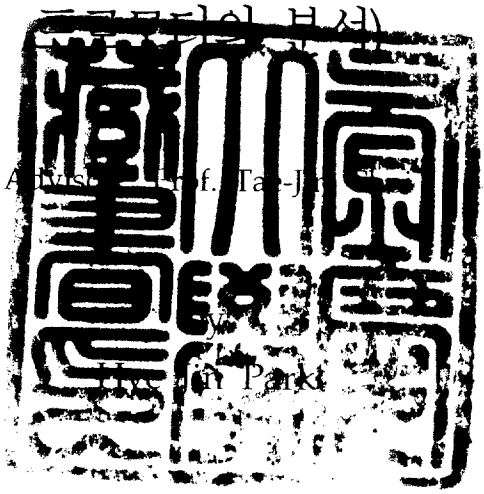
Characterization of *Chlorella* virus tRNA gene cluster promoters for the development of microalgae transformation vectors

(미세조류 형질전환 벡터 개발을 위한 클로렐라 바이러스의 tRNA 유전자 집단 프로모터의 분석)



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Characterization of *Chlorella* virus tRNA gene cluster promoters for the development of microalgae transformation vectors

A dissertation

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Characterization of *Chlorella* virus tRNA gene cluster promoters for the development of microalgae transformation vectors

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Abstract

Chlorella is a very attractive organism as a new bioreactor for foreign protein overexpression because it is an eukaryote that can be inexpensively cultured in large scale, grow relatively fast and can synthesize complex proteins that require post-translational modification to be biologically active. Recently, the flounder growth hormone (fGH) gene was stably introduced and expressed in a functional form in transformed *Chlorella ellipsoidea* with a modified plant transformation vector. In an attempt to improve the expression system, we replaced the CaMV 35S promoter with promoters from the tRNA gene clusters of chlorella viruses. Chlorella viruses SS-1, SS-2, KH-1 and KH-2 isolated from freshwater in Korea are similar to the *Paramecium bursaria* chlorella virus (PBCV-1), the prototype of the *Phycodnaviridae* family, and encode 11, 14, 16 and 14 tRNA genes, respectively. Analysis of the promoter region of these tRNA gene clusters of these viruses revealed repeated and conserved DNA motifs called Box A of 33 nucleotides and Boxes B or C of 84 nucleotides, which are arranged as AA in

SS-1, CABA in KH-1, KH-2 and ABABA in SS-2. These promoters and their derivatives of serial box deletion were fused to the green fluorescence protein (GFP) gene in chlorella transformation vector, and introduced in *C. vulgaris*. The ABABA-GFP fusion construct showed the highest GFP expression followed by the CABA and AA-GFP fusion, which were stronger than the most commonly used cauliflower mosaic virus (CaMV) 35S promoter. Serial deletion of the conserved boxes revealed that the activity decreased as the number of deletion increased and that minimum sequence with ABA box is required for the promoter activity similar to that of 35S promoter.

Introduction

Molecular farming is the growing of transgenic plants to produce pharmaceutical or industrial compounds instead of food or fiber. The possibilities range from the manufacture of medical products, such as pharmaceuticals and vaccines, to the production of products like biodegradable plastics and industrial chemicals. Since 1980s, increasing number of reports have shown that it is possible to express and purify proteins of commercial interest from transformed plant tissues (Hiatt *et al.*, 1989; Salmon *et al.*, 1998). According to the development of plant transformation technique, it is possible now to produce valuable proteins in industrial scale (Zhong *et al.*, 1999). Although it is not used commercially yet, interest and investment in molecular farming with plants is accelerating rapidly.

From a commercial point of view, transgenic plants have the highest safety and cost-effective production system for heterologous protein in quantities. It is an eukaryote that can synthesize complex proteins that require post-translational modification. However, it is difficult to purify the expressed protein since plants have hard structure such as cell wall. In addition, the release of transgenic plants into the environment has raised concerns over the spread of transgenes by cross-pollination to related wild species.

In contrast to plants, microalgae show several important advantages as large-scale bioreactor for proteins production. It can be inexpensively cultured in large scale, grow relatively fast. Also, it would be easy to purify the expressed protein because microalgae have simpler structure than complex plants. Since 1990s, there have been many attempts for chlorella transformation to express foreign proteins (Javis and Brown, 1991; Dowson *et al.*, 1997; Hawkins and Nakamura, 1999). In 1999, human growth hormone was expressed in transformed chlorella, but the expression was transient and the biological activity of the expressed protein

was not tested (Hawkins and Nakamura, 1999). In 2001, Chen *et al.* produced rabbit neutrophil peptide-1 gene in *C. ellipsoidea* by electroporation. Recently, the flounder growth hormone (fGH) gene was stably introduced and expressed in a functional form in transformed C. *ellipsoidea* by utilizing a modified plant transformation vector that contains cauliflower mosaic virus (CaMV) 35S promoter and the phleomycin resistant *Sh ble* gene as a selection marker (Kim *et al.*, 2002).

When the plants are used bioreactor, the accumulation of the highest level of the proteins is desirable. Hence, for production purposes, generally strong and constitutive promoters such as the CaMV 35S promoter have been used. However, the CaMV 35S promoter expression in plants is variable (Lam *et al.*, 1989).

In contrast to a wealth of information gained in the past 10 years regarding chlorella transformation technique, only a few promoters, such as CaMV 35S, RBCS2 have been used (Javis and Brown, 1991; Dowson *et al.*, 1997; Hawkins and Nakamura, 1999; Kim *et al.*, 2002; Mayfield *et. al.*, 2002). The efficiency of this promoter has not been compared with other promoters that function in chlorella.

Recently, the entire genome of a chlorella virus, PBCV-1, has been cloned and sequenced (Kutish *et al.*, 1996). This virus encodes many useful genes including restriction/modification enzyme, topoisomerase, chitinase and hyaluronan synthase (Zhang *et al.*, 1998; Xia *et al.*, 1986; Lavrukhin *et al.*, 2000; Sun *et al.*, 1999; Graves *et al.*, 1999). In addition, PBCV-1 has tRNA gene clusters in their genome. Interestingly, the tRNA gene cluster promoter is known to be active, and it was found that the tRNA gene cluster of CVK2 was transcribed as one unit and processed into small tRNAs (Nishida *et al.*, 1999). Although this shows that the 5' upstream region of tRNA gene clusters is active, this region has not been studied in detail.

In this study, the promoter activity of the upstream region of the tRNA gene clusters of chlorella viruses isolated in Korea was test in an trial to improve the

chlorella transformation system for higher expression of foreign protein. Also, constructs containing serial deletion of this region were constructed to find minimum region required for promoter activity.

Material and Method

Viruses strains

Twenty three chlorella virus strains have been isolated from fresh water samples collected from ten cites in Korea and their tRNA gene clusters have been characterized (Cho *et al.*, 2002). Among these strains, the tRNA gene cluster upstream regions of SS-1 and SS-2 that have been isolated from a pond in Seusan, and KH-1 and KH-2 that have been isolated from a lake in Daegu were analyzed.

Sequencing of tRNA gene cluster promoter

The initial trial for the direct sequencing of purified genomic DNA was unsuccessful probably due to the large size of the genomic DNA. Therefore, 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 follow: purified genomic DNA (3-5 μg) was digested overnight with BamH I and extracted with phenol/chloroform mixture. The DNA was precipitated with ethanol and dissolved in distilled water. These digested genomic DNA was used as template of PCR reaction for sequencing.

A primer for the tRNA gene cluster promoters (Ile-C, 5' TTGGCTCATAAG AGACCAATGC-3') was designed based on the isoleucine tRNA gene that located second in the tRNA gene cluster (Fig. 1). The tRNA gene cluster promoters of 3 other strains were determined by cut and walk method with this primer.

Sequencing reaction mixture (1-1.5 μg template DNA, 1 pmol of primer, 4 μl of terminator ready reaction mixture, and distilled water to 20 μl) 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 product was precipitated with ethanol and dissolved in 25㎕ 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^{TM} 310 Analyzer (Perkin Elmer).

PCR amplification of the tRNA gene cluster promoters

Direct sequencing results showed that the promoter regions of KH-1 and KH-2 have same sequence. Therefore, the tRNA gene cluster promoters were amplified by PCR from 3 strains, SS-1, SS-2 and KH-1 for promoter assay. Primers for the SS-1 (tRNApro-N, SS1pro-C), SS-2 and KH-1 (tRNA pro-N, tRNApro-C) were designed based on the sequence of tRNA gene cluster upstream region (Table 2).

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 (5 u/㎕), and 100 pmol of forward primer and reverse primer. PCR reaction condition were predenaturation at 95℃ for 5 min, 40 cycle of 1 min denaturation 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.

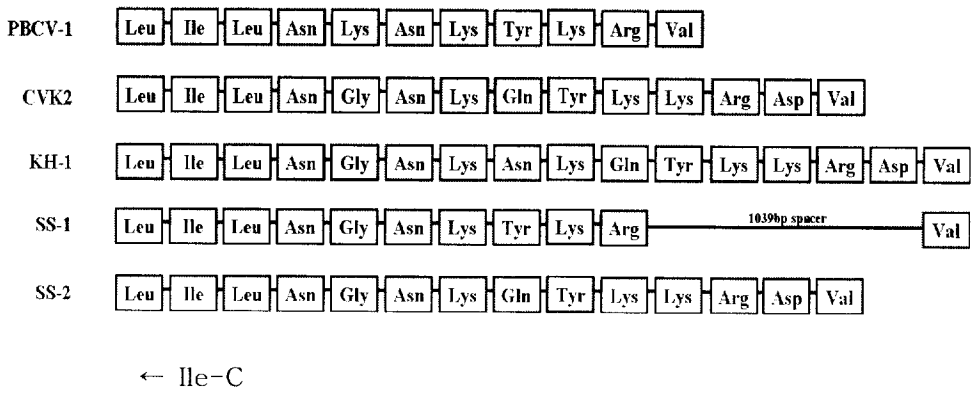

Fig. 1. Arrangement of tRNA gene clusters in *Chlorella* virus strains and location of primer used for the direct sequencing of promoter regions. PBCV-1 is the prototype of *Chlorella* viruses and CVK2 is isolated in Japan. KH-1, SS-1, SS-2 are virus strain isolated in Korea. Primer for direct sequencing is located in the Ile gene.

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, USA). *E. coli* XL1-Blue strain was transformed with the ligated DNA and clones containing the insert were screened with Macconkey plates. White colonies were inoculated to LB broth containing ampicillin (100 ㎍/㎖), and plasmid DNA was extracted with alkaline lysis method. Purified DNA were digested with EcoR I restriction enzyme and electrophoresed in 1% agarose gel.

Sequence analysis

Cloned plasmid DNAs were purified with Wizard plus SV minipreps DNA purification system (Promega) and used for DNA sequencing. Sequencing reaction mixture (500 ng template DNA, 1 pmol of primer, 8 ㎕ of terminator ready reaction mixture, and distilled water to 20 ㎕) was prepared and place into a thermal cycler. Twenty five cycle 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 ㎕ 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). Data about protein binding factors were obtained through the PLACE (Plant cis-acting regularoty DNA element) databases.

Table. 2. Oligonucleotide primers used for the PCR of tRNA gene cluster promoter regions.

Name	Sequence	Remark
tRNA-proN	5'-CGTAAGCTTGAAAGAATGTAT-3'	HindIII
tRNA-proC	5'-GCATACGGATCCGCGCGCGTGTAT-3'	BamHI
SS1-proC	5'-ATACGGATCCGCGCGATTAGAGTC-3'	BamHI

Construction of chlorella transformation vector

The transformation vector pMinGFPble had been constructed by modifying a plant transformation vector pMinGFP for expression of GFP (Kim *et al.*, 2002). This vector contains ori V for replication in both *E. coli* and *Agrobacterium*, npt III gene for kanamycin resistance, trf A for replication support, right and left border of T-DNA for integration and *Sh ble* gene for transformant selection with phleomycin. The GFP gene is under the control of the 35S promoter of CaMV (Fig. 2).

In order to compare the effect of the each promoters (35S, KH-1, SS-1, SS-2) on the expression of the GFP gene in transformed Chlorella, three different expression vectors were constructed as follows. The pTGFP vector contains the tRNA gene cluster promoter of SS-2 and GFP gene, the pSGFP vector contains the tRNA gene cluster promoter of SS-1 and GFP gene, the pKGFP vector contains the tRNA gene cluster promoter of KH-1 and GFP gene. The tRNA gene cluster promoters in the T-easy vector were digested with HindIII and BamHI, and cloned into the HindIII and BamHI digested pMinGFPble to replace the CaMV 35S promoter (Fig. 3).

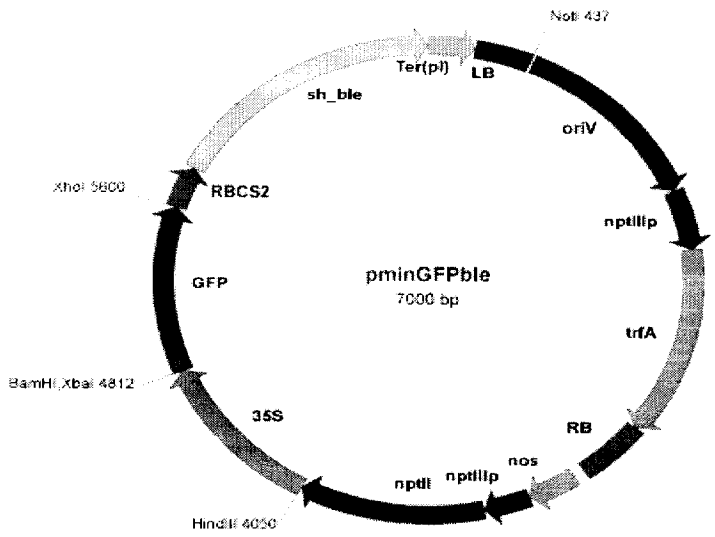

Fig. 2. Chlorella transformation vector pMinGFPble. In this vector, the GFP gene is under the control of cauliflower mosaic virus 35S promoter and the sh ble gene is under the control of *Chlamydomonas* RBCS2 gene promoter. Ori V, board host range replication origin, RB, right border region; LB, left border region; nos-nptIIIp npt II, nptII gene under the control of nos-npt III double promoter; ter(pI), 3' region of the potato protease inhibitor II gene. The 35S promoter was replaced with tRNA gene cluster promoters in this study.

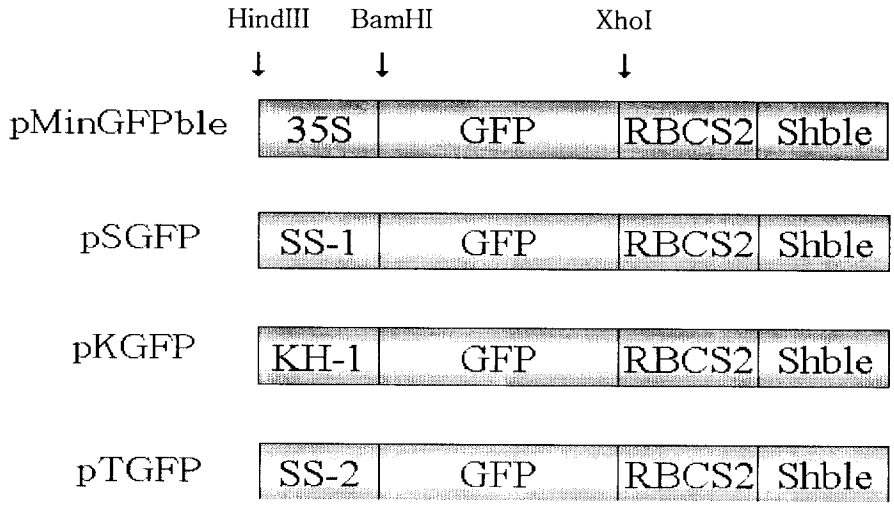

Fig. 3. Structure of Chlorella transformation vectors. The transformation vector was constructed by replaced the 35S promoter with promoters from tRNA gene cluster of indicated viruses.

Serial deletion of the promoter region from the SS-2 strain

In order to find the effect of the repeated sequence on the promoter activity, serial deletion constructs were made from the promoter region of SS-2 strain containing ABABA repeat. Serial deletions containing BABA, ABA, BA, and Box A fused to the GFP reporter gene were constructed by PCR amplification with AB box prime or BA box primer in conjunction with GFP-C primer (Table. 3). The location of the primers are shown in Fig. 4. The serially deleted promoter-GFP fragments were amplified in 30 $\mu\ell$ PCR mixture containing 1 $\mu\ell$ template DNA (pTGFP), 3 $\mu\ell$ of 10 × buffer (100 mM Tris.Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.25 mM dNTP, 0.5 $\mu\ell$ Taq DNA polymerase (2.5 $\mu\ell$/U) and 50 pmole AB-box/GFP-C or BA-box/GFP-C specific primer. The reaction conditions were: 35 cycles of 30 sec denaturation at 95℃, 30 sec annealing at 45℃, 60 sec extension at 72℃ followed by 5 min extension at 72℃. The amplified PCR product was analysed on 1% agarose gel electrophoresis after the gel was stained by ethidium bromide. The PCR product was digestion with HindIII and XhoI, and cloned into pTGFP digested with HindIII and XhoI.

Table. 3. Oligonucleotide primers used for the PCR of box deletion

Name	Sequence	Remark
AB-Box	5'-CACCAAAAGCTTCATTTATTACAGG-3'	HindIII
BA-Box	5'-TATATAAAGCTTAACTTAAGAAATA-3'	HindIII
GFP-C	5'-CTCGAGCTCTTAAAGCTCATCATG-3'	XhoI

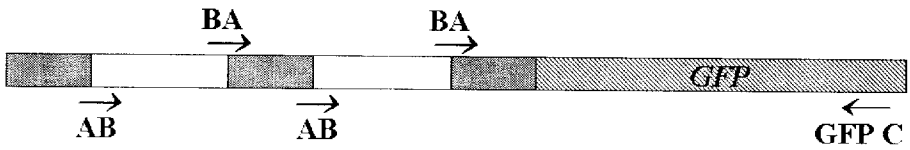

Fig. 1. Location of primers used for the of conserved sequence motif. PCR products containing GFP gene fused to serially deletion promoter were amplified by PCR with AB-Box or BA-Box primer and GFP-C primer. Filled and white boxes indicate Box A and Box B, respectively.

Chlorella Strain and medium

Chlorella vulgaris was provided from Korea Marine Microalgae Culture Center of Pukyong National University (Strain No. KMCC FC-001). Culture medium for *C. vulagris* (DW f/2) was made by replacing sea water of the f/2 medium with fresh water (Guillard and Ryther, 1962). Cells were cultured in DW f/2 containing 50 ㎍/㎖ each of chloramphenicol and streptomycin without shaking. Cells were inoculated at initial concentration of 1×10^6 cells/㎖ and cultured under 3000 lux fluorescent lamp at 25℃ with 18:6 h light:dark cycles.

Protoplast preparation

The cells were harvested for protoplast formation when cells reach $1\text{-}2\times10^8$ cells/㎖, usually 8-9 days after inoculation. Chlorella cells in 50㎖ of above culture were harvested by centrifuging for 5 min at 3,000 ×g. Cells were washed once with 25 mM phosphate buffer (pH 6.0) and suspended in 5 ㎖ of same buffer containing 0.6 M sorbitol, 0.6 M mannitol, 4% (w/v) cellulase (Calbiochem, USA), 2% (w/v) Macerase (Calbiochem), and 1% (w/v) pectinase (Sigma). The cell suspension was incubated at 25℃ for 16 h in the dark with gentle shaking.

Chlorella transformation

Protoplasts were centrifuged at 400 ×g for 5 min and the supernatant was decanted. Protoplasts were gently suspended with 5㎖ of DW f/2 medium containing 0.6 M each of sorbitol and mannitol, and washed by centrifugation at 400 ×g for 5 min. The pellet was suspended in 1 ㎖ 0.6 M sorbitol/mannitol with 0.05 M $CaCl_2$. Protoplasts in 0.4 ㎖ ($10^7 \sim 10^8$ cells) were placed into new microcentrifuge tube and 5 ㎍ of vector DNA was added with 25 ㎍ calf thymus DNA (Sigma) as carrier. After 15 min incubation at room temperature, 200 ㎕ of PNC [0.8 M NaCl, 0.05 M $CaCl_2$, 40% PEG 4000 (Sigma)] was added with gentle mixing. After 30 min incubation at room temperature, 0.6 ㎖ of DW f/2 medium containing 0.6 M sorbitol/mannitol, 1 % yeast extract and 1 % glucose was added, and the cells were incubated at 25℃ for 12 h in dark.

Selection of transformants

The transformed cells were transferred to fresh DW f/2 containing 1 ㎍/㎖ of phleomycin and cultured under 3000 lux fluorescent lamp at 25℃ with 18:6 h light:dark cycle. After 2 weeks culture, cells were transformed to fresh medium containing same concentration of phleomycin.

Measurement of the GFP gene expression

After 2 weeks culture, the transformed cells were observed for GFP expression with a fluorescence microscope (Olympus BH2-RFL-T3) using WB filter. In order to determine the fluorescence intensity, 1 ml of samples containing same number of cells that had been transformed with different constructs were measured using a fluorescence spectrometer (Perkin Elmer LB-500). Before the measurement, cells were first observed with the fluorescence microscope to confirm that there is no wild type cell in the samples. In order to get same number of cells for each sample, the cells were counted using a hemocytometer chamber over 5 times. The cells were centrifuged at 400 ×g for 5 min and the supernatant was decanted. The pellet was washed once with phosphate buffer (pH 6.0) and the cell pellet was resuspended to the final cell count of 3.0×10^6 cells/㎖ with same buffer. One milliliter of the resuspended cells were used for measurement, and five samples were measured from each preparation. Absorption spectrums of non-transformed and transformed cells were obtained first to determine the excitation wave length. Emission spectrums were obtained with an excitation wave length at 375 nm, and the GFP intensity values at 435 nm emission wave length were taken for comparison.

Results

Sequence analysis the tRNA gene cluster promoters

DNA fragments containing the upstream region of the tRNA gene clusters of chlorella virus SS-1, SS-2 and KH-1 were amplified. PCR products with expected size were obtained, which were 370bp in SS-1 and KH-1, and 490bp in SS-2 (Fig. 5). The PCR products were cloned into the pGEM-T vector and their sequences were determined, which shown in Fig. 6. Analysis of these promoter regions revealed repeated and conserved DNA motifs that we named as Box A, Box B and Box C (Fig. 7). Box A is composed of 33 nucleotides while boxes B or C are composed of 84~100 nucleotides. Although Box B and Box C were similar in size, they showed only 40% sequence homology, and they were regarded as different motifs. Fig. 8 is the simplified version of the promoter regions. PBCV-1 and SS-1 have two Box Aes, the KH-1 strain has CABA arrangement and the SS-2 strain has ABABA arrangement.

Analysis of the tRNA gene cluster promoter regions with PLACE (Plant cis-acting regulatory DNA element) program revealed the presence of a number of putative cis-acting elements for transcription factor binding (Fig. 6).

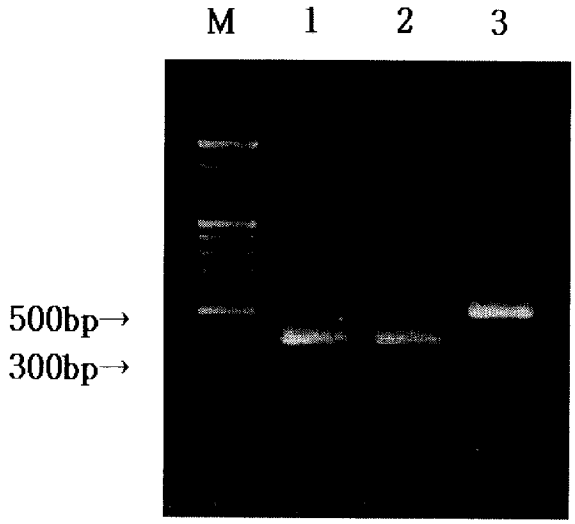

Fig. 5. PCR amplification of the tRNA gene cluster promoter from chlorella virus isolated in Korea. Lane M, 100bp DNA ladder size marker; lane 1, KH-1; lane 2, SS-1; lane 3, SS-2.

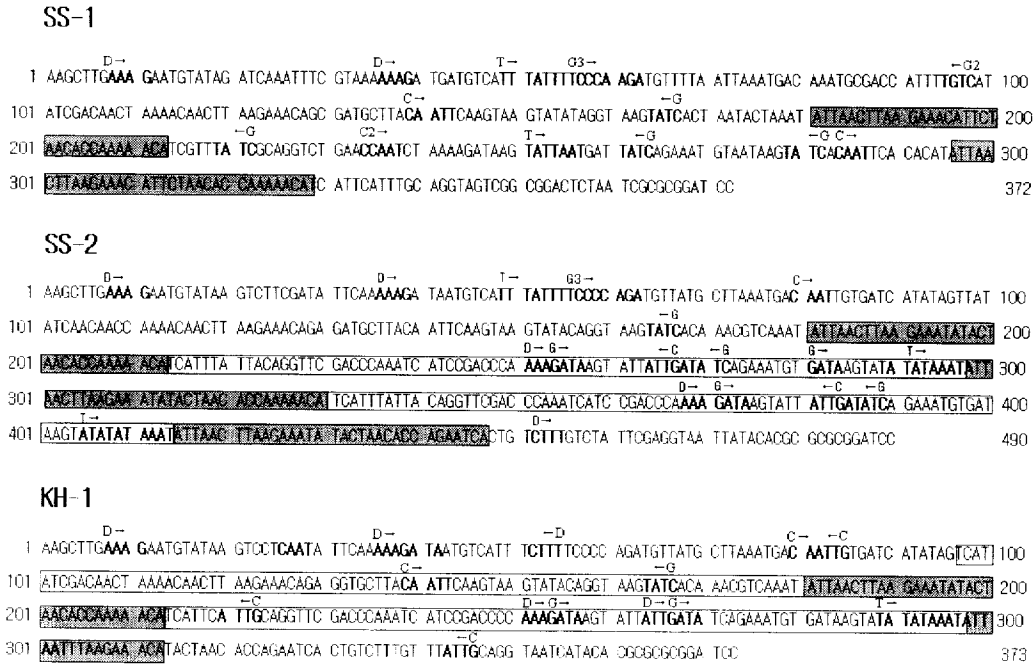

Fig. 6. Analysis of the tRNA gene cluster promoter regions of the chlorella viruses. The putative transcriptional factor binding sites known in plants are indicated (C: CAAT box, C2: CCAAT box, D: Dof protein binding site, G: GATA box, G2: GATA box, C3: GC like motif, T: TATA box, in bold). Repeated sequences described in the text are boxed (Box A: black, Box B, C: white.).

Box A

```
PBCV-1-A1:  ATTAACTTAAGAAACATACTAACACCAAAAACA
PBCV-1-A2:  ATTAACTTAAGAAACATTCTAACACCAAAAACA
 SS-1-A1 :  ATTAACTTAAGAAACATTCTAACACCAAAAACA
 SS-1-A2 :  ATTAACTTAAGAAACATTCTAACACCAAAAACA
 SS-2-A1 :  ATTAACTTAAGAAATATACTAACACCAAAAACA
 SS-2-A2 :  ATTAACTTAAGAAATATACTAACACCAAAAACA
 SS-2-A3 :  ATTAACTTAAGAAATATACTAACACCAGAATCA
 KH-1-A1 :  ATTAACTTAAGAAATATACTAACACCAAAAACA
 KH-1-A2 :  ATTAATTTAAGAAACATACTAACACCAGAATCA
            ***** ******** ** ********* ** **
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Fig. 7. Comparison of the repeated Box A sequences of tRNA gene cluster promoters of chlorella viruses. Names and locations of the boxes are shown in Fig. 9.

<A>

Box B

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SS-2-B1:TCATTTATTACAGGTTCGACCCAAATCATCCGACCCAAAAGATAAGTATTATTGATATCAGAAATG
SS-2-B2:TCATTTATTACAGGTTCGACCCAAATCATCCGACCCAAAAGATAAGTATTATTGATATCAGAAATG
KH-1-B1:TCATTCATTGCAGGTTCGACCCAAATCATCCGACCCCAAAGATAAGTATTATTGATATCAGAAATG
        ***** *** ************************** ******************************

        TGATAAGTATATATAAAT
        TGATAAGTATATATAAAT
        TGATAAGTATATATAAAT
        ******************
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<B>

Box C

```
KH-1-B1:TCAT_TCATTGCAGGTTCG_ACCCAAATCATCCGACCCCAAAGATAAGTATTATTGATATCAGAAA
KH-1-C1:TCATATCGACAACTAAAACAACTTAAGAAA_CAGAGGTGCTT_ACAATTCAAGTAAGTAT_ACAGG
        **** **            **  **   * * **       * ** *    *   *** * *

        TGTG_ATAAGTATATATAAAT
        TAAGTATCACAAACGTCAAAT
        *  * ** *  *     ***
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Fig. 8. Comparison of the repeated Box B sequences of tRNA gene cluster promoters of different chlorella viruses (A) and comparison of B and C box of KH-1 (B). Names and locations of the boxes are shown in Fig. 9.

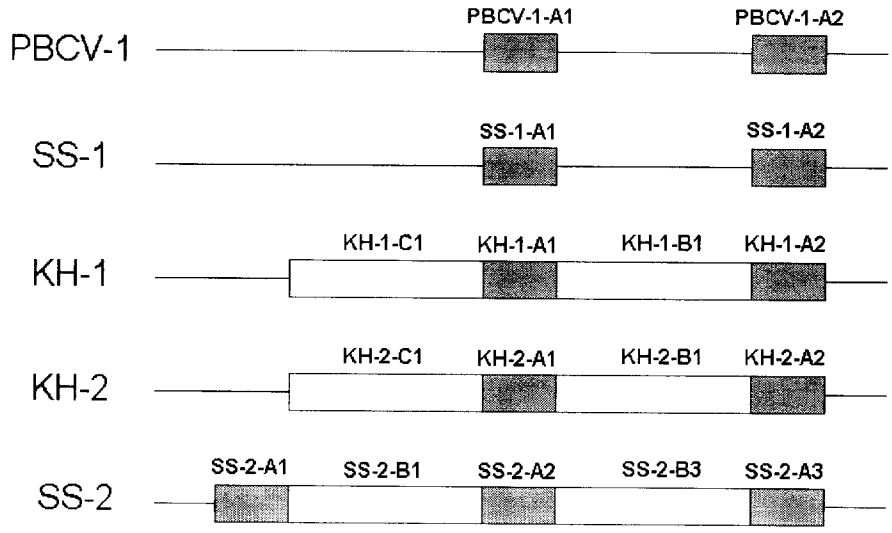


Fig. 9. The arrangement of repeated motifs in the putative promoter region of tRNA gene cluster in different chlorella viruses. Name of boxes cited in Fig. 7 and 8 are shown above the boxes. Black boxs, Box A; white boxs, Box B or C.

Test for promoter activity

In order to find whether the upstream region of the tRNA gene cluster functions as real promoters in transformed chlorella, DNA vector containing the GFP gene under the control of these regions were constructed. The constructs were confirmed by restriction enzyme digestion (Fig. 10), PCR and sequence analysis of the cloning junctions, and then protoplasts prepared from *C. vulgaris* were transformed with these vector. The transformed cells were selected by culturing in the presence of 1 ㎍/㎖ of phleomycin for 2 weeks. As shown in Fig. 12, chlorella cells transformed with the constructed vectors exhibited green fluorescence (Fig. 12B-E), in contrast to non-transformed cells that did not emit any green fluorescence (Fig. 12A).

For quantitative comparison of promoter activity between these constructs, fluorescence was measured using a fluorescence spectrometer. The excitation wave length of 375nm was chosen from the absorption spectrum analysis. GFP fluorescence values at the emission wave length of 435nm were chosen for comparison because the emission was the highest at this wave length (Fig. 13). Five samples were prepared from each preparation including non-transformed cells and grouped into 5 groups representing five repeats. In each group, the GFP intensity values of transformed cells were subtracted with the intensity of non-transformed cells to correct the autofluorescence. Than, relative intensity values for each samples against that from the cells transformed with 35S-GFP fusion (pMinGFPble vector) were calculated. Average intensity for each constructs were calculated with the relative intensity of corresponding construct in 5 groups, which are shown in Table 4.

As shown in Table 4, all of the cells transformed with tRNA gene cluster promoter-GFP fusion showed stronger intensity than those transformed with

CaMV 35S-GFP fusion construct. The cells transformed with ABABA-GFP fusion derived from the SS-2 strain showed the strongest (110%), followed by AA-GFP fusion (108%) and CABA-GFP fusion (102%) derived from SS-1 and KH-1 strain, respectively.

Promoter activity of constructs with serial deletion

In order to find the importance of the repeated boxes in the promoter activity, constructs containing serial deletion were constructed from the ABABA-GFP fusion construct derived from SS-2 strain. The partially deleted promoter-GFP fusion DNA was amplified by PCR with AB or BA box primer in combination with GFP-C primer to obtain PCR products of 800~1000 bp which includes ABA-GFP and A-GFP or BABA-GFP and BA-GFP fragment (Fig. 11). Although these PCR products contain DNA fragments of two different size, they appeared as one band because of small size difference, 33bp and 84bp, respectively. This was confirmed by cloning and sequence analysis of these PCR products. The PCR products were cloned into pGEM-T vector for sequence confirmation, and than the promoter-GFP region in pTGFP vector was replaced with the serial deletion containing BABA, ABA, BA or A-GFP fusion.

After transformation and selection of transformants for 2 weeks, GFP intensity of chlorella cells transformed with each construct was measured as described above and relative intensity against ABABA-GFP construct (100%) were calculated using same method. As shown in Table 5, GFP intensity of the cells decreased as the deletion of the conserved boxes increased. Chlorella cells transformed with BABA, ABA, BA or A-GFP fusion constructs showed 94%, 89%, 87% and 73% GFP intensity, respectively.

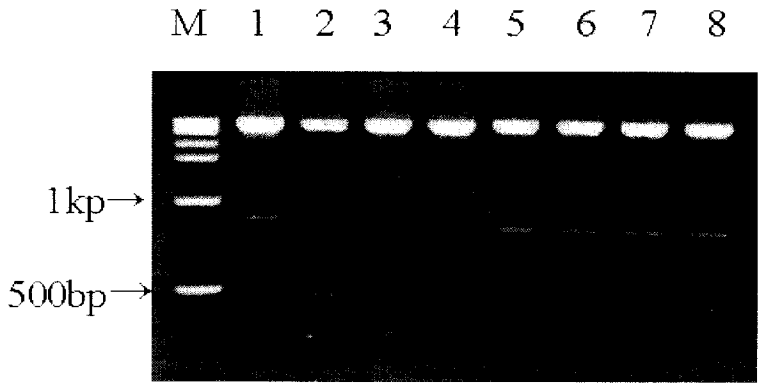


Fig. 10. Detection of promoter and GFP gene from the constructed vectors. Plasmid DNAs were digested with BamHI/XhoI to detected the promoter region (lane 1-4) and with HindIII/BamHI to detected the GFP gene (lane 5-8). Lane M, 1kb plus DNA ladder; lane 1 and 5, pMinGFPble; lane 2 and 6, pTGFP; lane 3 and 5, pSGFP; lane 4 and 8, pKGFP.

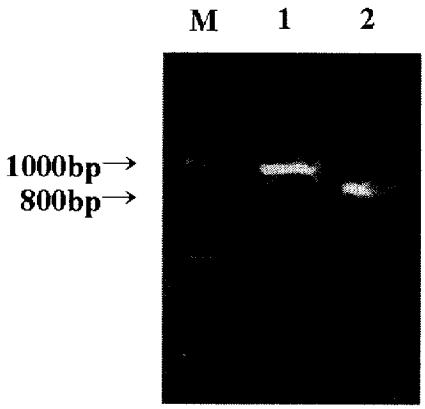


Fig. 11. PCR amplification of the box deletion promoters from pTGFP. Lane M, 100bp DNA ladder size marker; lane 1, AB box PCR ; lane 2, BA box PCR.

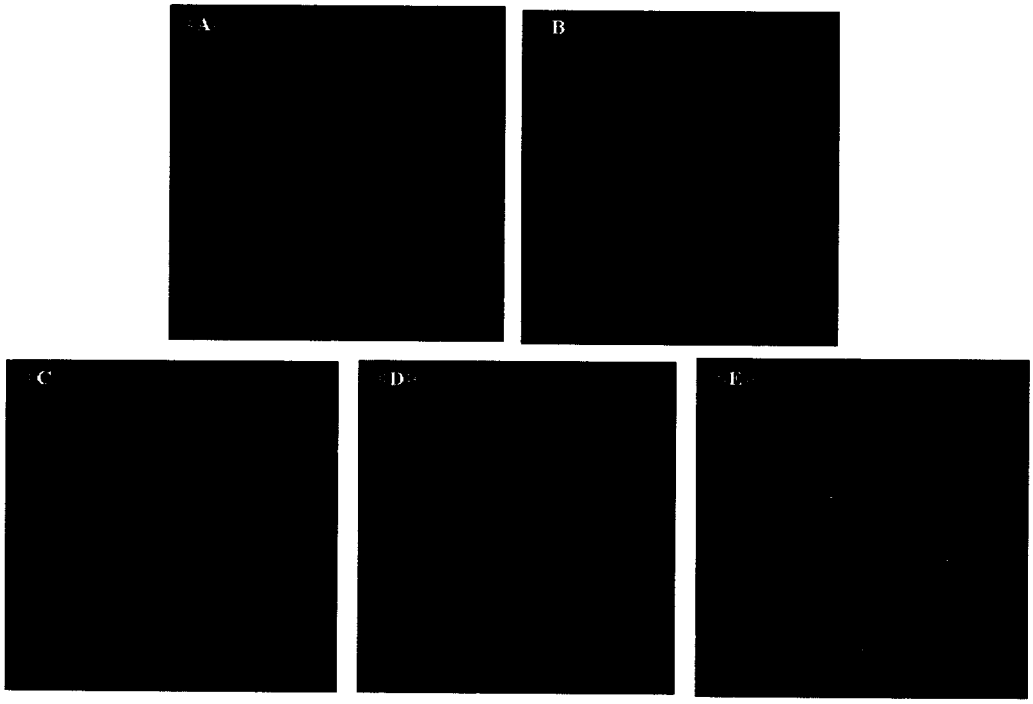


Fig. 12. Expression of the GFP gene in transformed C. *vulgaris*. A: wide type, B-E: *C. vulagaris* cells transformed with plasmid vector containing GFP gene fused to 35S promoter, tRNA gene cluster promoter from SS-1, KH-1 and SS-2 respectively.

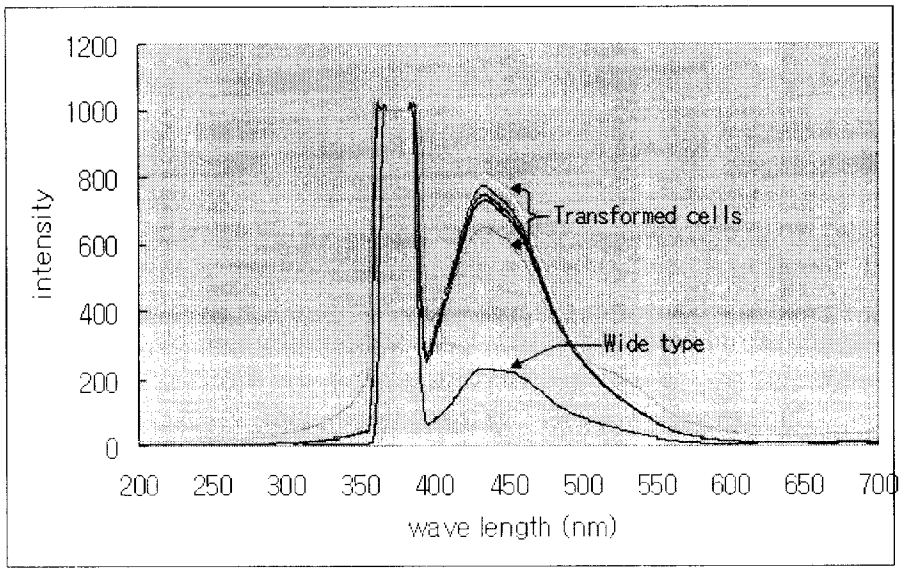
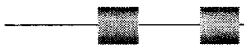


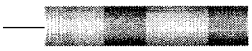
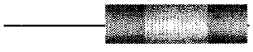
Fig. 13. Fluorescence spectrum of chlorella cells transformed with the constructs containing GFP gene. The spectrums were observed at the excitation wave length of 375nm.

Table. 4. Relative activity of promoters from tRNA gene clusters of four different chlorella viruses.

construct name	arrangement	GFP Intensity a)	relative intensity
MFP(35S)		510.17	100%
SFP(SS-1)		548.04	108%
TFP(SS-2)		558.9	110%
KFP(KH-1)		519.05	102%

a) The values are means from 5 measurements.

Table. 5. Relative promoter activity of serial deletion constructs derived from ABABA-GFP vector.

construct name	arrangement	GFP intensity a)	relative intensity
ABABA-GFP(SS-2)		558.9	100%
BABA-GFP		524.44	94%
ABA-GFP		499.7	89%
BA-GFP		488.46	87%
A-GFP		408.38	73%

a) The values are mean values from 5 measurement and excitation wave length of 375 and emission wave length of 435.

Discussion

Although the CaMV 35S promoter that has been used in may plant transformation system was also proven to function in microalgae (Mitra *et al.*, 1994: Ying *et al.*, 2001), promoters derived from chlorella or their virus would be suitable in the chlorella expression system. PBCV-1 encodes a number of transcriptional factors in its 340kb genome (Anne *et al.*, 1990) but it does not encode its own RNA polymerase and it is believed to use host transcription machinery to make its transcripts (Schuster *et al.*, 1986).

In addition to over 300 ORF encoded in the genome of PBCV-1, this virus and other related chlorella viruses contain 10~16 tRNA gene cluster, which suggests the adaptation of this virus to a wide host range in nature (Nishida *et al.*, 1999b: Van Etten *et al.* 1999). Aside from chlorella virus, the tRNA gene have also been identified in the genomes of murine gamma herpesvirus 68 (Bowden *et al.*, 1997) and bacteriophages T4 and T5 (Desai *et al.*, 1986). The presence of viral tRNA genes can be explained by the codon usage regulation of the chlorella viruses and their hosts (Van Etten *et al.*, 1995). Nishida *et al.* (1999b) identified tRNA gene cluster consist of 14 tRNA in the chlorella virus CVK2, and showed that these tRNAs were expressed and aminoacylated, which indicated that they involved in the viral protein synthesis.

Generally, eukaryotic tRNA genes are transcribed by RNA polymerase III and these genes contain two internal promoters designated as the Box A and Box B elements that present inside the tRNA coding region (Plamer and Folk, 1990). However, the tRNAs of chlorella virus CVK2 were cotranscribed as a large precursor of about 1.0kb and processed into individual mature tRNAs (Nishida *et al.*, 1999b). Exceptional cotranscription of tRNA gene clusters in eukaryotes have been known only from yeast so far (Schmit *et al.*, 1980).

Cotranscription of the tRNA gene cluster in virus infected chlorella suggest that the 5' upstream region of this cluster functions as a promoter. Sequence analysis of

the 5' region of the tRNA gene cluster of PBCV-1 revealed a typical promoter for chlorella viral genes that is composed of 65bp with 75% A+T (Schuster *et al.*, 1990). However, there has been no detail analysis for this region for the presence of a promoter.

Analysis of the genomes of chlorella viruses isolated in Korea showed the presence of tRNA gene clusters containing 14-16 tRNA genes (Cho *et al.*, 2002). The 5' upstream regions of these gene clusters were cloned, sequenced and analyzed for their promoter activity in transformed chlorella in the present study. Sequence analysis of the putative promoter regions revealed repeated and conserved DNA motifs called Box A of 33 nucleotides and Boxes B or C of 84 nucleotides (Fig. 7 and 8), which are arranged as AA in PBCV-1 and SS-1, CABA in KH-1 and KH-2, and ABABA in SS-2 (Fig. 9). This region also showed high A/T content of over 80%. Many promoter genes contain AT rich sequence as enhancer elements responsible for regulated gene expression. AT rich sequence have been shown to be important for quantitative expression of many plant genes (Yamamoto *et al.*, 1994). Several reports have previously suggested that the AT rich sequence might act as a general positive regulatory element (Bustos *et al.*, 1989). AT-rich sequence from the pea plastocyanin gene(*PetE*) promoter has been reported to act as quantitative and non-tissue-specific enhancer elements in transgenic plants. In *PetE* promoter, a single copy of the 31bp AT rich sequence increased the expression nearly 2-fold over the minimal promoter, whereas triplication increased the expression 4-5 fold (Sandhu *et al.*, 1998). This motif also has been shown to be responsible for the enhancer-like function in the maize *gpc1* promoter (Manjunath and Sachs, 1997).

Although the importance of repeated sequences in promoter activity has been acknowledged, repeated sequences with more than 30bp has not been reported from promoters of plant or plant viruses so far. In order to test the importance or possible role of this repeated sequence in the promoter activity of this region,

DNA fragments of about 400bp were amplified from chlorella viruses and cloned upstream of the GFP gene in a chlorella transformation vector, and then introduced into chlorella protoplasts. As shown in Table 4, the activity of these regions as promoters determined by the fluorescence from same number of transformed and selected cells was higher than that of CaMV 35S promoter. The putative promoter region from the chlorella virus SS-2 that containing ABABA arrangement showed the highest promoter activity, followed by those from KH-1 and SS-1 that have CABA and A-A arrangement, respectively.

A relationship between the number and arrangement of the conserved motifs and the promoter activity of the regions was expected. This was confirmed by serial deletion of the repeated motifs from the ABABA arrangement in the tRNA gene cluster of SS-2 strain. As shown in Table 5, the promoter activity decreased as the repeated motifs are removed from the promoter region, and promoter containing one A motif showed 73% activity compared to the complete ABABA arrangement. However, the promoter activity of only one Box A (73%) is higher than expected. The length one Box A is only 15% of ABABA and contains only one out of five repeated motifs. This suggest the importance of Box A in the promoter activity. Analysis of the Box A showed the presence of 'AGAAA' (Bate *et al.*, 1999), 'YACT' (Gowik *et al.*, 2004) and the 'RTTTTTR' (Lessard *et al.* 1991) sequences that have been identified as binding sites for transcriptional factors in tomato, flaveria and soybean, respectively. The importance of the Box A also can be observed in Table 4. The promoter with the A-A arrangement (SS-1) showed very similar (108% of CaMV) activity as ABABA (110% of CaMV), which is higher than that with CABA arrangement (102% of CaMV).

Table 5 also suggest that the Box B is important in promoter activity. There was sharp increase in promoter activity (73% to 87%) when the Box B was added to Box A. Box B also contains binding sites for transcriptional factors 'GATAAG' (Rose *et al.*, 1999), 'CAAT' (Shirsat *et al.*, 1989), 'GATA' (Teakle *et al.*, 2002)

(Fig. 6). This results suggest that both Box A and B are both important in the promoter activity of this region but Box A is more important. One possible explanation for the importance of the Box A would be the positional effect of this sequence, which could be tested with a construct containing AB arrangement.

In this study, eight promoters directly cloned from chlorella virus or obtained by serial deletion of the cloned promoter have been tested for their activity in transformed chlorella. The results showed that these promoters are comparable or better than the most commonly used CaMV 35S promoter and that they could be used in the expression of foreign protein using transformed chlorella.

국문초록

최근 형질전환 식물체중에서도 단세포 진핵 녹조류인 클로렐라가 새로운 생물반응기로서 각광받고 있다. 클로렐라는 일정량의 빛과 탄소원만 있으면 값싸게 대량 배양이 가능하며 하루에 2~9번 분열하기 때문에 성장속도가 빠르다. 또한 진핵생물인 클로렐라는 일반 진핵생물들과 마찬가지로 유전자를 전사한 후 변형 과정을 거치므로, 이종 진핵생물의 유전자로 형질전환시킬 경우, 생물학적 활성을 지닌 단백질을 만들 수 있는 등 이종 단백질의 발현에 적합한 시스템을 지니고 있다. 클로렐라에서의 동종 또는 이종 구성형 단백질의 일시적인 발현은 1990년대부터 보고되었으며, 최근에는 클로렐라 엘립소이데아에서 변형된 식물 형질전환 벡터를 이용하여 콜리플라워 모자이크 바이러스의 35S 프로모터 하에서 넙치 성장 호르몬을 안정적으로 발현시킨 연구가 보고되었다.

형질전환된 미세조류를 이용하여 유용 단백질을 생산하려는 노력이 시도되고 있는 시점에서 이에 적합한 프로모터의 개발이 동시에 수반되어야하며 이에 본 연구에서는 클로렐라 내에서 35S 프로모터를 대체할 프로모터의 개발을 위해 클로렐라 바이러스에 주목했다. 클로렐라 바이러스 SS-1, SS-2, KH-1, KH-2는 한국의 담수에서 분리되었으며 *Phycodnaviridae* family의 *Paramecium bursaria* chlorella virus(PBCV-1)와 유사하다. 이들은 각기 11, 14, 16, 14개의 tRNA gene을 가지고 있으며 이는 집단의 형태로 존재한다.

tRNA 유전자 집단의 프로모터 영역을 분석한 결과 유사한 서열이 반복되고 있었으며 33개의 뉴클레오타이드로 구성된 Box A와 84개의 뉴클레오타이드로 구성된 Box B와 C로 이루어져있었고 SS-1 strain의 경우 AA, KH-1과 KH-2 strain의 경우 CABA, SS-2 strain은 ABABA로 조합되어져있었다. 이들 프로모터들과 SS-2 strain의 Box deletion 시리즈 프로모터들이 녹색 형광 단백질(GFP)유전자에 연결하여 *Chlorella vulgaris*에 형질전환되었다.

분리된 tRNA 집단 프로모터와 Box deletion 시리즈 프로모터들은 CaMV 35S 프로모터와 유사하거나 그 이상의 활성을 보였다. 이중에서도 ABABA의 조합을 가지는 SS-2의 프로모터가 가장 높은 활성을 보였으며 SS-1과 SS-2 strain의 프로모터

는 35S 프로모터보다 높은 활성을 보였다. Serial box deletion에 의한 분석 결과 반복되는 박스 서열이 늘어날수록 프로모터의 활성은 증가하였으며 35S 프로모터와 유사한 활성을 가지기 위해서는 최소한 ABA 프로모터가 필요하였다.

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Reference

Anne, M.S., Michael, G., Kenneth, K., Marcia, Z., Jhon, B., Daniel, G. and Russel, H.M. (1990) Transcription and sequence studies of a 4.3-kbp fragment from a ds-DNA eukaryotic algal virus. *Virology*. 176: 515-523.

Bate, N. and Twell, D. (1998) Functional architecture if a late pollen promoter : pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant. Mol. Biol* 37:859-869.

Beck, C.F. and Haring, M.A. (1997) A promoter trap for *Chlamydomonas reinhardtii*: development of a gene cloning method using 5'RACE-based probes. *The Plant Journal*. 11(6): 1341-1348.

Bhattachar-Prakaxi, M., Peng, J., Elmer, J.S., Laco, G., Shen, P., Kaniewska, M.B., Kononowicz, H., Wen, F., Hodges, T.K. and Beachy, R.N. (1993) Specificity of a promoter from the rice tungro bacilliform virus for expression in phloem tissues. *Plant J.* 4: 71-79.

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

Brough, C.L., Sunter, G., Gardiner, W.E. and Bisaro, D.M. (1992) Kinetics of tomato golden mosaic virus DNA replication ad coat protein promoter activity in *Nicotiana tabacum* protoplast. *Virology* 187: 1-9.

Bustors, M.M., Guiltinan, M.J., Jordano, J., Begum, D., Kalkan, F.A. and Hall, T.C. (1989) Regulation of β-glucuronidase expression in transgenic tobacco plants by an A/T-rich, *cis*-acting sequence found upstream of a French bean β-phaseolin gene. *Plant Cell.* 1: 839-853.

Cho, H.-H., Park, H.-H., Kim, J.-O. and Choi, T.-J. (2002) Isolation and characterization of *Chlorella* viruses from freshwater sources in Korea. *Mol.Cells.* 14: 168-176.

Chen, Y., Wang, Y., Sun, Y., Zhang, L. and Li, W. (2001) Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. *Curr Genet.* 39: 365-370.

Dawson, H.N., Burlingame, R. and Cannons, A.C. (1997) Stable transformation of chlorella: Rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. *Curr Microbiol.* 35(6): 356-362.

Desai, S.M., Vaughan, J. and Weiss, S.B. (1986) Identification and location of nine T5 bacteriophage tRNA genes by DNA sequence analysis. *Nucleic Acids Res.* 14: 4197-4205.

Fukuoka, H., Ogawa, T., Mitsuhara, I., Iwai, T., Isuzugawa K., Nishzawa, A., Gotoh, Y., Nishizawa, Y., Tagiri, A., Ugaki, M., Ohshima, M., Yano, H., Murai, N., Niwa, Y., Hibi T. and Ohashi, Y. (2000) Agrobacterium-mediated transformation of monocot and dicot plants using the NCR promoter derived from soybean chlorotic mottle virus. *Plant Cell Reports.* 19(8): 815-820.

Gowik, U., Burscheidt, J., Akyildix, M., Schlue, U., Koczor, M., Streuble, M. and Westhoff, P. (2004) Cis-Regulatory element for mesophyll-specific gene expression in the C4 plant Flaveria trinervia, the promoter of the C4. *Plant Cell.* 16: 1077-1090.

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

Guillard, R.R. and Ryther, J.H. (1962) Studies on marine planktonic diatoms. I. Cyclotella nana Hustedt, and Detonula confervacea (cleve). *Gran. Can. J. Microbiol.* 8: 229-39.

Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards, K.E. (1982) Transcription of cauliflower mosaic virus DNA: Detection of promoter sequences, and characterization of transcripts. *Cell.* 30: 763-773.

Hawkins, R.L. and Nakamura, M. (1999) Expression of human growth hormone by the eukaryotic alga, Chlorella. *Curr Microbiol.* 38(6): 335-341.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies In transgenic plants. *Nature.* 2;342(6245): 76-78.

Jarvis, E.E. and Brown, L.M. (1991) Transient expression of firefly luciferase in protoplasts of the green alga *Chlorella ellopsoidea*. *Curr Genet.* 19: 317-321.

Kim, D.-H., Kim, Y.-T., Cho, J.-J., Bae, J.-H., Hur, S.-B., Hwang, I.-H. and

Choi, T.-J. (2002) Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*. *Mar.Biotechnol.* 4: 63-73.

Kutish, G.F., Li, Y., Lu, Z., Furuta, M., Rock, D.L. and Van Etten, J.L. (1996) Analysis of 76 kb of the chlorella virus PBCV-1 330-kb genome: map positions 182 to 258. 223(2): 303-17.

Lam, E., Benfey P.N., Gilmartin, P.M., Fang, R.X. and Chua, N.H. (1989) Site-specific mutations alter in vitro factor binding and change promoter expression pattern in transgenic plants. *PANS.* 86(20): 7890-7894.

Lavrukhin, O.V., Fortune, J.M., Wood, T.G., Burbank, D.E., Van Etten, J.L., Osheroff, N. and Lloyd, R.S. (2000) Topoisomerase II from Chlorella virus PBCV-1. Characterization of the smallest known type II topoisomerase. *J Biol Chem.* 275(10): 6915-21.

Lessard, P.A., Allen, R.D., Bernier, F., Crispino, J.D., Fujiwara, T. and Beachy, R.N. (1991) Mlutiple nuclear factor interact with upstream seqences of differentially regulated beta-conglycinin genes. *Plant Mol. Biol.* 16: 397-413.

Liu, Z.Z., Wang, J.L., Huang, X., Xu, W.H., Liu, Z.M. and Fang, R.X. (2003) The promoter of a rice glycine-rich protein gene, *Osgrp-2*, confers wascular-specific expression in transgenic plants. *Planta.* 216: 842-833.

Manjunath, S. and Sachs, M.M. (1997) Molecular characterization and promoter analysis of the maize cytosolic glyceraldehyde 3-phosphate dehydrogenase

gene family and its expression during anoxia. *Plant Mol Biol.* 33(1): 97-112.

Mayfield, S.P., Franklin, S.E. and Lerner, R.A. (2002) Expression and assembly of a fully active antibody in algae. *PNAS.* 100(2): 438-442.

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

Nishida, K., Kimura, Y., Kawaski. T., Fujie, M. and Yamada, T. (1999a) Genetic variation of *Chlorella* viruses: variable regions localizedd on the CVK2 genome DNA. *Virology.* 255: 376-384.

Nishida, K., Kawasaki. T., Fujie, M., Usami, S. and Yamada, T. (1999b) Aminoacylation of tRNAs Encoded by *Chlorella* Virus CVK2. *Virology.* 263: 220-229.

Norre, F., Peyrot, C., Garcia, C., Rance, I., Drevet, J., Theisen, M. and Gruver, V. (2002) Powerful effect of an atypical bifactorial endosperm box from wheat *HMWG-Dx5* promoter in maize endosperm. *Plant Mol Biol.* 50: 699-712.

Palmer, J.M. and Folk, W.R. (1990) Unraveling the conplexiites of transcription by RNA polymerase III. *Trands Biochem.* Sci. 15: 300-304.

Pobjecky, N., Rosenberg, G.H., Dinter-Gottlieb, G. and Kaufer, N.F. (1990) Expression of the beta-glucoronidase gene under the control of the CaMV 35S promoter in Schizosaccharomyces pombe. *Mol. Gen. Genet.* 220:

314-316.

Rose, A., Meier, I. and Wienand, U. (1999) The tamato I-box binding factor LeMYBI is a member of a novel class of Myb-like proteins. *Plant J.* 20: 641-652.

Salmon, V., Legrand, D, Slomianny, M.C., Yazidi, I., Spik, G., Gruber, V., Bournat, P., Olagnier, B., Mison, D., Theisen, M. and Merot, B. (1998) Production of human lactoferrin in transgenic tobacco plants. *Protein Expr Purif.* 13(1): 127-135.

Sandhu, J.S., Webster, C.I. and Gray, J.C. (1998) AT-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. *Plant. Mol. Biol.* 37: 885-896.

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

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

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

Schweizer, P., Christofferl, A. and Dudler, R. (1999) Transien expression of members of the germin like gene family in epidermal cells of wheat

confers disease resistance. *The Plant Journal.* 20(5): 541-552.

Shirsat, A., Wilford, N., Croy, R. and Boulter, D. (1989) Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. *Mol Gen Genet* 215: 326-331.

Sun, L., Adams, B., Gurnon, J.R., Ye, Y. and Van Etten, J.L. Characterization of two chitinase genes and one chitosanase gene encoded by Chlorella virus PBCV-1. *Virology.* 263(2): 376-87.

Teakle, G.R., Manfield, I.W., Graham, J.F. and Gilmartin, P.M. (2002) Arabidopsis thaliana GATA factors: organisation, expression and DNA-binding characteristics. *Plant Mol Biol.* 50: 43-57.

Tyagi, A.K. (2001) Plant genes and their expression. *Current science.* 80(2): 161-169.

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

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

Van Etten, J.L. (2003) Unusual life style of giant *Chlorella* viruses. *Ann.Rev.Genet.* 37: 153-195.

Van Etten, J.L, Graves, M.V., Muller, D.G., Boland, W. and Delaroque, N. (2002) Phycodnaviridae-large DNA algal viruses. *Arch Virol.* 147(8): 1479-516.

Verdaguer, B., Kochko, A., Beachy, R.N. and Fauquet, C. (1996) Isolation and expression in transgenic tobacco and rice plant, of cassava vein mosaic virus (CVMV) promoter. *Plant Mol. Biol.* 31: 1129-1139.

Vierstra, R.D. and Davis, S.J. (1998) Soluble, highly fluorescent variants of green fluorescent protein(GFP) for use in higher plants. *Plant Molecular Biology.* 36: 521-528.

Xia, Y.N. and Van Etten, J.L. (1986) DNA methyltransferase induced by PBCV-1 virus infection of a Chlorella-like green alga. *Mol Cell Biol.* 6(5): 1440-5.

Xie, Y., Liu, Y., Meng, M., Chen, L. and Zhu, Z. (2003) Isolation and identification of a super strong plant promoter from cotton leaf curl Multan virus. *Plant Mol. Biol.* 53: 1-14.

Xu, Y., Zhan, X. and Morris, B. (1998) Activity and its regulation of the bi-directional promoter from tobacco yellow dwarf geminivirus. *Chin. J. Virol.* 14: 68-74.

Yamamoto, S., Niciara, M., Morikawa, H., Yamauchi, D. and Minamikawa, T. (1995) Promoter analysis of seed storage protein genes from *Canavalia gladiat* a D.C. *Plant Mol Biol.* 27: 729-741.

Ying, C., Yiqin, W., Yongru, S., Limimg, Z. and Wenbin, L. (2001) Highly efficient espression of rabbit neutrophil peptide-1 gene in Chlorella ellipsoidea cells. *Curr Genet.* 39: 365-370.

Zhang, Y., Nelson, M., Nietfeldt, J., Xia, Y., Burbank, D., Ropp, S. and Van Etten,

J.L. (1998) Chlorella virus NY-2A encodes at least 12 DNA endonuclease/methyltransferase genes. *Virology*. 240(2):366-75.

Zhong, R., Taylor, J.J. and Ye, Z.H. (1999) Transformation of the collateral vascular bundles into amphivasal vascular bundles in an *Arabidopsis* mutant. *Plant Physiol.*, 120(1): 53-64.