Detection of *Cucurbit chlorotic yellows virus* from *Bemisia tabaci* captured on sticky traps using reverse transcription loop-mediated isothermal amplification (RT-LAMP) and simple template preparation

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Abbreviations: RT-LAMP, reverse transcription loop-mediated isothermal amplification; CCYV, *Cucurbit chlorotic yellows virus*; Tt, threshold time.

Abstract

Cucurbit chlorotic yellows virus (CCYV) of the genus *Crinivirus* within the family *Closteroviridae* is an emerging infectious agent of cucurbits leading to severe disease and significant economic losses. Effective detection and identification methods for this virus are urgently required. In this study, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed to detect CCYV from its vector *Bemisia tabaci*. LAMP primer sets to detect CCYV were evaluated for their sensitivity and specificity, and a primer set designed from the HSP70h gene with corresponding loop primers were selected. The RT-LAMP assay was applied to detect CCYV from viruliferous *B. tabaci* trapped on sticky traps. A simple extraction procedure using RNAsecureTM was developed for template preparation. CCYV was detected in all of the *B. tabaci* 0, 1, 7 and 14 days after they were trapped. Although the rise of turbidity was delayed in reactions using RNA from *B. tabaci* trapped for 7 and 14 days compared with those from 0 and 1 day, the DNA amplification was sufficient to detect CCYV in all of the samples. These findings therefore present a simple template preparation method and an effective RT-LAMP assay, which can be easily and rapidly performed to monitor CCYV-viruliferous *B. tabaci* in the field.

Keywords: *Cucurbit chlorotic yellows virus*; Virus detection; Sticky trap; Whitefly

1. Introduction

Cucurbit chlorotic yellows virus (CCYV) is a newly identified virus species of the genus *Crinivirus* within the family *Closteroviridae* (Okuda et al., 2010). CCYV infects melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and watermelon (*Citrullus lanatus*) plants (Gyoutoku et al., 2009) under natural conditions, while it infects many plant species, such as *Nicotiana benthamiana* and *Datura stramonium*, under experimental conditions (Okuda et al., 2010). Infected crops exhibit severe chlorotic yellow lesions on the leaves, and lower yields and quality of fruit. The fruit on CCYV-infected melon plants contain less sugar (Gyoutoku et al., 2009), leading to economic losses. In recent years, disease caused by CCYV has been reported in several Asian countries including China (Gu et al., 2011) and Taiwan (Huang et al., 2010), and in the African country of Sudan (Hamed et al., 2011). Diseases caused by *Beet pseudo-yellows virus* (BPYV), which is another cucurbit infecting crinivirus, previously known as *Cucumber yellows virus* (CuYV), have been reported in Japan. The symptoms of CCYV-infected and BPYV-infected cucumber plants are similar which requires molecular or serological methods for proper identification.

CCYV is semi-persistently transmitted by tobacco whitefly (*Bemisia tabaci*)

Mediterranean (MED, commonly known as biotype Q) and Middle East-Asia Minor 1 (MEAM1, commonly known as biotype B). In Japan, areas inhabited by *B. tabaci* MED have increased rapidly since this pest was first identified (Ueda and Brown, 2006). Since *B. tabaci* MED is highly resistant to several insecticides, eradication is difficult. Once the insect has colonized a cropping field, CCYV is rapidly spread throughout the surrounding area. Therefore, rapid and sensitive detection methods for CCYV are required so that appropriate countermeasures to control the spread of the disease and vector can be implemented. Sticky traps, which are thin blue or yellow plastic boards coated with adhesive substrates, are often used to estimate the number of insects in the field (Gerling and Horowitz, 1984). Insects captured on sticky traps can be identified by using a stereo microscope and viruses harbored within the captured insects can be detected by enzyme-linked immunosorbent assay (ELISA) or reverse transcriptase-polymerase chain reaction (RT-PCR) (Boonham et al., 2002; Okazaki et al., 2011). Thus, a survey to estimate the incidence and percentage of viruliferous insects in fields or greenhouses can predict the risk of virus infection in a particular area (Okazaki et al., 2007; Zen et al., 2008).

Loop-mediated isothermal amplification (LAMP) is a newly developed DNA amplification method, based on auto-cycling strand displacement DNA synthesis by a

Bst DNA polymerase using a set of specially designed inner and outer primers (Notomi, 2000). RNA can also be amplified by adding reverse transcriptase to the reaction mixture (RT-LAMP, Notomi, 2000). The primer set consists of four primers to recognize six distinct regions in a target sequence. In addition, two loop primers containing sequences complementary to the loop regions accelerate the amplification (Nagamine et al., 2002). The LAMP reaction can be completed within 60 min at a temperature ranging between 60 and 65 °C.

Several methods have been established to identify whether a LAMP or RT-LAMP reaction is positive or negative without separating the DNA fragments by agarose gel electrophoresis (Mori et al., 2001; Tomita et al., 2008). These methods are advantageous in the diagnosis of pathogens, enabling quick decisions to be made on control measures. Thus, LAMP and RT-LAMP have been applied to detect various plant pathogens such as *Tomato yellow leaf curl virus* (Fukuta et al., 2003) and *Liberibacter asiaticus* (Okuda et al., 2005).

The extraction of nucleic acid (DNA or RNA) is an essential step in all molecular identification procedures. The extraction method needs to provide DNA or RNA of sufficient purity, whilst remaining fast and easy to perform. Purity is especially important for a sensitive diagnosis because the presence of polymerase inhibitory

substances such as polysaccharide in the extracted nucleic acid solution may inhibit reactions and affect detection sensitivity (Monteiro et al., 1997). Moreover, RNA is not stable in solution, so an appropriate extraction method must be employed to ensure reliable results. In this study, a simple template preparation method for RT-LAMP to detect CCYV from *B. tabaci* captured on a sticky trap was developed.

2. Materials and methods

2.1 Virus source and whitefly population

A population of CCYV-viruliferous *B. tabaci* MED collected from a CCYV affected melon plants in Kumamoto Prefecture, Japan, was maintained on cucumber seedlings (cv. ‘Shimoshirazu-jibai’) in a netted cage (180 × 180 × 180 cm) inside a glasshouse. Cucumber seedlings were regularly added or replaced to maintain CCYV infection and the viruliferous *B. tabaci* population. Seedlings of melon (cv. ‘Earl’s Seine’) and cucumber (cv. ‘Shimoshirazu-jibai’) at the first to second true leaf stage were put in the cage for 4 days to prepare CCYV-infected plants. The seedlings were taken from the cage and *B. tabaci* were killed by applying an insecticide (50 ppm nitenpyram). The seedlings were then transplanted to pots (18 cm in height, 12 cm in diameter) and grown for 25 to 30 days until symptoms appeared on the upper

non-inoculated leaves. As a negative control, a non-viruliferous population of *B. tabaci* MED was maintained on cabbage (*Brassica oleracea*) plants in a plastic cage set in a growth cabinet (CLE-303, TOMY SEIKO, Tokyo, Japan) at 25°C under a photoperiod control of 16-h light and 8-h dark.

2.2 Preparation of RNA templates from plants

Total RNA was extracted from 0.1 g of the CCYV-infected melon and cucumber leaves, displaying clear symptoms, using ISOGEN (NIPPON GENE, Toyama, Japan) according to the manufactures' protocols. RNA was dissolved in 50 µl of ribonuclease free water. The concentration was quantified using the Qubit[®] RNA BR Assay Kit (Life Technology, Carlsbad, CA, USA) and adjusted to 10 ng/µl. A series of 10-fold serial dilution from 10 to 10⁻⁴ ng/µl were prepared to determine the sensitivity and density dependence of the reaction. RNA of cucumber leaves infected with BPYV was extracted to examine if the RT-LAMP assay using the selected primer set could distinguish CCYV from other closely related virus species.

2.3 Primer design and RT-LAMP reaction conditions

Candidate primer sets, which consisted of two inner primers (FIP and BIP), two

outer primers (F3 and B3) and two loop primers (LF and LB) suitable for the RT-LAMP reaction, were designed from the nucleotide sequences of the p4.9, HSP70h, p6, p59, p9, CP and CPm coding regions in the CCYV RNA2 sequence (GenBank accession number, AB523789) using a JAVA based web application, PrimerExplore V4 (<http://primerexplorer.jp/e/>). If any primer set including FIP, BIP, F3, B3, LF and LB was not proposed for a coding region by PrimerExplore V4 under the recommended conditions, the coding region was excluded from the analysis.

RT-LAMP reactions were conducted using the Loopamp[®] RNA Amplification Kit (Eiken Chemical, Tokyo, Japan) with 1.6 μ M of each FIP and BIP primers, 0.2 μ M of each F3 and B3 primers, 0.8 μ M of each LF and LB primers, together with 2 μ l of template RNA solution. The final volume of the reaction mixture was 25 μ l. The reaction mixture was incubated at 63°C for 60 min. The turbidity of the reaction mixture, which is caused by insoluble magnesium pyrophosphate, a byproduct of DNA amplification (Mori et al., 2004), was measured using a LA-200 turbidimeter (Teramecs, Kyoto, Japan) during the course of the reaction. The elapsed time in second after the reaction started at which the turbidity value was greater than 0.1 was defined as the time threshold (Tt).

2.4 Comparison of the sensitivity of RT-LAMP with conventional RT-PCR

The sensitivity of RT-LAMP to detect CCYV was compared with that of RT-PCR using PrimeScript™ One Step RT-PCR Kit Version 2 (Takara, Shiga, Japan). Total RNA was extracted from CCYV-infected melon leaves and a series of 10-fold serial dilution from 10^2 to 10^{-5} ng/μl were prepared as described above. The reaction mixture contained 2 μl of template RNA solution along with 0.4 μM of each of the F3 and B3 primers of the HSP70h primer set used in the RT-LAMP assay. The other reagents were added according to the manufacturer's protocol. The final volume of the reaction mixture was 25 μl. Reverse transcription of RNA was performed at 50°C for 30 min followed by 94°C for 2 min, and the PCR consisted of 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 10 min. RT-PCR products were fractionated by 2.5% agarose gel electrophoresis, stained with ethidium bromide (50 μg/ml) for 30 min, and visualized under ultraviolet light. Samples in which a DNA fragment of expected size was detected were regarded as positive. RT-LAMP was performed using the same serial dilutions of total RNA. The same composition of reaction mixture was used as above, except that 1 μl of Fluorescent Detection Reagent (Eiken Chemical) was added for visual detection (Tomita et al., 2008). The reaction was regarded as positive if fluorescence was observed after

incubation for 60 min at 63°C.

2.5 Preparation of RNA templates from viruliferous *B. tabaci*

B. tabaci MED adults were collected from CCYV-infected melon plants. The adults were individually placed into a microtube containing 100 µl of RNasequre™ (Applied Biosystems, Foster City, CA, USA), homogenized using a pestle, and heated at 60°C for 5 min. To examine the effect of RNasequre™ on the stability of the extracted RNA, 100 µl of RNase-free water was used to substitute the RNasequre™. Non-viruliferous *B. tabaci* MED adults were used as negative controls. Prepared samples were kept for 24 h at 37°C to evaluate the longer-term stability of the RNA in the samples. A sample was considered to be positive if the turbidity of the RT-LAMP reaction excess 0.1 within 60 min at 63°C.

To determine how long CCYV is detectable after its vector *B. tabaci* MED was captured on a trap, viruliferous *B. tabaci* MED were placed on a yellow sticky trap (Arysta LifeScience, Tokyo, Japan) and kept in a greenhouse without temperature control (the temperature ranged from 25 to 38°C). Seven to 12 *B. tabaci* were removed from the trap using a sterilized toothpick 0, 1, 7 and 14 days after being placed. They were homogenized with 100 µl of RNasequre™, heated at 60°C for 5 min, and used as

templates for RT-LAMP reactions.

2.6 Statistical analysis

The t-test and one way analysis of variance (ANOVA) were performed on the data using Prism 6 for MacOSX (Graph Pad Software, La Jolla, CA, USA). When an ANOVA result was significant, a post hoc test of the means was performed (Dunnett's multiple comparison test).

3. Results

3.1 Evaluation of RT-LAMP primer sets

LAMP primer sets including FIP, BIP, F3, B3, LF and LB designed by PrimerExplore V4 were only available for the HSP70h gene and the CPm regions. The primer sequences are shown in Table 1.

The turbidity of the RT-LAMP reaction mixture containing FIP, BIP, F3 and B3 of the HSP70h primer set and RNA extracted from CCYV-infected melon leaves increased 10 min after the reaction started (Fig. 1A). The addition of loop primers (LF and LB) accelerated the reaction (Fig. 1A). No turbidity increase was observed when the RNA of healthy melon leaves was used. The turbidity of the RT-LAMP reaction mixtures containing the CPm primer set, with or without the corresponding loop primers,

and RNA extracted from CCYV-infected melon leaves increased earlier than those containing the HSP70h primer set (Fig. 1B). However, an unspecific rise in turbidity was observed in the reaction with the CP primer set for the healthy control reaction (Fig. 1B). Therefore, the HSP70h primer set was used in the following study.

3.2 Sensitivity and specificity of RT-LAMP

RT-LAMP reactions performed with the HSP70h primer set and serial dilutions of the RNA template demonstrated an inverse correlation between Tt values and the concentration of RNA template (Fig. 2). RT-LAMP could detect CCYV ranging from 10 to 10^{-2} ng/ μ l of RNA extracted from CCYV-infected melon and cucumber leaves. No amplification was observed when the RNA concentration was 10^{-3} ng/ μ l or less. RT-LAMP with the HSP70h primer set and RNA extracted from BPYV-infected cucumber leaves did not show any turbidity increase during the course of the reaction even when the RNA concentration was 10 ng/ μ l.

3.3 Comparison of the sensitivity with conventional RT-PCR

An approximately 200-bp DNA fragment of the expected size was detected by RT-PCR using F3 and B3 of the HSP70h primer set and a 10-fold serial dilution of

template RNA ranging from 10^2 to 10^{-2} ng/ μ l (Fig. 3A). RT-LAMP reaction mixture including the HSP70h primer set and 10-fold serial dilution ranging from 10^2 to 10^{-2} ng/ μ l showed green fluorescence after 60 min at 63°C (Fig. 3B). No amplification was observed for both RT-PCR and RT-LAMP when the template RNA concentration was 10^{-3} ng/ μ l or less. Therefore, the detection sensitivity of the two assays was identical.

3.4 Detection from *B. tabaci*

The turbidity of the RT-LAMP reaction mixture with the HSP70h primer set and RNA extracted with RNAsecure™ from CCYV-viruliferous *B. tabaci* increased approximately 10 min after the reaction was initiated. The average Tt value was 741 (Table 2). No increase in turbidity was observed when RNA extracted from non-viruliferous *B. tabaci* was used. The average Tt value became slightly higher when the extracted RNA was kept at 37°C for 24 h; however, CCYV was successfully detected in all of the samples tested. The Tt values were higher when RNAsecure™ was substituted with water (Table 2). CCYV was detected in all of the samples if the RT-LAMP assay was conducted immediately after RNA was extracted using water. However, the average Tt values became much higher after 24 h at 37°C regardless of heat treatment. In addition, one out of four RNA samples gave a negative result (Table

2).

The RT-LAMP assay using the HSP70h primer set successfully detected CCYV from all of the *B. tabaci* removed from the sticky traps, and detection of the virus was possible up to 14 days after the insects were trapped (Fig. 4). Although the Tt values obtained from *B. tabaci* trapped for 7 and 14 days were significantly higher than those from day 0, the Tt values from these late time points were sufficient to consider the reactions positive.

4. Discussion

In this study, RT-LAMP was used for the detection of CCYV from an insect vector, *B. tabaci*. Nucleotide sequences of seven gene loci (p4.9, HSP70h, p6, p59, p9, CP, CPm) in the RNA2 region of the CCYV genome were used to design primer set by a JAVA-based web application PrimerExplore V4. Many incomplete sets of primers were proposed for each region, however, most lacked loop primers to accelerate the LAMP reaction (Nagamine et al., 2002). Loop primers must be located in a stem-loop region in the amplified sequence. The PrimerExplore V4 software takes into account the specific annealing temperature (Tm) and the 5' and 3'-end stability of the potential primer sequences. The low guanine-cytosine content of CCYV (37.6% and 35.7% for

RNA1 and RNA2, respectively) might make it difficult to obtain loop primers. Consequently, RT-LAMP primer sets including loop primers were only identified for the CPm and HSP70h regions. Real-time turbidity analysis revealed faster amplification in reactions containing the CPm primer set compared with the HSP70h primer set. However, the primer set for the CPm region was discarded because of weak nonspecific amplification.

Wang et al. (2014) reported a procedure for CCYV detection from melon plants using RT-LAMP with a primer set constructed from the CP region. Despite high sensitivity, the reaction needed at least 45 min to obtain visible amplification products which appeared as ladder-like DNA fragments. In this study, reactions using the primer set for the HSP70h region gave a positive result within 30 min using as little as 10^{-2} ng/ μ l of RNA from CCYV-infected cucumber and melon leaves. One possible reason for this increased sensitivity is that the primer set tested in this study included loop primers. Loop primers have been reported not only to accelerate reactions but also to increase sensitivity (Nagamine et al., 2002) and they are therefore more commonly being employed in diagnostic LAMP assays (Mori and Notomi, 2009). The availability of loop primers is therefore an important consideration in the development of virus detection assays.

The RT-LAMP assay used in this study successfully detected CCYV in all of the viruliferous *B. tabaci* vectors trapped on sticky traps for 14 days. Viruses in the genus *Crinivirus* are semipersistently transmitted by whiteflies (Wisler et al., 1998). The amount of CCYV in the vector body gradually decreases after the vector leaves infected plants, and the viruses are barely detectable on day 14 or later (Moriyama, unpublished data). In this experiment, Tt values, which were inversely correlated with the amount of virus RNA, did not drastically change over days post capture. Although the reason for this has not yet been confirmed, CCYV may decrease more slowly in dead *B. tabaci* bodies or the fragmented viral RNA may still be detectable by RT-LAMP. Future studies on how the virus is maintained and translocated in the vector body may provide further insight.

Estimating the population density of whitefly and the incidence of viruliferous vectors in the field is a key measure to protect crops from virus infection (Castle et al., 2009). Populations of two biotypes, MED and MEAM1, of *B. tabaci*, whose host preferences and tolerance to insecticides are distinct (Ueda and Brown, 2006; Iida et al., 2009), have been detected in Japan. Because both biotypes are morphologically indistinguishable, differences in the nucleotide sequences of the nuclear or mitochondrial genomes are used to identify these biotypes, as reviewed by De Barro et

al. (2011). In addition to the emergence of CCYV in cucurbit crops, a devastating disease caused by *Tomato leaf curl virus* (TYLCV) belonging to the genus *Begomovirus* in the family *Geminiviridae*, on tomato plants has become widespread throughout Japan. Farmers need to identify which biotypes are present in their fields and whether the whiteflies are viruliferous with CCYV, TYLCV or other criniviruses or begomoviruses to protect crops from virus infections. The samples prepared in this study contained stable DNA as well as RNA, and can therefore be used as templates for TYLCV detection and biotype determination by PCR (data not shown). The extraction method developed in this study therefore has the additional advantage that it could be used to determine the biotype of *B. tabaci* present and detect crinivirus (RNA virus) and begomovirus (DNA virus) from one sample.

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Figure legends

Figure 1. Changes in turbidity during the course of the RT-LAMP reaction using the HSP70h (A) and CPm (B) primer sets. Solid and dotted lines indicate the turbidity of the reaction mixtures containing RNA from CCYV-infected and healthy melon leaves, respectively. Lines in bold indicate reactions that included the corresponding loop primers.

Figure 2. Relationship between the threshold value of turbidity (T_t) in the RT-LAMP reaction and the concentration of RNA extracted from leaves of CCYV-infected melon plants. T_t values are defined as the time in seconds when the turbidity of the reaction mixture reached in excess of 0.1. Filled circles and triangles represent RNA extracted from different plants.

Figure 3. Comparison of the sensitivity of the two detection methods, RT-PCR and RT-LAMP. (A) An image of agarose gel electrophoresis of RT-PCR products amplified using the F3 and B3 primers of the HSP70h primer set and 10-fold serial dilutions of RNA from CCYV-infected cucumber leaves. The concentration of RNA ranges from

100 ng/μl (lane 1) to 10⁻⁵ ng/μl (lane 8). (B) Detection of CCYV based on fluorescence in an RT-LAMP assay using the HSP70h primer set. The same templates were used, as indicated by the numbers above the tubes.

Figure 4. Detection of CCYV from *B. tabaci* bodies trapped on a sticky trap. Dots indicate the mean threshold value of turbidity (Tt) in the RT-LAMP reaction. Bars represent the 95% confidence intervals of the means. Asterisks indicate a significant difference ($p < 0.05$) when compared with the mean Tt value of *B. tabaci* 0 days after being trapped according to one-way analysis of variance and Dunnett's multiple comparison tests. The numbers above each dot indicate the number of positive samples / number of samples tested.

Figure(s) revised
Figure 1

Figure 2

Figure 3

Figure 4

Table 1

List of primers for CCYV amplification

	Primer	Sequence (5'–3')	Nucleotide position ^{a)}
HSP70h	FIP	ACGGATGAGAATTACAATCGATGT-TTGGTTGTT CTATGTCACACG	(2341-2318) - (2270-2290)
	BIP	GTAATCCTAAAGTCTTGGTCCGTAA-AGTGAGA TTTGAAAACTCCTC	(2363-2387) - (2440-2419)
	F3	TTCGATCAGCAGTGTCTT	2252-2269
	B3	TTCATATACATTCAAAATGGTGC	2466-2444
	LF	CCATATTACCTGTGTCTCTTGAG	2314-2291
	LB	ACCAATGGCGATACCATACACATA	2388-2411
CPm	FIP	GGATGTTTCACTTTCTCCTCCGG-AATTGAATAA AGACCCACCTCT	(6417-6395) - (6348-6369)
	BIP	CTCCAGAACCACTAAAACCAGTAC-TTTAATCTT ATCAGATTCCCCTG	(6426-6449) - (6508-6486)
	F3	TGGCTGATTTAAACATTGAGG	6327-6347
	B3	TTGGAATATCTACTGGTGAGG	6542-6522
	LF	GGTCTGGTTTAACTGGTTCTGGA	6392-6370
	LB	CACCAGTCCCTAAACCGAGTGA	6450-6471

^{a)} Nucleotide positions correspond to those in the CCYV RNA2 sequence deposited in the GenBank database under accession number AB523789. FIP and BIP primers consist of two regions and their sequences are shown linked by a hyphen. Numbers in an inverted order indicate that the primer sequence is located on the complementary strand.

Table 2

Stability of RNA extracted for the detection of CCYV from *Bemisia tabaci* using LAMP

Treatment	Percentage of CCYV detected samples ^{a)}		Average Tt value ^{b)} of positive reactions	
	0 hr	24 hr	0 hr	24 hr
RNAsecure, 60°C 5min	4/4	4/4	741	837
Water, 60°C 5 min	4/4	3/4	871	1432
Water, no temperature treatment	4/4	3/4	880	1466

^{a)} Number of positive samples/total. Samples for which the turbidity was in excess of 0.1 in the LAMP reaction after 60 min were regarded as positive.

^{b)} Elapsed time (seconds) when the turbidity reached in excess of 0.1 in the LAMP reaction.