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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

Mitsuru Okuda[a,*], Shiori Okuda[b], Hisashi Iwai[c]

[a] *Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Kannondai, Tsukuba-City, Ibaraki 305-0856, Japan*
[b] *National Institute for Environmental Studies, Onogawa, Tsukuba-City, Ibaraki 305-8506, Japan*
[c] *The United Graduate School of Agricultural Sciences, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan*

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A B S T R A C T

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 RNAsecure™ 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.

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

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

* Corresponding author. Tel.: +81 29 838 8838; fax: +81 29 838 8837.
E-mail addresses: mokuda@affrc.go.jp (M. Okuda), topcrop@agri.kagoshima-u.ac.jp (H. Iwai).

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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, while 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 cm × 180 cm × 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 25–30 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 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 primers, 0.8 μM of each LF and LB primers, together with 2 μl of template RNA solution. The final volume of the reaction mixture was 25 μl. 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 s, 55 °C for 30 s, and 72 °C for 30 s, 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