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Multiplex RT-PCR detection of two orchid viruses with an internal control of plant *nad5* mRNA

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ABSTRACT

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A multiplex RT-PCR method was developed for the detection of *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) in orchids. Specific primers were designed based on the available sequences of CymMV and ORSV from the GenBank and were used to RT-PCR amplify the respective viral coat protein genes. In addition, one primer pair derived from the plant mitochondrial NADH dehydrogenase gene (*nad5*) was tested for the amplification of *nad5* mRNA as internal control in multiplex RT-PCR. The fragment of internal control mRNA was constantly amplified from total RNA of healthy and infected plants. The specificity of three designed primer pairs for CymMV, ORSV and *nad5* mRNA was confirmed by means of simplex and multiplex RT-PCR assays. The detection sensitivity of multiplex RT-PCR was 1 pg for CymMV and 10 pg for ORSV no mater one or two kinds of viral RNAs existing in samples. The quantity difference of viral RNA seemed to have no influence on the result of multiplex RT-PCR. Application of multiplex RT-PCR could greatly reduce the cost and false negative results for routine detection; therefore, it may have great help to the certification program of orchids.

Key words: *Cymbidium mosaic virus*, *Odontoglossum ringspot virus*, multiplex RT-PCR, *nad5*, internal control.

INTRODUCTION

Cymbidium mosaic virus (CymMV) and *Odontoglossum ringspot virus* (ORSV) are two of the most important viruses in orchids [23, 31, 32]. Although symptoms induced by either virus can be severe, leaves produced by infected plants often look normal [32]. To solve the problem of reliable diagnosis for CymMV and ORSV in orchids, detection methods such as enzyme-linked immunosorbent assay (ELISA) [2, 30], reverse transcription-polymerase chain reaction (RT-PCR) [15, 22, 24], immunocapture-PCR (IC-PCR) [4], digoxigenin (DIG)-labelled cRNA probes [13], immuno-capillary zone electrophoresis (I-CZE) [9], liquid chromatography/mass spectrometry (LC/MS) and matrix-assisted laser desorption-ionization (MALDI) mass spectrometry [26], TaqMan real-time RT-PCR [11], molecular beacons [8], and quartz crystal microbalance (QCM) immunosensors [10] have been employed. Among these methods, ELISA is easy to handle and suitable for large amounts of samples for routine detection. However, it is laborious and less sensitive than other approaches. Moreover, ELISA and other detection methods, such as IC-PCR, I-CZE, and QCM immunosensors, require time-consuming production of virus-specific antibodies. Although LC/MS and MALDI detection methods allow automated analysis of multiple samples with simple preparation steps [26], it needs to obtain and maintain the expensive equipments. On the other hand, the detection methods based on RT-PCR are more sensitive, reliable and rapid for detecting small amounts of viral templates [11, 22, 24]. Most of these methods can detect only single virus in one

reaction and need double detritions for both CymMV and ORSV, except the touch-down RT-PCR [24] and TaqMan real-time RT-PCR [11], which required sequence similarity between two viruses for single primer pair design and well-trained personnel coupled with expensive commercial kits, respectively.

The use of different primer pairs to detect several targets in one reaction is the advantage of multiplex RT-PCR over simplex RT-PCR. Accordingly, multiplex RT-PCR provides a quick, reliable and cost effective method for routine diagnosis of viruses and has been used for plant virus detection successfully during past few years [5, 12, 19, 25, 27]. The incorporation of plant mRNA specific primer pair in multiplex RT-PCR assay provides a useful indicator of the quality of plant extract and the effectiveness of RT-PCR [3]. Performance of multiplex RT-PCR with an internal control can also minimize the risk of obtaining false negative results [17, 27]. Although several plant internal control primers have been published [3, 18], but none of them can distinguish DNA from RNA, so that the complete removal of DNA is required before performing RT-PCR [17, 18]. To solve this problem, Menzel *et al.* [17] designed the primers for amplifying apple mitochondrial NADH dehydrogenase subunit 5 (*nad5*) gene in this way that the sense primer spans the splice junction with the last three nucleotides being in the second exon, and the antisense primer locates in the second exon. Therefore, the primers could only amplify the spliced mRNA-derived cDNA but not the intron-containing DNA. Therefore, there is no need to eliminate contaminating DNA from plant extract.

This study set out to develop a multiplex RT-PCR method for the detection of two orchid viruses, CymMV and ORSV. The coat protein (CP) genes of CymMV and ORSV were selected for RT-PCR amplification because high CP sequence conservation was reported among different isolates of individual virus [1]. In addition, one pair of plant mRNA specific primers was designed and also tested for their use to amplify internal controls in this multiplex RT-PCR assay.

MATERIALS AND METHODS

Virus and plant materials

Phalaenopsis orchids collected from growers were verified to be healthy, CymMV-, ORSV- or mixed-infected by ELISA using CymMV and ORSV antibodies (Agdia, Elkhart, IN, USA), and treated as experimental standards. CymMV and ORSV were isolated from these orchids and maintained in *Nicotiana benthamiana* for virion purification. Virus particles were purified from infected tissues according to the method of Lin and Chen [16] for CymMV and the method of Chapman [7] for ORSV. Orchid plants, collected from Chien Kuo holiday flower market in Taipei, were used as detection samples.

Plant total RNA and viral RNA extraction

Total RNA was extracted from leaf tissue of *Phalaenopsis* orchid with Plant Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA) according to the manufacture's manual. In brief, 100 mg of orchid leaf tissue was ground to fine powder with liquid nitrogen and then transferred to a microfuge tube. After mixed with 450 μl RX buffer, the solution was filtrated by Shearing tube column. The clear filtrates were mixed with 230 μl absolute ethanol, and then filtrated by Plant Total RNA tube column. This column was then washed once with WF solution and twice with WS solution. Finally, plant total RNA was eluted with 50 μl ddH_2O. The concentration of RNA was measured by GeneQuant II RNA/DNA Calculator (Pharmacia Biotech Ltd., Piscataway, NJ, USA). Plant total RNA was used directly for simplex and multiplex RT-PCR or stored at -80℃ for future use. Viral RNA was purified from virions by ammonia carbonate buffer and phenol/chloroform extraction [6]. After ethanol precipitation, viral RNA was resuspended by ddH_2O and the RNA concentration was determined.

Primer design

Sequence data of CymMV (AF016914, AY571289 and U62963) and ORSV (NC_001728, AY571290, S83257 and U34586) were collected from the GenBank of the National Center for Biotechnology Information (NCBI). Nucleotide sequences were aligned using the PILEUP and PRETTY programs in the Wisconsin GCG Package version 10.3 (Accelrys Inc., San Diego, CA, USA). Based on the results of sequence alignment, CP genes of CymMV and ORSV were selected to design virus specific primers for RT-PCR amplification. The primer names, targets, oligonucleotide sequences, locations within the genome and the expected size of amplified products are shown in Table 1. There are degenerate nucleotides appearing in three positions of CymMV CP-F1 primer to ensure the amplification of different CymMV isolates. Plant mitochondrial *nad5* mRNA was selected as the internal control for RT-PCR assay. Sequence data of *nad5* gene (D37958 and X07566) were used to design the internal control primers. Because the *nad5* gene consists of two exons (a and b) separated by an 848-bp intron [14, 17], the forward primer (mt-F2) was designed to locate across the splice junction of these two exons. That is the first 17 nucleotides of mt-F2 primer are homologous to the

Table 1. Primer names, sequences and position in the respective genomes, and expected size of RT-PCR product for each primer pair

Target	Primer[a]	Sequence[b] 5'- 3'	Position	Product size
CymMV	CymMV CP-F1	ATGGGAGAGYCCACTCCARCYCCAGC	CP gene 1-26 nt	669 bp
	CymMV CP-R1	TTCAGTAGGGGGTGCAGGCA	CP gene 669-650 nt	
ORSV	ORSV CP-F1	ATGTCTTACACTATTACAGACCCG	CP gene 1-24 nt	474 bp
	ORSV CP-R1	GGAAGAGGTCCAAGTAAGTCC	CP gene 474-454 nt	
Plant *nad5* mRNA	mt-F2	GCTTCTTGGGGCTTCTT*GTTCGATA*	*nad5* gene exons a+b	185 bp
	mt-R1	ATCTCCAGTCACCAACATTGGCAT	*nad5* gene exon b	

[a] F1 and F2 indicate forward primers, whereas R1 indicates reverse primer.

[b] Nucleotide at degenerate positions are represented by a single letter code; R = A and G; Y = C and T. For mt-F2 primer, the sequences present in *nad5* gene exon b are shown in italic font.

sequences at the 3'-end of exon a and the last 8 nucleotides of the primer are identical to the first 8 nucleotides of exon b (Table 1). The reverse primer of *nad5* gene, mt-R1, is positioned in exon b. The primers were synthesized by MDBio, Inc. (Taipei, Taiwan).

Simplex reverse transcription-polymerase chain reaction (RT-PCR)

For 12.5 μl RT reaction, 2 μl of extracted plant total RNA (200 ng) with/without viral RNA was mixed with 0.5 μl of 5 μM reverse primer, CymMV CP-R1, ORSV CP-R1 or mt-R1, which specifically targets to CymMV, ORSV, or *nad5* RNA, respectively. This mixture was then added ddH_2O to 5 μl. The solution was heated for 10 min at 70 ℃, instantly cooled down on ice for 5 min and then an RT mixture including 3.25 μl ddH_2O, 2.5 μl 5X first strand buffer (Promega, Madison, WI, USA), 1.25 μl dNTPs (10 mM), 0.25 μl rRNasin (40 U/ μl, Promega) and 0.25 μl AMV reverse transcriptase (10 U/ μl, Promega) were added and incubated at 42 ℃ for 60 min. For PCR, 20 μl reaction containing 2 μl RT product, 2 μl 10X DyNAzyme™ II DNA polymerase buffer (Finnzymes Inc., Espoo, Finland), 2 μl forward and reverse primers (5 μM CymMV CP-F1/CymMV CP-R1, ORSV CP-F1/ORSV CP-R1, or mt-F2/mt-R1), 2 μl dNTPs (2 mM), 0.4 μl DyNAzyme™ II DNA polymerase (2 U/ μl, Finnzymes Inc., Espoo, Finland) and 11.6 μl ddH_2O was performed. The amplification was carried out using GeneAmp® PCR system 2400 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with a 5 min denaturation step at 96 ℃, followed by 30 cycles of 96 ℃ for 30 sec, 52 ℃ for 30 sec, 72 ℃ for 30 sec, and a final extension step at 72 ℃ for 7 min. RT-PCR products were then analyzed by agarose gel electrophoresis.

Multiplex RT-PCR

RT reaction was performed as described above except that three reverse primers (CymMV CP-R1, ORSV CP-R1 and mt-R1) were added simultaneously. For 20 μl PCR reaction, 11.6 μl ddH_2O, 2 μl RT product, 2 μl 10X DyNAzyme™ II DNA polymerase buffer, 2 μl multiplex primer set (including 2.5 μM mt-F2/mt-R1, 1.25 μM CymMV CP-F1/CymMV CP-R1, 1.25 μM ORSV CP-F1/ORSV CP-R1), 2 μl dNTPs (2 mM) and 0.4 μl DyNAzyme™ II DNA polymerase was mixed and assayed in the same PCR condition. RT-PCR products were then analyzed by agarose gel electrophoresis. A 10-fold serial dilutions of viral RNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) which were separately mixed with healthy orchid total RNA (200 ng) were used to assess the detection limit of multiplex RT-PCR.

Agarose gel electrophoresis

Ten μl of RT-PCR products were used for electrophoresis analysis. The PCR fragments were separated in a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The amplified DNA fragments were visualized by staining the gel with ethidium bromide (0.5 μg / ml) and then illuminating with UV light. Sizes of fragments were determined with the Kodak Digital Science™ 1D image analysis software (Eastman Kodak Company, R℃hester, NY, USA) by comparison with DNA molecular weight markers (1 kb Plus DNA Ladder, Invitrogen, Carlsbad, CA, USA).

RESULTS

Specificity of designed primers

To test the specificity of designed primers, total RNA extracted from healthy and virus-infected orchids were

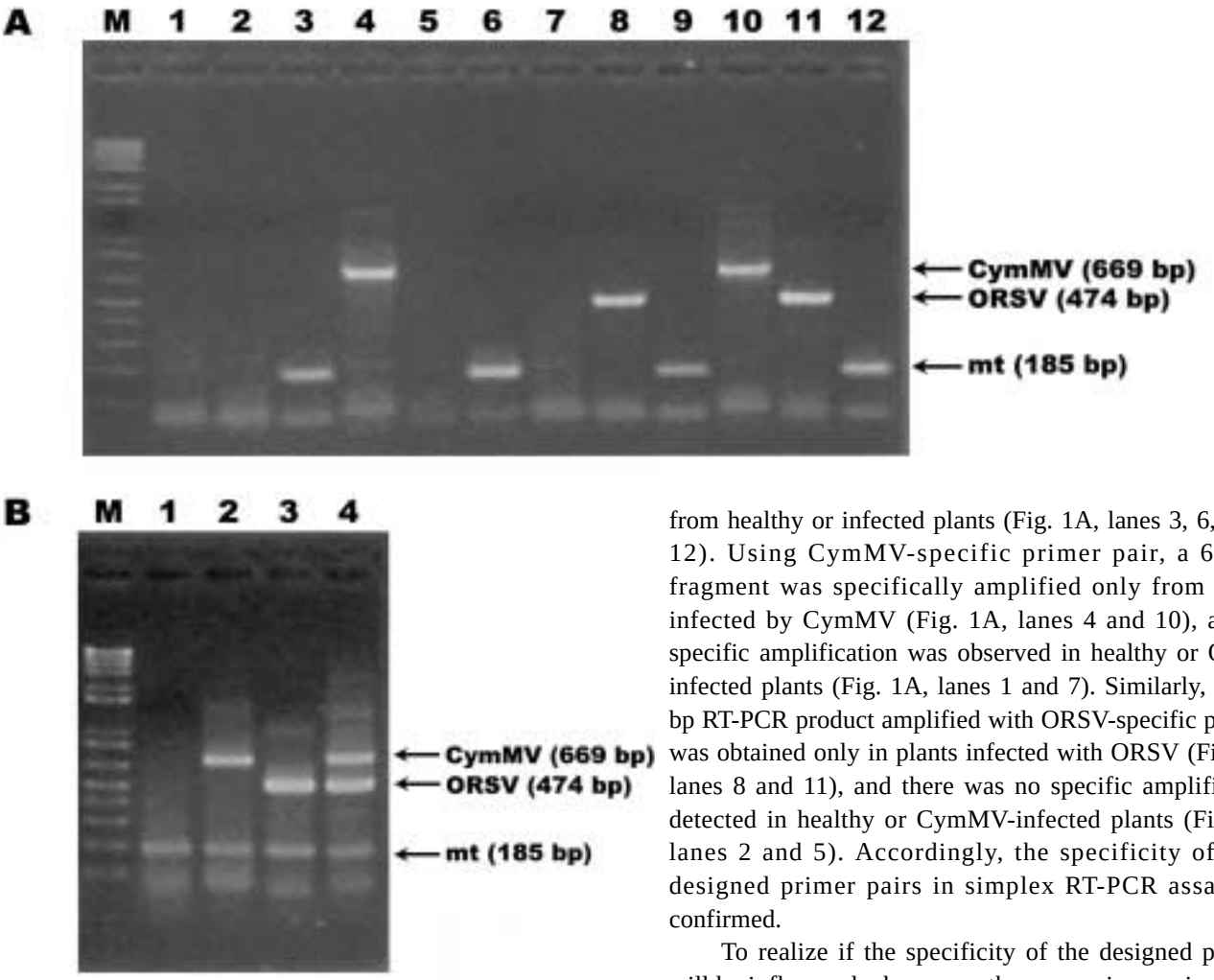

Fig. 1. The specificity of each primer pair in simplex and multiplex RT-PCR. A: Total RNA of healthy (lanes 1-3), CymMV-infected (lanes 4-6), ORSV-infected (lanes 7-9), and mix-infected (lanes 10-12) orchid plants were used for simplex RT-PCR amplification. The assay was performed with the primer pair of CymMV CP-F1/CymMV CP-R1 (lanes 1, 4, 7 and 10), ORSV CP-F1/ORSV CP-R1 (lanes 2, 5, 8 and 11), or mt-F2/mt-R1 (lanes 3, 6, 9 and 12), respectively. B: Total RNA of healthy (lane 1), CymMV-infected (lane 2), ORSV-infected (lane 3) and mix-infected (lane 4) orchid plants were used for multiplex RT-PCR amplification. The assay was performed with the multiplex primer set including CymMV CP-F1/CymMV CP-R1, ORSV CP-F1/ORSV CP-R1 and mt-F2/mt-R1. The RT-PCR products of CymMV, ORSV and internal control (mt) identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

used for RT-PCR amplification. A cDNA fragment of the expected size (mt, 185 bp) was consistently amplified by *nad5* mRNA-specific primers (mt-F2/mt-R1) as plant internal control, irrespective of whether total RNA came from healthy or infected plants (Fig. 1A, lanes 3, 6, 9 and 12). Using CymMV-specific primer pair, a 669-bp fragment was specifically amplified only from plants infected by CymMV (Fig. 1A, lanes 4 and 10), and no specific amplification was observed in healthy or ORSV-infected plants (Fig. 1A, lanes 1 and 7). Similarly, a 474-bp RT-PCR product amplified with ORSV-specific primers was obtained only in plants infected with ORSV (Fig. 1A, lanes 8 and 11), and there was no specific amplification detected in healthy or CymMV-infected plants (Fig. 1A, lanes 2 and 5). Accordingly, the specificity of three designed primer pairs in simplex RT-PCR assay was confirmed.

To realize if the specificity of the designed primers will be influenced when more than one primer pair present in RT-PCR solution, the multiplex primer set containing CymMV CP-F1/CymMV CP-R1, ORSV CP-F1/ORSV CP-R1 and mt-F2/mt-R1 were used for RT-PCR assay. As previously described, total RNAs extracted from healthy, CymMV-infected, ORSV-infected and mix-infected orchids were tested for multiplex RT-PCR amplification. The expected RT-PCR product of internal control was amplified from all orchid samples (Fig. 1B) although the level of amplification seemed better in simplex RT-PCR than in multiplex RT-PCR. However, the amplification of CymMV and ORSV specific fragments from single and mix-infected plants were very well by multiplex RT-PCR (Fig. 1B, lanes 2-4). From the above RT-PCR results, it clearly indicated that both single- and triple-pair of primers could produce specific cDNA fragments of plant *nad5* mRNA, CymMV and ORSV. Therefore, the specificity of these designed primers in multiplex RT-PCR was as good as that in simplex RT-PCR.

Optimization of multiplex RT-PCR reaction

For better results of multiplex RT-PCR detection, parameters such as the concentration of each primer pairs

and PCR annealing temperature were evaluated using mix-infected orchid. The concentration ratio of CymMV, ORSV and internal control (mt) specific primers were tested from 0.1:0.1:1 to 1:1:3 (1 is equal to 0.25 μM). The final concentrations of each viral specific primers were from 0.025 to 0.25 μM, and those of internal control primers ranged from 0.25 to 0.75 μM. The amplification efficiency of viral genes was stable in spite of primer concentration changed (Fig. 2). By contrast, the amplification efficiency for plant *nad5* mRNA was decreased while the primer concentration increased (Fig. 2). For the balance between the virus detection and plant internal control mRNA amplification, multiplex RT-PCR was then carried out with 0.125 μM of each viral specific primer and 0.25 μM of each plant *nad5* internal control primer (that is 0.5:0.5:1 in Fig. 2). In PCR reaction, the annealing temperature was tested from 50 ℃ to 57 ℃ (by 1 ℃ interval), but no significant difference in amplification efficiency was observed among treatments (data not shown). An annealing temperature of 52 ℃ was then used for further multiplex RT-PCR detection condition.

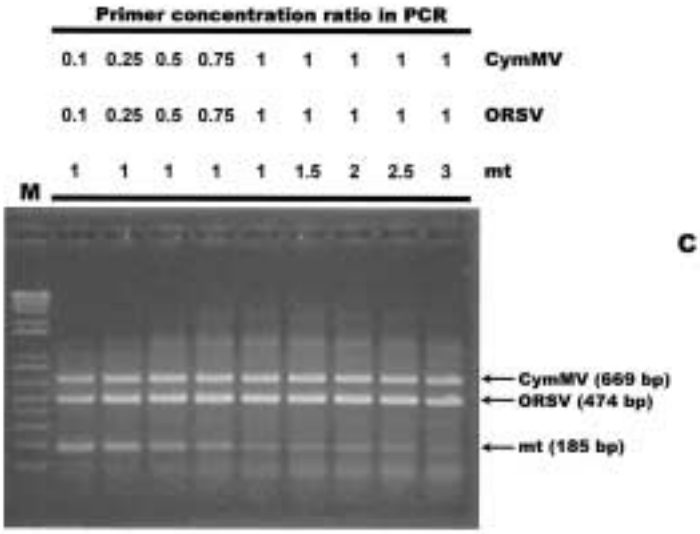

Fig. 2. Optimization of multiplex RT-PCR reaction with different concentration ratios of primer set. Total RNA of mix-infected orchid plant was used for multiplex RT-PCR amplification. The assay was performed with the primer pair of CymMV CP-F1/CymMV CP-R1 (CymMV), ORSV CP-F1/ORSV CP-R1 (ORSV) and mt-F2/mt-R1 (mt). The primer concentration ratio of each treatment was shown on the top of the figure. The number indicates the ratio of each primer pair, and 1 is equal to a final concentration of 0.25 μM primer in PCR reaction. The RT-PCR products of CymMV, ORSV and internal control (mt) identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

Detection sensitivity of multiplex RT-PCR

To determine the detection limits of this multiplex RT-PCR, a 10-fold serial dilutions (10 ng - 1 fg) of CymMV, ORSV or both viral RNAs were added into total RNA (200 ng) extracted from healthy orchid. In the multiplex RT-PCR, when only one type viral RNA present in plant total RNA, 1 pg of CymMV RNA and 10 pg of ORSV RNA were detected with constant amplification of plant mRNA *nad5* (Figs. 3A and 3B). When templates contained plant total RNA and both viral RNAs, the

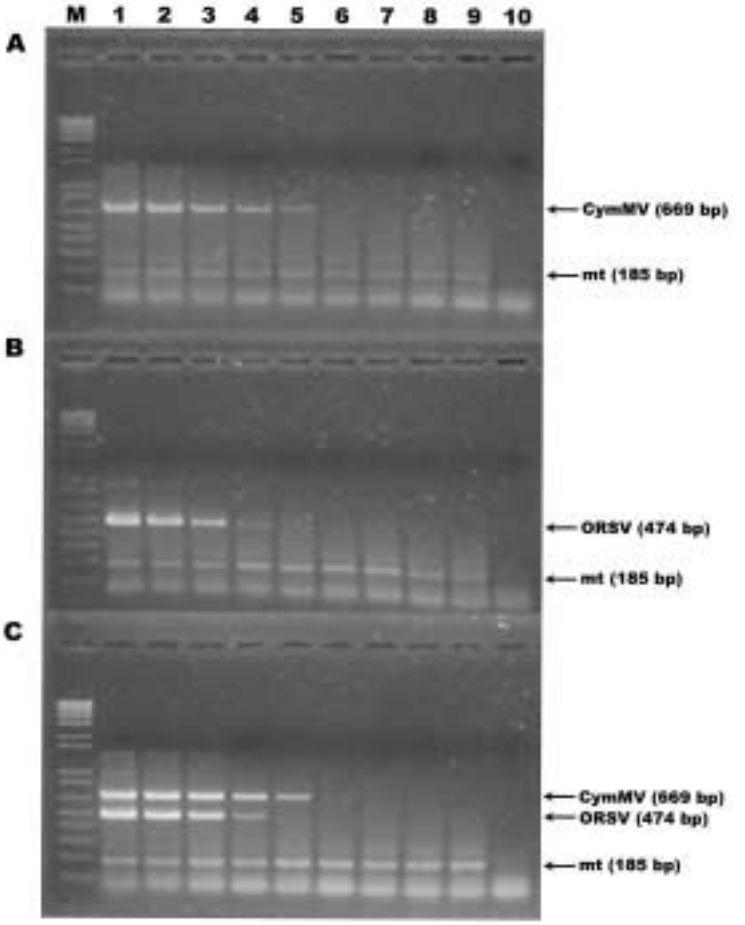

Fig. 3. Detection sensitivity of multiplex RT-PCR. The assay was performed with the multiplex primer set as Fig. 1B. CymMV (panel A), ORSV (panel B) or both (panel C) viral RNAs diluted in total RNA (200 ng) from healthy orchid was used for multiplex RT-PCR. The amounts of viral RNA added were 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg (lanes 1-8) in panels A and B, and double in panel C, respectively. Total RNA from healthy orchid (lane 9) and water (lane 10) were used as negative controls. The RT-PCR products of CymMV, ORSV and internal control (mt) identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

amplification efficiency of multiplex RT-PCR remained the same (Fig. 3C). These results revealed that when same amount of CymMV and ORSV RNAs were added alone or together to plant total RNA detection sensitivity of multiplex RT-PCR was similar.

To understand whether different quantities of viral RNAs have influence on the detection result, another set of multiplex RT-PCR assays were performed. Total RNA (200 ng) from healthy orchid contained either 10 ng of CymMV viral RNA mixed with 1 ng, 100 pg or 10 pg of ORSV viral RNA, or 10 ng ORSV viral RNA mixed with 1 ng, 100 pg or 10 pg of CymMV viral RNA were used as templates of multiplex RT-PCR. High amount of RT-PCR product was consistently amplified from 10 ng of viral RNA when it was mixed with different amounts of counter virus (Fig. 4). However, the lower amount of counter virus used in multiplex RT-PCR resulted in decreased signals correlated with the reduced viral RNAs. Nevertheless, the result revealed that even when CymMV and ORSV RNAs had 1000 times difference, the amplification products of virus in low amount were still visible in multiplex RT-PCR (Fig. 4, lanes 3 and 6).

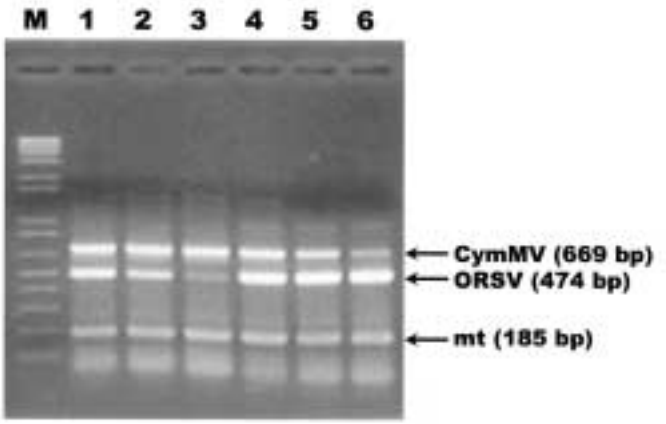

Fig. 4. Multiplex RT-PCR detection with different amounts of two viral RNAs. Different amounts of CymMV and ORSV viral RNAs were mixed with healthy orchid total RNA (200 ng). Ten ng of CymMV viral RNA were mixed with 1 ng, 100 pg or 10 pg of ORSV viral RNA (lanes 1-3), and 10 ng of ORSV viral RNA were mixed with 1 ng, 100 pg and 10 pg of CymMV viral RNA (lanes 4-6), respectively. The RT-PCR products of CymMV, ORSV and internal control (mt) identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

Detection of orchid samples from market using multiplex RT-PCR

The multiplex RT-PCR method developed was then applied to detect the existence of CymMV and ORSV in *Phalaenopsis* orchids sold in the flower market. Orchid plants with no obvious symptom were selected randomly and total RNA extracted from these orchid samples were used for multiplex RT-PCR detection. The amplification efficiency of multiplex RT-PCR was confirmed by the internal control. Three fourth of the market plants were free from infections by CymMV and ORSV (Fig. 5). Among three virus-infected plants, one was mix-infected with CymMV and ORSV, and the other two samples were either infected with CymMV or ORSV, respectively (Fig. 5). The expected cDNA fragment of plant *nad5* mRNA

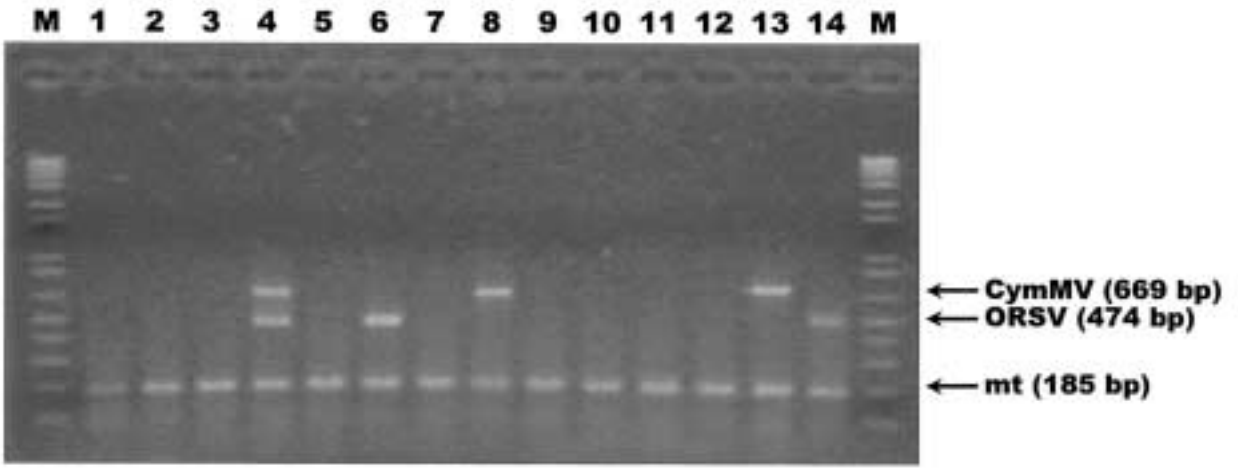

Fig. 5. Simultaneous detection of CymMV and ORSV in orchid plants using multiplex RT-PCR. Twelve orchid samples collected from holiday flower market were tested (lanes 1-12). Orchid plants infected with CymMV (lane 13) or ORSV (lane 14) were used as positive controls, respectively. The RT-PCR products of CymMV, ORSV and internal control (mt) identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

was amplified in all tested samples indicating the effectiveness of the application of internal control in multiplex RT-PCR.

DISCUSSION

This paper describes a multiplex RT-PCR assay for the simultaneous detection of two important orchid viruses, CymMV and ORSV, and the specific coamplification of plant *nad5* mRNA as internal control. In addition to the mRNA of apple mitochondrial NADH dehydrogenase subunit 5 (*nad5*) gene [17], mRNA transcribed from chloroplast NADH dehydrogenase ND2 subunit (*ndhB*) gene of *Atropa belladonna* [27] was used as the internal control in the multiplex RT-PCR assay. Both plant mRNA specific primer pairs were designed to amplify the target mRNA from total nucleic acids without the requirement of removing contaminating DNA. Our plant internal control primers were also designed for *nad5* gene but the extension of homologous nucleotide, which is identical to the sequence of exon b, from 3 to 8 nucleotides at 3' end of the forward primer (mt-F2) greatly increased the primer specificity in RT-PCR assay (data not shown). This result indicated that when it was required to design a primer across the splice junction, the PCR amplification of the primer could be improved as long as the sequence length at the second exon could be increased.

The specificity of three designed primer pairs for CymMV, ORSV and *nad5* mRNA was confirmed by means of simplex and multiples RT-PCR assays (Fig. 1). Interaction between primers which competed for polymerase and templates during multiplex PCR might be different from simplex PCR due to the nature of the primers [20]. Therefore, it is necessary to determine empirically the optimal proportion of each primer for multiplex RT-PCR. According to the results, the amplification of plant *nad5* mRNA reduced when the concentration of the primers was over 0.25 μM (Fig. 2). This may be attributed to the interferences that occur in PCR amplifications at high primer concentrations [28]. Nevertheless, the primer pair corresponding to CymMV and ORSV gave constant results when the concentrations of the primers were changed.

In competitive multiplex RT-PCR the detection sensitivity for two viral RNAs set at equivalent concentrations was less than when only one virus was present [28]. The reason for the decrease in detection sensitivity might be that the universal degenerate primer in multiplex PCR competed for the two viral templates rather than for one and therefore lowered the detection limit [21, 28]. In contrast, the detection sensitivities of our multiplex RT-PCR were the same no matter single or both viruses appearing in samples (Fig. 3). This difference might result from specific primers instead of universal degenerate primer used in our assays. This is the advantage of common multiplex PCR over the competitive multiplex PCR although the latter needs fewer primers than the former.

Recently, Vidalakis *et al.* [29] showed that the biological indexing in a mixed infection can be seriously impaired and the presence of different co-infecting pathogens might affect the detection efficacy. In addition, titer or distribution of some viruses may be affected in mixed-infected plants and thus decrease the efficacy of molecular detection methods such as multiplex PCR [21]. Furthermore, if one pathogen masks the presence of another, the false negative result may lead to inappropriate procedures to establish disease-free materials [29]. To mimic the uneven distribution and accumulation of viruses in orchid plants, a multiplex RT-PCR assay was performed with plant total RNA containing different amounts of CymMV and ORSV RNAs. Even when the amount of these two viruses was 1000 times different, the presence of virus was not masked because of low amount based on the results of multiplex RT-PCR (Fig. 4). That is the detection limit of ORSV remained 10 pg when 10 ng of CymMV RNA occurred in total RNA sample (Fig. 4, lane 3).

When detecting the orchid samples with multiplex RT-PCR, one third of them were infected by CymMV or/and ORSV. Since these orchid plants did not show obvious symptoms, it is possible to spread the viruses when handling the plants. For this reason, orchid certification program is being established in Taiwan. Viruses other than CymMV and ORSV may also be considered as detection targets in the certification program since there are more than 23 viruses have been identified in orchids [32]. The multiplex RT-PCR assay has been successfully used for simultaneously detection of up to seven different viruses in a single test [21]. As requested viruses are characterized and primers are developed, they can be added to this assay with appropriate confirmation. Apart from ELISA, this multiplex RT-PCR method may greatly reduce the cost and avoid the false negative results, which is desirable for routine virus diagnosis, may have great help to the certification program of orchids.

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摘要

李淑娟[1]、張雅君[1,2]. 2006. 以多對引子反轉錄聚合酶連鎖反應同時檢測兩種蘭花病毒和植物內在對照*nad5* mRNA. 植病會刊15:187-196. ([1]台北市 國立台灣大學植物病理與微生物學系；[2]聯絡作者，電子郵件：ycchang@ntu.edu.tw；傳眞：+886-2-23620271)

針對感染蘭科植物的蕙蘭嵌紋病毒 (*Cymbidium mosaic virus*, CymMV) 及齒舌蘭輪斑病毒 (*Odontoglossum ringspot virus*, ORSV) 開發出一套利用多對引子反轉錄聚合酶連鎖反應 (multiplex RT-PCR) 爲基礎的檢測方法。由基因資料庫 (GenBank) 中蒐集此二病毒的基因體序列，進行序列比對後，分別設計CymMV 和ORSV 之專一性引子對，以增幅個別病毒的鞘蛋白基因。除此之外，針對植物粒線體煙醯胺腺嘌呤二核酸去氫酶 (NADH dehydrogenase, *nad5*) 基因也設計另一組引子對，在進行反轉錄聚合酶連鎖反應時，可以專一性增幅出 *nad5* 的訊息RNA (mRNA) 片段，以此作爲檢測時的反應內在對照組。利用 *nad5* 專一性引子對，不論在健康或是病毒感染的植物全 RNA 樣品中，皆可以穩定的增幅出 *nad5* mRNA 片段。上述三組引子對不論是利用單對引子及多對引子反轉錄聚合酶連鎖反應進行測試，結果證實皆具有高度專一性。當以多對引子反轉錄聚合酶連鎖反應測試方法的靈敏度時，不論樣品含有單一或兩種病毒，針對 CymMV 檢測靈敏度皆可達 1 pg，對ORSV的檢測靈敏度則爲 10 pg。在複合感染的樣品中，此二病毒存在量的差異似乎不會影響檢測的結果。本檢測方法的研發可以應用於例行的病毒篩檢，同時可以降低檢測成本與避免僞陰性反應，期望對於國內蘭花種苗病毒驗證作業有所助益。

關鍵詞： 蕙蘭嵌紋病毒、齒舌蘭輪斑病毒、多對引子反轉錄聚合酶連鎖反應、*nad5*、反應內在對照組