Electronic Journal of Biotechnology 26 (2017) 64–68

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

# A highly sensitive method for the detection of Chrysanthemum virus B

Zhiyong Guan *, Dan Wu, Aiping Song, Fadi Chen, Sumei Chen, Weimin Fang

*College of Horticulture, Nanjing Agricultural University, Nanjing, China*

ARTICLE INFO

*Article history:*
Received 12 October 2016
Accepted 11 January 2017
Available online 17 January 2017

*Keywords:*
Coat protein
Diagnosis
Early stage diagnosis
Improved sensitivity
Polymerase chain reaction
Plant diseases
Real-time PCR
Sensitivity
Specific primer
Viral diseases
Viruses

ABSTRACT

*Background:* Chrysanthemum plants are subject to serious viral diseases. The viruses cause severe losses of the quantity and quality of chrysanthemum. The most problematic pathogen of chrysanthemum is typically considered Chrysanthemum virus B (CVB). Thus, a method for the simultaneous detection of CVB is needed.
*Results:* We used gene-specific primers, which were derived from the coat protein gene region of the virus, for reverse transcription to obtain cDNA. Nested amplification polymerase chain reaction (PCR) was employed to detect the viral gene. This method was sensitive enough to detect the virus at up to $10^{-9}$ dilution of the cDNA.
*Conclusion:* A highly specific and sensitive nested PCR-based assay has been described for detecting CVB. This new method is highly specific and sensitive for the detection of CVB, which is known to infect chrysanthemum plants in the fields. Further, this protocol has an advantage over traditional methods as it is more cost-effective. This assay is ideal for an early stage diagnosis of the disease.



## 1. Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is a frequently used ornamental species, second only to the rose in terms of its market value [1]. Because elite cultivars are typically propagated vegetatively, the risk of disseminating viral pathogens is high. A number of viruses are known to infect chrysanthemum that cause benign, stunt, chlorosis, mosaic symptoms, and mottle [2,3]. Consequently, this has led to qualitative and quantitative losses, resulting in serious problems in chrysanthemum production worldwide [4]. Chrysanthemum virus B (CVB), a member of the genus *Carlavirus*, is a single-stranded RNA virus and the causal agent of a severe disease in chrysanthemum. The length of the genomic RNA of CVB is 8000–9000 nucleotides, excluding the poly(A) tail, containing six open reading frames [5]. The virus particles are slightly flexuous, rod shaped, 685 nm long, and 12 nm in diameter [6,7]. Chrysanthemums infected with CVB have various symptoms; usually, the foliage of heavily infected plants is highly maticized and their flowers are malformed. However, occasionally, no symptoms are seen [8]. Thus, it is a serious potential threat to the floriculture industry worldwide [9].

A number of methods have been developed to detect plant viruses. The most commonly employed platform is the enzyme-linked immunosorbent assay (ELISA) [10], but it suffers from both a high rate of false negatives and a relatively low level of sensitivity. The sensitivity of DAS-ELISA and DOT-ELISA to detect potato viruses just for 10 ng $mL^{-1}$ [11]. The loop-mediated isothermal amplification assay is vulnerable to contamination, and primer design is not easy [12,13]. In samples with low viral RNA concentration, DNA amplification is not always sufficient enough to be detected by the visual assessment of turbidity. More recently, reverse transcription polymerase chain reaction (RT-PCR) is widely used for the detection of virus in plants because of its sensitivity, specificity, and rapidity [14], including nested PCR [15], multiplex RT-PCR [16], and real-time quantitative PCR (qRT-PCR) [17]. Song et al. [18] showed the end-point dilution limit to be $10^{-5}$ by single and multiplex RT-PCR. However, further research on whether using more primers would affect the detection sensitivity of multiplex RT-PCR assay is needed. Quantitative analysis of viral DNA by real-time PCR may become a valuable tool for monitoring virus infection and progression. Chen et al. developed a fluorescent qRT-PCR assay for the detection of Impatiens necrotic spot virus [19]; Agindotan et al. [20] described an assay in which four common potato-infecting viruses, Potato leafroll virus, Potato virus A, Potato virus X, and Potato virus Y, were detected simultaneously in real-time RT-PCR. The early diagnosis of CVB for chrysanthemum viral disease prevention and control requires a further increase in sensitivity over what is currently achievable.

In this study, a modified sensitive and specific approach to the molecular typing of CVB is described. We used gene-specific primers that were derived from the coat protein gene region of the virus for

* Corresponding author.
*E-mail address:* guanzhy@njau.edu.cn (Z. Guan).

http://dx.doi.org/10.1016/j.ejbt.2017.01.003

reverse transcription to obtain cDNA. We evaluated the assay for detecting CVB and improved the detection sensitivity. The method in this study can guarantee the accuracy of the test results and can be used to more effectively control the proliferation and spread of the virus and prevent further development of infection.

## 2. Materials and methods

### 2.1. Plant materials and RNA extraction

A survey of the incidence of viruses was performed during the late spring to late autumn in 2014 in Nanjing, China. Diseased leaves from 15 cultivars were collected from an experimental field at the National Chrysanthemum Germplasm Resources Preservation Center (Nanjing, China). The plants expressed CVB-like symptoms, including leaf chlorosis, mosaic symptoms, mottle, severe stunting with prematurity, and even severe symptoms of leaf spot. Diseased leaves were also sampled from different cultivars of *C. morifolium*. Both naturally available and detoxified virus-free plants were sampled to provide a negative control. Total RNAs were extracted from 0.2 g (fresh weight) of either symptomless or diseased leaves (Fig. 1) using the RNAiso reagent (TaKaRa, Tokyo, Japan). The resulting precipitated RNAs were dissolved in RNase-free distilled water.

### 2.2. Primer design

Primers targeted to the CVB coat protein gene sequence were designed using PRIMER PREMIER V5.0 software (Premier Biosoft Int., CA, USA). Their sequences and expected amplicon sizes are given in Table 1.

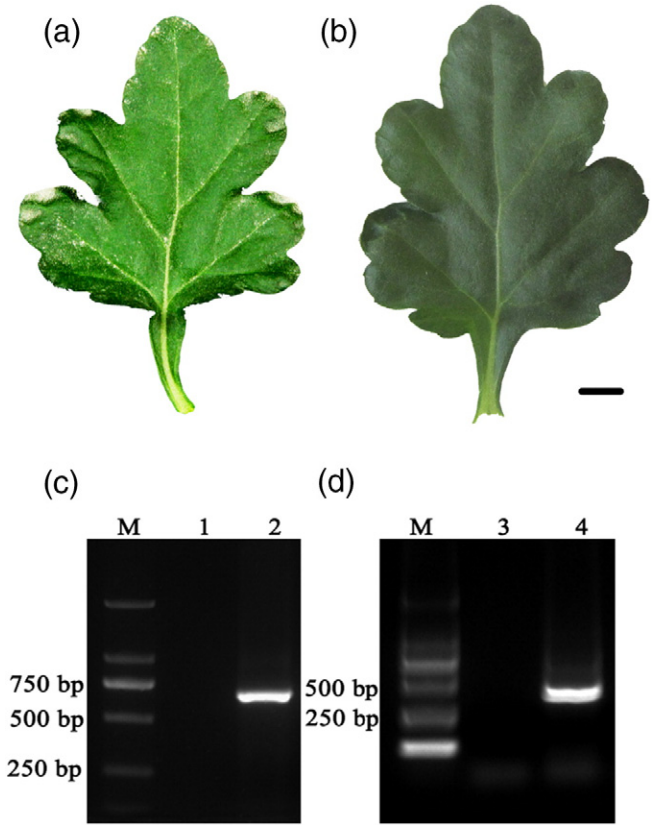


**Fig. 1.** (top) Symptoms of healthy plants and infected plants: (a) infected plants; (b) healthy plants. (bottom) Detection of CVB by nested PCR using gene-specific primers: lane M: DL2000 marker (TaKaRa); lane 1, lane 3: healthy control; lane 2: 621-bp fragment obtained from first ground; lane 4: 381-bp fragment obtained from second ground. (c) The first ground of nested RT-PCR; (d) second ground of nest PCR.

### 2.3. Reverse transcription performance

The cDNA synthesis reaction was conducted in a total volume of 20 μL. One microliter of 1 μg $\mu L^{-1}$ RNA template and either 2 μL of oligo-dT primer and 2 μL of random hexamers (25 μmol $L^{-1}$) or a combination of both or gene-specific primers were brought to a final volume of 12 μL with RNase-free water. The RNA was denatured at 70°C for 10 min and cooled to room temperature for 2 min. Then 8 μL of cDNA synthesis mastermix, 1 μL 10 μM dNTP, 4.0 μL 5× M-MLV buffer, 0.5 μL of 40 U $\mu L^{-1}$ RNase inhibitor (TaKaRa), 0.8 μL of 200 U $\mu L^{-1}$ M-MLV reverse transcriptase (TaKaRa), and 1.7 μL of RNase-free distilled water were added. The reactions were incubated at 42°C for 1 h and heated to 70°C for 15 min to inactivate the enzyme.

### 2.4. Nest PCR performance

The initial round of the nested PCR protocol involved a 25-μL reaction comprising 2.5 μL of 10× PCR buffer, 2.0 μL of 2.5 μM dNTP, 1 μL of 20 μM each forward and reverse primer (CVB-$F_1$ and CVB-$R_1$, respectively), 1 μL of cDNA template, 0.2 μL of 5 U $\mu L^{-1}$ *Taq* DNA polymerase and 17.5 μL of double-distilled water ($ddH_2O$). The second round was performed by taking a 1-μL aliquot of the first-round PCR product as template, replacing the CVB-$F_2$ and CVB-$R_2$. The thermal cycling protocol comprised an initial denaturation step (94°C for 3 min), followed by 35 cycles of 94°C for 30 s, 53°C (first round) and 57°C (second round) for 45 s each, 72°C for 45 s and completed by a final extension step (72°C for 7 min). The PCR products were separated by electrophoresis through a 1.75% TAE agarose gel and visualized by EtBr staining [21].

### 2.5. Real-time quantitative PCR and nested quantitative PCR

qPCRs were performed using a Real-Time PCR System (Eppendorf, Hamburg, Germany). Each 20 μL reaction comprised 5 μL of cDNA template, 1 μL of 10 μM primers (forward and reverse, YCVB-$F_1$, YCVB-$R_1$, respectively; see Table 1), 10 μL of SYBR, and 3 μL of $ddH_2O$. The thermal cycling protocol comprised an initial denaturation step (93°C for 3 min), followed by 40 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 20 s. For the nested qPCRs, the first round was performed as for the nested PCR (see above), and in the second round, the cDNA was replaced by 1 μL of the first-round product, replacing the primers YCVB-$F_2$ and YCVB-$R_2$. Following the PCR, a melting curve analysis was generated over the temperature range 58–95°C.

### 2.6. Sensitivity of RT-PCR

To determine the detection limit of the gene-specific primer PCR assay, both nested and qPCRs were performed using the cDNA derived from all the four methods, i.e., oligo-dT, random hexamer primers (25 μmol $L^{-1}$), a combination of random hexamer and oligo-dT primers, and the coat protein gene of the virus as specific primers. A 10× serial dilution ($1–10^{-10}$) of the cDNA preparation was used a template for the nested PCR assay. The cDNA was generated in four different ways to allow a direct comparison of the assay's performance with the same dilution series templates.

**Table 1**
List of primers used in the RT-PCR.

| Primer name | Sequence 5′–3′ |
|---|---|
| CVB-$F_1$ | AGTCACAATGCCTCCCAAAC |
| CVB-$R_1$ | CATACCTTTCTTAGAGTGCTATGCT |
| CVB-$F_2$ | TCTGAAGGTGAGCCAAGCG |
| CVB-$R_2$ | CATATCCTCGGAAGTAGCCATG |
| YCVB-F | GGTTCCTACCGAGTCAGTCAAAGA |
| YCVB-R | ATCGCACCACCGTCCCAT |