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Complementary DNA Cloning and Restriction Mapping of Nuclear Inclusion Body and Coat Protein Genes of Turnip Mosaic Virus-Ca Strain Genomic RNA

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순무모자이크 바이러스 Ca계통 핵봉입체와 외피단백질 유전자의 cDNA 클로닝 및 제한효소 지도작성

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ABSTRACT : Viral RNA was extracted from purified Chinese cabbage strain of turnip mosaic virus (TuMV-Ca) from infected leaves of turnip. Polyadenylated genomic viral RNA was recovered by oligo (dT) cellulose column chromatography and used as a template for the synthesis of complementary DNA (cDNA). Recombinant plasmids contained cDNAs ranged from about 900 bp to 2,450 bp were synthesized. Among the selected 41 transformants, pTUCA31 and pTUCA35 had over 2 Kbp cDNA insert. Restriction endonuclease patterns of the clones examined were very similar among them. Clones pTUCA23 and pTUCA31 were overlapped with pTUCA35. The longest clone pTUCA35, encoding 3′-end, showed that it contained two sites for *Eco*RI, and one site for *Bam*HI, *Cla*I, *Hin*cII, *Sac*I and *Xba*I, respectively. The restriction mapping indicated that the clone pTUCA35 contained partial nuclear inclusion body gene, complete coding region of the coat protein and 3′ untranslated region of TuMV-Ca genomic RNA.

***Key words* : Turnip mosaic virus, cDNA cloning, nuclear inclusion body & coat protein, restriction mapping.**

Turnip mosaic virus (TuMV), a member of the potyvirus group, has a worldwide geographical distribution everywhere cruciferous crops are present (4, 6). It causes diseases on economically important plants, particularly the *Brassica* species, including Chinesecabbage (*B. campestris* L. ssp. *pekinensis*) and radish (*Raphanus sativus*) in Korea. It is sap-transmissible and spread by over 40 species of aphids in a non-persistent manner (4, 9).

The genomic organization of potyvirus has been well characterized (1). The genome of potyviruses is composed of a positive-sense single-stranded RNA (ssRNA) of about 10 Kb, linked covalently at its 5′ end to a virus-encoded protein (VPg) and polyadenylated at its 3′ end (1). The genomic RNA is translated into a large polyprotein precursor which is proteolytically processed into at least eight translation products by three encoded proteinases (1, 10).

The purposes of this research are the cDNA synthesis and cloning of the 3′ terminal region for TuMV-Ca RNA and comparisons of restriction endonuclease map with those of several TuMV isolates (3, 7, 9, 10, 15) which are geographically distributed.

MATERIALS AND METHODS

Virus source. A viral isolate, designated as TuMV-Ca, was originally obtained from naturally infected Chinese cabbage leaves showing severe mosaic and small black necrotic spots symptoms in the high altitude area of Daekallyeong, Kangwon-Do, in Korea (11). Inoculum was prepared by

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homogenizing the infected leaves with a chilled mortar and pestle in inoculation buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% polyvinyl pyrroridone Mr 10,000 and 1% sodium sulfite). It was rubbed onto Carborundum-dusted leaves with a sterilized cotton stick. After three successive single-lesion transfers on *Chenopodium amaranticolor*, the virus was maintained on turnip (*Brassica campestris* spp. rapifera) plants in a light and temperature controlled greenhouse.

Virus purification and viral RNA extraction. The virus was purified by the method of Choi *et al.* (5) with some modifications. Purity and yield of the virus were determined by spectrometry and electron microscopy. Viral RNA was extracted from incubating the purified virus suspension (1 mg/ml) with 1% SDS, proteinase K (0.5 mg/ml) and 150 mM NaCl at 37℃ for 30 min. After disrupting the virus particles, the viral RNA was extracted three times with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) and once with chloroform/isoamyl alcohol (24 : 1, v/v). After lithium chloride fractionation, the viral RNA was collected and precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of cold absolute ethanol. After centrifugation, the RNA precipitates were resuspended in a small volume of diethyl pyrocarbamate-treated distilled water. Further purification of viral RNA for cDNA synthesis, polyadenylated genomic viral RNA was recovered by oligo (dT) cellulose column chromatography using mRNA Quick-Prep Kit (Pharmacia) according to the manufacture's specification.

cDNA synthesis and cloning. Most of DNA manipulations were performed essentially as described by Sambrook *et al.* (14). Purified polyadenylated viral RNA (1 μg) and 100 pmols of *Not*I-primer adaptor (Promega) (5′-d[AATTCGCGGCCGCT15]-3′), which contains an *Not*I restriction site (underlined) were combined, incubated to 70℃ for 10 min and then quickly chilled on ice. First-strand cDNA was synthesized using Moloney murine leukemia virus SuperScript reverse transcriptase (RNase H^-, BRL) at 37℃ for 60 min. Second-strand cDNAs were made by RNase H (Promega), *E. coli* DNA ligase (BM) and *E. coli* DNA polymerase I (Promega) by nick translation. The generated double-stranded (ds) cDNAs were ligated to their termini with *Sal*I adapter (BRL) at 16℃ for 16 hrs and then digested with *Not*I restriction endonuclease for different terminal residues. The ds-cDNA fragments were passed through Sephacryl S-500 HR chromatography column for size-fractionation. The resulting ds-cDNA products were ligated into the *Not*I-*Sal*I-cut, dephosphorylated pSPORT1 phagemid vector (BRL) and transformed into $CaCl_2$ treated competent *E. coli* strain JM109 cells. Recombinant colonies were selected on McConkey agar plates containing ampicillin (100 μg/ml). The insert sizes of selected recombinant cDNA clones were determined by restriction endonuclease digestion.

Restriction endonuclease map. Small-scale plasmid DNA isolation was performed by alkaline-lysis, and large-scale preparations weredone by alkaline-lysis plus polyethylene glycol method (14). In order to construct the restriction endonuclease map, the clones were digested to completion with eight restriction endonucleases (BM). Enzymes used were as follows; *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Nde*I, *Hin*cII, *Sac*I 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 mM EDTA, pH 8.0) at 50 volts. The patterns of separated DNA fragments were visualized with ultraviolet transilluminator after staining gels in freshly prepared ethidium bromide solution (100 ng/ml) and destaining them with running water. Photographs of the gels were taken using a red filter with Polaroid films (Type 667, Kodak). Sizes of all DNA fragments were estimated using lambda DNA-*Hin*dIII and 1 Kb DNA ladder (BRL) as a molecular size standard marker.

RESULTS

cDNA cloning of TuMV-Ca RNA. Polyadenylated genomic RNA of TuMV-Ca purified by oligo dT cellulose chromatography was used as a template for the synthesis of cDNA. Forty one recombinant DNA plasmids containing cDNA inserts of the 3′ terminal region of TuMV-Ca were generated. Recombinant plasmids contained cDNAs ranged from about 900 bp to 2,450 bp. Among the selected transformants, pTUCA31 (2.0 kbp insert) and pTUCA35 (2.45 kbp insert) had long insert cDNAs.

Restriction mapping. Oligo (dT) priming was used during the initial cDNA synthesis, the 3′ terminus of the viral genome would be represented in our library. Restriction endonuclease patterns of

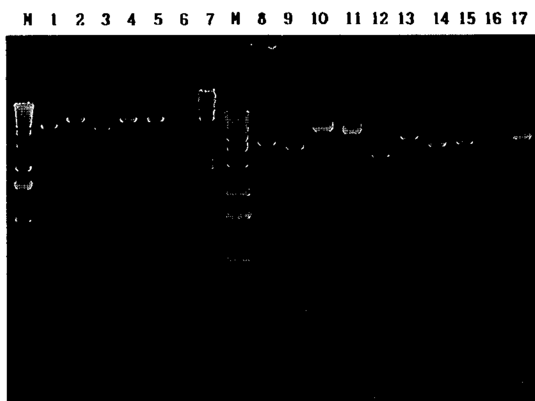

Fig. 1. Agarose gel electrophoresis (1.2%) for restriction pattern of pTUCA31 clone from TuMV-Ca RNA. Lane 1 : *Bam*HI, 2 : *Cla*I, 3 & 8 : *Eco*RI, 4 : *Hin*dIII, 5 : *Nde*I, 6 : not digested, 7 : Lambda-*Hin*dIII, 9 : *Hin*cII, 10 : *Sac*I, 11 : *Xba*I, 12 : *Eco*RI/*Hin*cII, 13 : *Eco*RI/*Sac*I, 14 : *Eco*RI/*Xba*I, 15 : *Hin*cII/*Sac*I, 16 : *Hin*cII/*Xba*I, 17 : *Sac*I/*Xba*I, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

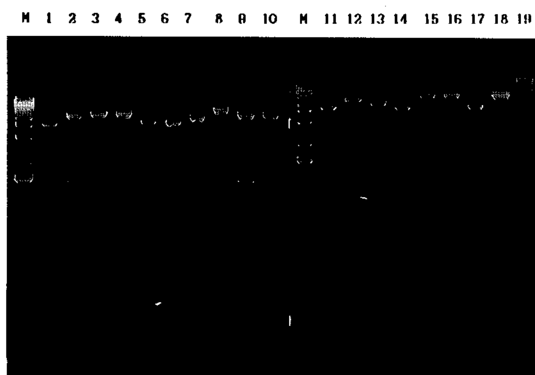

Fig. 2. Agarose gel electrophoresis (1.2%) for restriction pattern of pTUCA35 clone from TuMV-Ca RNA. Lane 1 : *Bam*HI/*Eco*RI, 2 : *Bam*HI/*Hin*dIII, 3 : *Bam*HI/*Xba*I, 4 : *Bam*HI/*Cla*I, 5 : *Eco*RI/*Hin*dIII, 6 : *Eco*RI/*Xba*I, 7 : *Eco*RI/*Cla*I, 8 : *Hin*dIII/*Xba*I, 9 : *Hin*dIII/*Cla*I, 10 : *Xba*I/*Cla*I, 11 : *Sma*I/*Bam*HI, 12 : *Sma*I/*Cla*I, 13 : *Sma*I/*Eco*RI, 14 : *Sma*I/*Hin*dIII, 15 : *Sma*I/*Nde*I, 16 : *Sma*I/*Sac*I, 17 : *Sma*I/*Xba*I, 18 : *Sma*I, 19 : Lambda-*Hin*dIII, and M : 1 Kb DNA Ladder (BRL) as a standard size marker.

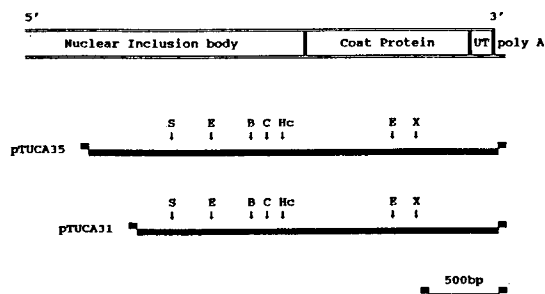

Fig. 3. Restriction endonuclease map of cDNA clones pTUCA31 and pTUCA35 containing the coding regions for the TuMV-Ca nuclear inclusion body and coat protein. The deduced location of the codingand noncoding regions of the nuclear inclusion body protein, coatprotein and 3′ untranslated (UT) regions are presented. Restriction endonucleases are abbreviated as follows; *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Hin*cII (Hc), *Sac*I (S) and *Xba*I (X).

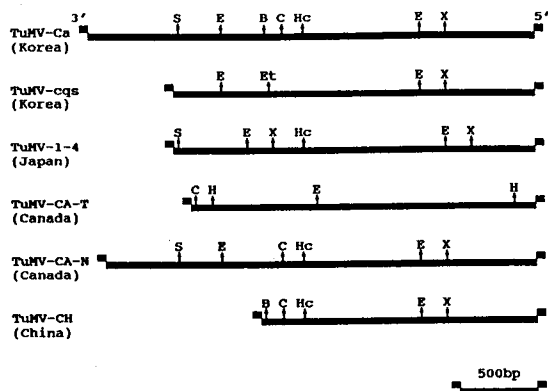

Fig. 4. Comparisons of restriction endonuclease maps between TuMV-Ca (TuMV-Ca) and other geographically distributed TuMV isolates. Restriction endonucleases are abbreviated as follows; *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Eco*T14I (Et), *Hin*cII (Hc), *Sac*I (S) and *Xba*I (X). Sequence data were obtained from as follows; cqs : Korean isolate by Choi *et al.* (3), 1-4 : Japanese isolate by Nakashima *et al.* (9), CA-T : Canadian isolate by Tremblay *et al.* (15), CA-N : Canadian isolate by Nicolas and Lalibéte (10), CH : Chinese isolate by Kong *et al.* (7).

the clones examined were very similar among them. Clones pTUCA23 and pTUCA31 were overlapped with pTUCA35. Fig. 1 showed the electrophoretic pattern of pTUCA31 clone. The longest clone pTUCA35, encoding 3′-end, showed that it contained two sites for *Eco*RI, one site for *Bam*HI, *Cla*I, *Hin*cII, *Sac*I and *Xba*I, respectively (Fig. 2). The estimated lengths of cDNA fragments of pTUCA35 measured from 3′ end were 450 bp at *Xba*I (vector site)-*Xba*I, 200 bp at *Xba*I-*Eco*RI, 740 bp at *Eco*RI-*Hin*cII, 100 bp at *Hin*cII-*Cla*I/*Bam*HI, 250 bp at *Cla*I/*Bam*HI-*Eco*RI, 250 bp at *Eco*RI-*Sac*I, and 450 bp at *Sac*I-*Sma*I (vector site) sites, respectively (Fig. 3).

As following the restriction endonuclease analysis, TuMV-Ca was distinguished from previously reported by Choi *et al.* (4), Nakashima *et al.* (9) and Tremblay *et al.* (15), and rather close to the reported results by Kong *et al.* (7) and Nicolas and Laliberte (10) (Fig. 4).

DISCUSSION

Chinese cabbage (*B. campestris* L. ssp. *pekinensis*), one of the major vegetables in Korea, have been produced about 2 million tons in Korea annually. We have cloned the 3′ terminal region of an Chinese cabbage isolate of TuMV (TuMV-Ca). Up to now, two isolates of TuMV have been reported in Korea (4). This is the second report of cDNA cloning for TuMV in Korea. TuMV-Ca exhibits different properties that may not be similar to two isolates of TuMV identified from same host in Korea by host range studies (4). Although TuMV-Ca seems to differ from the five Taiwan strains of TuMV (6) based upon the host range studies, the physical and serological properties were indistinguishable. Our aims were to present a characterization of TuMV isolates on the basis of geographical distribution.

Most isolates of TuMV resemble the type strain in biological and serological properties. For TuMV strain grouping has been classified as 2 groups by 3 subgroups based on the differential reactions to indicator plant (*Nicotiana glutinosa*) (16). However, no distinct symptoms produced on leaves of *N. glutinosa* inoculated with cqs and cql isolate have been noticed (4). It has been suggested that *C. quinoa* and *N. clevelandii* were more reliable and differential hosts rather than *N. glutinosa* (4).

As following the restriction endonuclease analysis, TuMV-Ca was distinguished from previously reported by Choi *et al.* (4), Nakashima *et al.* (9) and Tremblay *et al.* (15), and rather close to the reported results by Kong *et al.* (7) and Nicolas and Lalibéte (10) (Fig. 4). The clone pTUCA35 contained partial nuclear inclusion body (NIb), complete coding region of the coat protein and 3′ untranslated region based on the restriction mapping. Recently, the nucleotide sequences of partial region of NIb and a full length of coat protein gene of the TuMV-Ca has been determined (12, 13). The homology between TuMV-Ca and previously reported sequence of TuMV RNA (Canadian isolate) was 95.0%, showing 51 nucleotides substitutions. And the NIb encoded by TuMV-Ca exhibits 94.1% amino acid identity to TuMV Canadian isolate, representing 20 amino acids substitutions.

Although many serological assays are useful for virus detection, several potyviruses share serological similarities such that they may not be distinguishable from each other in these assays (8). Nucleic acid probes can be utilized to circumvent the problems of serological cross reactivity. Assays using the nucleic acid probe scan be as sensitive as assays with specific antibodies. When targeted to unique regions such as 3′ untranslated region of the potyvirus RNA, specific viruses and even strains of a virus can be distinguished. Knowing the sequence of the virus will facilitate the development of highly specific probes. Sensitive and accurate detection of virus in infected plant tissue is required for effective control of virus dissemination. Some confusion has resulted because antibodies may cross react with virus coat proteins of many viruses within the potyviruses (8). Therefore, nucleic acid probes such as interviral-specific that will be highly sensitive and specific for the detection of TuMV.

요 약

배추에서 분리한 순무 모자이크 바이러스(TuMV-Ca)를 순무에서 증식 후 순화하여 이로부터 바이러스 RNA를 추출하였다. 추출된 TuMV-Ca RNA 중 poly (A) tail를 가지는 게놈 RNA는 oligo dT 셀룰로오스 크로마토그래피법으로 정제하였고, 이를 cDNA 합성시 주형으로 사용하였다. cDNA 합성 후 바이러스 게놈 RNA에 대한 cDNA를 포함하는 재조합 플라스미드는 900~2,450 bp 범위였다. 선발된 41개의 재조합 유전자 중에서 pTUCA31 및 pTUCA35는 2 Kbp 이상 크기의 cDNA가 삽입되어 있었다. 선발된 클론들은 제한효소 패턴에서 서로 매우 유사하였으며, pTUCA23과 pTUCA31은 pTUCA35 클론과 중복부위를 포함하였다. 선발된 클론 중 바이러스 RNA 3′ 말단부위의 가장 긴 cDNA를 가지는 pTUCA35는 두개의 *Eco*RI 절단부위와 *Bam*HI, *Cla*I, *Hinc*II, *Sac*I 및 *Xba*I 각각 한개의 절단부위를 포함하였다. pTUCA35 클론은 제한효소지도 작성결과 순무 모자이크 바이러스 Ca계통 게놈 RNA중에서 핵봉입체 유전자의 일부분과, 전장의 바이러스 외피단백질 유전자 및 3′ 말단의 비번역 부위를 포함하였다.

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