

## Detection of Cucumber Mosaic Virus Using the Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

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### Abstract

A simplified and reproducible method in plant breeding using the reverse transcription-polymerase chain reaction (RT-PCR) was developed for the selection of plants infected with cucumber mosaic virus (CMV). Total RNA was extracted from CMV-infected leaves of *Nicotiana glutinosa* by the simplified SDS/phenol method. The RNA was initially reverse-transcribed into cDNA, then the CMV coat protein-coding sequence was amplified by the PCR method. The detection limit of CMV by the RT-PCR method ranged from 10 to 100 ng of CMV particles per 10 mg of fresh leaf weight. The sensitivity of the method was similar to that of the ordinary ELISA method.

**Key words:** simplified detection method·RT-PCR·cucumber mosaic virus·*Nicotiana glutinosa*

### I. Introduction

The recent development of biotechnology has made it possible to produce virus-resistant plants using plant transformation techniques and to generate virus-free plants using tissue culture techniques. For applying these techniques it is becoming more important to develop early diagnosis method of virus-infected plants. Bioassays, electron microscopic observation and/or serological methods have been used for selecting virus-infected plants. While ELISA, one of the serological methods, has seemed to be the most sensitive and most reproducible method for virus detection, this is not applicable unless any antibody for the virus studied is available. Bioassays are also sensitive and reproducible, but time consuming and laborous as well as electron microscopic observation. Thus, an alternative simplified and reproducible method to select virus-infected plants is required to promote frequent and easy use of the above advanced techniques such as plant transformation and tissue culture.

Due to the recent development of molecular techniques, it is possible to detect viruses by using nucleic acid hybridization<sup>6)</sup> or polymerase chain reaction (PCR)<sup>11)</sup>. Especially, PCR has been used successfully to detect human infectious viruses<sup>8)</sup>, plant viruses<sup>10,15)</sup>, viroids<sup>5)</sup>, plant pathogenic bacteria<sup>3)</sup> or fungi<sup>12)</sup>, because it offers the following advantages: ① only a small fragment of a leaf is sufficient to detect the virus, ② detection by the PCR is more sensitive than other conventional methods so far used.

In this report we describe a simplified and reproducible method using reverse transcription-PCR (RT-PCR) for the detection and selection of tobacco plants infected with cucumber mosaic virus (CMV), which is one of the most prevalent plant viruses causing mosaic, mottling or necrotic spot symptoms on leaves of more than 190

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plant species including Cucurbitaceae, Solanaceae, Compositae, Cruciferae, Rosaceae and Liliaceae<sup>7</sup>.

## II. Materials and Methods

CMV pepo-isolate<sup>9</sup> was propagated in *Nicotiana glutinosa*. Purified virus particles and virus RNA were obtained by the method described by Takanami<sup>13</sup>. Virus RNA was obtained from the purified CMV preparation by three cycles of deproteinization with phenol in the presence of 1% SDS followed by ethanol precipitation. Purified virus and virus RNA were stored at  $-80^{\circ}\text{C}$  as suspensions in 10 mM phosphate buffer and 70% ethanol suspensions, respectively.

Total RNA was recovered by SDS/phenol extraction from CMV-infected or uninfected leaves of *N. glutinosa*. The leaves (10 mg or 100 mg) were homogenized in 750  $\mu\text{l}$  of RNA extraction buffer (0.1 M Tris-HCl, pH 9.0; 0.1 M NaCl; 1% SDS; 14 mM  $\beta$ -mercaptoethanol) and 750  $\mu\text{l}$  of phenol/chloroform with mortar and pestle. The aqueous phase was collected after centrifugation followed by two extractions with phenol/chloroform. Nucleic acid in the aqueous phase was precipitated with ethanol, dissolved in 100  $\mu\text{l}$  of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and total RNA was precipitated with 2.5 M LiCl. The pellet was dissolved in 100  $\mu\text{l}$  of TE buffer after centrifugation. After the first, second and third extractions with phenol/chloroform and after LiCl precipitation, total RNA in each sample was precipitated with ethanol, dissolved in 20  $\mu\text{l}$  of TE buffer and used for RT-PCR as a template.

Oligonucleotide primers designed for amplification of the coat protein (CMV-CP)-coding sequence were synthesized based on the nucleotide sequence of CMV-O reported by Hayakawa *et al.*<sup>4</sup>. The 5'-primer (26-mer, 5'-AAGGATCCATGGACAAATCTGAATCA-3') contains 18 nucleotide sequence identical to 5'-end of CMV-CP (CMV-O RNA3, No. 1261–1278) and *Bam*HI restriction site at the 5'-end portion. The 3'-primer (26-mer, 5'-AAGGTACCTCAGACTGGGAGTACTCT-3') contains 18 nucleotide sequence complementary to 3'-end of CMV-CP (CMV-O RNA3 No. 1900–1917) and *Kpn*I restriction site at the 5'-end portion. About 670-bp length cDNA was expected to be amplified by the primers.

RT-PCR was performed in a thermal cycler JAPH-90 (Ricoh Chemical Co.) according to the procedure described in Table 1. Five microliters of each product were analyzed on 1.5% agarose gel after PCR amplification.

## III. Results and Discussion

The efficiencies of RT-PCR were investigated under several conditions using purified virus RNA as a template. No differences were found in electrophoresis pattern when 1, 2 and 4  $\mu\text{g}$  of CMV RNA were applied. The sensitivity was not improved when reamplification was carried out using 1  $\mu\text{l}$  of the RT-PCR product or the 670-bp RT-PCR amplified cDNA extracted from agarose gel as the template. On the other hand, the amount of amplified product significantly increased when the 10x buffer for *Taq* DNA polymerase (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM  $\text{MgCl}_2$ ; 0.1% gelatin) was used instead of the 10x buffer for *Tth* DNA polymerase.

No significant effects of the amount of *Tth* DNA polymerase (1, 2, 4 or 8 units) on the DNA amplification were observed, indicating that the smallest amount of enzyme (1 unit) was sufficient to detect CMV. There was no significant difference between  $50^{\circ}\text{C}$  and  $55^{\circ}\text{C}$  for the annealing temperature, although a little better results were given by  $50^{\circ}\text{C}$  than  $55^{\circ}\text{C}$ .

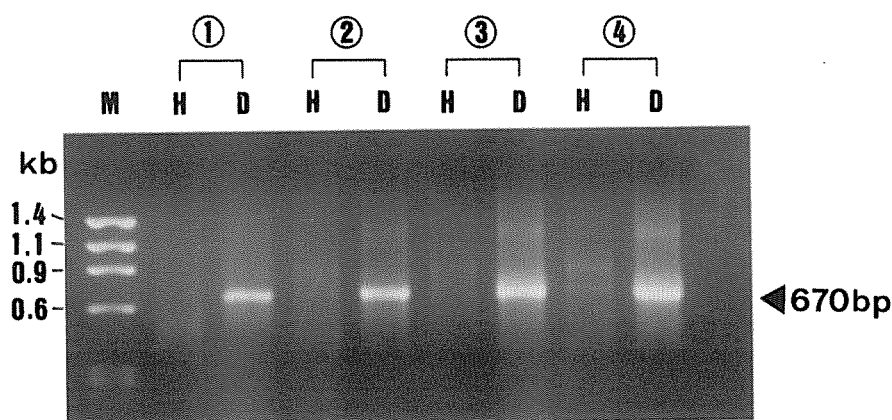


Fig. 1. Detection of CMV RNA by RT-PCR in tobacco leaves. Total RNA was extracted after the first (①), second (②) and third (③) treatment with phenol/chloroform and after LiCl precipitation (④) from CMV-infected (D) or uninfected (H) tobacco leaves (10 mg), and used as a template for RT-PCR. Lane M indicates DNA size marker.

The procedures of total RNA extraction from CMV-infected or uninfected leaves of *N. glutinosa* were improved in order to detect CMV RNA in leaves. Since similar results were obtained using total RNA extracted from 10 mg or 100 mg of leaves, the results obtained by the use of 10 mg were shown in Fig. 1. Amplified viral sequences were readily detected in all of four samples extracted from infected leaves but not in those extracted from uninfected leaves. These findings indicate that the crude RNA extract obtained from 10 mg leaves after one phenol/chloroform extraction followed by ethanol precipitation can be used as a template for RT-PCR. The simplified RNA extract procedure enables us to extract RNA from leaves within one hour and to detect CMV within a day.

As a result, the following procedures were developed for simple and reproducible detection of CMV using RT-PCR. The sample (10 mg fresh weight) was homogenized with 750  $\mu$ l of RNA extraction buffer and 750  $\mu$ l of phenol/chloroform, and the aqueous phase was collected after centrifugation. Total RNA in the aqueous phase was precipitated with ethanol, dissolved in 20  $\mu$ l of TE buffer and used as a template for RT-PCR. The procedure based on cDNA synthesis for PCR is shown in Table 1. Five microliters of the RT-PCR product were analyzed by 1.5% agarose gel, and CMV infection was detected by the presence of 670-bp length cDNA.

Figure 2 shows the sensitivity of CMV detection by RT-PCR using total RNA extracted from 10 mg of uninfected leaves mixed with 1 pg~10  $\mu$ g of virus particles. The detectable limit of CMV by RT-PCR ranged from 10 to 100 ng of virus particles (about 0.5~5.5 pM as virus RNA) per 10 mg of leaves which corresponded to 0.001~0.0001% of fresh leaf weight. The sensitivity of the RT-PCR procedure was similar to that of the double-antibody sandwich method of ELISA<sup>1)</sup>. Cuzzo *et al.*<sup>2)</sup> reported that the CMV concentrations in transgenic tobacco plants were 0.001~0.0001% and 0.1~0.01% of fresh leaf weight in CMV coat protein expressing (CMV resistant) and nonexpressing (non-resistant) ones 8 days after inoculation, respectively. The current RT-PCR method was sensitive enough to distinguish CMV resistant plants from nonresistant ones. The sensitivity of RT-PCR could be further improved when the DNA hybridization was used after PCR amplification.

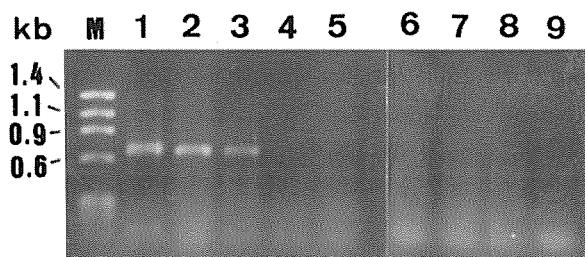
The present results indicated that the current RT-PCR procedure is useful to detect CMV not only from

**Table 1.** Procedure of RT-PCR for the detection of CMV RNA in tobacco leaves.

(cDNA synthesis)	
total RNA extract	1 $\mu$ l
3'-primer (20 pmol/ $\mu$ l)	1 $\mu$ l
dNTPs (5 mM each)	2 $\mu$ l
5x buffer <sup>1)</sup>	2 $\mu$ l
H <sub>2</sub> O	3 $\mu$ l
Total	9 $\mu$ l
(Mineral oil 2–3 drops)	
70°C, 2 min; then slowly decrease temperature to 37°C	
M-MLV reverse transcriptase 1 $\mu$ l (200 units)	
37°C, 1 h	
98°C, 5 min	
(PCR)	
cDNA synthesis product	10 $\mu$ l
10x buffer <sup>2)</sup>	5 $\mu$ l
dNTPs (5 mM each)	2 $\mu$ l
3'-primer (20 pmol/ $\mu$ l)	1 $\mu$ l
5'-primer (20 pmol/ $\mu$ l)	1 $\mu$ l
H <sub>2</sub> O	30 $\mu$ l
<i>Tth</i> DNA polymerase (1 unit/ $\mu$ l)	1 $\mu$ l
Total	50 $\mu$ l
94°C, 1 min	
50°C, 2 min	
72°C, 2 min	
30 cycles	

<sup>1)</sup> 250 mM Tris-HCl, pH 8.3; 375 mM KCl; 50 mM DTT; 15 mM MgCl<sub>2</sub>

<sup>2)</sup> 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.1% gelatin



**Fig. 2.** Detection of CMV RNA by RT-PCR in tobacco leaves containing various amounts of CMV. Total RNA was extracted from 10 mg of leaves containing 10  $\mu$ g (lane 1), 1  $\mu$ g (lane 2), 100 ng (lane 3), 10 ng (lane 4), 1 ng (lane 5), 100 pg (lane 6), 10 pg (lane 7) and 1 pg (lane 8) CMV by the simplified SDS-phenol method and used as a template for RT-PCR. Lane 9: healthy leaves. Lane M: DNA size marker.

artificially inoculated tobacco plants but also from naturally infected lily plants. We successfully applied the current method for detection of several other viruses including lily symptomless virus and tulip breaking virus<sup>14)</sup>. RT-PCR method could be applied to detect other viruses whose antibodies are not available, since primers can be easily synthesized if only a part of the virus nucleotide sequence is known. This method also can be applied for viruses which can be detected with ELISA, because simultaneous use of both methods will increase the

reliability of selection of virus-resistant or virus-free plants. It is also applicable to identify strains of viruses, because only one nucleotide mutation can be detected by the properly designed primer.

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## 逆転写-polymerase chain reaction (RT-PCR) による キュウリモザイクウイルスの検出

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逆転写-polymerase chain reaction (RT-PCR) 法によるキュウリモザイクウイルス (CMV) の遺伝子診断法を開発した。SDS/フェノール法により供試葉から全 RNA を抽出し、逆転写酵素により 1 本鎖 cDNA を合成したのち、PCR によって CMV の外被タンパク質遺伝子を増幅した。本法による CMV の検出限界はタバコ葉 10 mg あたり 10~100 ng で、ELISA 法と同等であった。RNA 抽出法の簡便化により 1 日で CMV の検出が可能になった。本法は CMV の新しい診断法としてウイルス抵抗性品種の選抜に用いることができる。

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