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One-step reverse transcription loop-mediated isothermal amplification assay for rapid detection of *Cymbidium mosaic virus*

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A B S T R A C T

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 1 h 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.

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; Zettler et al., 1990) and is considered to have spread widely and is generally present among orchids worldwide (Zettler et al., 1990). Some special orchid species that belong to the genus *Dendrobium* have been used in traditional Chinese medicine for the treatment of various diseases (Chinese Materia Medica Dictionary, 1985; Tang and Eisenbrand, 1992). It is reasonable that CymMV may infect these *Dendrobium* sp. and be detrimental to the growth of these valuable orchids. In addition, among ornamental orchids, CymMV usually causes breakdown of flower color, size reduction and stunted growth, thus reducing the quality and quantity of these orchids (Zettler et al., 1990). Therefore, the diagnosis of CymMV for the control of virus infection is important as it will help to reduce the economic impact of this virus on the orchid industry.

To date, 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, time-consuming or requiring expensive equipment. Recently, loop-mediated isothermal amplification (LAMP) and reverse transcription-LAMP assay have been shown to be a rapid, simple, sensitive and inexpensive approach to DNA and RNA detection in various organisms, such as bacteria, parasites and viruses (Notomi et al., 2000; Fukuta et al., 2004; Buates et al., 2010; Huang et al., 2010). Nevertheless, to date, this 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 aim was to develop a RT-LAMP assay that allowed 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 new RT-LAMP assay for the diagnosis of CymMV will not only be useful for the local orchid industry but also will help orchid growers worldwide and make plant quarantine by governments simpler and easier.

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Table 1
Primers used in this study.

Name CyF2	Sequence 5′-CAAGGCTGGTTTCCAGGAGG-3′
CyB2	5′-TGATGAGGTTGCCGTTTTGG-3′
CyFIP	5′-GGCAGTGGAATCGACGGCATCGCAAGGCTGGTTTCCAGGAGG-3′
CyBIP	5′-GCCCTACTGACCGTGAACGTGCTGATGAGGTTGCCGTTTTGG-3′
CyF3-1	5′-GCTGGCCACTAACGATCC-3′
CyF3-2	5′-TGCTGGCCACTAACGATCC-3′
CyB3-1	5′-GATGGCCCTTGGTGACCTC-3′
CyB3-2	5′-AGATGGCCCTTGGTGACCTC-3′
CyB3-3	5′-AAGATGGCCCTTGGTGACCTC-3′

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 the 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, Tokyo, Japan), the primers used for the RT-LAMP assay were designed based specifically on the sequence of the CymMV capsid protein gene (Fig. 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 the RT-LAMP product

After the RT-LAMP reaction was performed, an aliquot of 2 μl 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, Carlsbad, CA). 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 h at 37 °C in a water bath.

2.6. Sensitivity and specificity of the RT-LAMP

The sensitivity of the RT-LAMP assay was evaluated by adding different amounts of viral RNA to the RT-LAMP. 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 the LAMP assay, various CymMV isolates were examined 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 (Fig. 1) and used subsequently for the evaluation of the specificity of CymMV LAMP assay.

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 resulting LAMP 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 the 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 the assay. Reverse transcription was combined in this case 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