Isolation and characterization of *Chlorella* virus from fresh water in Korea

국내 담수로부터 클로렐라 바이러스의 분리 및 특성

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by

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Isolation and characterization of *Chlorella* virus from fresh water in Korea

A Dissertation

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CONTENTS

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Abstract

Viruses infecting microalgae are of interest because of their impact on aquatic ecosystem and recent discovery of many useful genes from these viruses. Twenty three *Chlorella* virus isolates were isolated from 10 cities in Korea. The viruses were first amplified in *Chlorella* strain NC64A, and pure virus isolates were obtained by repeated plaque isolation. The isolated virus isolates have 300 to 350kb genomes. Digestion of purified genomic DNAs with 10 restriction enzymes revealed different DNA fragment patterns among these isolates. One of isolate, SS-1, was resistant to Hind III, Pvu II, Alu I and HaeIII digestion, which indicated possible methylation at AGCT or GC sequences. The major capsid protein (MCP) of all the isolated viruses did not react to antiserum against *Chlorella* virus EPA-1 but 5 isolates reacted to antiserum against PBCV-1. Nucleotide sequences of the MCP genes of 3 isolates determined by cut-and-walk method showed over 90% homology among the isolates. The tRNA coding regions of 8 selected isolates were amplified by PCR and cloned. All of the isolates contain 14-16 tRNA genes in 1.2-2kb region except for the SS-1 isolate that has a 1039bp spacer in the middle. The spacer of SS-1 contains an open reading frame (ORF) of 294 amino acids. Although the spacer shows 51% amino acid sequence

similarity to PBCV-1 ORF A478L, southern blot analysis suggested that it is a novel gene not present in PBCV-1. The promoter of the tRNA genes of *Chlorella* virus are composed by repetition of AT-rich motifs. However, the number and sequences of the motifs were vary in different isolates.

Introduction

Viruses infecting algae are widely distributed in nature, and they have been isolated from fresh water and seawater throughout the world. It is known that as much as 10^6-10^8 particles/㎖ of virus or virus-like particles are present in natural sea water (Fig. 1). Although a large fraction of these viruses are bacteriophages, many viruses are from eukaryotic algae. Viruses or virus-like particles have been reported from at least 44 taxa of eukaryotic algae since 1970s (Van Etten *et al.*, 1999). However, most of these viruses are not well characterized because they were difficult to obtain in large quantities.

In contrast to most algae virus, *Paramecium bursaria Chlorella* virus (PBCV-1) is the most studied virus among the algal viruses because this virus can be produced in large quantities by using exsymbiotic chlorella-like green algae, *Chlorella* strain NC64A, isolated from *P. bursaria*. This virus is the prototype of a group (family Phycodnaviridae, genus Chlorovirus) of large, polyhedral, plaque forming algae virus (Van Etten, 2000). DNA sequence analysis of PBCV-1 genome of 330,742bp showed that this virus has 376 protein encoding genes and 10 transfer RNA genes (Li *et al.*, 1995, 1997; Lu *et al.*, 1995, 1996; Kutish *et al.*, 1996). PBCV-1 virion contains at least 50 proteins and lipid component located inside the capsid shell. The major capsid protein (MCP) is one of four proteins located on the viral surface and account for about 40% of the total virion protein (Skrdla *et al.*, 1984). The MCP is one of glycosylated viral proteins, which are glycosylated by virus encoded putative glycosyltransferases gene (Graves *et al.*, 2001).

Viruses infecting algae are of interest because of their impact on aquatic ecology, possible application for the control of blooming of toxic red or

green algae. In addition, recent DNA sequence analysis of *Chlorella* viruses indicated that these viruses encode many useful proteins such as restriction/modification enzymes, topoisomerase, chitinase, hyaluronan synthase (Zhang *et al.*, 1998; Xia *et al.*, 1986; Lavrukhin *et al.*, 2000; Sun *et al.*, 1999; Graves *et al.*, 1999). They also encode transcriptional and translation factors (Yamada *et al.*, 1993a). In addition, the *Chlorella* viruses are new source of promoters for expressing genes in foreign hosts. For example, the upstream region of a eukaryotic algal viral adenine DNA methytransferase genes functions as a strong promoter both in plant and bacteria (Mitra *et al.*, 1994). Most noteworthy is that the components of the translational machinery are encoded by the viral genome and some components of the host protein synthesis machinery might be replaced by viral gene products (Nishda *et al.*, 1999). *Chlorella* viruses encode their own tRNA for protein synthesis (Li *et al.*, 1997; Nishda *et al.*, 1998a). This tRNA gene was clustered in the genome and contribute to the preferential translation of viral proteins during the virus replication cycle. Interestingly, the tRNA clusters are cotranscribed into a large precursor of about 1.0kb in size in the virus-infected *Chlorella* cell (Nishda *et al.*, 1999). Cotranscribed tRNA gene cluster in eukaryotes have been known only for yeast so far (Schmidt *et al.*, 1980). These facts suggest that upstream of tRNA gene cluster might be include strong promoter.

Despite of these importance, researches on algae virus in our country is very limited. In this study, several isolates of algae viruses were isolated from fresh water from 10 cities in Korea and some characteristics of these viruses were examined.

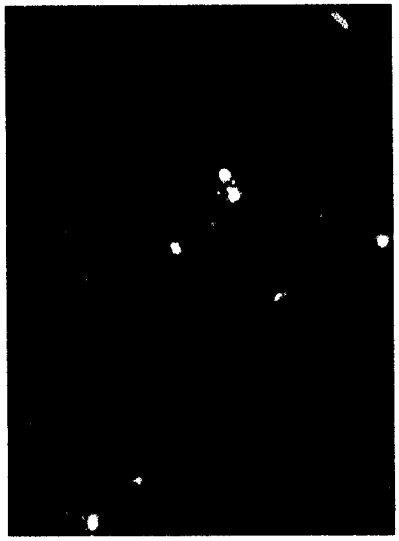


Fig. 1. Fluorescence photomicrography of sea water sample stained with SYBR Green I. The larger green particles are bacterial cells and the smaller numerous green spots are virus particles. Water sample was collected from Namhae, Kyungnam, Korea and filtered through 0.02 um Anodisc filters backed up by 0.8 um mixed-ester membrane filter, and stained with 2.5% SYBR.

Materials and Methods

Sampling

Fresh water samples were collected from 10 cities in Korea, list in the Table 4. The sample water was filtered through a 0.22 ㎛ pore size Al_2O_3 anodisk 25 membrane filter (Whatman), backed by a glass fiber filter at approximately 20 kPa vacuum. The filtrates were kept in 4℃ before inoculation into *Chlorella* host.

Virus Amplification

The *Chlorella* strain NC64A was kindly provided by Dr. James Van Etten in the Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, USA. Cells of *Chlorella* strain NC64A were cultured in the modified Bold's basal medium (MBBM) as described (Van Etten *et al.*, 1983). Initially, virus isolation from these samples was attempted using plaque isolation method as described below. No plaque was recovered from this trial, probably because of low virus titer in the sample and virus was amplified for plaque isolation.

For the amplification of virus in the samples, 5㎖ of filtered water was added to 100㎖ of *Chlorella* strain NC64A grown in MBBM medium and incubated for five days at 25℃ with continuous light and shaking at 150 rpm (Van Etten *et al.*, 1983).

Single Plaque Isolation

After complete lysis of the *Chlorella*, the supernatant was diluted to 10^{-6}-10^{-7} and 5㎕ of diluted virus was mixed with 4㎖ of fresh *Chlorella* (4.0 $\times 10^{8}$ cells/㎖), 2㎖ of 0.75% agar and overlayed on 1.5% agar plate made with MBBM medium (Van Etten *et al.*, 1983). The plate was incubated at 25℃ for five days. Single plaque was isolated from the plate and inoculated to 100㎖ of fresh *Chlorella*. Plaque isolation was repeated three times after complete lysis (Fig. 2).

Electron Microscopy

Chlorella strain NC64A infected with *Chlorella* viruses for 5 h were pelleted at 5,000rpm for 10 min. The pellets were fixed with 2% glutaraldehyde in 0.1M sodium carcodylate buffer (pH 7.4) for 2 h and then post fixed with 1% OsO_4 in 0.2M sodium carcodylate buffer for 30 min. The fixed pellets were embedded in epoxy resin, sectioned and stained with 2% uranylacetate and lead citrate. Purified virus particles were negatively stained with 2% uranylacetate on collodion coated grids.

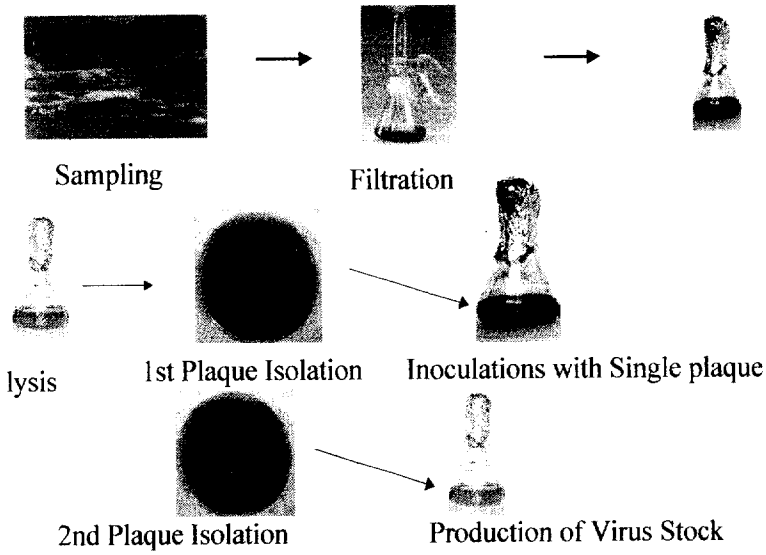


Fig. 2. Overall procedures of *Chlorella* virus isolation from fresh water samples.

Virus Purification

One hundred milliliter of actively growing *Chlorella* strain NC64A was inoculated with single plaque and incubated until complete lysis. The lysate was centrifuged in a Sovall GS-3 rotor at 5,000 rpm for 5 min at 4℃. Triton-X100 was added to the supernatant at 0.1% final concentration and stirred for 20 min at 4℃. Virus particles were pelleted in a Sorvall T-880 rotor at 20,000 rpm, for 60 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8 and centrifuged through a 10-40% discontinuous sucrose gradient (20,000 rpm, 20 min, 4℃). The virus band was collected from 30-40% interface, and pelleted 3 h at 27,000 rpm with a T-880 rotor. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8 (Van Etten *et al.*, 1983).

Polyacrylamide Gel Electrophoresis of Viral Proteins

Purified virus was suspended in sample loading buffer (1 mM EDTA, 250 mM Tris, pH 6.8, 4% SDS, 50% glycerol, 2% β-mercaptoethanol, and 0.2% bromophenol blue) and heated at 100℃ for 10 min. After cooling, the samples were loaded onto 15% acrylamide gels and electrophoresed under constant current of 20 mA for 1.5 h. The proteins were visualized by staining with Coomassie brilliant blue (R-250) and relative mobilities of the protein was determined by comparison with internal standard protein of known molecular weight (Bio Rad).

Isolation and Analysis of Viral Genomic DNA

Isolated virus (400㎕) was mixed with 10XTEN (100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 M NaCl) buffer (60㎕), 1% Na-sarcosyl (60㎕) and 60% (w/w) CsCl (0.6㎖) and trace amount of EtBr. After heating at 75℃ for 15 min, the mixture was loaded on a performed 40-60%(w/w) CsCl gradient and centrifuged in a Sovall TH-641 rotor at 35,000 rpm, 18 hours, 25℃ (Van Etten *et al.*, 1981). DNA band was collected and EtBr was removed by butanol extraction. DNA was precipitated with EtOH, dried and resuspended in 1XTE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). These viral genomic DNAs were digested with 10 DNA restriction endonucleases and the products were separated on 0.7% agarose gels in 0.5×TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0).

Isolation of Major Capsid Protein (MCP)

Total proteins of purified *Chlorella* virus HS-2 isolate were separated on a 12.5% polyacrylamide gel and stained with 0.3M $CuCl_2$. The MCP band was sliced out, and the protein was eluted using an electro-separation system (S&S Elutrap, Schleicher & Schuell) and quantified by Bradford assay.

For the N-terminal sequencing of the MCP of HS-2, the MCP band on the SDS-PAGE gel was transferred onto PVDF paper (Amersham pharmacia biotech) and N-terminal sequencing was performed from Korea Basic Science Institute.

Production of Polyclonal Antibodies

Polyclonal antibodies against purified HS-2 virion and eluted MCP were produced from mouse. Antigens (25㎍) were mixed with 50㎕ of Freund's complete adjuvant (Sigma) and the emulsion was injected intradermally into 8-week-old female BALB/C mice. Three booster injection with 25㎍ of antigen mixed with Freund's incomplete adjuvant were given at 1 week intervals. One week after the final booster, 1×10^7 sarcoma cells in 1㎖ were intraperitoneally injected. Ascitic fluid was collected after 10 to 15 days, clarified by overnight incubation at 4℃ followed by centrifugation at 3000 × g for 15 min. The supernatant was collected and kept in -20℃.

Western Blot Analysis

The antibodies against purified PBCV-1, EPA-1 and NY-2A were kindly provided by Dr. James Van Etten in the Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, USA. Electrophoretically separated polypeptides were transferred to nitrocellulose membrane at 30mA for 16 h using transfer buffer (120 mM glycine, 15.6 mM Tris, and 20% methanol). The membrane was washed in TTBS buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) and blocked with TBS buffer containing 5% skim milk for 30 min. After washing for 5 min in TTBS buffer (1×TBS containing 0.05% Tween-20), the membrane was treated for 1.5 h with polyclonal antibody (PBCV-1 and EPA-1) diluted 1: 10,000 in TTBS containing 5% skim milk. After three washes for 5 min in TTBS, the membrane was treated for 1 h with alkaline phosphatase conjugated anti-mous IgG in TTBS containing 5% skim milk. The membrane was washed three times

for 5 min in TTBS and developed by NBT (43$\mu\ell$) and BCIP (33$\mu\ell$) solution in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$).

Sequencing of the MCP Gene by Cut and Walk Method

For the direct sequence analysis of viral genomic DNA without cloning, purified genomic DNA was first digested with restriction enzyme, extracted with phenol:chloroform and used as template for sequencing, which was named as "Cut and Walk method". The details for this method was as folllow; Purified genomic DNA (3-5μg) was digested with BamH I overnight and extracted with phenol/chloroform mixture. The DNA was precipitated with ethanol and dissolved in distilled water. These digested genomic DNAs were used with template of sequencing PCR reaction.

Sequencing reation mixture (1-1.5μg template DNA, 1 pmol of primer, 4 $\mu\ell$ of terminator ready reation mixture, and distilled water to 20$\mu\ell$) was prepared and placed into a thermal cycler. Twenty five cycles of PCR reation composed of 10 sec denaturation at 96℃, 5 sec annealing at 50℃ and 4 min extension at 60℃ was performed. After reaction was finished, the products were precipitated with ethanol and dissolved in 25$\mu\ell$ of Template Suppressing Reagent (Perkin Elmer). The DNAs were denatured for 2 min at 95℃, cooled down on ice and analyzed with the ABI PRISM™ 310 Analyzer (Perkin Elmer). The oligonucleotide used as first primer (MCP-N) for MCP gene sequencing was designed based on the N-terminal amino acid sequencing of purified MCP. The second primer of same sense (MCP-2N) was designed by comparing the published PBCV-1 MCP sequence and the MCP sequence of KH-2 isolate determined with the MCP-N primer by cut

and walk method (Table 1). The location of these primers are shown in Figure 3. The MCP genes of 3 other isolates were determined by cut and walk method with these primers.

PCR Amplification of the tRNA Coding Region

The tRNA coding regions of 8 *Chlorella* virus isolates were amplified by PCR with primers designed based on the published PBCV-1 sequence. The primers were forward primer and reverse primer corresponding to the 5′ end of the first isolucine tRNA gene (GATAGGGTATGCAAGTGGTCAAAGCAG CTGGTCTCAAGATCCCAGTCCGTCGGGTTCGCGGGTTCGACTCCCGTCT CTATCA) and the 3′ end of the last valine tRNA gene (GATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTG GCAGGATCGAAACCTGCAGGGATCA) of the PBCV-1 gene cluster. The lle-c primer and the val-n primer were designed to identify the upstream and downstream of the tRNA region. These primers are listed in Table 2 and their relative locations of these primers are shown in Fig. 4.

The tRNA regions were amplified in 30㎕ of PCR mixture containing 5㎕ of 10×buffer (100 mM Tris.Cl, pH 8.3, 500 mM KCl, 15 Mm $MgCl_2$), 0.2 mM dNTP, 0.5㎕ of Taq DNA polymerase (5u/㎕), and 100 pmol of forward primer and reverse primer. PCR reaction condition were predenaturation at 95℃ for 5 min, 40 cycles of 1 min denatiration at 94℃, 1 min annealing at 50℃, and 1.5 min extension at 72℃, followed by a 7 min postextension at 72℃. The PCR products were analyzed by electrophoresis in 1% agarose gels and visualized by UV irradiation.

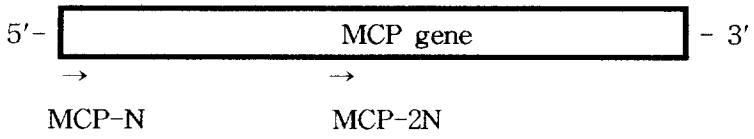


Fig. 3. Locations of primers used for sequencing of major capsid protein.

Table 1. Oligonucleotide primers used for sequencing of major capsid protein

Oligo name	Sequence
MCP-N	5′-ATGGCCGGAGGACTTTCACAGCT-3′
MCP-2N	5′-TGCCCCTCATCTTCTTCTTC-3′

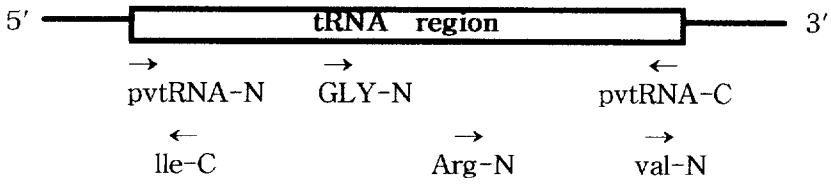


Fig. 4. Locations of primers used for the PCR amplification and direct sequencing of tRNA region.

Table 2. Oligonucleotide primers used for the PCR amplification and direct sequencing of tRNA region

Oligo name	Sequence
pvtRNA-N	5′-GATAGGGTATGCAAGTGGTC- 3′
pvtRNA-C	5′-GATCCCTGCAGGTTTCGATC- 3′
GLY-N	5′-TAACAGCCTTCCAAGCTGTA- 3′
Ile-C	5′-TTGGCTCATAAGACCAATGC- 3′
val-N	5′-GATCGAAACCTGCAGGGATC- 3′
Arg-N	5′-GCGACAGACTTCTAATCTGT- 3′

pGEM-T Vector Cloning

The PCR products were eluted from the agarose gel with DE81 filter paper (Watman) and cloned into pGEM-T vector system (Promega). *E. coli* XL1-Blue strain was transformed with the ligated DNA and clones containing the insert were screened with X-gal and isopropylthio-β-D-galactoside (IPTG). White colony was inoculated to LB broth containing ampicillin (100μg/mℓ) and plasmid DNA was extracted with alkaline lysis method. Purified DNA were digested with EcoR I restriction enzyme and electrophoresed in 1% agarose gel.

Sequencing

Cloned plasmid DNAs were purified with Wizard plus SV minipreps DNA purification system (Promega) and sequencing was performed. Sequencing reaction mixture (500ng template DNA, 1 pmol of primer, 8$\mu\ell$ of terminator ready reaction mixture, and distilled water to 20$\mu\ell$) was prepared and place into a thermal cycler. Twenty five cycles of PCR reaction composed of 10 sec denaturation at 96℃, 5 sec annealing at 50℃ and 4 min extension at 60℃ was performed. The products were precipitated with ethanol and dissolved in 25$\mu\ell$ of Template Suppressing Reagent (Perkin Elmer). The DNAs were denatured for 2 min at 95℃, cooled down on ice and analyzed with the ABI PRISM™ 310 Analyzer (Perkin Elmer).

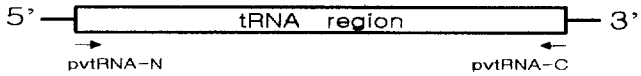
Probe Preparation for Southern Blot Analysis of SS-1 Spacer Gene

The tRNA genes of PBCV-1 and SS-1 isolate, the A478L ORF of PBCV-1 used as probes for Southern blot analysis were amplified by PCR using synthetic oligonucleotides as primers listed Table 3. The relative location of these primers are shown in Fig. 5. The probes were synthesized using DIG (digoxigenin) oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Germany). The PCR products were denatured by heating for 10 min in a boiling water bath and quickly chilled on ice. These probes were mixed with 2$\mu\ell$ of hexanucleotide mix, 2$\mu\ell$ of dNTP mixture and 1$\mu\ell$ of Klenow enzyme and incubated for 18h at 37℃. The quentity of the probes were immunologically determined using DIG-DNA detection kit (Boehringer mannheim).

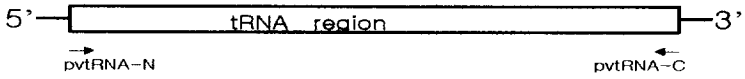
Genomic DNA Preparation from SS-1 Isolate and Southern Blotting

Genomic DNA was isolated from the purified viruses on CsCl equilibrium gradients (Van Etten *et al.*, 1981). Purified DNA was digested with BamH I, Xho I and electrophoresed on 0.7% agarose. The gel was transferred to the depurination buffer (0.2N HCl) and incubated for 15 min. The gel was transferred to the denaturation solution (0.5 N NaOH, 1.5 N NaCl) and incubated for 45 min with gentle agitation. The gel was transferred to distilled water for 10 sec and incubated in neutralization solution (0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 30 min. The DNAs were transferred onto nitrocellulose (NC) membrane for 10 h using capillary transfer method and cross-linked by using UV cross-linker (Hoefer, USA) with preset condition

PBCV-1 tRNA region



SS-1 tRNA region



PBCV-1 A478L

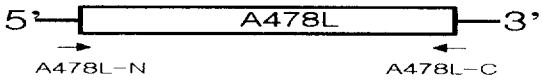


Fig. 5. Location of primer used for the preparation of templates for the Southern blot of SS-1 spacer gene.

Table 3. Oligonucleotide primers used for the preparation of templates for the southern blot of SS-1 spacer gene

Oligo name	sequence
pvtRNA-N	5′-GATAGGGTATGCAAGTGGTC- 3′
pvtRNA-C	5′-GATCCCTGCAGGTTTCGATC- 3′
A478L-N	5′-TTAAGATGGACGATTTCGAG- 3′
A478L-C	5′-AAATGTTGTGCACTCGTTGC- 3′

(1200 μJ/㎠ at 254nm). The NC membrane was prehybridized with standard hybridization buffer (5×SSC, 0.1% N-laurolsarcosine, 0.02% SDS, 1% Blocking reagent) for 1 h. DIG-labelled probe was heat-denatured in boiling water bath for 10 min before added to NC membrane. Hybridization was performed with the probe at 68℃ for 16 h in the same solution and the membrane was washed with 2× SSC containing 0.1% SDS at room temperature for 5 min, then was washed with 0.1× SSC containing 0.1% SDS at 68℃ for 15 min.

After hybridization, the membrane was rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and blocked with blocking solution (1/10 volume of 10% blocking buffer into maleic acid) for 30 min at room temperature. Anti-DIG antibody conjugate was diluted to 1:10,000 in blocking solution and incubated for 1.5 h. The NC membrane was washed twice for 15 min in maleic acid buffer and equilibrated in detection buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 2 min. Then, 43$\mu\ell$ of NBT and 33$\mu\ell$ of BCIP were added to 10㎖ of detection buffer and NC membrane was developed in a dark room.

Sequence Analysis of the Putative Promoter Region of tRNA Genes

Promoter of tRNA gene was sequenced by cut and walk method with internal primer Ile-C of tRNA region shown Fig 4. The purified viral genomic DNA was digested with BamH I and extracted with phenol/chloroform. The product was precipitated with ethanol and dissolved in distilled water. These digested genomic DNAs were used with template of sequencing PCR reaction.

Sequencing reaction mixture (1-1.5μg template DNA, 1 pmol of primer, 4

$\mu\ell$ of terminator ready reaction mixture, and distilled water to 20$\mu\ell$) was prepared and place into a thermal cycler. twenty five cycles of PCR reaction composed of 10 sec denaturation at 96℃, 5 sec annealing at 50℃ and 4 min extension at 60℃ was performed. After reaction was finished, the products were precipitated with ethanol and dissolved in 25$\mu\ell$ of Template Suppressing Reagent (Perkin Elmer). The DNAs were denatured for 2 min at 95℃, cooled down on ice and analyzed with the ABI PRISM™ 310 Analyzer (Perkin Elmer).

Results and Discussion

Isolation of *Chlorella* Viruses

Virus or virus-like particles are known to present as much as 10^6-10^8 particles/mℓ in nature (Noble *et al.*, 1998), but most of them are bacteriophages and typical *Chlorella* virus titer in nature is about 1 to 100 PFU/mℓ, but fluctuates with the seasons (Van Etten *et al.*, 1985, Yamada *et al.*, 1991). No plaque was recovered when the filtered water was directly used for plaque assay, which was acceptable considering the low virus titer in nature. Therefore, the viruses in the samples were first amplified using *Chlorella* host NC64A. The inoculated *Chlorella* cells were completely lysed after 5 day (Fig. 6). Considering the low titer and the amount of inoculum, the fast lysis of inoculated *Chlorella* is quite surprising. It is known that the life cycle of typical *Chlorella* virus is 480-600 minutes and the burst size is 200-350 PFU per cell (Van Etten *et al.*, 1983).

Twenty three *Chlorella* virus isolates which formed plaques on the *Chlorella* strain NC64A were obtained by single plaque isolation from samples collected from 10 cities in Korea (Table 4). As shown in the table, the origins of these isolates are ponds, streams and river. Most of the sampling sites were greenish but the collected samples were not too much turbid. To date, the only known host of *Chlorella* virus are the *Chlorella* strain NC64A and *Chlorella* strain Pbi (Van Etten *et al.*, 1991). The *Chlorella* strain NC64A exist as hereditary endosymbionts in green isolates of the protozoan *P. bursaria* (Van Etten *et al.*, 1985). The presence of virus infecting the *Chlorella* strain NC64A indicates that the presence of same or

related *Chlorella* strains in fresh water in Korea. However, isolation of the host *Chlorella* strain in the water sample, which is time and labor taking procedure, was not performed and the *Chlorella* strain NC64A was used as the sole host throughout this study.

Although several molecular characteristics such as the genome size, G+C content and resistance to restriction enzymes are used for the classification of *Chlorella* viruses (Van Etten *et al.*, 1991), the plaque size produced by the virus at standard condition has been used for primary grouping of these viruses (Van Etten *et al.*, 1983, 1999). The plaque size produced by the Korean isolates was compared with that of PBCV-1. The plaque ranged from 2 to 5mm in diameter after 7 days at 25℃ in sealed agar plate (Fig. 7). The plque size of PBCV-1 was about 3mm and the Korean isolates could be separated into three groups; a group producing smaller plaques than PBCV-1 (YK, HD-2, KH-2, SS-2, BM-2, BL-2, HOS-1, JH-2), a group producing same size as PBCV-1 (BM-1, NW-2, SW) and the other group producing larger plaques (DJ, HS-1, HS-2, HD-1, KH-1, SS-1, BL-1, HOS-1, JH-1, NW-1, ES-1, ES-2). It has been reported that the plaque size of *Chlorella* virus is related with the time required for replication of the virus, and the viruses replicate fast make larger plaques (Van Etten *et al.*, 1991). Virus isolates producing different size of plaques were used in further study.

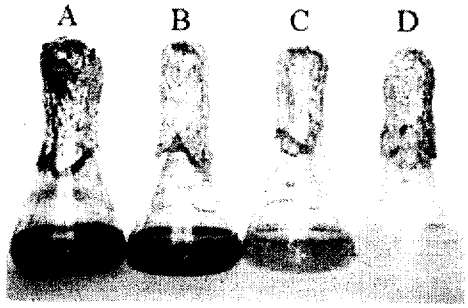


Fig. 6. Lysis of *Chlorella* strain NC64A by *Chlorella* virus isolate DJ-1. The photographs show normal chlorella strain NC64A (A) and 1 day (B), 3 days (C), and 5 days (D) after infection with *Chlorella* virus isolate DJ-1 the MOI of 0.4.

Table 4. Source of *Chlorella* viruses in Korea

Locations	Water Source	Code Number
Pusan	Lake	DJ
		YK
	Stream	HS-1
		HS-2
	Pond	HD-1
		HD-2
Daegu	Lake	KH-1
		KH-2
Seusan	Pond	SS-1
		SS-2
Buyeo	Lake	BM-1
		BM-2
Boryung	Pond	BL-1
		BL-2
Hongsung	Pond	HOS-1
		HOS-2
Changwon	Pond	JN-1
		JN-2
Namwon	Pond	NW-1
		NW-2
		ES-1
		ES-2
Suwon	Pond	SW

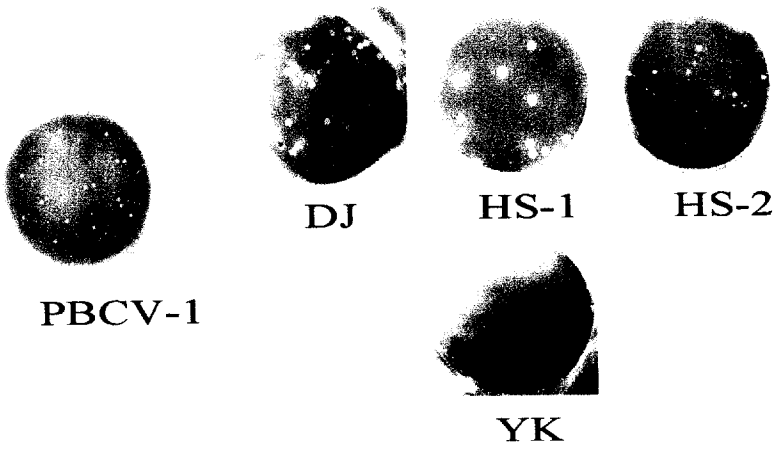


Fig. 7. Plaque assay of *Chlorella* viruses on a lawn of *chlorella* strain NC64A. Plaques are 6 days after plating and show the difference of plaque size produced by different isolates of *Chlorella* viruses isolated in Pusan.

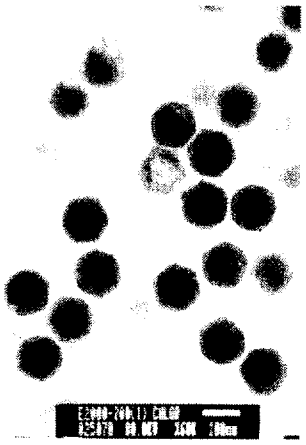
Morphology of *Chlorella* Virus DJ-1

The structure of the isolated virus was observed with a electron microscope and the micrographes of the DJ-1 strain are shown in Figure. 8. Virus particles in infected *Chlorella* cell were polyhedra, with diameters of 150-190nm, which is typical morphology of viruses belong to Phycodnaviridae. Virus particles with thin outer shell and full of electron dense core were observed from thin section and negative staining. Virus particles bound on the surface of *Chlorella* cells were also observed. It is known the virus attaches to the cell surface receptor via one of its hexagonal vertices, digests the wall at the attachment point, and release viral DNA into the host leaving an empty capsid on the cell surface (Van Etten *et al.*, 1999). Accumulated virus particles were also observed in the cytoplasm.

Analysis of Viral Structure Proteins and Genomic DNA

Structural proteins of purified virus were analyzed by SDS-PAGE and viral proteins ranging from 10 to over 200kDa were observed (Fig. 9). The PBCV-1virus particles contain more than 50 polypeptides, which range in size from 10 to 280kDa (Skrdla *et al.*, 1984). The 54kDa major capsid protein which is known to compose about 40% of total viral proteins was distinctive with a number of minor proteins and the size and relative content was similar in all of the tested isolates. However, the viruses could be divided into several groups when the protein bands with MW 25-35kDa were compared. The PBCV-1, DJ-1, SS-1 and HS-2 have two proteins in this range, but the HS-2 isolate has bigger protein than rest of isolates. The

A.



B.

C.

D.

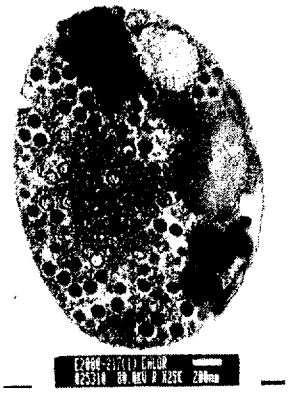


Fig. 8. Electron micrograph and negatively staining of *Chlorella* virus isolate DJ-1. (Bar=100nm). A. Vrions in an infected *Chlorella* cell, B. negatively stained purified virions, C. Electron micrograph of ultrathin section shows the entering of *Chlorella* virus into host cell, D. Accumulated virus particles in cytoplasm of infected *Chlorella* cell.

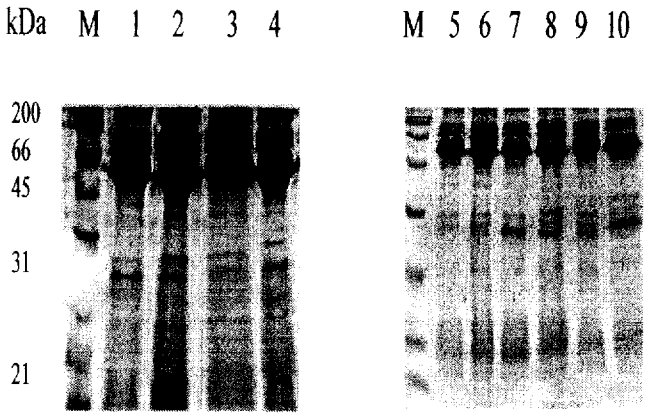


Fig. 9. SDS-PAGE analysis of viral structure proteins. Structural proteins of *chlorella* viruses are analyzed on 15% polyacrylamide gel. M, broad range size marker; Lane 1,5 PBCV-1; Lane 2, DJ-1; Lane 3, HS-1; Lane 4, HS-2; Lane 6, YK, lane 7, SS-1; Lane 8, SS-2; Lane 9, KH-1; Lane 10, KH-2.

isolates HS-1, YK, SS-2, KH-1 and KH-2 have three proteins in this range but the KH-2 isolate shows different band pattern compare to other isolates. The difference in the molecular weight reflects the size difference of the gene encoding the proteins. However, it is not clear at this moment whether these proteins with similar molecular weight are same protein or not. Therefore, immunological and molecular analysis of these proteins are necessary to identify these proteins and genes encoding them.

Genomic DNAs of 8 selected viruses were treated with 10 restriction enzymes (Table 5). The DNAs showed different DNA fragment pattern among these isolates (Fig. 10). One unusual feature of *Chlorella* virus DNA is that they contain relatively high levels of methylated bases (Van Etten *et al.*, 1985). As shown in Table 5, the genomic DNA of the SS-1 isolate was resistant to the restriction enzymes Hind Ⅲ, Alu I and PvuⅡ. These three restriction endonucleases recognize a common sequence AGCT, and are inhibited by the methylation of the first cytosine residue. The SS-1 DNA was also resistant to Hae Ⅲ that recognizes and cleaves GGCC sequence, but inhibited by the methylation of the cystein residue. The gene coding a GpC methylase has been cloned from *Chlorella* virus (Xu *et al.*, 1998). The gene encoding a AGCT methylase has been cloned from *Arthrobacter luteus* (Zang *et al.*, 1993), but not from any *Chlorella* virus. Because all of above enzymes are inhibited by the methylation of the cytosine residue, it is not clear whether SS-1 has GC or AGCT methylase, and further investigation is necessary.

Although the virus isolates were morphologically similar and had a common host, the results of protein patterns, and sensitivity of their genomic DNAs to restriction enzymes suggest that these viruses are PBCV-1 related but distinct viruses.

Table 5. Sensitivity of *Chlorella* viruses to DNA restriction endonucleases

Restriction endonuclease	PBCV-1	DJ-1	HS-1	HS-2	YK	SS-2	SS-1	KH-1	KH-2
BamH I	+	+	+	+	+	+	+	+	+
EcoR I	+	+	+	+	+	+	+	+	+
Hind III	+	+	+	+	+	+	−	+	+
Pst I	+	+	+	+	+	+	+	+	+
Alu I	+	+	+	+	+	+	−	+	+
Xho I	+	+	+	+	+	+	+	+	+
Cla I	+	+	+	+	+	+	+	+	+
Pvu II	+	+	+	+	+	+	−	+	+
Kpn I	+	+	+	+	+	+	+	+	+
Hae III	+	+	+	+	+	+	−	+	+

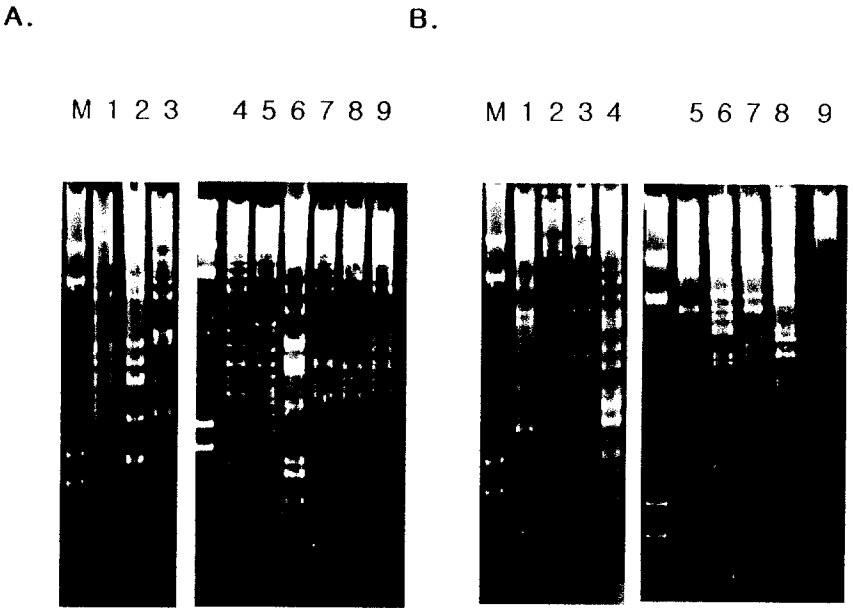


Fig. 10. Electrophoresis of *Chlorella* viruses DNA after digestion with BamH I (A)and Hind III (B). Lanel, PBCV-1; Lane 2, HS-1; Lane 3, HS-2; Lane 4, KH-2; Lane 5, KH-1; Lane 6, SS-2; Lane 7, DJ; Lane 8, YK, Lane, SS-1.

Western Blot Analysis and Sequencing of the Major Capsid Proteins (MCP)

Western blot analysis of viral proteins of 8 virus isolates were performed with polyclonal antisera prepared against 2 American viruses, PBCV-1 and EPA-1. The MCP of all the isolates did not react to antiserum against *Chlorella* virus EPA-1 but 5 isolates reacted to antiserum against PBCV-1 (Fig. 11). In other to determine whether the three PBCV-1 antibody non-reacting isolates are related each other, a polyclonal antibody against purified HS-2 virion was prepared and Western blot was conducted with this antiserum. As shown in Figure 11C, the MCP of three isolates that did not react to antiserum against PBCV-1 reacted to antiserum against HS-2. It is known that the immunological characteristics of *Chlorella* virus MCP are determined not by the amino acid sequence but by the polysaccharide attached to the MCP (Graves *et. al.*, 2001). Viral glycoprotein of PBCV-1 contains seven neutral sugars: glucose, fucose, galactose, mannose, kylose, rhamnose, and arabinose (Wang *et al.*, 1993), and are glycosylated not by host-encoded glycosyltransferase but by virus-encoded glycosyltransferase (Graves *et. al.*, 2001). In order to confirm this explanation with the Korean isolates, a polyclonal antiserum was prepared against gel purified MCP of HS-2. As shown in Figure. 11D, MCP of all of the isolates reacted to this antiserum, which indicated high sequence homology of these proteins. This was further confirmed by nucleotide sequencing of MCP gene of selected isolates. In order to design a sequencing primer, the N-terminal amino acid sequence of HS-2 isolate MCP was determined with gel purified protein, One interesting feature of *Chlorella* virus MCP is the absence of the N-

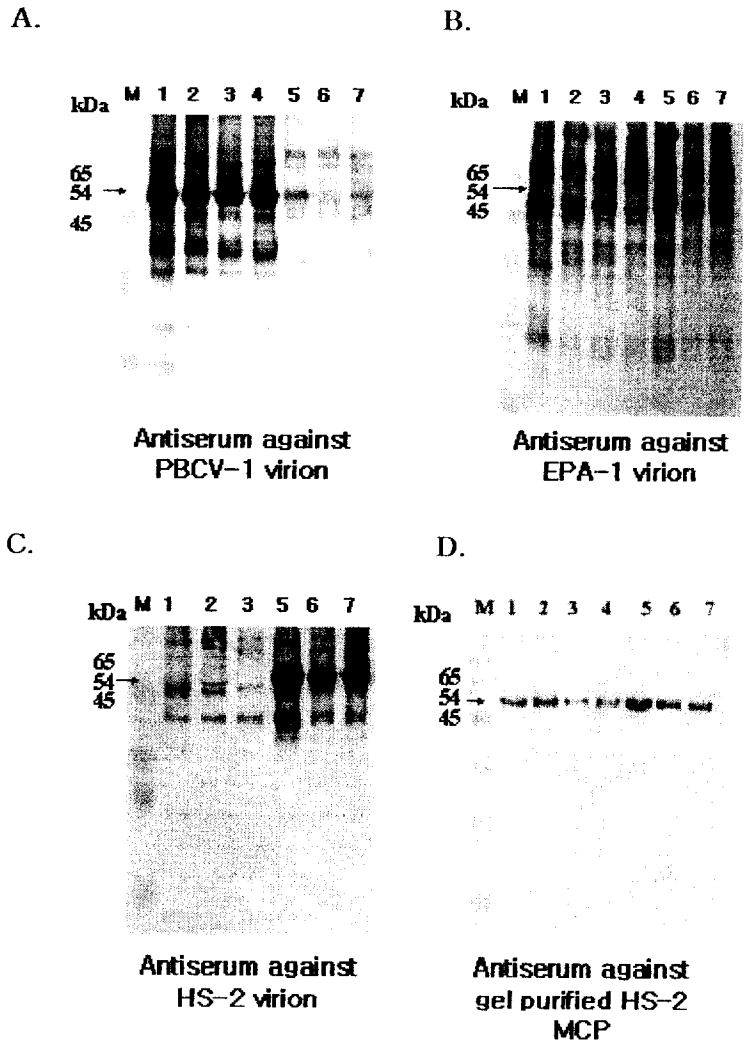


Fig. 11. Western blot analysis of purified *Chlorella* viruses. Total viral proteins of selected isolates were analyzed with antiserum against whole virion (A, B, and C) and gel purified HS-2 MCP (D). M, broad range size marker; Lane 1, PBCV-1; Lane 2, DJ; Lane 3, HS-1; Lane 4, SS-1; Lane 5, HS-2; Lane 6, SS-2; Lane 7, KH-2.

terminal methionine residue which is belived to be removed by methionine aminopeptidase (Van Etten *et al.*, 1999), The determined N-terminal sequence of HS-2 MCP was AGGLSQ, in which the first methionine residue is deleted. A sequencing primer, MCP-N (ATGGCCGGAGGACTTTCACAGC T), was designed based on this amino acid sequence, and sequencing of HS-2, SS-1, KH-1 and KH-2 genomic DNA was conducted with the cut and walk method described above. Another primer, MCP-2N (TGCCCCTCATCTTCTTCTTC), was synthesized to complete the sequencing of the rest of the MCP gene. The amino acid sequence of MCP of these isolates is shown in Figure 12. Multiple alignment of these sequences showed over 98% sequence identity, which explain the Western blot analysis result shown in Figure 11D.

Sequence Analysis of *Chlorella* Viruses tRNA Genes

The tRNA coding regions of 8 isolates were amplified by PCR with primers designed based on the PBCV-1 sequence. The primers are located in the first leucine tRNA and the last valine tRNA of PBCV-1 genome. However, there is another leucine residue at the third place and two PCR products of 1. 2 -2kb were detected from all of the tested isolates, which indicated the presence of more than one leucine or valine tRNA in this region (Fig. 13). Both of the PCR products were cloned in pGEM-T vector and their sequence were determined (Fig. 14). The sequence analyse of this

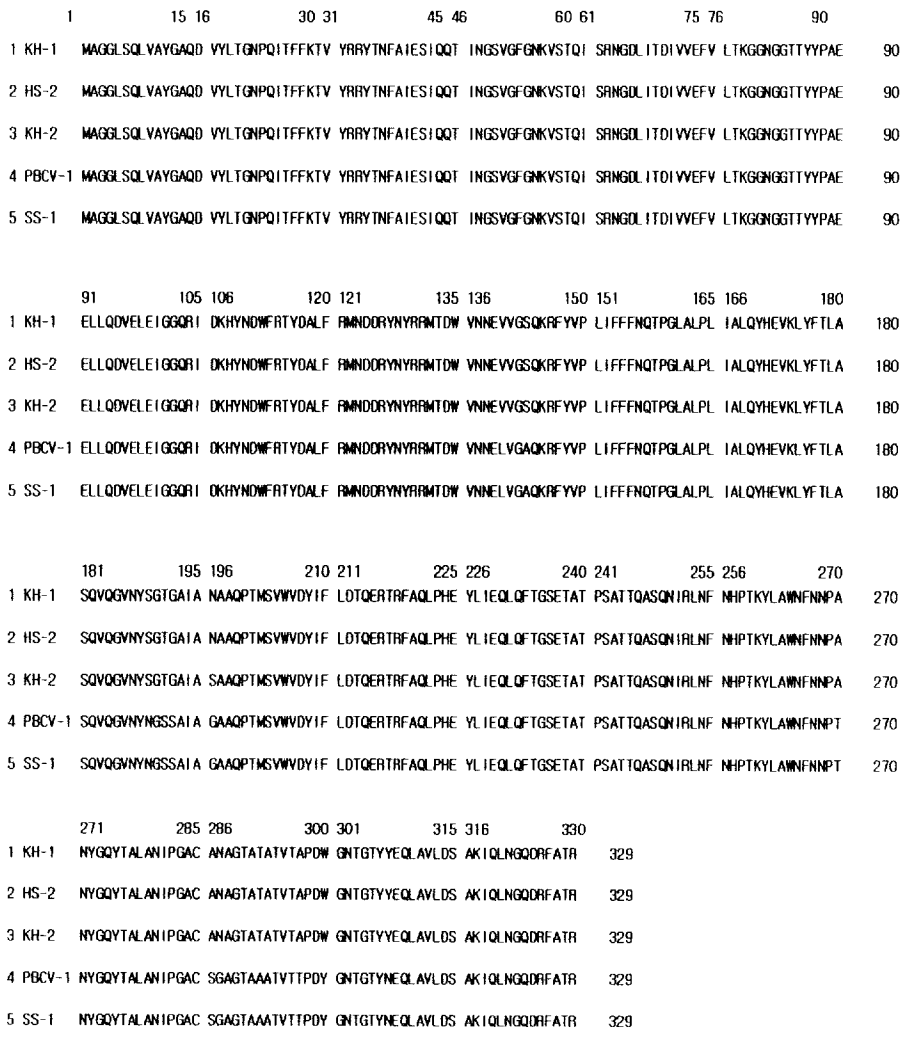


Fig. 12. Comparison of amino acid sequence of major capsid protein (MCP) of different isolates. Amino acid sequence of the MCP genes of 3 isolates were deduced from the nucleotide sequence determined by cut and walk method, and showed over 99% homology among the isolates.

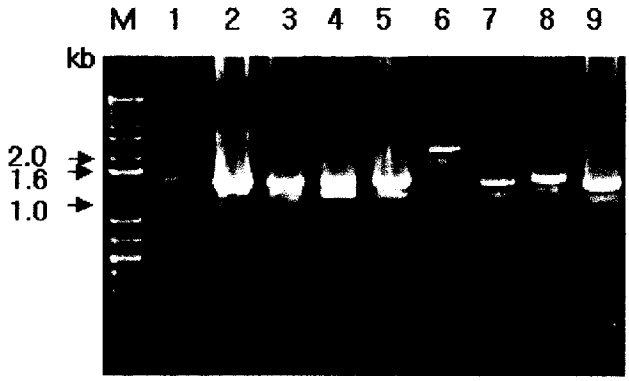


Fig. 13. PCR amplification of tRNA coding gene of 8 *Chlorella* virus isolates. M, 1kb size marker; Lane 1, PBCV-1; Lane 2, DJ-1; Lane 3, HS-1; Lane 4, HS-2; Lane 5, YK; Lane 6, SS-1; Lane 7, SS-2; Lane 8, KH-1; Lane 9, KH-2.

SS-1

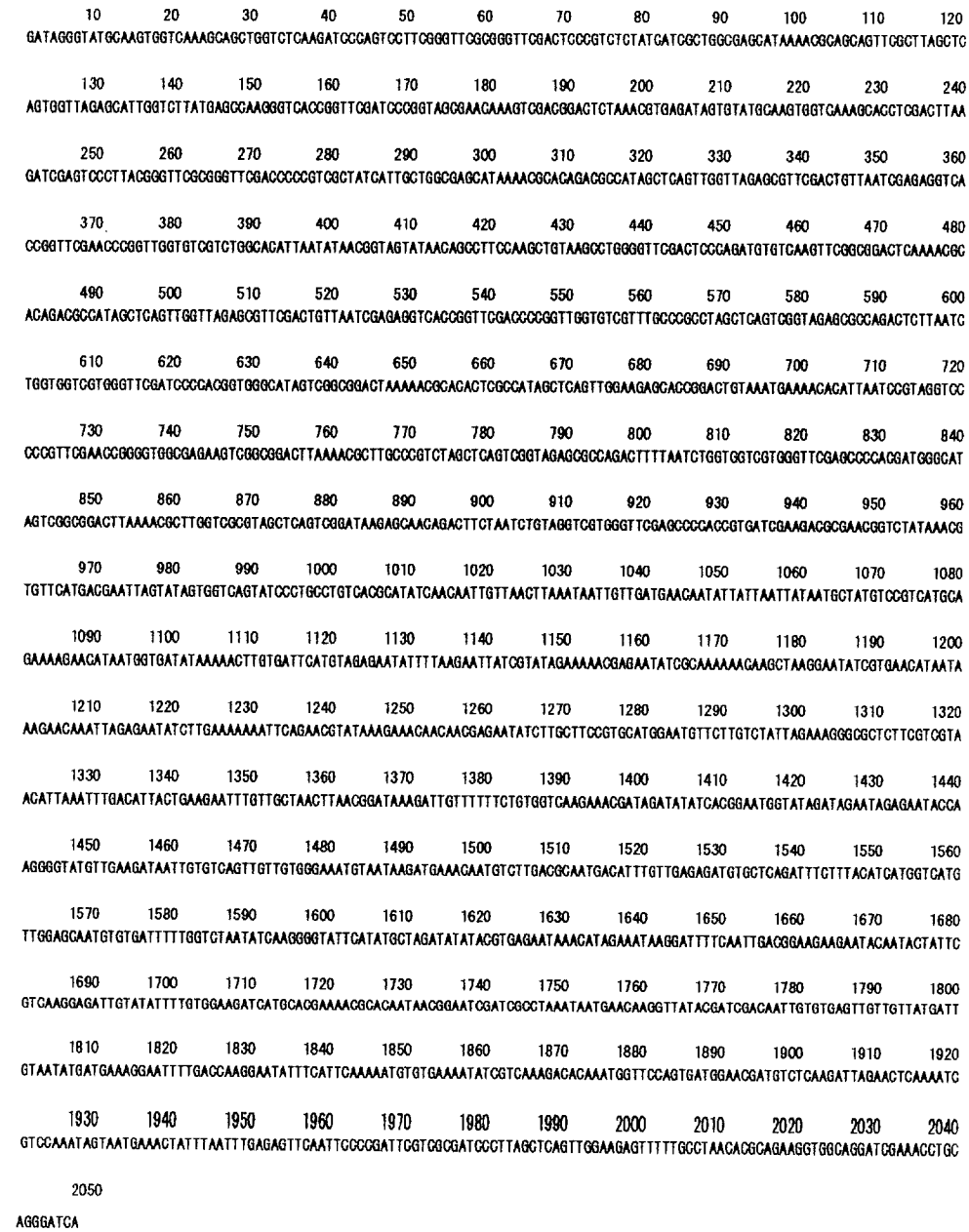


Fig. 14. Nucleotide sequences of tRNA region in genome of 8 virus isolates.

HS-1

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        10         20         30         40         50         60         70         80         90        100        110        120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCCAGTCCTTCGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTC

       130        140        150        160        170        180        190        200        210        220        230        240
AGTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAA

       250        260        270        280        290        300        310        320        330        340        350        360
GATCGAGTCCCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAACCGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCA

       370        380        390        400        410        420        430        440        450        460        470        480
CCGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCA

       490        500        510        520        530        540        550        560        570        580        590        600
TAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCT

       610        620        630        640        650        660        670        680        690        700        710        720
GGTGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCATAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCG

       730        740        750        760        770        780        790        800        810        820        830        840
GTTGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCA

       850        860        870        880        890        900        910        920        930        940        950        960
TAGTGTAGTGGTATCACAGTGGCCTCTGAATCCACTAACCGCAGTTCGAATCTGCGTGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACACAATAAT

       970        980        990       1000       1010       1020       1030       1040       1050       1060       1070       1080
CCGTAAGTCCCCCGTTCGAACCGGAGTGGCGAGAAGTCGGCGGACTCTAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCA

      1090       1100       1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
CGATGGGCATAGTCGGCGGACCTAAAACGCTTGGTCGCGTAGCTCAGTTGGATAGAGCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGT

      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300       1310       1320
CTATAAACGTGTTCATGGCGAATTAGTATAGTGGTCAGTATCCCTGCCTGTCACGCAGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGC

      1330       1340       1350       1360       1370
CTAACACGCAGAAGGTGGCAGGATCGAAACCTGCAGGGATCA
```

Fig. 14. - continued

YK-1

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        10        20        30        40        50        60        70        80        90       100       110       120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCAGTCCTTCGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTCA

       130       140       150       160       170       180       190       200       210       220       230       240
GTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTGGACTTAAG

       250       260       270       280       290       300       310       320       330       340       350       360
ATCGAGTCCCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCAC

       370       380       390       400       410       420       430       440       450       460       470       480
CGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCATA

       490       500       510       520       530       540       550       560       570       580       590       600
GACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGG

       610       620       630       640       650       660       670       680       690       700       710       720
TGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCATAGTGTAGTGGTATCACAGTGGCCTCTGAATCCACTAACCGCAGTTCGAATCTGCG

       730       740       750       760       770       780       790       800       810       820       830       840
TGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACAGAATAATCCGTAAGTCCCCCGTTCGAACCGGAGTGGCGAGAAGTCGGCGGACTCTAAACGCTT

       850       860       870       880       890       900       910       920       930       940       950       960
GCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACCTAAAACGCTTGGTCGCGTAGCTCAGTTGGATAGA

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGTCTATAAACGTGTTCATGGCGAATTAGTATAGTGGTCAGTATCCCTGCCTGTCACGC

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190
AGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTGGCAGGATCGAAACCTGCAGGGATCA
```

Fig. 14. - continued

DJ-1

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      10        20        30        40        50        60        70        80        90       100       110       120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCAGTCCTTCGGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTCA

     130       140       150       160       170       180       190       200       210       220       230       240
GTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAAG

     250       260       270       280       290       300       310       320       330       340       350       360
ATCGAGTCCCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCAC

     370       380       390       400       410       420       430       440       450       460       470       480
CGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCATA

     490       500       510       520       530       540       550       560       570       580       590       600
GACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGG
     610       620       630       640       650       660       670       680       690       700       710       720
TGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCATAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGT
     730       740       750       760       770       780       790       800       810       820       830       840
TGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGATCCCCACGGTGGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCATA
     850       860       870       880       890       900       910       920       930       940       950       960
GTGTAGTGGTATCACAGTGGACTCTGAATCCACTAACCGCAGTTCGAATCTGCGTGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACACAATAATCC
     970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GTAAGTCCCCCGTTCGAACCGGAGTGGCGAGAAGTCGGCGGACTCTAAAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCA
     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CGATGGGCATAGTCGGCGGACTCTAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACTTA
     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
AAACGCTTGGTCGCGTAGCTCAGTTGGATAGAGCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCGCACCGTGATCGAAGACGCGAACGGTCTATAAACGTGTTCATGGCGAATT
     1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
AGTATAGTGGTCAGTATCCCTGCCTGTCACGCAGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTGGCAGGAT
     1450      1460

CGAAACCTGCAGGGATCA
```

Fig. 14. - continued

KH-2

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      10        20        30        40        50        60        70        80        90       100       110       120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCAGTCCTTCGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTCA
     130       140       150       160       170       180       190       200       210       220       230       240
GTGGTTAGAGCATTGGTCTTATGAGCGAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAAG
     250       260       270       280       290       300       310       320       330       340       350       360
ATCGAGTCCCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCAC
     370       380       390       400       410       420       430       440       450       460       470       480
CGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCATA
     490       500       510       520       530       540       550       560       570       580       590       600
GACGCCTAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCCGAGAGGTCACCGGTTCGAACCCGGTTGGTAGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTG
     610       620       630       640       650       660       670       680       690       700       710       720
GTGGTCGTGGGTTCGATCCCCACGGTGGGCAAAGTCGGCGGACTCTAAACGCACAATTGGCTGCATAGTGTAGTGGTATCACAGTGGACTCTGAATCCACTAACCGCAGTTCGAATCTGC
     730       740       750       760       770       780       790       800       810       820       830       840
GTGTGGCCTCCACTCGCCATAGGTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACACAATAATCCGTAGGTCCCCCGTTCGAACCGGGGTGGCGAGAAGTCGGCGGACTAAAAACGCT
     850       860       870       880       890       900       910       920       930       940       950       960
TGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTTTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACTCTAAACGCTTGCCCGTCTAGCTCAGTCGGTAGA
     970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACTTAAAACGCTTGGTCGCGTAGCTCAGTTGGATAGAGCGACAGACTTCTAATCTGTAGG
     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGTCTATAAACGTGTTCATGGCGAATTAGTATAGTGGTCAGTATCCCTGCCTGTCACGCAGGAGACCGGGGTTCAATTCCCC
     1210      1220      1230      1240      1250      1260      1270      1280      1290
GATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTGGCAGGATCGAAACCTGCAGGGATCA
```

Fig. 14. – continued

HS-2

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      10        20        30        40        50        60        70        80        90       100       110       120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCGAGTCCTTCGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTC
     130       140       150       160       170       180       190       200       210       220       230       240
AGTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAA
     250       260       270       280       290       300       310       320       330       340       350       360
GATCGAGTCCCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCA
     370       380       390       400       410       420       430       440       450       460       470       480
CCGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCAT
     490       500       510       520       530       540       550       560       570       580       590       600
AGACGCCATAGCTCAGTTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCCCCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCT
     610       620       630       640       650       660       670       680       690       700       710       720
GGTGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCATAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCG
     730       740       750       760       770       780       790       800       810       820       830       840
GTTGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCA
     850       860       870       880       890       900       910       920       930       940       950       960
TAGTGTAGTGGTATCACAGTGGACTCTGAATCCACTAACCGCAGTTCGAATCTGCGTGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACACAATAAT
     970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
CCGTAGGTCCCCCGTTCGAACGGGAGTGGCGAGAAGTCGGCGGACTCTAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTTTTAATCTGGTGGTCGTGGGTTCGAGCCGGA
     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CGATGGGCATAGTCGGCGGACCTAAAACGCTTGGTCGCGTAGCTCAGTTGGATAGAGCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGT
     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CTATAAACGTGTTCATGGCGAATTAGTATAGTGGTCAGTATCCCTGCCTGTCACGCAGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGC
     1330      1340      1350      1360      1370
CTAACACGCAGAAGGTGGCAGGATCGAAACCTGCAGGGATCA
```

Fig. 14. – continued

KH-1

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        10        20        30        40        50        60        70        80        90       100       110       120
GATAGGGTATGCAAGTGGTCAAAGCAGCTGGTCTCAAGATCCAGTCCTTCGGGTTCGCGGGTTCGACTCCCGTCTCTATCATCGCTGGCGAGCATAAAACGCAACAGTTCGCTTAGCTGA
       130       140       150       160       170       180       190       200       210       220       230       240
GTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAAG
       250       260       270       280       290       300       310       320       330       340       350       360
ATCGAGTCCCTTAGGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCAC
       370       380       390       400       410       420       430       440       450       460       470       480
CGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCATA
       490       500       510       520       530       540       550       560       570       580       590       600
GACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGG
       610       620       630       640       650       660       670       680       690       700       710       720
TGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCATAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGT
       730       740       750       760       770       780       790       800       810       820       830       840
TGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCATA
       850       860       870       880       890       900       910       920       930       940       950       960
GTGTAGTGGTATCACAGTGGACTCTGAATCCACTAACCGCAGTTCGAATCTGCGTGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACACAATAATCC
       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GTAAGTCCCCCGTTCGAACCGGAGTGGCGAGAAGTCGGCGGACTCTAAAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCA
      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CGATGGGCATAGTCGGCGGACTCTAAACGCTTGCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACTTA
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
AAACGCTTGGTCGCGTAGCTCAGTTGGATAGAGCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGTCTATAAACGTGTTCATGGCGAATT
      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
AGTATAGTGGTCAGTATCCCTGCCTGTCACGCAGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTGGCAGGAT
      1450      1460

CGAAACCTGCAGGGATCA
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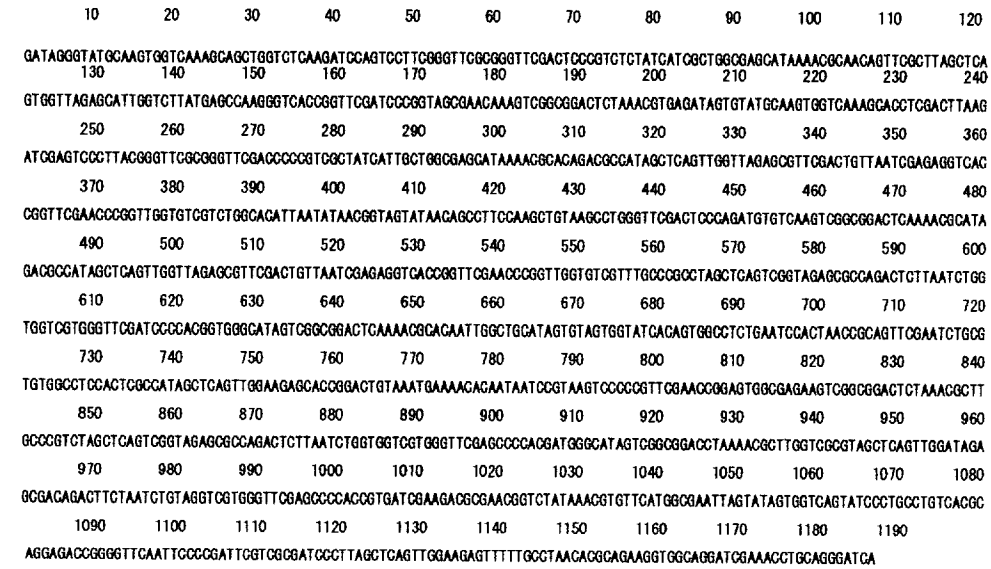
Fig. 14. - continued

SS-2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130 140 150 160 170 180 190 200 210 220 230 240
GTGGTTAGAGCATTGGTCTTATGAGCCAAGGGTCACCGGTTCGATCCCGGTAGCGAACAAAGTCGGCGGACTCTAAACGTGAGATAGTGTATGCAAGTGGTCAAAGCACCTCGACTTAAG
250 260 270 280 290 300 310 320 330 340 350 360
ATCGAGTCCGCTTACGGGTTCGCGGGTTCGACCCCCGTCGCTATCATTGCTGGCGAGCATAAAACGCACAGACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCAC
370 380 390 400 410 420 430 440 450 460 470 480
CGGTTCGAACCCGGTTGGTGTCGTCTGGCACATTAATATAACGGTAGTATAACAGCCTTCCAAGCTGTAAGCCTGGGTTCGACTCCCAGATGTGTCAAGTCGGCGGACTCAAAACGCATA
490 500 510 520 530 540 550 560 570 580 590 600
GACGCCATAGCTCAGTTGGTTAGAGCGTTCGACTGTTAATCGAGAGGTCACCGGTTCGAACCCGGTTGGTGTCGTTTGCCCGCCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGG
610 620 630 640 650 660 670 680 690 700 710 720
TGGTCGTGGGTTCGATCCCCACGGTGGGCATAGTCGGCGGACTCAAAACGCACAATTGGCTGCATAGTGTAGTGGTATCACAGTGGCCTCTGAATCCACTAACCGCAGTTCGAATCTGCG
730 740 750 760 770 780 790 800 810 820 830 840
TGTGGCCTCCACTCGCCATAGCTCAGTTGGAAGAGCACCGGACTGTAAATGAAAACAGAATAATCCGTAAGTCCCCCGTTCGAACCGGAGTGGCGAGAAGTCGGCGGACTCTAAACGCTT
850 860 870 880 890 900 910 920 930 940 950 960
GCCCGTCTAGCTCAGTCGGTAGAGCGCCAGACTCTTAATCTGGTGGTCGTGGGTTCGAGCCCCACGATGGGCATAGTCGGCGGACCTAAAACGCTTGGTCGCGTAGCTCAGTTGGATAGA
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
GCGACAGACTTCTAATCTGTAGGTCGTGGGTTCGAGCCCCACCGTGATCGAAGACGCGAACGGTCTATAAACGTGTTCATGGCGAATTAGTATAGTGGTCAGTATCCCTGCCTGTCACGC
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190
AGGAGACCGGGGTTCAATTCCCCGATTCGTCGCGATCCCTTAGCTCAGTTGGAAGAGTTTTTGCCTAACACGCAGAAGGTGGCAGGATCGAAACCTGCAGGGATCA

Fig. 14.

regions showed that there are 11 to 16 tRNA genes in this region that are separated with small spacers (Fig. 15). Genes coding tRNA have been identified in the genome of murine gammaherpesvirus 68 (Bowden *et al.*, 1997). The presence of tRNA genes in a virus genome is unusual considering the dependence of translation processes of viruses on the host translational machinery. However, this can be explained by the bias in the codon usage of the *Chlorella* virus and it host (Van Etten *et al.*, 1991), which has been predicted from the difference in the G+C contents of the two organisms; 40% G+C in the PBCV-1 DNA contrast to 67% G+C content of host *Chlorella* NC64A (Van Etten *et al.*, 1985).

One interesting feature in the tRNA regions of the 8 isolates is the presence of a 1039bp spacer between (UCU) tRNA and (AAC) tRNA of the SS-1 isolate. This spacer comprises an open reading frame of 885bp and encodes a putative protein of 294 amino acids with a molecular weight of about 29 KDa. A sequence of the spacer and the published PBCV-1 genome sequence showed 51% amino acid sequence similarity to PBCV-1 ORF a478L (Fig. 16). It has been suggested that rearrangements of *Chlorella* virus genomes during replication in natural environment could occur dynamically and frequently (Nishida *et al.*, 1999). For example, a total of 29 of the PBCV-1 ORFs resemble 1 or more other PBCV-1 ORFs, suggesting that they might be either gene families or derived by gene duplication (Van Etten *et al.*, 1999).

Although the sequence homology between the identified spacer and the PBCV-1 a478L ORF was low, possible duplication or translocation of a478L ORF in SS-1 genome was investigated Southern blot analysis (Fig. 17). Hybridization of the restriction enzyme digested genomic DNA of both PBCV-1 and SS-1 with PBCV-1 tRNA probes (Fig. 17A) resulted in one hybridizing band with same pattern, which indicated the presence of only

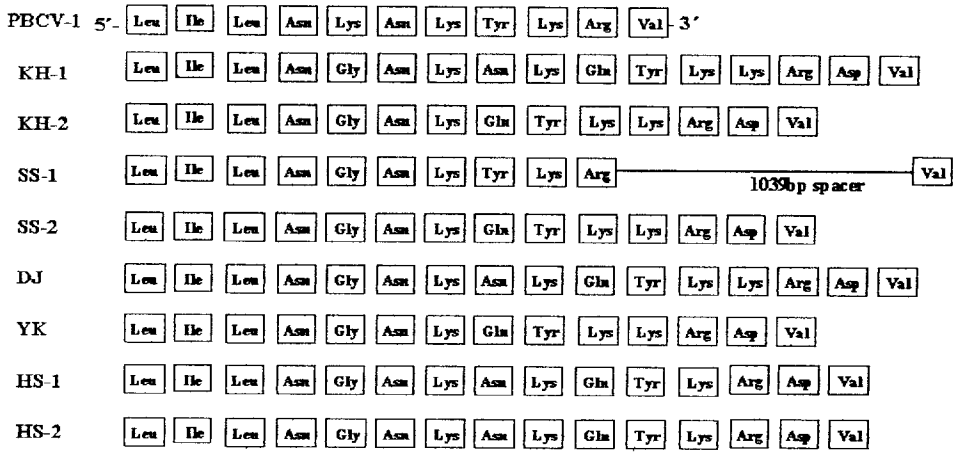

Fig. 15. Structure of tRNA coding regions of *Chlorella* viruses isolated in Korea. The tRNA regions of 8 selected isolates were amplified and cloned. They contain 14-16 tRNA genes in 1.2- 2kb region except the SS-1 isolate that has 1039bp.

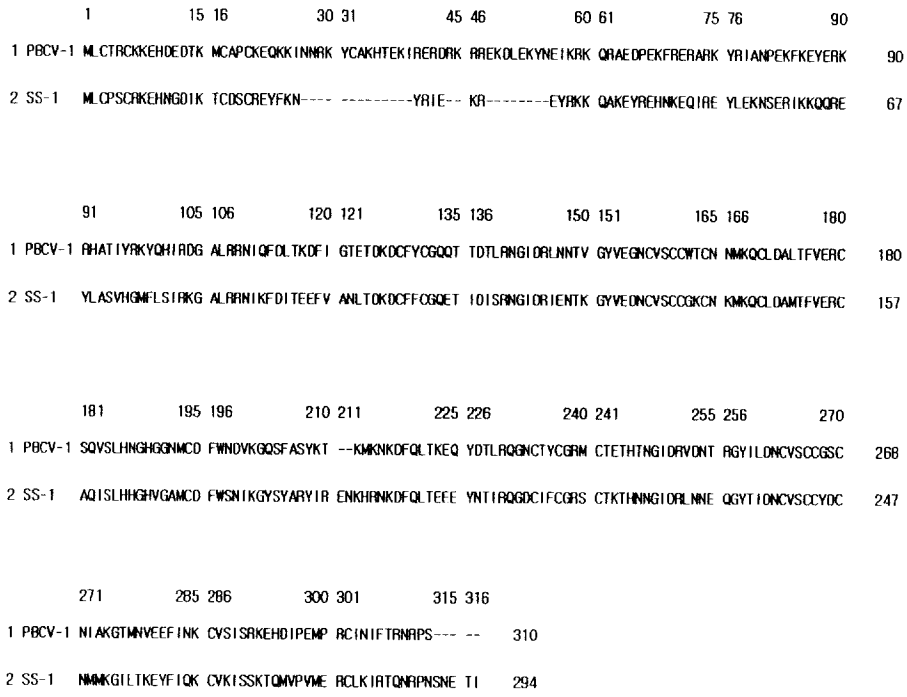

Fig. 16. Comparison of amino acid sequence of the SS-1 spacer and the PBCV-1 a478L gene.

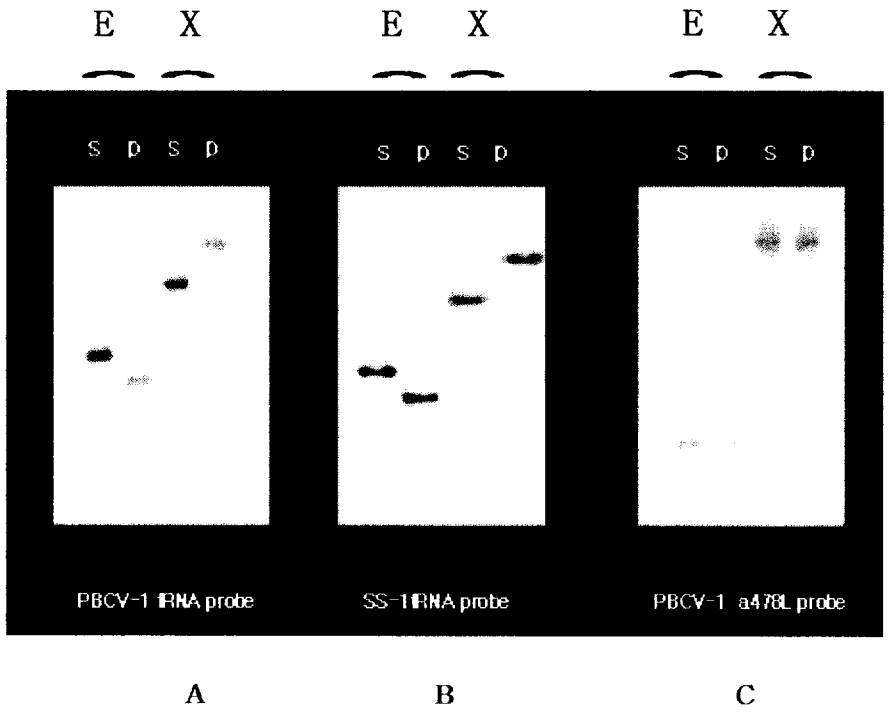

Fig. 17. Southern blot analysis of the PBCV-1 (P) and SS-1 (S) digested with EcoR I (E) and Xho I(X). The used probes are shown at the bottom and their locations are shown in Figure 5.

one tRNA region in the two viruses. Hybridization of same DNA with SS-1 tRNA probe (Fig. 17B) resulted in same result as with the PBCV-1 tRNA probe region. Hybridization of the same DNA with a PBCV-1 a478L probes resulted in only one hybridizing band from both viruses but different location from the tRNA region (Fig. 17C). This result indicted that there is a gene corresponding to the PBCV-1 a478L ORF on both of the PBCV-1 and SS-1 genome and that this gene presents at a different location from the tRNA coding region. Therefore, the low sequence homology between the SS-1 tRNA spacer and PBCV-1 a478L ORF, the presence of a DNA fragment corresponding to the PBCV-1 a478L ORF in both of the viruses indicated that the ORF in the SS-1 tRNA spacer is a novel gene that does not present in the PBCV-1 genome.

Analysis of the tRNA Genes and their Putative Promoter Regions

All of the tRNA genes encoded by the *Chlorella* viruses contain putative tRNAs that has features common to most tRNA: a 7-bp acceptor stem, an internal promoter consisting of a conserved YGG sequence in the D loop and conserved size and consensus sequences in the TΨC loop, a 5-bp anticodon stem, and a 7-b anticodon loop containing the consensus YU- anticodon-R (Geiduschek and Tocchini-Valentini, 1988; Dirheimer *et al.*, 1995). In the tRNA genes of 8 Korean *Chlorella* viruses, a 14-b intron-like sequence resides between the first and second nucleotide 3′ to the anticodon of tyrosine tRNA (GUA) (Fig. 18).

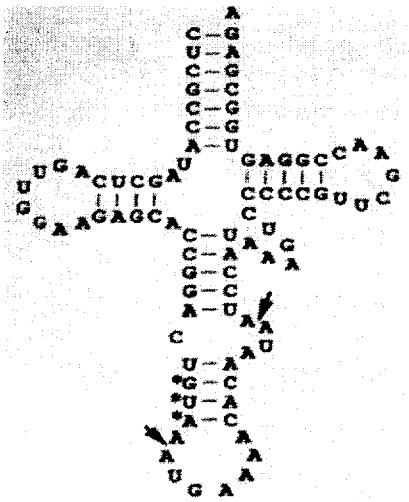

Fig. 18. Secondary structure model for Chlorella viral Tyr tRNA precursors containing insertion. Arrows indicate the sites where the tRNA-splicing machinery should cut and region the pre-tRNA to form the mature tRNA molecule.

It is known that eukaryotic RNAs are transcribed by conserved polymerase III promoter elements called A box and B box that present inside the tRNA coding region as shown in Figure. 19. However, it has been demonstrated that the viral tRNAs of Japan *Chlorella* virus K2 is cotranscribed as a large precursor of about 1.0kb that is precisely processed into individual mature tRNA species (Nishda *et al.*, 1999). In addition, the upstream sequence of the PBCV-1 tRNA gene cluster is a typical promoter for *Chlorella* viral genes that is composed of 65bp with 75% A+T (Schuster *et al.*, 1990). These results suggest that upstream of tRNA gene cluster might contain a major acting promoter. In order to find whether the *Chlorella* viruses isolated in Korea contain a common sequence similar to the putative promoter, the upstream regions of these regions were sequenced by cut and walk method with a primer (ll-Cprimer) running toward the 5′ from the inside of the tRNA coding region (Fig. 4) and the results are shown in Figure 20 and 21.

One interesting feature in the sequence analysis of this region is the repeat of conserved sequences. In case of the PBCV-1, a conserved motif of 27 bp is repeated twice and they are separated by 96 pb (sequnce A in Fig. 20). One Korean isolate, the SS-1, has same putative promoter structure as the PBCV-1. However, another conserved motif of 89 bp (sequnce B in Fig. 20) was identified in the putative promoter region of the SS-2, KH-1 and KH-2 isolates. In addition to the presence of the second conserved motif in this region, the arrangement of these conserved motifs was vary among these viruses. The PBCV-1 and SS-1 have A-A arrangement, the KH-1 and KH-2 isolates have B-A-B-A arrangement and the SS-2 isolate has A-B-A-B-A arrangement (Fig. 21).

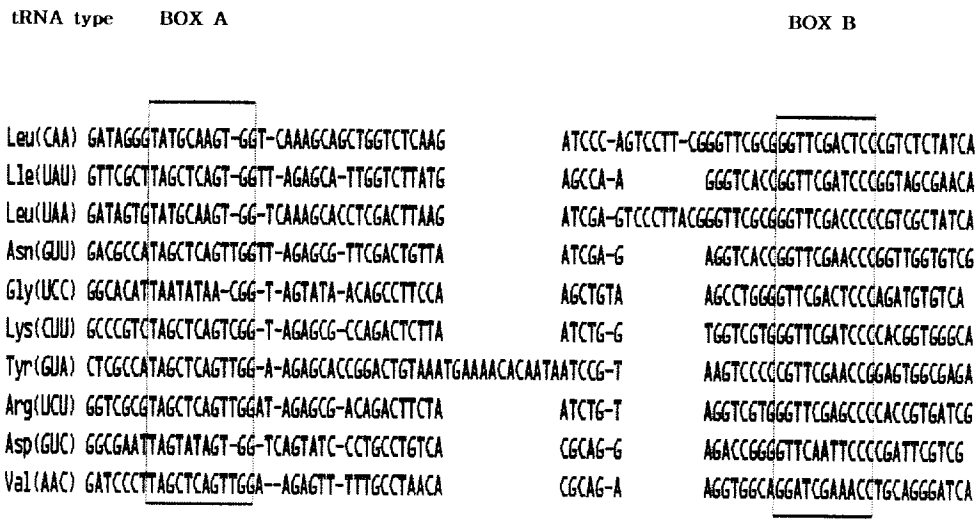

Fig. 19. Alignment of 10 tRNA sequences encoded on the HS-2 genome. The conserved A and B boxes comparising the RNA polymeraseIII promoter are boxed.

Although it was shown that the tRNAs of *Chlorella* virus are trasncribed as a cotranscript and the 5′ upstream was suggested as possible promoter region, no detail analysis of this region has not been performed. However, the multiple presence of conserved A+T rich motifs in this region is of noteworthy, and further analyses of this regions as promoters of the tRNA are necessary.

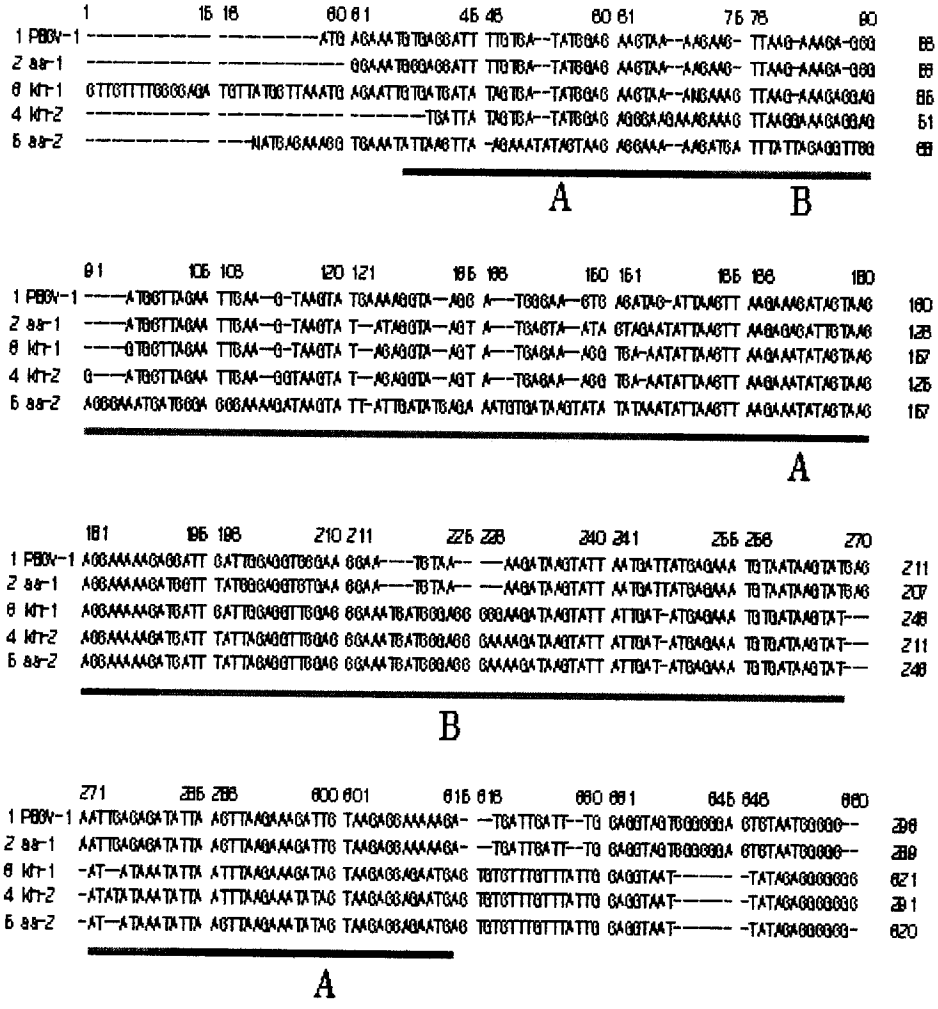

Fig. 20. Sequence comparison of the putative tRNA gene promoters. Repeated sequences in the SS-2 isolate are indicated as A and B.

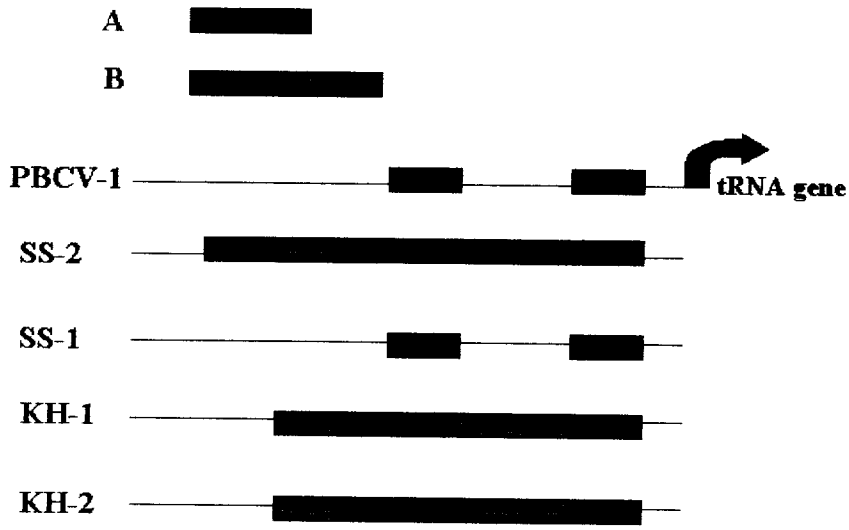

Fig. 21. Arrangement of the repeated sequences in the putative tRNA gene promoters of different *Chlorella* viruses.

국문 초록

미세조류에 발생하는 바이러스는 수중 생태계에서의 그들의 영향력과 최근 이들 바이러스들로부터 많은 유용한 유전자들의 발견으로 관심의 대상이 되고 있으나 이에 대한 국내에서의 연구는 이루어지지 않았었다. 본 연구를 통하여 국내에서는 처음으로 배양된 클로렐라 strain NC64A 세포의 융해를 일으키는 바이러스 23 균주가 국내의 10 지역으로부터 수집한 담수에서 분리되어졌다.

이들 분리된 바이러스들은 300 - 350kbp에 달하는 genome을 가지고 있으며, 분리된 genomic DNA는 10개의 restriction enzyme으로 처리한 결과 이들 바이러스마다 서로 다른 DNA fragment 배열을 보여주었다. 분리된 바이러스 중 SS-1은 Hind III, Pvu II, Alu I 그리고 HaeIII 제한효소로 절단하였을 때 genomic DNA가 잘리지 않은 것으로 보아 AGCT 또는 GC 염기서열에 메틸화되어 있음을 제시하였다.

분리된 모든 바이러스의 외피 단백질(major capsid protein, MCP)은 미국에서 분리된 클로렐라 바이러스 EPA-1의 항체에 반응하지 않았으며, 클로렐라 바이러스 PBCV-1 항체에는 5종의 바이러스가 반응하였다. 3종의 바이러스의 MCP의 염기서열을 cut and walk 방법으로 분석한 결과 이들 바이러스 사이에 90% 이상의 상동성을 확인하였다.

분리된 바이러스 중 8 균주의 tRNA 유전자를 PCR에 의해 증폭하고 클로닝한 후 그 염기서열을 분석하였다. 그 결과, 이들 바이러스 중 tRNA 유전자 안에 1039bp의 삽입 유전자를 가지는 SS-1 바이러스를 제외하고는 1.2 - 2kb 크기의 DNA에 14-16개의 tRNA 유전자를 가지고 있음을 확인하였다. 이 SS-1 바이러스의 삽입 유전자는 294개의 아미노산으로 구성되어 있었고, 클로렐라 바이러스 PBCV-1의 A478L 유전자와 51%의 상동성을 가지지만 Southern blot을 이용한 분석결과 PBCV-1에는 존재하지 않는 새로운 유전자인 것으로 확인되었다. 이들 클로렐라 바이러스 tRNA 유전자의 promoter로 여겨지는 부분에 대한 클로닝 및 염기서열 분석 결과 A+T 염기의 비율이 높은 반복된 염기서열이 발견되었으며 이들 반복된 염기서열의 종류 및 배열은 바이러스 균주에 따라 다양하였다.

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실험실 생활에 잘 적응할 수 있게 도와준 대현 선배, 은영이 언니와 정임이 언니, 나의 동기 종오와 현향이, 바이러스 실험실의 후배 지연, 수미, 선미, 재경, 경호, 경규, 혜진이에게 고마움을 전하며, 대학원 과정동안 함께 했던 여러 선배님과 동기들, 유전방의 이쁜이 혜은이 언니, 사랑하는 나의 친구들에게 고마움을 전합니다.

언제나 사랑과 격려를 아낌없이 주신 나의 사랑하는 부모님과 외국에서 힘든 생활을 하면서도 항상 걱정해준 하나뿐인 나의 현욱 오빠에게 이 작은 결실을 바칩니다. 마지막으로, 힘들고 지칠 때 정신적으로나 학문적으로 도움을 준 나의 연인 철호 오빠에게 고마움을 전합니다.

References

Becker, B., D.E. Lesemann, and W. Reisser. (1993). Ultrastructural studies on a chlorella virus from Germany. Arch. Virol. 130:145-155.

Bornemann, C., and H. Follmann. (1997). Large-scale production and plaque titration of European chlorella viruses. J. Virol. Methods 67:119-125.

Bowden, R. J., Simas, J. P., Davis, A. J., and Efstathiou, S. (1997). Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency. *J. Gen. Virol.* 78, 1675-1687.

DeAngelis, P.L., W. Jing, M.V. Graves, D.E. Burbank, and J.L. Van Etten. (1997). Hyaluronan synthase of chlorella virus PBCV-1. Science 278:1800-1803.

Driheimer, G., Keith, G., Dumas, P., and Westhof, E. (1995). Primary, secondary, and tertiary structures of tRNAs. In " tRNA" (D. R.Soll, and U. Raj Bhandary, Eds),pp.93-126. American Society for Microbiology, Washington, DC.

Garvish, J.F., and R.S. Lloyd. (1999). The catalytic mechanism of a pyrimidine dimer specific glycosylase (pdg)/abasic lyase, chlorella virus-pdg. J. Biol. Chem. 274:9786 9794.

Geiduscek, E.P., and Tocchini- Valentini, G.P. (1988). Transcription by RNA polymerase III. Annu. Rev. Biochem. 57, 873-914.

Girton, L., and J.L. Van Etten. (1987). Restriction site map of the Chlorella virus PBCV-1 genome. Plant Mol. Biol. 9:247-257.

Grabherr, R., P. Strasser, and J.L. Van Etten. (1992). The DNA polymerase genes from chlorella viruses PBCV-1 and NY-2A contain an intron with nuclear splicing sequences. Virology 188:721-731.

Graves, M.V., and R.H. Meints. (1992). Characterization of the major capsid protein and cloning of its gene from algal virus PBCV-1. Virology 188:198-207.

Graves, M.V., Bernadt, C.T., Ronald Cerny, and Van Etten, J. L. (2001). Molecular and genetic evidence for a virus-encoded glycosyltransferase involded in protein glycosylation. Virology 285, 332-345.

Graves, M.V., D.E. Burbank, R. Roth, J. Heuser, P.L. DeAngelis, and J.L. Van Etten. (1999). Hyaluronan synthesis in virus PBCV-1 infected chlorella-like green algae. Virology 257:15-23.

Higashiyama, T., and T. Yamada. (1991). Electrophoretic karyotyping and chromosomal gene mapping of Chlorella. Nucl. Acids Res. 19:6191-6195.

Hiramatsu, S., M. Fujie, S. Usami, K. Sakai, and T. Yamada. (2000). Two catalytic domains of chlorella virus CVK2 chitinase. J. Biosci. Bioeng. 89:252-257

Hiramatsu, S., M. Ishihara, M. Fujle, S. Usami, and T. Yamada. (1999). Expression of a chitinase gene and lysis of the host cell wall during chlorella virus CVK2 infection. Virology 260:308-315.

Kapaun, E., and W. Reisser. (1995). A chitin-like glycan in the cell wall of a Chlorella sp. (Chlorococcales, Chlorophyceae). Planta 197:577-582.

Kapaun, E., E. Loos, and W. Reisser. (1992). Cell wall composition of virus-sensitive symbiotic Chlorella species. Phytochemistry 31:3103-3104.

Kutish, G.F., Y. Li, Z. Lu, M. Furuta, D.L. Rock, and J.L. Van Etten. (1996). Analysis of 76 kb of the chlorella virus PBCV-1 330-kb genome: Map positions 182 to 258. Virology 223:303-317.

Kvitko, K., and B.V. Gromov. (1984). New finding of a titrable infectious zoochlorella virus. Dokl. Akad. Nauk. SSSR 279:998-999.

Lavrukhin, O.V., J.M. Fortune, T.G. Wood, D.E. Burbank, J.L. Van Etten, N. Osheroff, and R.S. Lloyd. (2000). Topoisomerase II from chlorella virus PBCV-1. Characterization of the smallest known type II topoisomerase. J. Biol. Chem. 275;6915-6921.

Li, Y., Z. Lu, D.E. Burbank, G.F. Kutish, D.L. Rock, and J.L. Van Etten. (1995). Analysis of 43 kb of the chlorella virus PBCV-1 330 kb genome: Map position 45 to 88. Virology 212:134-150.

Li, Y., Z. Lu, L. Sun, S. Ropp, G.F. Kutish, D.L. Rock, and J.L. Van Etten. (1997). Analysis of 74 kb of DNA located at the right end of the 330-kb

chlorella virus PBCV-1 genome. Virology 237:360-377.

Lu, Z., Y. Li, Q. Que, G.F. Kutish, D.L. Rock, and J.L. Van Etten. (1996). Analysis of 94 kb of the chlorella virus PBCV-1 330 kb genome: Map position 88 to 182. Virology 216:102-123.

Lu, Z., Y. Li, Y. Zhang, G.F. Kutish, D.L. Rock, and J.L. Van Etten. (1995). Analysis of 45 kb of DNA located at the left end of the chlorella virus PBCV-1 genome. Virology 206:339-352.

Meints, R.H., D.E. Burbank, J.L. Van Etten, and D.T.A. Lamport. (1988). Properties of the Chlorella receptor for the virus PBCV-1. Virology 164:15-21.

Meints, R.H., K. Lee, and J.L. Van Etten. (1986). Assembly site of the virus PBCV-1 in a Chlorella-like green alga: Ultrastructural studies. Virology 154:240-245.

Mitra, A., and D.W. Higgins. (1994). The chlorella virus adenine methyltransferase gene promoter is a strong promoter in plants. Plant Mol. Biol. 26:85-93.

Mitra, A., D.W. Higgins, and N.J. Rohe. (1994). A chlorella virus gene promoter functions as a strong promoter both in plants and bacteria. Biochem. Biophys. Res. Comm. 204:187-194.

Narva, K.E., D.L. Wendell, M.P. Skrdla, and J.L. Van Etten. (1987). Molecular cloning and characterization of the gene encoding the DNA methyltransferase, M.CviBIII, from Chlorella virus NC-1A. Nucl. Acids Res. 15:9807-9823.

Nelson, M., D.E. Burbank, and J.L. Van Etten. (1998). Chlorella viruses encode multiple DNA methyltransferases. Biol. Chem. 379:423-428.

Nishida, K., S. Suzuki, Y. Kimura, N. Nomura, M. Fujie, and T. Yamada. (1998). Group I introns found in chlorella viruses: Biological implications. Virology 242:319-326.

Nishida, K., T. Kawasaki, M. Fujie, S. Usami, and T. Yamada. (1999). Aminoacylation of tRNAs encoded by chlorella virus CVK2. Virology 263:220-229.

Nishida, K., Y. Kimura, T. Kawasaki, M. Fujie, and T. Yamada. (1999). Genetic variation of chlorella viruses: Variable regions localized on the CVK2 genomic DNA. Virology 255:376-384.

Plugge, B., S. Gazzarrini, M. Nelson, R. Cerana, J.L. Van Etten, C. Derst, D. DiFrancesco, A. Moroni, and G. Thiel. (2000). A potassium channel protein encoded by chlorella virus PBCV-1. Science 287:1641-1644.

Que, A., Y. Zhang, M. Nelson, S. Ropp, D.E. Burbank, and J.L. Van Etten. (1997). Chlorella virus SC-1A encodes at least six DNA methyltransferases. Gene 190:237-244.

Que, Q., Y. Li, Y. Wang, L.C. Lane, W.G. Chaney, and J.L. Van Etten. (1994). Protein glycosylation and myristylation in chlorella virus PBCV-1 and its antigenic variants. Virology 203:320-327.

Schmidt, O., Mao, J., Ogden, R., Beckmann, J., Sakano, H., Abelson, J., and Soll, D. (1980). Direct tRNA precursors in yeast. Nature 287,750-752.

Schuster, A.M., D.E. Burbank, B. Meister, M.P. Skrdla, R.H. Meints, S. Hattman, D. Swinton, and J.L. Van Etten. (1986). Characterization of viruses infecting a eukaryotic Chlorella-like green alga. Virology 150:170-177.

Schuster, A.M., J.A. Waddle, K. Korth, and R.H. Meints. (1990). The chloroplast genome of an exsymbiotic Chlorella-like green alga. Plant Mol. Biol. 14:859-862.

Schuster, A.M., L. Girton, D.E. Burbank, and J.L. Van Etten. (1986). Infection of a Chlorella-like alga with the virus PBCV-1: Transcriptional studies. Virology 148:181-189.

Schuster, A.M., M. Graves, K. Korth, M. Ziegelbein, J. Brumbaugh, D. Grone, and R.H. Meints. (1990). Transcription and sequence studies of a 4.3 kbp fragment from a dsDNA eukaryotic algal virus. Virology 176:515-523.

Skowron, P.M., N. Swaminathan, K. McMaster, D. George, J.L. Van Etten, and D.A. Mead. (1995). Cloning and applications of the two/three-base restriction endonuclease R.CviJI from IL-3A virus-infected chlorella. Gene 157:37-41.

Sriskanda, V., and S. Shuman. (1998). Chlorella virus DNA ligase: nick recognition and mutational analysis. Nucl. Acids Res. 26:525-531.

Sun, L., B. Adams, J.R. Gurnon, Y. Ye, and J.L. Van Etten. (1999). Characterization of two chitinase genes and one chitosanase gene encoded by chlorella virus PBCV-1. Virology 263:376-387.

Sun, L., J.R. Gurnon, B.J. Adams, M.V. Graves, and J.L. Van Etten. (2000). Characterization of a beta-1,3-glucanase encoded by chlorella virus PBCV-1. Virology 276:27-36.

Van Etten, J. L.(2000). phycodnaviridae. *In* "Virus Taxonomy"(M. H. V. Van Regenmortel, C. M. Fauaquet, d. h. l. bISHOP, et al. Eds.), pp.183-193. Academic Press, San Diego.

Van Etten, J.L. (1994). Phyconaviridae - Chlorella viruses. In: Encyclopedia of Virology. R.G. Webster and A. Granoff (eds). Saunders Scientific Publ., London. pp. 35-40.

Van Etten, J.L. (1995). Minireview: Giant chlorella viruses. Mol. Cells. 5:99-106.

Van Etten, J.L. (2000). Genus Chlorovirus (Phycodnaviridae). In: The Springer Index of Viruses, C.A. Tidona and G. Darai (eds). Springer-Verlag, Berlin (in press)

Van Etten, J.L. (2000). Phycodnaviridae. Virus Taxonomy, Classification and

Nomenclature of Viruses, Seventh Report. M.H.V. Van Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carsten, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, R.B. Wickner (eds). pp. 183-193. Academic Press, San Diego, CA.

Van Etten, J.L. and R.H. Meints. (1999). Giant viruses infecting algae. Ann. Rev. Microbiol. 53:447-494

Van Etten, J.L., A.M. Schuster, L. Girton, D.E. Burbank, D. Swinton, and S. Hattman. (1985). DNA methylation of viruses infecting a eukaryotic Chlorella-like alga. Nucl. Acids Res. 13:3471-3478.

Van Etten, J.L., C.H. Van Etten, J.K. Johnson, and D.E. Burbank. (1985). A survey for viruses from fresh water that infect a eukaryotic Chlorella-like green alga. Appl. Environ. Microbiol. 49:1326-1328.

Van Etten, J.L., D.E. Burbank, A.M. Schuster, and R.H. Meints. (1985). Lytic viruses infecting a Chlorella-like alga. Virology 140:135-143.

Van Etten, J.L., D.E. Burbank, and R.H. Meints. (1986). Replication of the algal virus PBCV-1 in UV-irradiated Chlorella. Intervirology 26:115-120.

Van Etten, J.L., D.E. Burbank, D. Kuczmarski, and R.H. Meints. (1983). Virus infection of culturable Chlorella-like algae and development of a plaque assay. Science 219:994-996.

Van Etten, J.L., D.E. Burbank, J. Joshi, and R.H. Meints. (1984). DNA synthesis in a Chlorella-like alga following infection with the virus PBCV-1.

Virology 134:443-449.

Van Etten, J.L., D.E. Burbank, Y. Xia, and R.H. Meints. (1983). Growth cycle of a virus, PBCV-1, that infects Chlorella-like algae. Virology 126:117-125.

Van Etten, J.L., L.C. Lane, and R.H. Meints. (1991). Viruses and virus-like particles of eukaryotic algae. Microbiol. Rev. 55:586-620.

Van Etten, J.L., R.H. Meints, D. Kuczmarski, D. E. Burbank, and K. Lee. (1982). Viruses of symbiotic Chlorella-like algae isolated from Paramecium bursaria and Hydra viridis. Proc. Natl. Acad. Sci. USA 79:3867-3871.

Van Etten, J.L., R.H. Meints, D.E. Burbank, D. Kuczmarski, D.A. Cuppels, and L.C. Lane. (1981). Isolation and characterization of a virus from the intracellular green alga symbiotic with Hydra viridis. Virology 113:704-711.

Van Etten, JL. (1999). Algal viruses (Phycodnaviridae). In: Encyclopedia of Virology, 2nd edition. R.G. Webster and A. Granoff (eds). Academic Press, London, pp. 44-50.

Wang, I-N., Y. Li, Q. Que., M. Bhattacharya, L.C. Lane, W.G. Chaney and J.L. Van Etten. (1993). Evidence for virus-encoded glycosylation specificity. Proc. Natl. Acad. Sci. USA 90:3840-3844.

Xia, Y., and J.L. Van Etten. (1986). DNA methyltransferase induced by PBCV-1 virus infection of a Chlorella-like green alga. Mol. Cell. Biol. 6:1440-1445.

Xu, M., M.P. Kladde, J.L. Van Etten, and R.T. Simpson. (1998). Cloning, characterization and expression of the gene coding for a cytosine-5-DNA methyltransferase recognizing GpC sites. Nucl. Acids Res. 26:3961-3966.

Yamada, T., S. Hiramatsu, P. Songsri, and M. Fujie. (1997). Alternative expression of a chitosanase gene produces two different proteins in cells infected with chlorella virus CVK2. Virology 230:361-368.

Yamada, T., T. Fukuda, K. Tamura, S. Furukawa, and P. Songsri. (1993). Expression of the gene encoding a translational elongation factor 3 homolog of chlorella virus CVK2. Virology 197:742-750.

Zang B, Tao T, Wilson GG, Blumenthal RM (1993). The M · AluI DNA-(cytosine C5)-methyltransferase has an unusually large, partially dispensable, variable region. Nucleic Acids Res 21:905-911

Zhang, Y., D.E. Burbank, and J.L. Van Etten. (1988). Chlorella viruses isolated in China. Appl. Environ. Microbiol. 54:2170-2173.

Zhang, Y., M. Nelson, J.W. Nietfeldt, D.E. Burbank, and J.L. Van Etten. (1992). Characterization of chlorella virus PBCV-1 CviAII restriction and modification system. Nucl. Acids Res. 20:5351-5356.

Zhang, Y., Nelson, M., Nietfeldt, J., Xia, Y., Burbank, D.E., Ropp, S., and Van Etten, J.L. (1998). Chlorella virus NY-2A encodes at least twelve DNA endonuclease/methyltransferase genes. Virology 240:366-375.