viruses with an internal control of plant *nad5* mRNA. Plant Pathology Bulletin. 15:187-196.

Lenarcic, R., Morisset, D., Mehle, N. and Ravnikar, M. (2013). Fast real-time detection of Potato spindle tuber viroid by RT-LAMP. Plant Pathology. 62:1147-1156.

Li, R. and Ling, K. S. (2014). Development of reverse transcription loop-mediated isothermal amplification assay for rapid detection of an emerging potyvirus: *Tomato necrotic stunt virus*. Journal of Virological Methods. 200:35-40.

Lin, L., Li, R., Mock, R. and Kinard, G. (2011). Development of a polyprobe to detect six viroids of pome and stone fruit trees. Journal of Virological Methods. 171:91-97.

Ling, K.-S., Li, R. and Bledsoe, M. (2013). *Pepino mosaic virus* genotype shift in North America and development of a loop-mediated isothermal amplification for rapid genotype identification. Virology Journal. 10:1-12.

Matsushita, Y. and Tsuda, S. (2015). Host ranges of Potato spindle tuber viroid, Tomato chlorotic dwarfviroid, Tomato apical stunt viroid and Columnea latent viroid in horticultural plants. European Journal of Plant Pathology. 141:193-197.

Matsushita, Y. and Tsuda, S. (2016). Seed transmission of Potato spindle tuber viroid, Tomato chlorotic dwarfviroid, Tomato apical stunt viroid and Columnea latent viroid in horticultural plants. European Journal of Plant Pathology. 145:1007-1011.

Matsushita, Y., Usugi, T. and Tsuda, S. (2010). Development of a multiplex RT-PCR detection and identification system for Potato spindle tuber viroid and Tomato chlorotic dwarf viroid. European Journal of Plant Pathology. 128:165-170.

Mohammad, A. A., and Dehabadi, S. H. (2013). Colorimetric immunocapture reverse transcription loop-mediated isothermal amplification assay for rapid detection of the Potato virus Y. Journal of Plant Pathology and Microbiology. 4:1-6.

Monger, W., Tomlinson, J., Booonham, N., Marn, M. V., Plesko, I. M., Molinero-Demilly, V. and Nielsen, S. L. (2010). Development and inter-laboratory evaluation of real-time PCR assays for the detection of pospiviroids. Journal of Virological Methods. 169:207-210.

Mori, Y. and Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. Journal of Infection and Chemotherapy. 15:62-69.

Nielsen, S. L. and Nicolaisen, M. (2010). First report of *Columnea latent viroid* (CLVd) in *Gloxinia gymnostoma*, *G. nematanthodes* and *G. purpurascens* in a botanical garden in Denmark. New Disease Reports. 22:4.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 28:1-7.

Olivier, T., Demonty, E., Fauche, F. and Steyer, S. (2014). Generic detection and identification of pospiviroids. Archives of Virology. 159:2097-2102.

Owens, R. A., Sano, T., Feldstein, P. A., Hu, Y. and Steger, G. (2003). Identification of a novel structural interaction in *Columnea latent viroid*. Virology. 313:604-614.

Papayiannis, L. C. (2014). Diagnostic real-time RT-PCR for the simultaneous detection of *Citrus exocortis viroid* and *Hop stunt viroid*. Journal of Virological Methods. 196:93-99.

Park, J., Jung, Y., Kil, E. J., Kim, J., Thi Tran, D., Choi, S. K. and Lee, S. (2013). Loop-mediated isothermal amplification for the rapid detection of *Chrysanthemum chlorotic mottle viroid* (CChMVd). Journal of Virological Methods. 193:232-237.

Reanwarakorn, K., Klinkong, S. and Porsoongnurn, J. (2011). First report of natural infection of *Pepper chat fruit viroid* in tomato plants in Thailand. New Disease Reports. 24:6.

Rizza, S., Nobile, G., Tessitori, M., Catara, A. and Conte, E. (2009). Real time RT-PCR assay for quantitative detection of *Citrus viroid III* in plant tissues. Plant Pathology. 58:181-185.

Singh, R. P., Lakshman, D. K., Boucher, A. and Tavantzis, S. M. (1992). A viroid from *Nematanthus wettsteinii* plants closely related to the *Columnea latent viroid*. Journal of General Virology. 73:2769-2774.

Stark-Lorenzen, P., Guitton, M. C., Werner, R. and Muhlbach, H. P. (1997). Detection and tissue distribution of *Potato spindle tuber viroid* in infected tomato plants by tissue print hybridization. Archives of Virology. 142:1289-1296.

Suzuki, R., Fukuta, S., Matsumoto, Y., Hasegawa, T., Kojima, H., Hotta, M. and Miyake, N. (2016). Development of reverse transcription loop-mediated isothermal amplification assay as a simple detection method of *Chrysanthemum stem necrosis virus* in chrysanthemum and tomato. Journal of Virological Methods. 236:29-34.

Tangkanchanapas, P., Reanwarakorn, K., Chanprame, S. and Hongprayoon, R. (2005). An RT-PCR primer pair for the detection of six *Pospiviroid* in tomato plants. Thai Phytopathol. 19:13-21.

Tangkanchanapas, P., Reanwarakorn, K. and Kirdpipat, W. (2013). Detection of *Columnea latent viroid* (CLVd) and *Pepper chat fruit viroid* (PCFVd) for plants in family *Solanaceae*. Thai Agricultural Research Journal. 31:108-122.

Thanarajoo, S. S., Kong, L. L., Kadir, J., Lau, W. H. and Vadamalai, G. (2014). Detection of *Coconut cadang-cadang viroid* (CCCVd) in oil palm by reverse transcription loop-mediated isothermal amplification (RT-LAMP). Journal of Virological Methods. 202: 19-23.

Tiberini, A. and Barba, M. (2012). Optimization and improvement of oligonucleotide microarray-based detection of tomato viruses and pospiviroids. Journal of Virological Methods. 185:43-51.

Torchetti, E. M., Navarro, B. and Serio, F. D. (2012). A single polyprobe for detecting simultaneously eight pospiviroids infecting ornamentals and vegetables. Journal of Virological Methods. 186:141-146.

Tsagris, E. M., Martinez de Alba, A. E., Gozmanova, M. and Kalantidis, K. (2008). Viroids. Cellular Microbiology. 10:2168-2179.

Tsutsumi, N., Yanagisawa, H., Fujiwara, Y. and Ohara, T. (2010). Detection of *Potato spindle tuber viroid* by reverse transcription loop-mediated isothermal amplification. Research bulletin of the plant protection service Japan. 46:61-67.

Walsh, H. A. and Pietersen, G. (2013). Rapid detection of *Grapevine leafroll-associated virus* type 3 using a reverse transcription loop-mediated amplification method. Journal of Virological Methods. 194:308-316.

Zhao, B., Yang, D., Zhang, Y., Xu, Y., Zhao, X., Liang, J. and Zhao, K. (2018). Rapid visual detection of *Lily mottle virus* using a loop-mediated isothermal amplification method. Archives of Virology. 163:545-548.

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