



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.

© 2017 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 mottled 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: guanzyh@njau.edu.cn (Z. Guan).

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.

2.3. Reverse transcription performance

The cDNA synthesis reaction was conducted in a total volume of 20 μL . One microliter of $1 \mu\text{g } \mu\text{L}^{-1}$ RNA template and either 2 μL of oligo-dT primer and 2 μL of random hexamers ($25 \mu\text{mol L}^{-1}$) 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 \times M-MLV buffer, 0.5 μL of 40 U μL^{-1} RNase inhibitor (TaKaRa), 0.8 μL of 200 U μL^{-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 \times PCR buffer, 2.0 μL of 2.5 μM dNTP, 1 μL of 20 μM each forward and reverse primer (CVB-F₁ and CVB-R₁, respectively), 1 μL of cDNA template, 0.2 μL of 5 U μL^{-1} Taq DNA polymerase and 17.5 μL of double-distilled water (ddH₂O). The second round was performed by taking a 1- μL aliquot of the first-round PCR product as template, replacing the CVB-F₂ and CVB-R₂. 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-F₁, YCVB-R₁, respectively; see Table 1), 10 μL of SYBR, and 3 μL of ddH₂O. 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-F₂ and YCVB-R₂. Following the PCR, a melting curve analysis was generated over the temperature range $58\text{--}95^\circ\text{C}$.

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 ($25 \mu\text{mol L}^{-1}$), a combination of random hexamer and oligo-dT primers, and the coat protein gene of the virus as specific primers. A $10\times$ serial dilution ($1\text{--}10^{-10}$) of the cDNA preparation was used as 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	CATACCTTTCITAGAGTGCTATGCT
CVB-F ₂	TCTGAAGGTGAGCCAAGCG
CVB-R ₂	CATATCCTCGGAAGTAGCCATG
YCVB-F	GGTTCCTACCGAGTCAGTCAAAGA
YCVB-R	ATCGCACCACCGTCCCAT

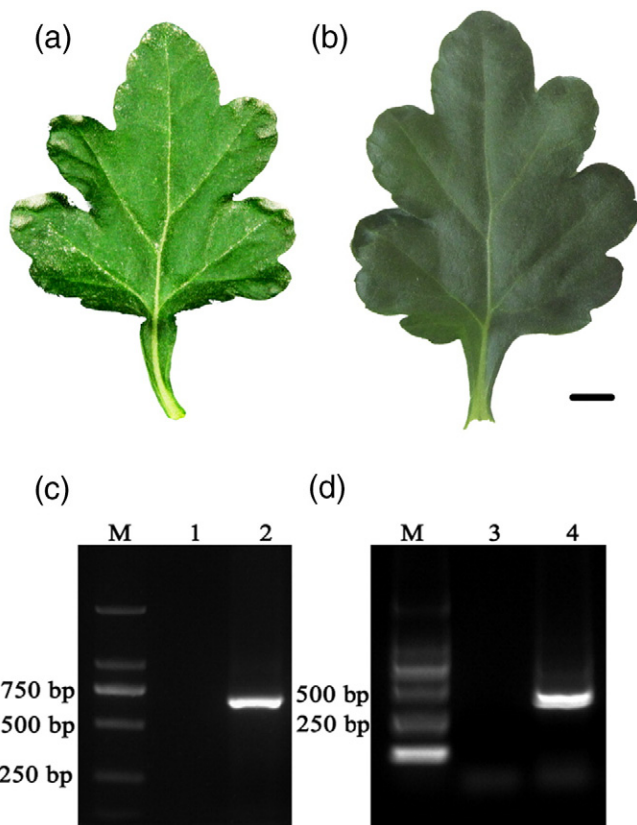


Fig. 1. (top) Symptoms of healthy plants and infected plants: (a) infected plants; (b) healthy plants. (bottom) Detection of CVB by nested PCR using gene-specific primers: lane M: DL2000 marker (TaKaRa); lane 1, lane 3: healthy control; lane 2: 621-bp fragment obtained from first ground; lane 4: 381-bp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

3. Results and discussion

In this study, CVB in diseased plant was detected by nested PCR by using gene-specific primers derived from the coat protein gene region of the virus. The length of the first-round nested RT-PCR amplicon was 621 bp and that of the second-round amplicon was 381 bp (Fig. 1). Samples of the healthy control with no symptoms were detected in lanes 1 and 3. A sample with symptoms of CVB infection is shown in lanes 2 and 4, showing that CVB can be detected in specific fragments of the first and second rounds in nested PCR. The PCR-amplified products from the second round were sequenced, and the sequences were compared with the genomic sequences of CVB deposited in NCBI. The resulting sequence was 98% homologous to that of the CVB coat protein gene in NCBI (GenBank accession EU499736.1).

To test the sensitivity of the assay, four different cDNAs, undergoing a series of dilutions, were analyzed by nested RT-PCR. Using gene-specific primers, we can detect the virus in up to 10^{-9} dilution (Fig. 2d); this was the most efficient method in this study. The oligo-dT and random hexamer primers' end-point dilution limit observed in the study was 10^{-4} (Fig. 2a–b). The cDNA obtained from oligo-dT/hexamer mix primers showed an end-point dilution limit of 10^{-3} (Fig. 2c).

It is very important to design gene-specific primers based on genetic variations for plant disease diagnosis and pathogenic bacteria species identification [22]. To validate the pathogen specificity of the assay, templates prepared from leaves infected with TAV or CMV were tested. A positive result was detected only with the template prepared

from CVB-infected leaf (Fig. 3). This shows a good specificity of the primers, which can be used to detect CVB in chrysanthemum-specific tests.

The amplification results of reverse-transcribed cDNAs by the four different methods in RT-qPCR and nested qPCR were as follows (Fig. 4). The efficiency of the gene-specific primer cDNA was significantly higher than that in the other methods. This shows that gene-specific primers may be used for cDNA synthesis to obtain higher target amounts of amplification. When extended to a panel of 15 of the most important current chrysanthemum cultivars, the assay proved to be effective in each case by using gene-specific primers.

In recent years, with the development of molecular biology, a number of molecular biology techniques applied in the field of plant pathology play an important role in the early diagnosis of plant diseases. Oligo-dT priming is the most widely used method for obtaining cDNA through the reverse transcription of mRNA. The oligo-dT primer is first annealed to the poly(A) tail that is universally present at the 3' end of almost all mRNAs by T–A base pairing. The reverse transcriptase then extends from the annealed oligo-dT primer along the mRNA template, resulting in the copying of the mRNA sequence into the cDNA sequence [23]. However, oligo-dT priming can also lead to truncated cDNAs through internal priming in adenine-rich regions, which occurs relatively frequent [24]. Random hexamers provide a cDNA that can be used for the detection of other RNA viruses by avoiding possible secondary structures such as loops and stems and resulting in a more even representation of the entire mRNA sequence. However, using random hexamers may overestimate the target mRNA copy number [25]. This loss of sensitivity is possibly caused by competition from other RNAs, such as the highly abundant rRNA, with the mRNA for primer binding. We used the coat protein gene of CVB as gene-specific primers for reverse transcription to obtain cDNA. When the PCR is conducted using two gene-specific primers, the 3' terminal of the primer with higher binding specificity to the mRNA will be extended, thus initiating the first strand synthesis. Thus, only the gene-specific primers generate the desired cDNA, resulting in more specific PCR amplification. This greatly amplifies the content of CVB gene, which in turn increases the sensitivity.

Of all the available types of PCR, RT-qPCR is simple and has high specificity and sensitivity. Its operating procedures are fully automated. RT-qPCR has become the mainstream technique in molecular biology technology studies and shows up prominently in the plant quarantine [26,27,28]. In real-time PCR, transcripts of low expression may be difficult to quantify; therefore, it is necessary to generate the best possible cDNA to start with as the entire subsequent experiment depends on its quality. As shown in this study, the rates of

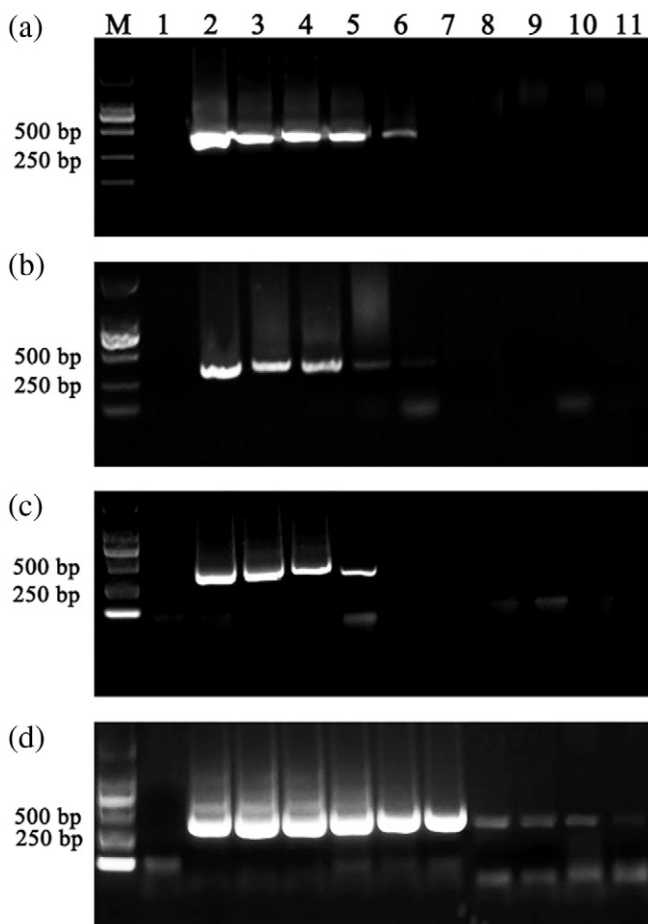


Fig. 2. Sensitivity of second round of nested PCR for each cDNA. M: DL2000 marker (TaKaRa); lane 1: healthy control; lanes 2–11: diluted to 10^{-10} dilution. (a) Oligo-dT; (b) random hexamer primers; (c) mixture of oligo-dT and random hexamer primers; (d) gene-specific primers.

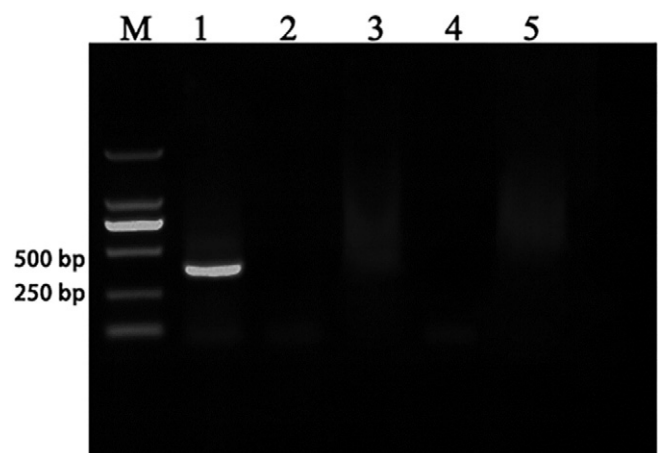


Fig. 3. Detection of viruses by gene-specific primers. M: DL2000 marker (TaKaRa); lane 1: CVB; lane 2: TAV; lane 3: CMV; lane 4: healthy control; lane 5: negative control.

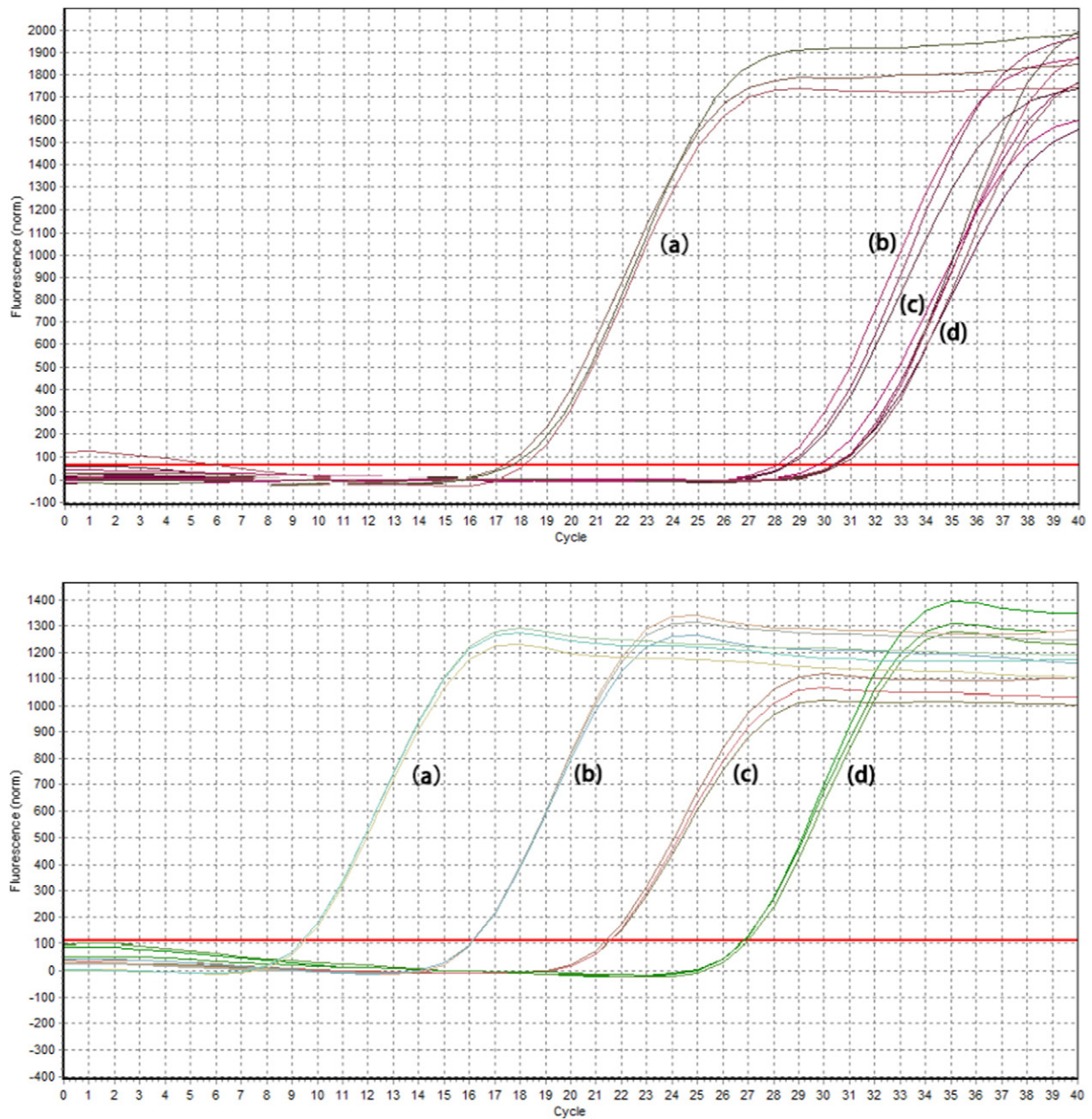


Fig. 4. Quantitative and nested qPCR amplification curve of four different methods. (top) Quantitative PCR; (bottom) nested qPCR. cDNA from (a) gene-specific primer; (b) mixture of oligo-dT/hexamer primers; (c) hexamer primer; (d) oligo-dT primer.

cDNA obtained using reverse transcription-specific primer can be substantially high in the qPCR assay.

It would be an important advantage to contain the disease if we can identify the presence of CVB before the onset of disease symptoms. The assay not only needs to be sensitive to a low viral titer but also should be both rapid and specific. However, current RT-PCR assays are incapable of distinguishing between CVB and related viruses. The usage of gene-specific primers is suitable for detecting CVB as it is more sensitive and specific than previously designed methods and also measures DNA characteristics with a clear non-hybrid zone.

In conclusion, we successfully provide a new method to detect CVB. We developed a nested PCR-based assay that uses gene-specific primers to obtain the most versatile cDNA for the detection of CVB in chrysanthemum. This method can detect the virus at up to 10^{-9} dilution of the cDNA obtained using the gene-specific primers, thus more accurately detecting the virus as soon as possible. In this study,

the use of gene-specific primers derived from the coat protein gene region was found to be the most suitable compared to the use of random hexamer and oligo-dT primers. The method provides a robust diagnostic tool for the early detection of CVB, which can be readily used to analyze field samples. It can play an active role in disease control and provide technical support for early treatment.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by grants from special Fund for Agroscientific Research in the Public Interest under Grant 201403039 New agricultural varieties; Technology and New Model Update Project of Jiangsu Province under Grant SXGC[2015]322.

References

- [1] Shinoyama H, Aida R, Ichikawa H, Nomura Y, Mochizuki A. Genetic engineering of chrysanthemum (*Chrysanthemum morifolium*): Current progress and perspectives. *Plant Biotechnol* 2012;29:323–37. <http://dx.doi.org/10.5511/plantbiotechnology.12.0521a>.
- [2] Palma ACF, Kota R, Charkowski AO. Optimization of a chemiluminescent dot-blot immunoassay for detection of potato viruses. *Am J Potato Res* 2013;90:306–12. <http://dx.doi.org/10.1007/s12230-013-9305-4>.
- [3] Verma N, Kumar K, Kulshrestha S, Raikhy G, Hallan V, Ram R, et al. Molecular studies on *Tomato aspermy virus* isolates infecting chrysanthemums. *Arch Phytopathol Plant Protect* 2009;42:99–111. <http://dx.doi.org/10.1080/03235400600951779>.
- [4] Rybicki EP, Pietersen G. Plant virus disease problems in the developing world. *Adv Virus Res* 1999;53:127–75. [http://dx.doi.org/10.1016/S0065-3527\(08\)60346-2](http://dx.doi.org/10.1016/S0065-3527(08)60346-2).
- [5] Singh L, Hallan V, Martin DP, Ram R, Zaidi AA. Genomic sequence analysis of four new chrysanthemum virus B isolates: Evidence of RNA recombination. *Arch Virol* 2012;157:531–7. <http://dx.doi.org/10.1007/s00705-011-1190-x>.
- [6] Levay K, Zavriev S. Nucleotide-sequence and gene organization of the 3'-terminal region of chrysanthemum virus-b genomic RNA. *J Gen Virol* 1991;72:2333–7. <http://dx.doi.org/10.1099/0022-1317-72-10-2333>.
- [7] Song A, Lou W, Jiang J, Chen S, Sun Z, Guan Z, et al. An isoform of eukaryotic initiation factor 4e from *Chrysanthemum morifolium* interacts with chrysanthemum virus b coat protein. *PLoS One* 2013;8:e57229. <http://dx.doi.org/10.1371/journal.pone.0057229>.
- [8] Hollings M, Stone OM. Serological and immunoelectrophoretic relationships among viruses in the tombusvirus group. *Ann Appl Biol* 1975;80:37–48. <http://dx.doi.org/10.1111/j.1744-7348.1975.tb01598.x>.
- [9] Singh L, Hallan V, Jabeen N, Singh AK, Ram R, Martin DP, et al. Coat protein gene diversity among *Chrysanthemum virus B* isolates from India. *Arch Virol* 2007;152:405–13. <http://dx.doi.org/10.1007/s00705-006-0854-4>.
- [10] Crosslin JM, Hamm PB, Shiel PJ, Hane DC, Brown CR, Berger PH. Serological and molecular detection of tobacco vein necrosis isolates of *Potato virus Y* (PVY^{AV}) from potatoes grown in the western United States. *Am J Potato Res* 2005;82:263–9. <http://dx.doi.org/10.1007/BF02871955>.
- [11] Kumar S, Singh S. Sensitivity of polyvalent DOT-ELISA in comparison to DOT and DAS-ELISA for detection of potato viruses. *J Indian Potato Assoc* 1999;26:39–42.
- [12] X-I Liu, X-t Zhao, Muhammad I, B-b Ge, Hong B. Multiplex reverse transcription loop-mediated isothermal amplification for the simultaneous detection of CVB and CSVD in chrysanthemum. *J Virol Methods* 2014;210:26–31. <http://dx.doi.org/10.1016/j.jviromet.2014.09.008>.
- [13] Mori Y, Kitao M, Tomita N, Notomi T. Real-time turbidimetry of LAMP reaction for quantifying template DNA. *J Biochem Biophys Methods* 2004;59:145–57. <http://dx.doi.org/10.1016/j.jbbm.2003.12.005>.
- [14] Hosokawa M, Matsushita Y, Uchida H, Yazawa S. Direct RT-PCR method for detecting two chrysanthemum viroids using minimal amounts of plant tissue. *J Virol Methods* 2006;131:28–33. <http://dx.doi.org/10.1016/j.jviromet.2005.07.001>.
- [15] Coiras MT, Aguilar JC, García ML, Casas I, Pérez-Brena P. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J Med Virol* 2004;72:484–95. <http://dx.doi.org/10.1002/jmv.20008>.
- [16] Zhao X, Liu X, Ge B, Li M, Hong B. A multiplex RT-PCR for simultaneous detection and identification of five viruses and two viroids infecting chrysanthemum. *Arch Virol* 2015;160:1145–52. <http://dx.doi.org/10.1007/s00705-015-2360-z>.
- [17] Papayiannis LC. Diagnostic real-time RT-PCR for the simultaneous detection of *Citrus exocortis viroid* and *Hop stunt viroid*. *J Virol Methods* 2014;197:93–9. <http://dx.doi.org/10.1016/j.jviromet.2013.11.001>.
- [18] Song A, You Y, Chen F, Li P, Jiang J, Chen S. A multiplex RT-PCR for rapid and simultaneous detection of viruses and viroids in chrysanthemum. *Lett Appl Microbiol* 2013;56:8–13. <http://dx.doi.org/10.1111/lam.12007>.
- [19] Chen X, Xu X, Li Y, Liu Y. Development of a real-time fluorescent quantitative PCR assay for detection of *Impatiens necrotic spot virus*. *J Virol Methods* 2013;189:299–304. <http://dx.doi.org/10.1016/j.jviromet.2013.02.012>.
- [20] Agindotan BO, Shiel PJ, Berger PH. Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan® real-time RT-PCR. *J Virol Methods* 2007;142:1–9. <http://dx.doi.org/10.1016/j.jviromet.2006.12.012>.
- [21] Schmutzhard J, Riedel HM, Wirtgart BZ, Grillner L. Detection of herpes simplex virus type 1, herpes simplex virus type 2 and varicella-zoster virus in skin lesions. Comparison of real-time PCR, nested PCR and virus isolation. *J Clin Virol* 2004;29:120–6. [http://dx.doi.org/10.1016/S1386-6532\(03\)00113-6](http://dx.doi.org/10.1016/S1386-6532(03)00113-6).
- [22] Cha RS, Thilly WG. Specificity, efficiency, and fidelity of PCR. PCR primer: A laboratory manual; 1995 37–51.
- [23] Nam DK, Lee S, Zhou GL, Cao XH, Wang C, Clark T, et al. Oligo(dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription. *Proc Natl Acad Sci U S A* 2002;99:6152–6. <http://dx.doi.org/10.1073/pnas.092140899>.
- [24] Resuehr D, Spiess AN. A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods. *Anal Biochem* 2003;322:287–91. <http://dx.doi.org/10.1016/j.ab.2003.07.017>.
- [25] Zhang JL, Byrne CD. Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR. *Biochem J* 1999;337:231–41. <http://dx.doi.org/10.1042/0264-6021:3370231>.
- [26] Manome A, Kageyama A, Kurata S, Yokomaku T, Koyama O, Kanagawa T, et al. Quantification of potato common scab pathogens in soil by quantitative competitive PCR with fluorescent quenching-based probes. *Plant Pathol* 2008;57:887–96. <http://dx.doi.org/10.1111/j.1365-3059.2008.01881.x>.
- [27] Schaad NW, Frederick RD. Real-time PCR and its application for rapid plant disease diagnostics. *Can J Plant Pathol* 2002;24:250–8. <http://dx.doi.org/10.1080/07060660209507006>.
- [28] Kim WS, Haj-Ahmad Y, Stobbs LW, Greig N. Evaluation of viroid extraction methods and application of a one-step reverse transcription real-time polymerase chain reaction assay (RT-qPCR) for the rapid detection of *Chrysanthemum stunt viroid* (CSVD) infection. *Can J Plant Pathol* 2015;37:221–9. <http://dx.doi.org/10.1080/07060661.2015.1012742>.