한국식물병리학회지 10(3) : 228-234(1994)
Korean J. Plant Pathol. 10(3) : 228-234(1994)

Construction of Complementary DNA Library and cDNA Cloning for Cy Strain of Odontoglossum Ringspot Virus Genomic RNA

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오돈토글로썸 윤문 바이러스 Cy계통 게놈 RNA의 cDNA 구축 및 유전자 클로닝

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ABSTRACT : Genomic RNA was extracted from Cy strain of odontoglossum ringspot tobamovirus (ORSV-Cy) isolated from infected leaves of tobacco cv. Samsun. Size of the genomic RNA was about 6.6 kb in length. The genomic RNA was fractionated using Sephadex G-50 column chromatography into 2 fractions. They were polyadenylated at their 3′-end using *E. coli* poly(A) polymerase. Polyadenylated viral RNA was recovered by oligo (dT) cellulose column chromatography and used as a template for the synthesis of complementary DNA (cDNA). First-strand cDNA synthesis was performed using the polyadenylated RNA, oligo (dT) primer adapter containing *Not*I restriction site and Moloney murine leukemia virus SuperScript reverse transcriptase (RNase H$^-$). Second-strand cDNA was synthesized by using *E. coli* DNA ligase, *E. coli* DNA polymerase I and *E. coli* RNase H. Recombinant plasmids containing cDNAs for ORSV-Cy RNA ranged from about 800 bp to 3,000 bp. Among the selected 238 recombinants, pORCY-124 clone was the largest one covering 3′-terminal half of the viral RNA. This clone contained two restriction sites for *Eco*RI and *Xba*I and one site for *Acc*I, *Ava*I, *Bgl*II, *Bst*XI, *Hin*dIII, *Pst*I, and *Tth*III 1, respectively. The clone contained partial viral replicase, a full-length movement protein and a complete coat protein genes followed by a 3′ untranslated region of 414 nucleotides based on restriction mapping and nucleotide sequencing analyses. Clones pORCY-028, -068, -072, -187 and -224 were overlapped with the pORCY-124. Clones pORCY-014 and -095 covered 5′ half upstream from the middle region of the viral RNA, which was estimated based on restriction mapping and partial sequence analysis. Constructed cDNA library covered more than 90% of the viral genome.

***Key words* : Odontoglossum ringspot virus, *Cymbidium goeringii*, cDNA synthesis, restriction mapping, cDNA library.**

Odontoglossum ringspot virus (ORSV) is a member of tobamovirus group of plant viruses. The ORSV, regarded by some virologists (4, 12, 13) as an orchid strain of TMV (TMV-O), which was first isolated from *Rossioglossum grande* (8), formerly called *Odontoglossum grande* (11). TMV is one of the most characterized plant viruses (16) and has an important position in the development of both virology and molecular biology. The genome of tobamoviruses is composed of a positive-sense single-stranded RNA of about 6,400 nucleotides. 5′-terminus of the genome is 'capped' with a 7-methylguanosine residue in a 5′ to 5′ triphosphate linkage to a guanosine called cap structure (m^7GpppG) and the 3′-terminus formed tRNA-like structure (16). The entire nucleotide sequence of the genomic RNA of the vulgare strain of TMV has been determined (6). Subsequenctly, the complete nucleotide sequences of other tobamoviruses, such as tomato mosaic (TMV-L or ToMV), tobacco mild green mosaic

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(TMGMV), pepper mild mottle (PMMV), cowpea strain of TMV (Cc TMV or sunn-hemp mosaic; SHMV) and cucumber green mottle mosaic (CGMMV) have been determined (1, 14, 15, 23, 24).

Unlike fungal and bacterial diseases of plants, chemical control of viral diseases have not been developed yet. This reality demonstrates the magnitude of the importance of virus diseases of plants. It is well known that it is difficult to diagnose specially and rapidly virus infection without considering the comparative biological, physicochemical, immunological and molecular biological characteristics of the virus.

ORSV is the most prevalent virus infecting orchids in Korea (3, 18, 25) and has been found in most regions of the world where orchids are cultivated (19, 26). Orchid viruses of more than 25 species infecting various orchids genera reduce severely the quality and quantity of the plants (2, 3, 26). Although biological and immunological assays are useful for virus identification, TMV, TMV-O and ORSV share several similarities and differences (17, 19, 20).

The purpose of this study is the cDNA synthesis to construct library of ORSV RNA and compare to the known other tobamoviruses for molecular characterization of the ORSV.

MATERIALS AND METHODS

Virus strain. The ORSV strain used in this study, previously named ORSV-Cy was originally isolated from naturally infected oriental cool-growing native orchid, *Cymbidium goeringii* Reichenbach which are popular in Korea (17). Its symptomatology, serology and other properties were previously reported (17, 18). The virus was propagated in *Nicotiana tabacum* cv. Samsun by mechanical inoculation. The virus-inoculated tobacco leaves were harvested 2 weeks 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.* (18). Viral RNA was extracted from purified virus particles by using SDS/proteinase K disruption and phenol extraction. After ethanol precipitation, the viral RNA precipitate was rinsed with 80% ethanol. The RNA was dried under vacuum desiccator, and then dissolved in a 100 μl of TEN buffer (10 mM tris, pH 8.0, 1.0 mM EDTA, 0.1 M NaCl). The genomic RNA was eluted by using Sephadex G-50 (Pharmacia) mini-column chromatography into 2 fractions (intact and fragmented forms). The viral RNAs were polyadenylated at their 3′-end by using *E. coli* poly (A) polymerase as following: The viral RNAs were incubated at 75℃ for 2 min and quickly chilled on ice water. One μl of RNase ihibitor (40 U, BM), 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 *in vitro* polymerase reactions were performed at 37℃ for 20 min and terminated by the addition of EDTA to a final concentration of 10 mM. The reactions were extracted by phenol and the polyadenylated RNAs were precipitated by ethanol and ammonium acetate, respectively. The native and polyadenylated viral RNAs were separated on native agarose gel in TBE buffer (89 mM tris, 89 mM boric acid, 2.0 mM EDTA) at 30 volt after formamide and formaldehyde treatment at 65℃ for 10 min and quickly chilled on ice-water. Sizes and integrity of the RNAs were estimated using pepper strain of TMV (synonym of pepper mild mottle virus, 6,355 bases in length) provided by Professor J. K. Choi, Kangwon National University and 1 Kb DNA ladder (BRL) as molecular size standard markers.

Synthesis and cloning of cDNA. Most of DNA manipulations were performed essentially as described by Sambrook *et al.* (22). First strand synthesis was performed with the oligo $(dT)_{15}$ primer 5′-pGACTAGTTCTAGATCGCGAGCGGCCGCCCT15-3′, 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 8.0), 75 mM KCl, 3.0 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). The primer and the RNA template were combined, incubated at 70℃ for 10 min to anneal the primer to the template 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 of the reaction mixture on ice. Second-strand cDNAs were synthesized in a 150 μl

reaction volume consisting of 20 μl first strand reaction mixture, 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5.0 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 hrs. Five units of T4 DNA polymerase was added to the reaction and incubated at 16℃ for an additional 5 min. The reaction was terminated by addition of EDTA (10 mM) and proteinase K (50 μg/ml), incubated at 42℃ for 10 min. The reaction mixture was extracted once with phenol/chloroform and cDNA was precipitated with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of cold absolute ethanol.

Double-stranded cDNAs (ds-cDNA) were annealed with *Sal*I adapter using T4 DNA ligase at 16℃ for 12 hrs. The ds-cDNAs were then digested with *Not*I by completion to generate different terminal sites for orientation. The ds-cDNA fragments were passed through Sephacryl S-500 HR chromatography column (BRL) for size fractionation. The resulting ds-cDNAs were ligated to pSPORT1 (BRL) vector using T4 DNA ligase at 16℃ for 16 hrs. Recombinant plasmids were transformed into competent *E. coli* strain JM109 cells by the $CaCl_2$ method (22) and selected on McConkey agar (Difco) plates containing ampicillin (100 μg/ml) or Luria-Bertani agar media containing ampicillin, 20 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, BRL) and 100 μl of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG, BRL). Ligation Expression Kit (Clontech Lab.) was used to increase the ligation and transformation efficiencies 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 (22). The cDNA lengths of selected recombinant clones of the viral genome 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. Restriction endonucleases (BM, Pharmacia) used were as follows; *Aat*II, *Acc*I, *Ava*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Nco*I, *Pst*I, *Sac*I, *Sma*I, *Sph*I, *Tth*III 1, and *Xba*I. The DNA fragments were separated by size using an agarose gel electrophoresis (1.2% agarose) in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0) at 50 volts. They were visualized with ultraviolet light after staining gels for 3 min in freshly prepared ethidium bromide solution (100 ng/ml) followed by destaining in running water for 5 min. Photographs 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 marker.

RESULTS

cDNA cloning. Viral genomic RNA was extracted from purified Cy strain of odontoglossum ringspot virus (ORSV-Cy). Size of the genomic RNA of ORSV-Cy was about 6.6 Kb in length (Fig. 1). Two

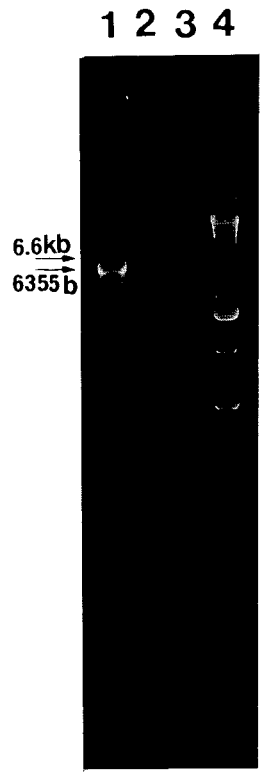

Fig. 1. Electrophoretic pattern of viral RNAs on 1.5% agarose gel. Lane 1 : RNA of pepper strain of TMV, 2 : native RNA of ORSV-Cy, 3 : polyadenylated RNA of ORSV-Cy, 4 : 1 Kb DNA ladder (BRL) as a standard size marker.

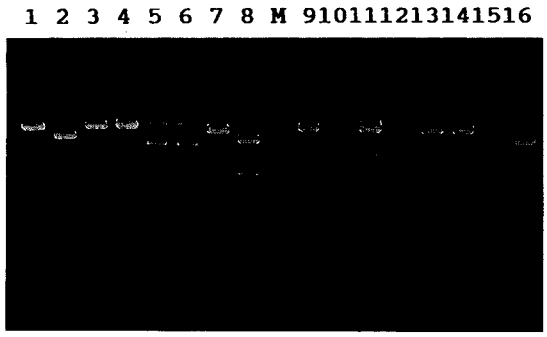

Fig. 2. Agarose gel electrophoresis (1.2%) for restriction pattern of pORCY-072 clone from ORSV-Cy RNA. Lane 1 : *Aat*II, 2 : *Acc*I, 3 : *Ava*I, 4 : *Bam*HI, 5 : *Bql*II, 6 : *Cla*I, 7 : *Eco*RI, 8 : *Hin*dIII, 9 : *Kpn*I, 10 : *Nco*I, 11 : *Pst*I, 12 : *Sac*I, 13 : *Sma*I, 14 : *Sph*I, 15 : *Tth*III 1, 16 : *Xba*I, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

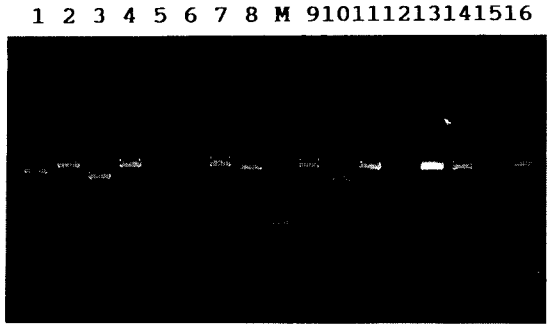

Fig. 3. Agarose gel electrophoresis (1.2%) for restriction pattern of pORCY-095 clone from ORSV-Cy RNA. Lane 1 : *Aat*II, 2 : *Acc*I, 3 : *Ava*I, 4 : *Bam*HI, 5 : *Bql*II, 6 : *Cla*I, 7 : *Eco*RI, 8 : *Hin*dIII, 9 : *Kpn*I, 10 : *Nco*I, 11 : *Pst*I, 12 : *Sac*I, 13 : *Sma*I, 14 : *Sph*I, 15 : *Tth*III 1, 16 : *Xba*I, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

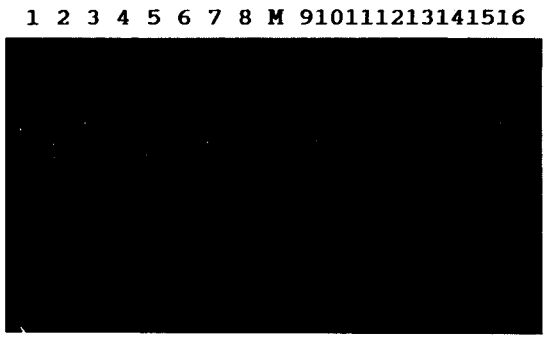

Fig. 4. Agarose gel electrophoresis (1.2%) for restriction pattern of pORCY-124 clone from ORSV-Cy RNA. Lane 1 : *Acc*I, 2 : *Ava*I, 3 : *Bam*HI, 4 : *Bgl*II, 5 : *Bst*XI, 6 : *Cla*I, 7 : *Eco*RI, 8 : *Hin*cII, 9 : *Hin*dIII, 10 : *Kpn*I, 11 : *Nco*I, 12 : *Pst*I, 13 : *Sac*I, 14 : *Sma*I, 15 : *Tth*III 1, 16 : *Xba*I, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

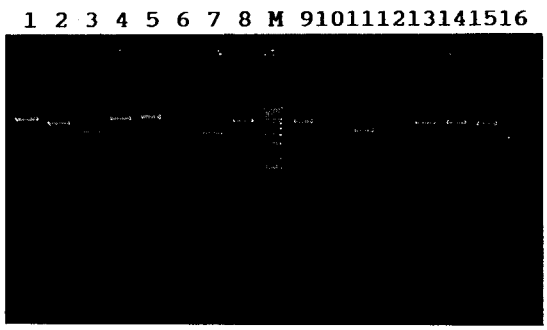

Fig. 5. Agarose gel electrophoresis (1.2%) for restriction pattern of pORCY-187 clone from ORSV-Cy RNA. Lane 1 : *Aat*II, 2 : *Acc*I, 3 : *Ava*I, 4 : *Bam*HI, 5 : *Bgl*II, 6 : *Cla*I, 7 : *Eco*RI, 8 : *HindIII, 9 : Kpn*I, 10 : *Nco*I, 11 : *Pst*I, 12 : *Sac*I, 13 : *Sma*I, 14 : *Sph*I, 15 : *Tth*III 1, 16 : *Xba*I, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

hundred and thirty eight recombinant clones for ORSV-Cy RNA were generated. The cDNAs for ORSV-Cy contained the recombinant plasmids ranged from about 800 bp to 3,000 bp. Among the selected transformants, pORCY-124 (3.0 Kb), pORCY-095, -072, -187 and -224 (2.4 Kb), pORCY-014 and -068 (1.8 Kb) and pORCY-028 (1.6 Kb) were used in this study.

Restriction mapping. The restriction fragments of recombinant clones are illustrated in Fig. 2, 3, 4 and 5. Among the selected 238 recombinants, clone pORCY-124 was the largest one covering 3′-terminal half of the viral RNA. It contained two sites for *Eco*RI and *Xba*I, and one site for *Acc*I, *Ava*I, *Bgl*II, *Bst*XI, *Hin*dIII, *Pst*I, and *Tth*III 1, respectively (Fig. 4). The clone pORCY-124 contained genes partial viral replicase, a full-length movement protein and a complete coat protein followed by 3′ untranslated region of 414 nucleotides (21). Clones pORCY-028, -068, -072, -187 and -224 were overlapped with the pORCY-124. Clones pORCY-014 and -095 covered 5′ half upstream from the middle region of the viral RNA based on restriction mapping and partial sequence analysis. Restriction map of ORSV-Cy is shown in Fig. 6. Constructed cDNA library covered more than 90% of the viral genome (Fig. 7).

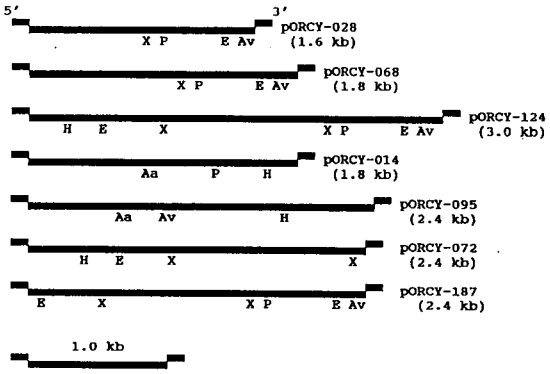

Fig. 6. Restriction endonuclease map of cDNA clones of odontoglossum ringspot virus Cy strain. Restriction endonucleases used are abbreviated as follows; *Aat*I (Aa), *Ava*I (Av), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Xba*I (X). 5′ and 3′ sides of lines (■) indicate *Sal*I adapter and *Not*I vector clining site, respectively.

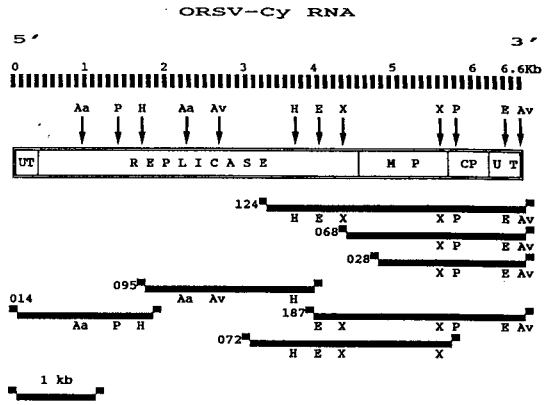

Fig. 7. Schematic representation of corresponding sites of recombinant clones of odontoglossum ringspot virus-Cy strain RNA. The numbers correspond to the pORCY cDNA clones. Restriction endonucleases used are abbreviated as follows; *Aat*I (Aa), *Ava*I (Av), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Xba*I (X). 5′ and 3′ sides of lines (■) indicate *Sal*I adapter and *Not*I vector cloning site, respectively. The locations of the coding and non-coding regions of the 183 K viral replicase (REPLICASE), 34 K movement protein (MP), 18 K coat protein (CP) genes and 5′ and 3′ untranslated (UT) regions are presented.

DISCUSSION

In this paper, we have described synthesis and cloning of cDNA of ORSV-Cy genomic RNA. The library constructed in this study was near full-length, lacking about 10% of the genome size from the 5′ terminus based on restriction mapping and 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 is necessary to produce a full-length cDNA clone of the genomic RNA. Previous attempt to obtain full-length cDNA clone of the genomic or mRNA has relied on deoxy cytosine (dC)-deoxy guanine (dG) tailing method (7). In the novel method reported here, poly(A) tail is synthesized to fractionate different sizes of the native virus genomic RNAs that were originally polyadenylated or not.

Nucleotide and amino acid sequences comparisons among tobamoviruses revealed some interviral relationships within a genus but not between viruses in different genera (5, 6, 14, 15, 16, 23, 24). The genome organization of TMV is well known (16). It is generally agreed that single-stranded, plus-sense RNA encodes four proteins, two of which, the 126 K viral replicase, and the rest is 30 K movement protein and 17 K coat protein (16). It has been proposed that a fifth protein, 54 K, is also encoded by TMV (6, 16). Recently, partial nucleotide sequences of German type strain, Japanese isolate and Korean isolate of ORSV were reported by Dubs and Van Regenmortel (5), Isomura *et al.* (10) and Ryu *et al.* (21), respectively. They suggested that the differences were found in the nucleotide and amino acid sequences of movement and coat proteins genes and 3′ non-coding regions of ORSV and other tobamoviruses.

The genomic organizations of the TMV-L and TMGMV are similar to that of TMV-vulgare, while the SHMV and the CGMMV are distinguished from the TMV-vulgare by the overlapped regions (10~20 nucleotides) between 30 K movement and 17 K coat proteins as well as by the origin of virion assembly in the capsid protein gene (5, 6, 14, 15, 16, 23, 24). Sub-genomic mRNAs are used to produce the 30 K and 17 K proteins. The mRNA for the 30 K protein is encapsidated and found in the intermediate (I) length particles which are present in purified virion particles of all known tobamoviruses. The mRNA for 17 K coat protein is not encapsidated by most TMV strains, but SHMV and CGMMV encapsidate the subgenomic mRNA in short (S) particles. The 3′ terminal adenosine of the vulgare st-

rain of TMV and most other tobamoviruses can be aminoacylated *in vitro* with histidine, while SHMV with valine. The nucleotide length of 3′ untranslated regions of tobamoviruses is variable.

Recently, the nucleotide sequence analysis of the 3′ terminal region containing movement and coat proteins genes and 3′ untranslated regions of ORSV-Cy has been determined (unpublished data). ORSV-Cy is distinguished from SHMV and CGMMV, and but close to TMV-L, TMV-vulgare and TMGMV. Interestingly, 3′ untranslated region of ORSV-Cy RNA includes nine pseudoknots and tRNA-like structures, which is the longest compared with those of other tobamoviruses or even tripartite plant viruses. Jensen and Gold (11) and Inouye (9) isolated ORSV from *R. grande* and *Cymbidium*, respectively, and Kado *et al.* (12), Corbett (4) and Lawson (13) isolated TMV-O from various orchids. The distinction between ORSV and TMV-O is still not clear. Our presumed structural model and sequence analysis data suggests that ORSV is the distinct tobamovirus. Therefore, comparison of molecular characteristics of the two viruses should be done in the future.

요 약

Tobamovirus의 하나인 오돈토글로썸 윤문 바이러스 Cy계통(ORSV-Cy)을 Samsun 담배에서 증식 순화하여 이로부터 바이러스 RNA를 추출하였다. 순화된 바이러스 입자로부터 분리된 ORSV-Cy 게놈 RNA는 약 6.6 Kb였다. 게놈 RNA를 Sephadex G-50 크로마토그래피에 통과시킨 후 이를 대장균에서 분리된 poly(A) polymerase를 사용하여 3′ 말단에 poly (A) tail을 합성하였다. Poly(A) tail시킨 바이러스 RNA는 oligo(dT) 셀룰로오스 컬럼 크로마토그래피법으로 정제하였고, 이를 cDNA 합성시 주형으로 사용하였다. 첫번째 가닥 cDNA 합성은 poly(A) tail된 RNA, *Not*I 제한효소 부위를 포함하는 oligo (dT) primer와 RNase H가 결여된 Mononey murine virus superscript 역전사 효소를 사용하였다. 두번째 가닥 cDNA는 대장균 DNA ligase, 대장균 RNase H 및 대장균 DNA polymerase I을 사용하여 nick translation에 의해 합성하였다. 합성된 ORSV-Cy RNA에 대한 cDNA를 포함한 재조합 플라스미드는 800~3,000 염기 크기 범위였다. 선발된 총 238개 재조합 클론 중에서 바이러스 RNA 3′ 말단을 포함하는 pORCY-124 클론이 가장 길었다. 이 클론은 *Eco*Ri 및 *Xba*I에 대한 인식부위가 2개씩 존재하였고, *Acc*I, *Ava*I, *Bgl*II, *Bst*XI, *Hin*dIII, *Pst*I 및 *Tth*III 1 인식부위가 각각 1개씩 존재하였다. 이 클론은 제한지도 및 염기서열결과를 토대로 할 때 바이러스 복제효소의 일부분과 전체크기의 이동단백질 및 외피단백질 그리고 3′ 말단의 414 염기로된 비번역부위를 포함하였다. pORCY-028, -068, -072, -187 및 -224 클론들은 -124 클론과 중복되는 부분이 존재하였다. pORCY-014 및 -095 클론은 제한효소 및 염기서열 분석결과를 토대로 하여 바이러스 RNA 중심으로부터 5′ 말단 방향을 포함하였다. 구축된 cDNA 유전자원 클론들은 본 바이러스 게놈 크기에 대해 90% 이상을 포함하였다.

ACKNOWLEDGEMENT

We would like to thanks to Professor J. K. Choi for supplying TMV-pepper strain and discussion. This work was supported by grants from Plant Molecular Biology & Biotechnology Research Center in Gyeongsang National Unversity, Chinju, Scientific Research Center of Korea Science & Engineering Foundation, Taejon, Korea.

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