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Short Communication

Multiplex Reverse Transcription Polymerase Chain Reaction for Simultaneous Detection of Viruses in Gentian

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ABSTRACT

The multiplex reverse transcription polymerase chain reaction (RT-PCR) was used to detect *Broad bean wilt virus 2*, *Cucumber mosaic virus* and *Clover yellow vein virus*. Using specific primers, the method enabled detection of the viruses with a high degree of sensitivity and specificity and identification of the virus species according to the size of the PCR product amplified. Each of the three viruses was also detected within a mixture of the viruses by this method. Of 247 field plants of gentian subjected to the multiplex RT-PCR, 88% were found to be infected with one or more of the viruses.

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Key words : multiplex RT-PCR, gentian, *Broad bean wilt virus 2*, *Cucumber mosaic virus*, *Clover yellow vein virus*.

Gentian (*Gentiana* spp.) is an important perennial ornamental flower crop, which is used as a cut or potted flower. The plants can produce flowers 3 to 6 years after seedlings are planted. Because of the long cultivation period, the incidence of viral disease can increase within the field. Viral symptoms on gentian plants, such as severe mosaic and necrotic spots on the leaf, vein necrosis, and malformation of the leaf, drastically decrease their economic value. In Japan, *Cucumber mosaic virus* (CMV), *Broad bean wilt virus 2* (BBWV-2), *Clover yellow vein virus* (ClYVV) and *Tobacco rattle virus* (TRV) have been reported to infect gentian plants in fields[17]. CMV, BBWV-2 and ClYVV are the major agents of viral disease and frequently result in mixed infections of gentian plants[8].

Virus detection and identification are of prime concern for disease control. Simple immunological methods, such as enzyme-linked immunosorbent assay (ELISA)[2], dot immunobinding assay (DIBA)[5] and tissue printing immuno assay (TPI)[11], are popular for virus detection. However, antiserum specific to each virus is required for success with these methods. Furthermore, they must be done independently for each virus species if multiple virus detections are required.

The polymerase chain reaction (PCR) method is a sensitive and rapid method for virus detection. In the case of *Tomato spotted wilt virus* detection, reverse transcription-PCR (RT-PCR) can detect lower levels of virus concentration than ELISA[9]. Furthermore, two or more targets can be discriminated using a multiplex RT-PCR, a method that simultaneously amplifies cDNA fragments of multiple sizes. Several previous reports have addressed the use of multiplex RT-PCR for multiple virus detection in a single reaction[1,4,6,13,15,16]. This method is well suited to detect viruses in gentian because most cultured plants in the field are infected with several viruses[8]. In this paper, we report the optimum conditions for multiplex RT-PCR to detect BBWV-2, CMV and ClYVV simultaneously. We also report a survey of the virus diseases of gentian detected using this method.

CMV-Y[23] was propagated in *Nicotiana tabacum* cv. Samsun NN plants and purified as described previously[3]. ClYVV-NFU[18] was propagated in *Vicia faba* and purified by the method of Uyeda *et al.*[24] BBWV-IA[10], an isolate of BBWV-2, was propagated in *Chenopodium quinoa* plants and purified essentially as described by Natsuaki *et al.*[14].

Virion RNAs were prepared from purified virus. Samples of purified virus were suspended in an extraction buffer (0.1 M Tris-HCl pH 7.6, 0.1 M NaCl, 1% SDS and 2% 2-mercaptoethanol added immediately before use), incubated at 65°C for 5 min, and treated two times with an equal volume of phenol : chloroform (1 : 1). Finally, RNA was precipitated with 2.5 volumes of ethanol and

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stored at −20°C.

Total RNAs were extracted from small amounts of leaves according to the method of Perry and Francki[19], after minor modifications as follows. Infected leaves were ground with double-strength STE buffer (0.1 M Tris-HCl pH 7.0, 0.2 M NaCl, 2 mM EDTA, 0.1% SDS and 2% 2-mercaptoethanol added before use), and RNA was extracted with an equal volume of phenol and chloroform. After centrifugation at 10,000×*g* for 5 min, the upper phase was transferred into a microtube containing one-third volume of 8 M LiCl and placed on ice. After 1 hour of incubation, insoluble RNA was pelleted by centrifugation at 10,000×*g*.

Approximately 10 ng of purified viral RNA or 500 ng of plant total RNA was used for the first-strand cDNA synthesis. The samples were primed with a random hexamer and reverse-transcribed using a First-strand cDNA Synthesis Kit (Amersham Pharmacia) in a 15 μl final reaction volume as described by the manufacturer. Reverse-transcription with random hexamers is reported to be efficient and consistent[7].

All specific primers for PCR were designed using a personal computer with the software DNASIS MAC v3.6 (Hitachi Software Co. Ltd.) and the sequences of the primers are listed in Table 1. The CMF primer and CMR primer were designed from the sequence of CMV-Y RNA3 (Accession no. M57602) and can hybridize to CMV cDNAs. Primers ClYF and ClYR, a modification of Bariana *et al.*[1], were used for ClYVV detection. For BBWV-2 cDNA amplification, primers BBF and BBR were designed from the nucleotide sequence of RNA2 of BBWV-IA (Accession no. AB032403). The target region of the PCR amplification, and the expected size of the PCR products are shown in Table 1. The size of the virus-specific RT-PCR fragments differed, facilitating the identification of the virus species through agarose gel electrophoresis. The PCR reaction mixture (50 μl) was composed of 1× *Ex Taq* buffer, 200 μM dNTP mix, 20 pmol of each of primers, 2.5 units of *Ex Taq* DNA polymerase (Takara Shuzo Co., Otsu, Japan) and 1 μl of first-strand reaction. PCR amplifications were performed using an incubation cycle of 94°C for 40 sec, 55°C for 10 sec, and 72°C for 30 sec, and these cycles were repeated 30 times. After the PCR reaction, products were resolved with agarose gel electrophoresis and visualized with ethidium bromide staining.

Under the PCR conditions stated, DNA fragments were well amplified from each viral RNA using the corresponding primer pairs or a primer mixture containing three primer pairs. Other isolates of BBWV-2 (IB and IC isolated from gentian, and MB7 isolate[12]), CMV (E5 and C7-2 isolates[20]) and ClYVV (NC isolate[22]) were also detected as corresponding cDNA fragments.

Only the BBWV-2 fragment was amplified from the mixed cDNA sample of BBWV-IA, CMV-Y and ClYVV-NFU (not shown), presumably, because of different amplification efficiencies for each of the fragments. Thus, different quantities of primers were used for each virus with these ratios of ClYVV primers (pmol) : BBWV-2 primers (pmol)-20 : 20, 20 : 10, and 20 : 5. When 10 ng of both ClYVV and BBWV-2 virion RNA were used as a template, the fragments of ClYVV and BBWV-2 were amplified together in the experiment using 20 pmol and 5 pmol primers, respectively (not shown). These results indicated that 20 pmol of each ClYVV primer and 5 pmol of each BBWV-2 primer were optimum for simultaneous

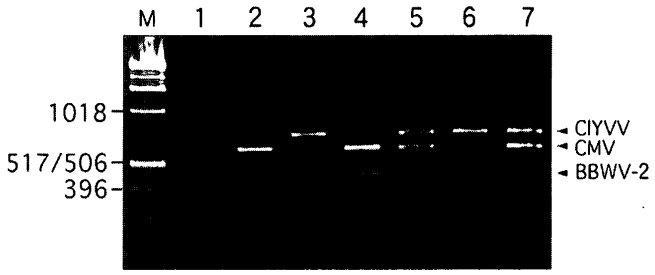

Fig. 1. Detection of viral cDNAs by multiplex DNA amplification. Complementary DNAs derived from various combination of purified virion RNAs were subjected to DNA amplification. PCR products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining. Lane M, 1 Kb DNA Ladder (Invitrogen Co., CA, USA) ; lane 1, BBWV-2 ; lane 2, CMV ; lane 3, ClYVV ; lane 4, BBWV-2+CMV ; lane 5, CMV+ClYVV ; lane 6, BBWV-2+ClYVV ; lane 7, BBWV-2+CMV+ClYVV.

Table 1. Primer sets for multiplex amplification of BBWV-2, CMV and ClYVV

Primer	Primer sequence	Target region/expected size (bp)
BBF	5′-GTACATGCTTGGTCAAGCAC-3′	BBWV-2 23 kDa coat protein gene/453
BBR	5′-AACTTCTTGTTACCACGCAC-3′	
CMF	5′-CGGTCTTTAGCACTTTGGTGCG-3′	CMV RNA3 intergenic region/607
CMR	5′-CATATTCCGTGACTGAATCAGG-3′	
ClYF	5′-CATTCCAGACAGAGACATCAATGCAG-3′	ClYVV coat protein gene/739
ClYR	5′-ACGGAGAATTTAAAGACGGATAC-3′	

Table 2. Number of gentian plants from which each virus was detected using multiplex RT-PCR

Symptoms[a]	Detected viruses								
	BBWV-2	ClYVV	CMV	BBWV-2 +CMV	BBWV-2 +ClYVV	ClYVV +CMV	BBWV-2 +ClYVV+CMV	ND[b]	Total
Severe symptoms	26	12	17	35	3	1	0	5	99
Mild symptoms	77	8	12	9	0	0	0	12	118
No symptoms	15	0	2	0	0	0	0	13	30
Total	118	20	31	44	3	1	0	30	247

a) Symptoms were rated as severe (chlorosis, mosaic, malformation, necrosis and stunt), mild (mild mottle) or no symptoms.
b) ND, no viruses detected.

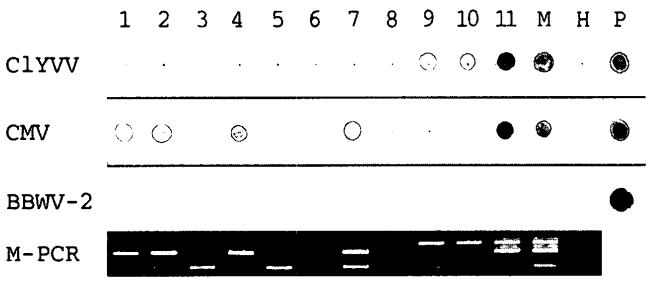

Fig. 2. Comparison of results obtained with dot-blot hybridization (ClYVV, CMV and BBWV-2) and multiplex RT-PCR (M-PCR). About 2 μg/dot of plant total RNAs were blotted onto a nylon membrane. Numbers at the top represent individual gentian plants. M is mixed RNA of samples Nos. 3, 4 and 10. H means RNA from virus-free gentian, and P indicates a positive control of each viral cDNA cloned in pUC18 (0.1 μg/dot). Labels at left indicate the probe specific to ClYVV, CMV or BBWV-2.

amplification. Then, 20 pmol, 10 pmol or 5 pmol of each CMV primer was added to 10 ng of each of the viral RNAs, and RT-PCR was performed. Figure 1 shows that all DNA fragments were well amplified simultaneously, when the PCR mixture contained 20 pmol of each ClYVV primer, 10 pmol of each CMV primer and 5 pmol of each BBWV-2 primer. Accordingly, this ratio of primer quantity was used for subsequent experiments.

When *N. benthamiana* plants infected with one or more of ClYVV-NFU, CMV-Y or BBWV-IA were subjected to multiplex RT-PCR analysis, the respective viruses were detected equivalently and simultaneously (not shown). These viruses could also be detected in field samples of gentian with this method (Fig. 2).

The sensitivity of multiplex RT-PCR detection was compared with that of dot-blot hybridization for the detection of CMV. Purified viral RNA of CMV-Y was serially diluted with extracts from BBWV-2- and ClYVV-infected gentian. One microliter of each dilution, ranging from 10 ng/μl to 1 fg/μl of CMV viral RNA, was subjected to multiplex RT-PCR and dot-blot hybridization. A photobiotin-labeled probe[25] was prepared from the clone containing the CMV-specific cDNA fragment. Hybridization was done as described by Sambrook *et al.*[21]. The detection endpoint for dot-blot hybridization was 100 pg/μl and 10 fg/μl for multiplex RT-PCR (not shown). These results indicate that multiplex RT-PCR is 10^4 times more sensitive than dot-blot hybridization.

Two hundred and forty-seven samples of field gentian plants with either viral symptoms or no symptoms were sampled in Iwate Prefecture, Japan from 1997 to 1999, then subjected to multiplex RT-PCR. Most of the gentian plants (88%) were infected with one or more of BBWV-2, CMV and ClYVV (Table 2). These viruses could not be detected in 17 gentian plants that had severe or mild viral symptoms. To verify the results, some of the samples were analyzed with dot-blot hybridization using specific probes for BBWV-2, CMV and ClYVV. To produce these probes, respective virus-specific fragments were amplified by multiplex RT-PCR, then cloned into pUC18 and labeled with photobiotin[25]. In Fig. 2, each probe detected the corresponding virus in gentian. For all of these samples, the results of multiplex RT-PCR corresponded well with those of the hybridization analyses. Multiplex RT-PCR could also detect each of the three viruses from a mixed sample of BBWV-2-, CMV- or ClYVV-infected gentian. Hybridization analyses were also performed on 30 samples in which no virus was detected by multiplex RT-PCR. No signal was detected in the hybridization analyses, confirming that BBWV-2, CMV and/or ClYVV did not infect these samples (data not shown). These results demonstrate that multiplex RT-PCR is useful for the detection of multiple viruses in gentian.

There are many reports of diagnostic techniques based on serology or gene technology. Simplicity, rapidity, high sensitivity and low cost are required for a diagnostic technique used for ecological and epidemiological study. We have designed primers and found the optimum conditions for multiplex RT-PCR in detecting BBWV-2, CMV and ClYVV. This method is quite useful not only for high sensitivity detection of viruses, but also for identification of virus species with one reaction.

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