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Complementary DNA Cloning of Genomic RNA in Orchid Strain of Tobacco Mosaic Virus

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Viral RNA was extracted from a purified orchid strain of tobacco mosaic virus (TMV-O) from *Cymbidium* "Grace Kelly". Polyadenylated viral RNAs were primed with *Not* I-oligo (dT) primer-adapter. First-strand cDNAs were reversely transcribed by Moloney murine leukaemia virus reverse transcriptase (RNase H^-), and then second-strand cDNAs were synthesized by RNase H and DNA polymerase I. The resulting double-stranded cDNAs were ligated into pSPORT1 vector and transformed into competent *E. coli* strain JM109 cells. The size of cDNAs within the recombinant plasmids was ranging from 0.9 to 3.9 kb. Among the selected clones, pTMO-0205 and -0210 covered the 3′ half and the 5′ half of the viral genomic RNA, respectively, which were covering more than 99% of the viral genome size based on sequencing analysis. Two cDNA fragments which were 3.1 kb *Bam*HI and *Not*I fragement released from pTMO-0205 and 3.3 kb *Sal*I and *Bam*HI fragment released from pTMO-0210 were ligated with T4 DNA ligase. The clone was almost entire length, lacking only 31 nucleotides from the 5′ terminus based on the sequencing result. This method was shown to be efficiently applicable to other plant viral genomic RNA for the construction of cDNA.

Keywords : TMV-O, tobamovirus, *Cymbidium* sp., cDNA synthesis and cloning

Virus infections reduce the yield severely and lower the quality of many crops and horticultural plants in all part of the world. Unlike fungal and bacterial diseases of plants, chemical control of viral diseases have not been developed yet. This reality demonstrates the magnitude of importance of virus diseases of plants. It is well known that it is difficult to diagnose virus infection specially and rapidly without considering the comparative biological, physicochemical, immunological and molecular biological characteristics of the virus involved.

Tobacco mosaic virus (TMV) is a type member of tobamovirus group and one of the best characterized plant viruses (Palukaitis and Zaitlin, 1986). Odontoglossum ringspot virus (ORSV) is another member of tobamovirus group (Paul, 1975). The ORSV, regarded by some virologists (Corbett, 1967; Kado *et al.*, 1968, Lawson, 1970) as an orchid strain of TMV (TMV-O), which was first isolated from *Rossioglossum grande* (Helmut *et al.*, 1992) formerly called *Odontoglossum grande* (Jensen and Gold, 1951). The genomic organization of the TMV has been well characterized. The genome of tobamoviruses is composed of a positive-sense single-stranded RNA of about 6.4 kb, cap structure (m^7GpppG) at its 5′ end and tRNA-like structure at its 3′ end (Palukaitis and Zaitlin, 1986). TMV is one of the best characterized plant viruses and has an important position in the development of both virology and molecular biology. Although biological and immunological assays are useful for virus identification and differentiation of specific variant, TMV, TMV-O and ORSV share several similarities and differences.

In method described here, poly(A) tail is synthesized to size-fractionated native virus genomic RNAs which were originally polyadenylated or not. This technique should be the method of choice for the construction of cDNA libraries, particularly for multipartite, long, or heterologous genome of plant viruses.

The purpose of this study was the cDNA synthesis

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to clone cDNA of TMV-O.

MATERIALS AND METHODS

Virus source

A tobamovirus was originally isolated from *Cymbidium* "Grace Kelly" showing mild mosaic symptoms on the leaves in 1993. After single local lesion transfer to *Nicotiana tabacum* cv. Xanthi-nc, an orchid strain, designated as TMV-O, was multiplied on *N. tabacum* cv. Samsun (Ryu *et al.*, 1993). Virus inoculated tobacco leaves were harvested in 2 wk after post-inoculation and stored at −70℃ until purification.

Viral RNA isolation and poly(A) tailing

The virus was purified by the method of Park *et al.* (1990b). Viral RNA was extracted from purified virus particles by using SDS/proteinase K disruption and phenol extraction. After ethanol precipitation, the precipitate of viral RNA was rinsed with 80% ethanol. The resulting precipitate was dried under vacuum dessicator, it was dissolved in 100 μL of TEN buffer (10 mM tris, pH 8.0, 1 mM EDTA, 0.1 M NaCl). The genomic RNA was size-fractionated by passing through Sephadex G-50 (Pharmacia) mini-column chromatography. Intact form of viral RNA and short-length viral RNAs were polyadenylated at their 3′-end by using *E. coli* poly(A) polymerase as follow: The viral RNAs were incubated at 75℃ for 2 min and quickly chilled on ice water, and then 1 μL of RNase inhibitor (40 units, BM) and freshly prepared poly(A) polymerase buffer (10× stock; 0.5 M tris, pH 8.0, 2.5 M NaCl, 25 mM $MnCl_2$, 100 mM $MgCl_2$, 10 mM DTT, 10 mmol/L ATP) and 2 μL of *E. coli* poly(A) polymerase (7 units, BRL) were added to the reaction tubes. The reactions were performed at 37℃ for 20 min and terminated the reaction by the addition of EDTA, to a final concentration of 10 mM. The reactions were phenol extraction and ethanol precipitation.

cDNA synthesis and cloning

Most of DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). First strand synthesis was performed with the oligo $(dT)_{15}$ primer (5′-pGACTAGTTCTAGATCGCGAGCGGCCGCCCT$_{15}$-3′), which contains an *Not*I restriction site (underlined), in a 20 μL reaction volume consisting of 3 μg polyadenylated RNA, 50 ng primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 0.5 mM dGTP and 600 units of Superscript RNase H^- Moloney murine leukaemia virus reverse transcriptase (RT, BRL). In order to anneal the primer to the template RNA, the primer and RNA were combined, incubated at 70℃ for 10 min and cooled rapidly on ice for 5 min. The remaining reagents containing RT were then added and incubated at 37℃ for 70 min. The reaction was terminated by the placement the reaction mixture on ice. Second-strand cDNAs were synthesized with the first strand reaction mixture, in a 150 μL reaction volume consisting of 20 μL first strand reaction mixture, 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM β-NAD^+, 1.2 mM DTT, 0.25 mM dATP, 0.25 mM dTTP, 0.25 mM dCTP, 0.25 mM dGTP, 10 units of *E. coli* DNA ligase, 40 units of *E. coli* DNA polymerase I, and 2 units of *E. coli* RNase H. The reaction was incubated at 16℃ for 2 h. To the reaction, 5 units of T4 DNA polymerase was added and incubated at 16℃ for an additional 5 min. The reaction was terminated by the addition of EDTA and proteinase K, incubated at 42℃ for 10 min, extracted once with phenol and chloroform and precipitated with 0.5 vol. of 7.5 M ammonium acetate and 2 vol. of cold absolute ethanol. Double-stranded cDNAs (ds-cDNA) were annealed with *Sal*I adapter and T4 DNA ligase at 16℃ for 16 h and then they were digested with *Not*I restriction endonuclease to completion to generate different terminal sites for directional orientation. The ds-cDNA fragments were passed through Sephacryl S-500 HR chromatography column (BRL) for size fractionation. The resulting ds-cDNAs were annealed to pSPORT1 (BRL) vector and recombinant plasmids were transformed into competent *E. coli* strain JM109 cells by the $CaCl_2$ method and selected for on McConkey agar plates containing ampicillin (100 μg/mL) or Luria-Bertani agar media containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, BRL) and isopropyl-β-D-thiogalactopyranoside (IPTG, BRL). For increasing the ligation and transformation efficiencies, ligation expression kit(Clontech Lab.) was used in ligation reactions for

cDNA and vector annealing by the manufacture's instruction.

Restriction endonuclease mapping

Small-scale plasmid DNA isolation was carried out by alkaline-lysis extraction procedure and large-scale preparation was done by alkaline-lysis plus polyethylene glycol method (Sambrook *et al.*, 1989). The cDNA lengths of selected recombinant clones to the viral RNA were determined by restriction endonuclease (*Not*I/*Sal*I) digestion. In order to construct the restriction map, the clones were digested to completion with several restriction endonucleases singly or doubly. The DNA fragments were separated by size using an agarose gel electrophoresis (1.2% agarose, 5 V/cm) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). They were visualized with ultraviolet light after staining gels in freshly prepared ethidium bromide solution (100 ng/mL) and destaining them with running water. Photogaphs of the gels were taken using a red filter with Polaroid films (Type 667, Kodak). Sizes of all DNA restriction endonuclease fragments were estimated using 1 kb DNA ladder (BRL) as a molecular size standard marker.

Nucleotide sequencing

cDNA sequencing was done by the direct sequencing of plasmid DNA based on dideoxynucleotide method (Sanger *et al.*, 1977) using [α-^{35}S] dCTP and Sequenase version 2.0 (USB).

Cloning of cDNA

For cloning purpose, the plasmid DNAs isolated by alkaline lysis extraction procedures were further purified on CsCl-ethidium bromide gradients by centrifugation at 50,000 rpm in a Beckman VTi 65 rotor for 16 h at 18℃. To generate full-length genomic cDNA clone of the virus, two large subclones, pTMO-0205 and pTMO-0210 which were covered the 3′ half and the 5′ half of the viral genomic RNA, respectively, were used. The ligated cDNAs were annealed to *Not*I/*Sal*I cut pSPORT1 vector and transformed into *E. coli* strain NM522 cells. The transformed colonies containing recombinant clones were selected from Luria-Bertani medium supplemented with ampicillin at 100 μg/mL. They were screened for the desired cloned fragment (6.4 kb) by using *Not*I-*Sal*I restriction enzymes digestions. The resulting plasmid DNA was confirmed by restriction analysis.

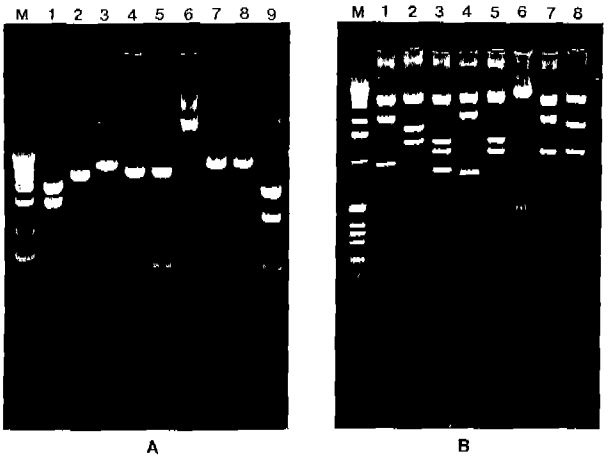

Fig. 1. Electrophoretic pattern of pTMO-0205. A: Lane 1, *Bam*HI; 2, *Bgl*II; 3, *Cla*I; 4, *Eco*RI; 5, *Hin*dIII; 6, *Nde*I; 7, *Sac*I; 8, *Xba*I; 9, *Pvu*II; and M, marker (1 kb DNA ladder). B: 1, *Bam*HI/*Eco*RI; 2, *Bam*HI/*Hin*dIII; 3, *Eco*RI/*Hin*dIII; 4, *Bam*HI/*Cla*I; 5, *Cla*I/*Eco*RI; 6, *Cla*I/*Sac*I; 7, *Eco*RI/*Xba*I; 8, *Eco*RI/*Sac*I and M, marker (1 kb DNA Ladder). The gels (A, 1.1%; B, 1.2% agarose gel) were electrophoresed in TAE buffer.

RESULTS

One hundred and twenty-one recombinant clones for TMV-O RNA were generated. The size of cDNAs within the recombinant plasmids was ranging from 0.9 to 3.9 kb. Among the selected transformants, pTMO-0205 (3.3 kb insert) and pTMO-0210 (3.9 kb insert), having long insert cDNAs, were analysed further.

The DNA fragments of recombinant clones from TMV-O 02 RNA obtained after digestion to completion with several restriction endonucleases are illustrated in Figs. 1 and 2. Restriction endonuclease patterns of the clone pTMO-0205 showed that it contained one reaction site for each *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I and *Acc*I, respectively. The pTMO-0210 was the largest clone covering 5′ half of the viral RNA. This clone contained one site for *Bam*HI, *Hin*dIII, *Xba*I, two sites for *Cla*I and *Eco*RI, and three site for *Nde*I, respectively. *Bam*HI site was coexisted at upstream and downstream of the two clones, this

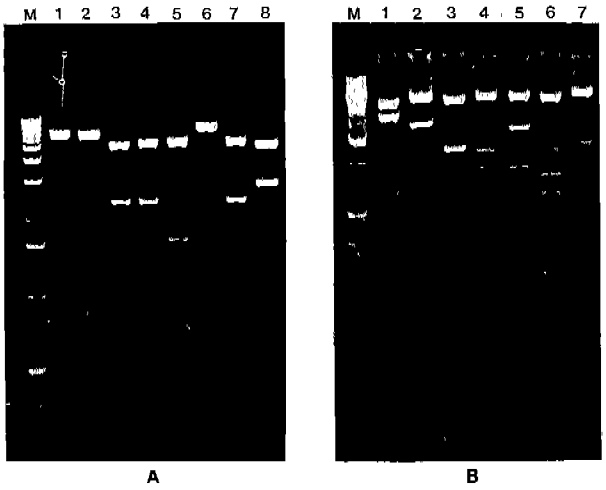

Fig. 2. Electrophoretic pattern of pTMO-0210. A: 1, *Bam*HI; 2, ClaI; 3, *Eco*RI; 4, *Hin*dIII; 5, *Nde*I; 6, *Sac*I; 7, *Sma*I; 8, *Xba*I; and M, marker (1Kb DNA ladder). B: 1, *Bam*HI/*Eco*RI; 2, *Bam*HI/*Hin*dIII; 3, *Eco*RI/*Hin*dIII; 4, *Bam*HI/*Cla*I; 5, *Cla*I/*Xba*I; 6, *Nde*I/*Xba*I; 7, *Bam*HI/*Sac*I and M, marker (1 kb DNA Ladder). The gels (A, 1.1%; B, 1.2% agarose gel) were electrophoresed in TAE buffer.

suggests that the two clones were overlapped in the middle region of the viral genome.

Partial nucleotide sequences of 5′- and 3′-ends of the virus were determined by using pTMO-0205 and -0210 (Fig. 3). The partial nucleotide sequences of the 3′ and 5′ terminal region of the clones pTMO-0205 and -0210, respectively, are shown Fig. 4. The nucleotide sequence homology between the virus and the TMV-vulgare (Goelet *et al.*, 1982) was 99%, showing 4 nucleotides substitutions. Clone pTMO-0205 contained the sequences for partial virus replicase, full-length movement protein, coat protein and 3′ untranslated regions. The 3′ end was

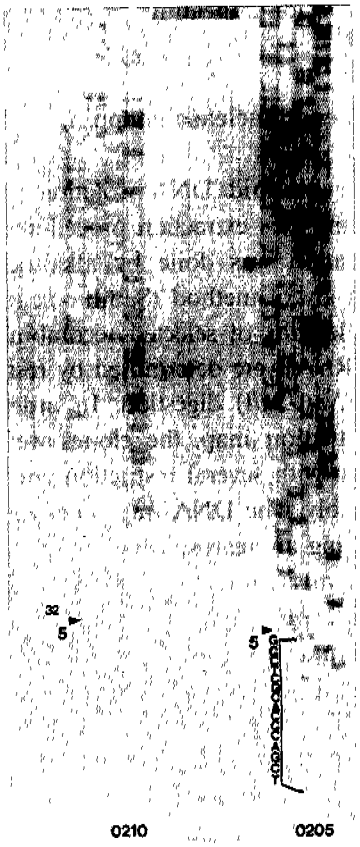

Fig. 3. Partial nucleotide sequences of pTMO-0205 and -0210 clones. Nucleotides denoted were *Sal*I adapter and arrows indicate the start nuleotide of cDNAs. The lane of each clonere presents A, G, C and T from left to right, respectively. Five percent PAGE containing 8 M urea was used and exposed onto X-ray film after electrophoresis.

pTMO-02105

5′-ACAACAAACAACATTACAATTACTATTTACAATTACAATGGCATACACACAGACAGCTAC
ACATCAGCTTTGCTGGACACTGTCCGAGGAGACAACTCCTTGGTCAATGATCTAGCAAAG
CGTCGTCTTTACGACACAGCGGTAGAAGAGTTTAACGCTCGTGACCGCAGGCCCAAAGTG
AACTTTTCAAAAGTAATAAGCGAG

pTMO-0205

TCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTTGAGGTAGTCAAGATGCATAATAAATA
CGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTTTTTCCCTCCAC
TTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCATATGTATATGGTTCATATACATC
AGCAGGCACGTAATAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCA-3′

Fig. 4. Nucleotide sequences of the 5′ and 3′ terminal regions of the cDNA clone pTMO-0210 and -0205 from TMV-O RNA. Thick underlines indicate repeated regions of the leader sequences of the tobamovirus group. Double-underline and asterisk indicate the initiation and termination codons for viral replicase and coat protein, respectively. Bold characters indicate the 3′ terminal untranslated region of the viral RNA. The underline in the 3′ terminal region of the pTMO-0205 indicates the conserved sequences of 3′-end of the tobamovirus group.

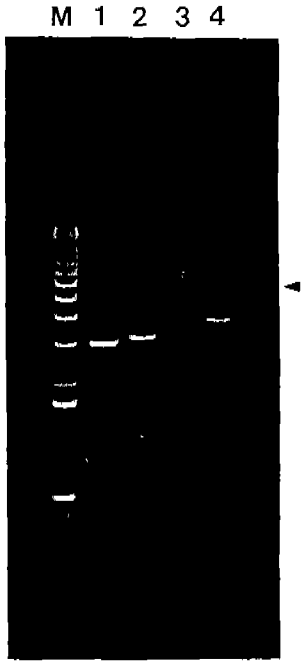

Fig. 5. Electrophoretic patterns of constructed near full-length cDNA of TMV-O RNA. Lane 1, *Bam*HI/*Not*I cDNA of pTMO-0205; 2, *Sal*I/*Bam*HI of pTMO-0210; 3, resulting cDNA of TMV-O RNA; 4, vector (pSPORT1) and M, 1 kb DNA ladder. Arrow indicates the site of a cDNA (6.4 kb).

terminated CCA. Clone pTMO-0210 contained the sequences for virus replicase gene and 5′ untranslated region. The clone pTMO-0210 contained most of 5′ leader sequences (ACAATTAC) and translational ATG start codon of viral replicase. They were covering more than 99% of the viral genome size.

For construction of the viral genomic cDNA, two cDNA fragments which were 3.1 kb *Bam*HI and *Not*I fragement released from pTMO-0205 and 3.3 kb *Sal*I and *Bam*HI fragment released from pTMO-0210 were ligated with T4 DNA ligase. The clone was almost entire size, lacking only 31 nucleotides from the 5′ terminus based on the sequencing result (Fig. 5).

DISCUSSION

In this paper, we have described synthesis of cDNA and construction of a cDNA recombinant of TMV-O genomic RNA. The clones pTMO-0205 and -0210 were sequenced at their 3′ and 5′ terminal regions, which revealed that these clones covered the 3′ half and 5′ half of the viral RNA, respectively. The clone pTMO-0210 contained the leader sequences of tobamovirus group. The two clones were ligated and selected. The clone was almost full-length, lacking only 31 nucleotides from the 5′ terminus based on the sequencing analysis. In order to study the functions of the various gene products as well as the interactions between viral proteins and their target RNA sequences, it was necessary to produce a full-length cDNA clone of the genomic RNA. Previous attempts to obtain full-length double-stranded (ds) cDNA have relied on deoxy cytosine (dC)-deoxy guanine (dG) tailing method (Gubler and Hoffman, 1983). In this approach first-strand cDNA transcripts are tailed with either dC or dG using terminal deoxynucleotidyl transferase and second-strand cDNA is primed with oligo (dC) or (dG), respectively and generated using DNA polymerase I or Klenow fragment. Such dC-dG regions of the cDNAs are some problems as follows. This region inhibit *in vitro* transcription, translation and sequencing. Synthesis of ds cDNA by self-priming or RNase H methods avoid the introduction of dC-dG tails but do not preserve the full-length character of cDNA constructs and therefore may not reveal the viral cap structure or the complete 5′ noncoding sequence. In the method reported here, poly(A) tail is synthesized to size-fractionated native virus genomic RNAs which were originally polyadenylated or not. This technique should be the method of choice for the construction of cDNA libraries, particularly for multipartite, long, or heterologous genome. This method was shown to be efficiently applicable to other plant viral genomic RNA for the construction of full-length cDNA.

The complete nucleotide sequence of TMV-vulgare by Goelet *et al.* (1982) was the first report among all plant viruses. Subsequently, the complete nucleotide sequences of other tobamoviruses, such as tomato mosaic (TMV-L or ToMV), tobacco mild green mosaic (TMGMV), pepper mild mottle (PMMV), cowpea strain of TMV (Cc TMV or sunn-hemp mosaic virus: SHMV) and cucumber green mottle mosaic (CGMMV), have been determined (Meshi *et al.*, 1982; Ohno *et al.*, 1984; Solis and Garcia-Arenal, 1990; Alonso *et al.*, 1991; Ugaki *et al.*, 1991). Nucleotide and amino acid sequences comparisons among tobamoviruses revealed some interviral relationships within a genus but not between viruses in different genera. The genome organization of TMV is well

known. It is generally agreed that single-stranded, plus-sense RNA encodes four proteins, two of which, the 126 K and 183 K viral replicase, and the rest is 30 K movement protein and 17 K coat protein. It has been proposed that a fifth protein, 54 K, is also encoded by TMV. Recently, partial nucleotide sequences of German type strain, Japanese isolate and Korean isolate of ORSV were reported by Dubs and Van Regenmortel (1990), Isomura *et al.* (1991) and Ryu *et al.* (1994). They suggest that the differences were found between the nucleotide and amino acid sequences of movement and coat proteins and 3′ non-coding regions of ORSV and TMV. The host range and serological property of TMV-O were similar to those of TMV-O 01 and 07 isolates reported by Kado *et al.* (1968) but distiguished from ORSV-type strain (Paul, 1975) and previously reported ORSV-Cy (Park *et al.*, 1990a). Inouye (1966) and Jensen and Gold (1951) and isolated ORSV from *Cymbidium* and *R. grande,* respectively, and Corbett (19 67), Kado *et al.* (1968) and Lawson (1970) isolated TMV-O from various orchids. The distinction between ORSV and TMV-O is still not clear. It would be interesting to compare the molecular characteristics of the two viruses. To make sure that the clone described represent a biologically active TMV molecules we are currently reconstructing the cDNA containing the T7 or T3 promoter for production of *in vitro* transcript. This will enable us to make RNA transcripts *in vitro* which can be tested for infectivity.

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담배 모자이크 바이러스 蘭系統 게놈 RNA의 cDNA 클로닝

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적 요

심비디움 그레이스 켈리로부터 분리한 담배 모자이크 바이러스 난계통을 순화하여 이로부터 바이러스 RNA를 추출하였다. Poly(A) tailing시킨 바이러스 RNA에 *Not*I-oligo (dT) primer를 결합시켰다. 첫번째 가닥 cDNA는 RNase H 작용이 결여된 Molony murine leukemia virus 역전사효소로 합성하였고, 두번째 가닥 cDNA 합성은 RNase H와 DNA polymerase I을 사용하였다. 최종 합성된 겹가닥 cDNA를 pSPORT1 벡터에 클로닝하여 이를 대장균 JM109 계통에 형질전환시켰다. 재조합 클론에 들어 있는 cDNA는 0.9-3.9 kb 길이였다. 선발된 클론중 pTMO-0205와 -0210은 3′ 및 5′ 말단으로부터 각각 바이러스 게놈 RNA의 반을 포함하였고, 염기서열 분석 결과를 토대로 할 때 바이러스 게놈 RNA 길이의 99%에 해당하였다. 바이러스 게놈 RNA에 대한 cDNA 합성을 위해 pTMO-0205 클론에서 3.1 kb의 *Bam*HI-*Not*I 절편과 pTMO-0210에서 3.3 kb의 *Sal*I-*Bam*HI 절편을 회수하여 T4 DNA ligase로 클로닝하였다. 선발된 클론은 염기서열 분석 결과 5′ 말단의 31 염기를 제외한 거의 전장에 해당하였다. 본 방법은 다른 식물 바이러스 게놈 RNA의 전장 cDNA 합성시에도 효과적인 방법임을 의미한다.

주요어: 담배 모자이크 바이러스 난계통, tobamovirus, 심비디움, cDNA 합성 및 클로닝

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