One-step Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Cymbidium Mosaic Virus

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Abstract

Cymbidium mosaic virus (CymMV) is the most prevalent orchid virus. A single-tube one-step betaine-free reverse transcription (RT) loop-mediated isothermal amplification (LAMP) assay was developed for the rapid and easy detection of orchid-infecting CymMV. Five sets of primers were designed based on the conserved regions among various virus isolates. The specificity and the sensitivity of the assay were then evaluated using the RT-LAMP reaction. Within one hour under isothermal conditions at 60°C the target viral gene was amplified successfully. This RT-LAMP assay was found to be quick, specific, sensitive and easy to perform assay that involved only one step and was simpler to carry out than alternative approaches. Thus this assay is an alternative for the rapid and easy detection of CymMV in orchids. This is first time that a RT-LAMP method for the detection of an orchid virus has been described.

Keywords: CymMV, RT-LAMP, orchid, detection

1. Introduction

Cymbidium mosaic virus (CymMV) is the most prevalent and economically important orchid virus. This virus belongs to the genus *Potexvirus* of the family *Flexiviridae* and contains a monopartite positive-sense single strand RNA genome (Adams et al., 2005). It infect numerous commercially important orchid genera (ICTVdB: The Universal Virus Database of the International Committee on Taxonomy of Viruses [http://www.ictvdb.iacr.ac.uk/Ictv/fr-index.htm.]; Adams et al., 2005 and Zettler et al., 1990) and is considered to have widely spread among and be generally present among orchids worldwide (Zettler et al., 1990). Some special orchid species that belong to the genus *Dendrobium* have been using as traditional Chinese

medicine for the treatment of various diseases, such as chronic atrophic gastritis, diabetes, and cardiovascular diseases (Chinese Materia Medica Dictionary, 1985; Tang and Eisenbrand, 1992). It is reasonable that CymMV may infect these *Dendrobium* spp. and be detrimental to the growth of those valuable medicinal orchids. In addition, among ornamental orchids, CymMV usually causes flower color breakdown, size reduction and stunted growth, thus reducing the quality and quantity of these orchids (Zettler et al., 1990). Therefore, diagnosis of CymMV in order to control virus infection is important as it will help to reduce the economic impact of this virus on the orchid industry.

Up to the present, a range of techniques based on immuno-interaction (Tanaka et al, 1997; Eun and Wong, 1999), nucleic acid hybridization (Hu and Wong, 1998; Eun et al., 2002) and polymerase chain reaction (Seoh et al., 1998) have been reported as useful for the detection of CymMV. However, all of these techniques have some intrinsic disadvantages, such as low sensitivity, being time-consuming or requiring expensive equipment. Recently, loop-mediated isothermal amplification (LAMP) and the reverse transcription-LAMP assay have been developed and shown to be a rapid, simple, sensitive and inexpensive approach to DNA and RNA detection in various organisms, such as bacteria, parasites and virus (Notomi et al., 2000; Fukuta et al., 2009; Buates et al., 2010; Hung et al., 2010; Shuanghui et al., 2010). Nevertheless, up to the present, this novel technique has been applied to the detection of plant RNA viruses in only a few cases (Fukuta et al., 2003; 2004; Varga and James, 2006).

In this study, the specific aim was to develop a RT-LAMP assay that allowed the rapid detection of orchids infected with CymMV. Using one of five sets of LAMP primers designed specifically, a RT-LAMP assay was established for the detection of CymMV in orchids that was shown to be highly sensitive, specific, rapid, easy to

carry out in one step, and had a minimal requirement in terms of equipment and staff. The application of this developed newly RT-LAMP assay for the diagnosis of CymMV will not only be useful to the local orchid industry but also will help orchid growers worldwide and make plant quarantine by governments simpler and easier.

2. Materials and methods

2.1. Sources of plant

Various different genera of orchids were used for *Cymbidium mosaic virus* (CymMV) detection, namely *Dendrobium crumenatum, Dendrobium dixanthum, Phalaenopsis* sp., *Oncidium* sp. and *Cymbidium ensifolium*. The orchids used were collected from the medicinal garden at China Medical University or purchased from a flower market in Taichung.

2.2. RNA extraction and viral RNA preparation

Total RNA was extracted from the leaves of the orchids using a method described previously (Lin *et al*., 2004; 2006). A total RNA-derived cDNA from the leaves of CymMV-infected *Nicotiana benthamiana*, a CymMV RNA-derived cDNA and a CymMV CP cDNA clone were used for the initial experiments. CymMV viral RNA was extracted from CymMV virions purified from the *N. benthamiana* infected with CymMV. The viral RNA was quantified and then used for the sensitivity assay.

2.3. Design of the primers for the RT-LAMP assay

Using Primer Explorer V3 software (http://primerexplorer.jp; Eiken Chemical Co. Ltd, Japan), the primers used for RT-LAMP assay were designed specifically based on the sequence of the CymMV capsid protein gene (Figure 1). A set of six RT-LAMP

primers comprising two outer primers (F3 and B3), two inner primers (F2 and B2), and two loop-forming primers (F1c and B1c) that recognize six distinct regions on the target sequence were used. The F2 and F1c primers connect together as the loop primer FIP. The B2 and B1c primers connect together as the loop primer BIP. Their primer sequences are shown in Table 1.

2.4. Reverse transcription–loop-mediated isothermal amplification

The betaine-free RT-LAMP reaction was carried out in a 25 μl reaction mixture containing 2 μmol (each) of the outer primers F3 and B3, 8 pmol (each) of the loop primers FIP and BIP, 2.5 mM deoxynucleoside triphosphates, 10 mM $(NH4)_2SO_4$, 12 mM $MgSO_4$, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 8 U of Bst DNA polymerase (New England Biolabs, Beverly, MA), 50 U of AMV reverse transcriptase (Promega, Madison, WI), and 2 μl of RNA or DNA. The mixture was incubated at 60°C for 60 min using a heating block; this was followed by heating at 80°C for 10 min to terminate the reaction.

2.5. Detection of RT-LAMP product

After the RT-LAMP reaction was performed, an aliquot of 2 ul of RT-LAMP product was subjected to DNA electrophoresis on a 1.0% agarose gel. The nucleic acid was visualized under ultraviolet light after ethidium bromide staining. Additionally, the RT-LAMP product could also be detected by naked eye by observing the color change at end of the RT-LAMP reaction when SYBR Green I reagent was added (Invitrogen, USA). In order to confirm the origin of the RT-LAMP products, the restriction enzyme Hpa II was used to digest the DNA products of the LAMP assay for 1 hr at 37 ℃ in a water bath.

2.6. Sensitivity and specificity of RT-LAMP

The sensitivity of the RT-LAMP assay was evaluated by adding different amounts of viral RNA to the RT-LAMP reaction. The sensitivity limit of the RT-LAMP assay was determined based on whether the DNA ladder-like product of the RT-LAMP reaction was produced or not. The specificity of the RT-LAMP assay was assessed by sequencing of the PCR produced DNA clones. The PCR product was amplified using the F2 and B2 primers and a 1000-fold diluted RT-LAMP product as template.

3. Results

3.1. Establishing a LAMP assay specific for the detection of CymMV

Since the subgenomic RNA encoding the coat protein (CP) has the highest level in infected tissue, the CP gene was chosen as the target gene for the CymMV LAMP assay. To design the primers for LAMP assay, various CymMV isolates were explored to identify the conserved regions of the virus genome. The CP sequences of five isolates from Taiwan (AY429021, AY571289 and EU314803), Japan (AB197937) and Korean (AF016914) were obtained from GenBank and aligned by Primer Explorer V3 software. A set of primers based upon the conserved regions among isolates was pinpointed (Figure 1) and used subsequently for the evaluation of CymMV LAMP assay specificity.

Initially, two cDNAs derived from total RNA extracted from CymMV-infected *Nicotiana benthamiana* and CymMV viral RNA and a CymMV CP cDNA clone were used as the temples for the primer testing. The LAMP reaction was conducted as described in the Materials and Methods using the primer set, F3-1, B3-1 FIP, and BIP, as shown in Fig 1. As illustrated in Fig 2A, the LAMP-resulting products consisted of ladder-like DNA fragments and were detected by gel electrophoresis. In addition, the products were detectable using the naked eye as green fluorescence on UV excitation

after adding SYBR Green I dye, as illustrated in Fig 2C. These results indicated that the primer set was able to amplify successfully the target DNA sequence. To confirm that the LAMP products were derived from the target gene sequence, the amplified products were digested with Hpa II, which has a site in the loop region as shown in Fig 1. The agarose gel analysis showed that the LAMP products were digested to give the expected DNA fragment of 290 nucleotides (Fig. 2). The correct size of the expected fragment revealed that the primer set used indeed was able to recognize and amplify specifically the target sequence of the CymMV CP gene. In addition, DNA sequencing results also confirmed that the LAMP products were derived from the target gene sequence as shown in Fig 4.

3.2. Optimization of one-step RT-LAMP assay for detection of CymMV

Cymbidium mosaic virus is a RNA virus. Reverse transcription is required to produce complementary DNA as a temple for further DNA amplification by LAMP assay. Here, reverse transcription was combined with the DNA amplification using AMV reverse transcriptase and *Bst* DNA polymerase simultaneously within the RT-LAMP assay; this created a one-step assay. In addition, the optimization of the LAMP primers sequences and the sensitivity of RT-LAMP assay were explored. In order to optimize the LAMP primer sequences, two new F3 and three new B3 primers, with or without 1 or 2 additional nucleotide(s) at the 5’ end, were created as shown in Table 1. The efficiency of the various RT-LAMP assays was then evaluated using five combinations of F3 and B3, namely CyF3-1 plus CyB3-1, CyF3-1 plus CyB3-2, CyF3-1 plus CyB3-3, CyF3-2 plus CyB3-2, and CyF3-2 plus CyB3-3. This was carried out using 4 pg of viral RNA as template. As shown in Fig 3A, when CyF3-1 plus CyB3-2, CyF3-1 plus CyB3-3, CyF3-2 plus CyB3-2, and CyF3-2 plus CyB3-3 were used, RT-LAMP products with ladder-like DNA fragments were detected by gel

electrophoresis analysis. Importantly, one primers set, CyF3-1 plus CyB3-3, performed better in the reaction of RT-LAMP assay than the other primer sets (efficiency: CyF3-1+CyB3-3>CyF3-1+CyB3-2>CyF3-2+CyB3-3>CyF3-2+CyB3-2). This finding indicated that the addition of one or two more nucleotides to the 5' end of B3 primer was able to improve the amplification efficiency of the RT-LAMP assay.

The sensitivity of the RT-LAMP assay was assessed using serial dilutions of the viral RNA as template in the RT-LAMP assay with the optimized outer primers, CyF3-1 plus CyB3-3. On examining Fig 3B, it can be seen that the LAMP products as ladder-like DNA fragments were amplified down to >1 pg, giving a sensitivity of about 1 pg of the viral RNA. Interestingly, moreover, when 1 pg of viral RNA was used, the sizes of the ladder-like DNA fragments were slightly smaller than those produced by a greater amount of viral RNA.

Finally, the RT-LAMP product was sequenced by cloning a PCR product produced by amplification using the F2 and B2 primers and a 1000-fold dilution of RT-LAMP products as template. Sequencing of the cloned PCR products from RT-LAMP product, as illustrated in Fig 4, showed that the product of the RT-LAMP assay was indeed a CymMV CP sequence. These results confirm that this newly developed and optimized one-step RT-LAMP assay is both highly sensitive and specific for the detection of CymMV.

3.3. Application in the field of the one-step RT-LAMP assay for the detection CymMV infection in various orchids

To examine the usefulness of this new one-step RT-LAMP assay for the detection of CymMV in orchids in the field, a series of randomly collected orchids were tested. Eight orchids belonging to four genera, *Dendrobium, Phalaenopsis*, *Oncidium* and *Cymbidium,* were used as the test samples for the detection of CymMV infection.

Total RNA was extracted from these orchids and used as template for the single tube RT-LAMP assay (primers: CyF3-1, CyB3-3, FIP and BIP). As shown in Fig 5A, ladder-like DNA fragments were produced by seven of the eight orchids tested after 60 min reaction time under the conditions used. This result suggests that about 78% of the field samples tested positive for CymMV infection. In spite of several lanes in Fig 5A showing up as a smear rather than a clear ladder, these smears probably represent a positive result. These results were in complete agreement with the conventional RT-LAMP using a two steps procedure that is shown in Fig 5B. These results demonstrate that the one-step RT-LAMP assay developed here is able to be applied successfully to the detection of CymMV in a range of orchid species.

4. Discussion

Cymbidium mosaic virus (CymMV) is the most prevalent and economically important orchid virus (Wong et al., 1994). Reverse transcription-polymerase chain reaction (RT-PCR) has been used for the detection of CymMV (Ryu et al., 1995). Rapid detection and identification of CymMV by real–time polymerase chain reaction amplification in orchids has also been reported (Eun et al., 2000). Therefore, RT-PCR is used currently as the “golden standard assay” for CymMV detection. However, one of major problem with RT-PCR is that the assay needs an accurately controlled thermocycling system to perform repeatedly the three temperature shifts needed to amplify using reverse transcriptase and *Taq* DNA polymerase over 2-3 hours. The assay developed in this study is one-step RT-LAMP assay, which can detected successfully CymMV in various orchids including *Phalaenopsis* sp., *Oncidium* sp., *Cymbidium* sp. and *Dendrobium* sp., and can be applicable for the rapid detection of the CymMV in orchids (about 1 hour). Thus, importantly compared to RT-PCR, this method carries out CymMV detection more easily and quickly. This is especially

important when detecting CymMV using a mobile laboratory or in a resource-limited situation in a developing country. There is no need for any expensive systems, only a temperature controlled water bath or a heating block. CymMV has spread widely throughout ornamental and medicinal orchids. Therefore, an easy method that can be used for virus surveillance will help to reduce economic losses in both the ornamental and traditional Chinese medicine orchid industries. With this convenient and cheap method, virus-infected orchid seedlings can be detected and removed as earlier as possible during cultivation, which will improve quality control.

In this study, because a set of four primers is required to perform LAMP, the specificity is relatively higher than with conventional PCR/RT-PCR which usually uses only two primers. Once the expected ladder-like DNA amplified pattern is presented on the agarose gel, this indicates that the target gene exists in the test sample. If necessary, the specificity of the RT-LAMP assay can be confirmed by sequencing these LAMP products or by enzyme digestion analysis.

For the development of this RT-LAMP assay, the abundant CP gene from the subgenomic RNA of CymMV was chosen and a specifically designed primer set was created to recognize a highly conserved region within the CP gene. While the characterized ladder-like pattern of LAMP product can be analyzed by gel electrophoresis (Fig. 1A, 1B), time can be saved by the use of SYBR Green I as the detection system during field operations. SYBR Green I interacts with the RT-LAMP DNA produced and this interaction can be detected when the tube is exposed to UV light, allowing positive or negative samples to be pinpointed by the naked eye (Fig. 1C). This eliminates the need for the detection system would help greatly when carrying out large scale screening.

Primer evaluation in terms of sensitivity involved adding nucleotides to the 5’ end of, the F3 and B3 primers. The results showed that when a longer F3 or B3 was used,

better amplification was obtained compared to shorter F3 or B3 primers. Interestingly, in Fig. 3A, the results suggest that outer primer B3 plays a more critical role in the RT-LAMP reaction than F3; this is probably because this primer not only is important to amplifying the LAMP amplicon during the initiation step, but is also essential to synthesizing the cDNA by reverse transcription.

To avoid problems due to variation in the priming sites of the four primers, the target sequence for this RT-LAMP assay was positioned within a conserved region of the CymMV genome. This approach was successful because during the field trial it was found that seven out of eight of the orchid from different genera tested positive for CymMV. This demonstrates that the optimized primer set was indeed suitable for detecting CymMV in orchid plants using RT-LAMP assay.

In Fig. 3B, interestingly, when 1 pg of viral RNA was used as template, the sizes of the ladder bands changed slightly. To investigate this change, the RT-LAMP products were sequenced. The results showed that this RT-LAMP product contained a loop-region deletion, which suggest that when the concentration of the target RNA is limiting and under these circumstances the RT-LAMP amplification may produce an incomplete product that results in smaller bands. This finding is similar to a previous report (Huang et al., 2010) and may be useful as an indicator when measuring the sensitivity limit of a particular LAMP system.

The present results show that the sensitivity of this method is proximally 1 pg of viral RNA under the conditions used. However, the assay has not been optimized fully and some optimal reaction parameters, such as buffer composition, temperature, and sample concentration/complexity need to be explored. Changes to these factors based on further investigations might increase the sensitivity further.

In conclusion, a diagnostic method for the detection of CymMV using a simple one-step RT-LAMP assay has been developed and this approach has the potential to

become a valuable diagnostic tool in the orchid industry. It will allow the creation of a cheap and simple-to-run diagnostic kit. To the best of our knowledge, this is the first study to demonstrate the application of one-step RT-LAMP amplification to the diagnosis of CymMV.

Acknowledgments

This work was supported by CMU grant CMU98-N1-02 and NSC grant NSC 98-2311-B-039-001-MY3 of Taiwan.

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Figure legends

Fig. 1. Alignment of the DNA sequence of the LAMP primer-recognized regions for five isolates of Cymbidium mosaic virus. Nucleotide sequences differing from that of AY429021 are shown as lower case letters, and identical nucleotides are shown as periods. The sequences of primer sites are boxed. The five isolates are from Taiwan (AY429021, AY571289, and EU314803), Japan (AB197937) and Korean (AF016914) and were obtained from GenBank.

Fig. 2. Analysis and specificity of the loop-mediated isothermal amplification (LAMP) assay. (a) Agarose gel electrophoresis of the LAMP products. LAMP was performed as described in the methods using different templates. Lane M, molecular markers; lane 1, negative control; lane 2, cDNA derived from total RNA extracted from a CymMV-infected *Nicotiana benthamiana*; lane 3, cDNA derived from CymMV RNA; lane 4, CymMV CP partial cDNA clone. (b) Agarose gel electrophoresis of the LAMP products digested with Hpa II. Lane 1, the digested product of lane 2 product in (a); lane 2, the digested product of lane 3 product in (a). (c) Visualization of the LAMP products using UV excitation. The tubes from left to right containing the LAMP products and SYBR Green I are the same as those in lanes 1, 2, 3, and 4 in (a), respectively.

Fig. 3. Evaluation of the five sets of primers (a) and of sensitivity (b). (a) RT-LAMP assays were performed using different F3 plus B3 primers, namely CyF3-1 plus CyB3-1 (lane 2), CyF3-1 plus CyB3-2 (lane 3), CyF3-1 plus CyB3-3 (lane 4), CyF3-2 plus CyB3-2 (lane 5), CyF3-2 plus CyB3-3 (lane 6), and no primer (lane1) with 4 pg of CymMV viral RNA template. (b) The sensitivity for the RT- LAMP assay was investigated using the CyF3-1 plus CyB3-3 primers and various amounts of CymMV

viral RNA: 12 pg (lane 2), 4 pg (lane 3), 1 pg (lane 4), 0.2 pg (lane 5), 0.04 pg (lane 6) pg Lane 1, negative control, no primer and 12 pg of CymMV viral RNA.

Fig. 4. Alignment of the DNA sequence of the PCR products amplified from the RT-LAMP products. The sequences of the F2 and F3 primers used for the PCR are boxed. Nucleotide sequences differing from that of AY429021 are shown as lower case letters. Nucleotides deleted are indicated as x.

Fig. 5. Detection of CymMV in orchid plants. (a) Agarose gel electrophoresis of the RT-LAMP products amplified using template obtained by total RNA extracted from the leave of *Dendrobium crumenatum* (lane 2), *Dendrobium dixanthum* (lane 3), two other *Dendrobium* sp. (lane 4 and 5), *Phalaenopsis* sp. (lane 6), *Oncidium* sp. (lane 7) and two *Cymbidium ensifolium* (lane 8 and 9). Lane M, molecular marker; lane 1, negative control, no RNA template. (b) Agarose gel electrophoresis of the LAMP products obtained using cDNA derived from the same total RNA sample that was used in (a). The total RNA used were extracted from the leave of *Dendrobium crumenatum* (lane 2), *Dendrobium dixanthum* (lane 3), two other *Dendrobium* sp. (lane 4 and 5), *Phalaenopsis* sp. (lane 6), and *Oncidium* sp. (lane 7). Lane M, molecular marker; lane 1, negative control, no cDNA template.