Development microalgae transformation vector using early gene promoters of *Chlorella* virus

클로렐라 바이러스의 초기 발현 유전자 프로모터를 이용한 미세조류 형질 전환 벡터 개발

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Development microalgae transformation vector using early gene promoters of *Chlorella* virus

A dissertation
by
Heoy Kyung Jung

Approved by:

(Chairman) : Won Jae Lee

(Member) : Gun Do Kim

(Member) : Tae Jin Choi

(Member)

(Member)

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CONTENTS

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Heoy Kyung Jung

Department of Microbiology, The Graduate school,
Pukyong National University

Abstract

Chlorella is an unicellular green algae that shares many metabolic pathways with higher plants, which offers many advantages as an expression system of foreign protein. Recent expression of a fish growth hormone in biologically active form using transformed *chlorella* suggested possible application of this system. In an heterologous expression system, strong and constitutive promoters are a prerequisite for efficient expression, which the scope of this study.

Viral genes encoded by PBCV-1, the prototype of *Phycodnaviridae*, can be grouped early and late genes depend on the time of their expression. The early genes that encode proteins required for virus replication are expressed before the virus replication possibly by host transcription machinery, which offers possible application of the promoters of these genes in the construction of *Chlorella* transformation vectors.

Putative promoter regions of the DNA polymerase, ATP-dependent DNA ligase and chitinase genes were amplified from 8 *Chlorella* viruses isolated in Korea using primer sets designed based on the sequence of PBCV-1 genome. Sequence analysis of these early gene promoters showed high sequence homology to the corresponding regions of PBCV-1 genome. Analysis of this putative promotor regions indicated the presence of many cis-acting elements for transcription factors including TATA box, CAAT box, NTBBF1 box, GATA box and CCAAT.

Chlorella transformation vectors containing three promoter regions from *Chlorella* virus HS-1 fused upstream of the green fluorescence protein (GFP) gene were constructed and introduced into protoplasts of *C. vulgaris*. Initial observation with a fluorescence microscope indicated the expression of GFP protein in transformed *Chlorella*. The transformed cells were selected in culture medium containing phleomycin, and GFP intensity of same number of cells transformed with different constructs were compared. Compared to GFP intensity from *Chlorella* cells transformed with CaMV 35S-GFP fusion (100%), cells transformed with the chitinase, the DNA polymerase and the DNA ligase gene promoter-GFP fusion construct showed 101.5%, 100.8%, 95.8% intensity, respectively.

These results indicate that *Chlorella* virus early gene promoters are active in transformed *Chlorella*, and *Chlorella* viruses can be useful sources of promoters in addition to many genes of interest.

INTRODUCTION

Chlorella is an attractive eukaryotic microalgae as a new system for foreign protein overexpression in many aspects. It has been widely used in aquaculture and industry such as health food production. It can be cultured inexpensively because it requires only limited amount of sunlight and carbon dioxide as energy sources. It can be cultured large scale in a pool by using sunlight. It also grows relatively fast and divide 2-9 times per day depend on the light intensity and temperature (Sorokin and Krauss, 1958). Also, *Chlorella* may produce complex protein that requires post-translational modification in biologically active form. These characteristics provide a rationale for the usage of *Chlorella* as a bioreactor for foreign protein overexpression. Recently, fish growth hormone has been expressed in biologically active form in transformed *Chlorella* (Kim *et al.*, 2002). Therefore, the purpose of this study was to isolate strong and constitutive promoters to improve for *Chlorella* expression system.

Chlorella viruses are large double-stranded DNA virus of about 330-380 kb, which contains many useful genes such as restriction/modification enzymes, topoisomerase, chitinase, hyaluronan synthase (Van Etten, 2000; Zhang *et al.*, 1998; Xia *et al.*, 1986; Lavrukhin *et al.*, 2000; Sun *et al.*, 1999; Graves *et al.*, 1999). In addition, the large size DNA contains many promoters that can be used in heterologous protein expression system. For example, the upstream region of *Chlorella* virus adenine methyltransferase gene functioned as a strong promoter both in plants and bacteria (Mitra *et al.*, 1994).

One consideration in choosing target promoters was the time of gene expression. *Chlorella* virus transcription can be divided into early and late stages. Early genes are expressed in host cells as early as 5-10 min post-inoculation, or immediate early. Early genes of PBCV-1 have been isolated and characterized (Schuster *et al.*, 1996). These include genes encoding proteins with significant

homology to transcriptional factors and mRNA processing protein, helicase, mRNA capping enzymes, factors influencing translational aminoacyl tRNA synthetase, ribosomal protein and unknown enzymes (Nishida *et al.*, 1999a; Yamada *et al.*, 1993). Early genes usually encode proteins required for virus replication. They are probably transcribed by host transcription system. Therefore, early gene promoters could be recognized by host transcription machinery. A typical TATA-box and a common element 5′-ATGACAA were identified in the promoter region of almost all of the immediate early genes, which may be recognized by host RNA polymerase and transcription factors (Kawasaki *et al.*, 2004). In contrast to them, late transcription begins 60-90 min p. i., after viral DNA synthesis starts and they usually encodes proteins required for virus structure.

Sequence analysis of the entire genome of PBCV-1, the prototype of the *Phycodnaviridae* family, indicates that there is no DNA dependent RNA polymerase required for viral RNA transcription (Van Etten, 2003). Considering the temporal regulation of early and late genes expression, it is possible that the late genes are transcribed by host RNA polymerase that has been modified by viral proteins. In fact, several genes encoding transcriptional factors have been identified from the genome of PBCV-1 (Van Etten, 1999).

Genes encoding a polymerase, an ATP-dependent DNA ligase and a chitinase have been identified as the early genes that are essential for the DNA replication and virus infection (Van Etten, 2003).

In this study, the promoter regions of the polymerase, ligase and chitinase gene of *Chlorella* viruses isolated from Korea were isolated and characterized. Also, promoters of these genes isolated from *Chlorella* virus HS-1 were tested for their activity in transformed *Chlorella* with green fluorescence protein (GFP) fused to these promoter regions by comparing their activities with that of cauliflower mosaic virus (CaMV) 35S promoter, the most commonly used promoter in plant transformation system and used in previous *Chlorella* transformation system.

MATERIALS AND METHODS

Virus culture and purification

Chlorella strain NC64A were cultured in a modified Bold's basal medium (MBBM) as described (Van Etten *et al.*, 1983). The *Chlorella* viruses used in this study, KH-1, KH-2, SS-1, SS-2, HS-1, YK, YK-1 and YK-2, have been isolated from fresh water in Korea (Cho *et al.*, 2002).

One hundred milliliter of actively growing *Chlorella* strain NC64A was inoculated with virus at an moi of 0.01 to 0.001 and incubated until completely lysed. The lysate was centrifuged in a Sorvall 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 by centrifuged 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 then pelleted for 3 hrs at 27,000 rpm with T-880 rotor. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8 (Van Etten *et al.*, 1983).

Isolation and analysis of viral genomic DNA

Isolated virus (400㎕) was mixed with 10X TEN (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 Sorvall TH-641 rotor at 35,000 rpm, 18 hrs, 25℃ (Van Etten *et al.*, 1981). DNA

band was collected and EtBr was removed by butanol extraction. DNA was precipitate with EtOH, dried and resuspended in 1X TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

PCR amplification of the *Chlorella* virus early gene promoters

DNA fragments containing about 420 bp upstream region of the DNA polymerase, the DNA ligase and the chitinase genes were amplified by PCR with primers designed based on the published PBCV-1 sequences. The locations of these genes in the PBCV-1 genome are indicated in Fig. 1 and the primers are listed in Table 1. The DNA fragments were amplified in 50$\mu\ell$ of PCR mixture containing 5$\mu\ell$ of 10X buffer (100 mM Tris-.Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.5 mM dNTP, 4$\mu\ell$ of Taq DNA polymerase(5u/$\mu\ell$), and 100 pmol of forward primer and reverse primer. PCR reaction condition were predenaturation at 94℃ for 10 min, 35 cycle of 1 min denaturation at 94℃, 1 min annealing at 50℃, and 1 min extension at 72℃, followed by a 7 min postextension at 72℃. The PCR products were analyzed by electrophoresis in 1.2% agarose gel and visualized by UV irradiation.

①	A181/182R	Chitinase
②	A185R	DNA polymerase
③	A544R	DNA ligase

Figure. 1 Location of early genes selected in this study on the PBCV-1 genome. The line represent the PBCV-1 genome of about 330 kb, which are indicated with the numbers above the line. The arrows indicate direction of transcription.

Table 1. Oligonucleotide primers used for the PCR amplification of the early gene promoters

Target gene	Primer name	Sequence	Remark
Polymerase	Pol-promoter-F	ATA AAG CTT CTA TTG ATA ACG CCA AGG	HindIII
	Pol-promoter-R	ATA GGA TCC GAT CAC TTG TAT TTC TAA	BamH I
Ligase	Lig-promoter-F	ATA AAG CTT CCA ACA ACA TTC GCT GCT	HindIII
	Lig-promoter-R	ATA GGA TCC AGA ACA CAC GTA ACT TCA	BamH I
Chitinase	Chit-promoter-F	ATA AAG CTT GAT AAT TTG AAT GTG TGT	HindIII
	Chit-promoter-R	ATA GGA TCC TAT ACT TTA AAC CAC CAT	BamH I

pGEM-T vector cloning and sequencing of early gene promoters

The PCR products were eluted from the agarose gel with gel extraction kit (Bioneer, Korea) and cloned into pGEM-T vector system (Promega, USA) for sequencing confirmation. *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/㎖) and plasmid DNA was extracted with alkaline lysis method. Purified DNA were digested with EcoR I restriction enzyme and electrophoresed in 1% agarose gel.

The plasmids containing appropriate inserts were purified with plasmid purification kit (Bioneer) and used as templates for PCR sequencing. Sequencing reaction mixtures [1000 ng template DNA, 4 pmol of T7 primer or SP6 primer for the early gene promoters, 2μℓ of terminator ready reaction mix] were prepared, and 25 cycles of PCR reaction composed of 10 sec denaturation at 96℃, 7 sec annealing at 50℃ and 4 min extension at 60℃ was performed with an Eppendorf Mastercycler 9600 (Eppendorf). The sequencing products were precipitated with ethanol and dissolved in 25μℓ of template suppressing reagent (Perkin Elmer). The DNAs were denatured for 2 min at 98℃ and cooled down on ice, and analyzed with a ABI PRISM™ 310 Analyzer (Perkin Elmer).

Vector construction and preparation for *Chlorella* transformation

Chlorella transformation vector containing the putative promoter regions were constructed by replacing the CaMV 35S promoter in the pMinGFP vector (Kim *et al*, 2002). The pMinGFP vector has green fluorescent protein (GFP) as a reporter gene, the ori V for replication in both *E. coli* and *Agrobacterium*, the nptIII gene

for kanamycin resistance, the trf A gene for replication support, right and left border of T-DNA for integration and Sh ble gene for trnasformant selection with phleomycin. The early gene promoters in the T-easy vector (Promega) was digested with Hind III and BamH I, and cloned into the Hind III and BamH I digested pMinGFP to replace the CaMV 35S promoter. The resultant transformation vector containing the DNA polymerase, the DNA ligase and the chitinase gene promoter were names as pPGFP, pLGFP and pCGFP, respectively (Fig. 2).

Chlorella strain and medium

Chlorella vulgaris was provided from Korea Marine Microalgae Culture Center of Pukyong National University (Strain No. KMCC FC-001). Cells were cultured in the D.W. f/2 medium in which sea water was replaced with fresh water (Guillard and Ryther, 1962) 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 hrs light:dark cycles.

Protoplast preparation

The cells were harvested for protoplast formation when cells were reached at $1\sim2\times10^7$ 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 the 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 hrs 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 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㎖ (10^7-10^8 cells) were placed into new microcentrifuge tube and 5㎍ of vector DNA was added with 25㎍ calf thymus DNA as carrier (Sigma). 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 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 hrs in dark. The transformed cells were transferred to fresh f/2 containing 1㎍/㎖ of phleomycin and cultured under 3000 lux fluorescent lamp at 25℃ with 18:6 hrs light:dark cycle. After 8 days culture, cells were transferred to fresh medium containing same concentration of phleomycin.

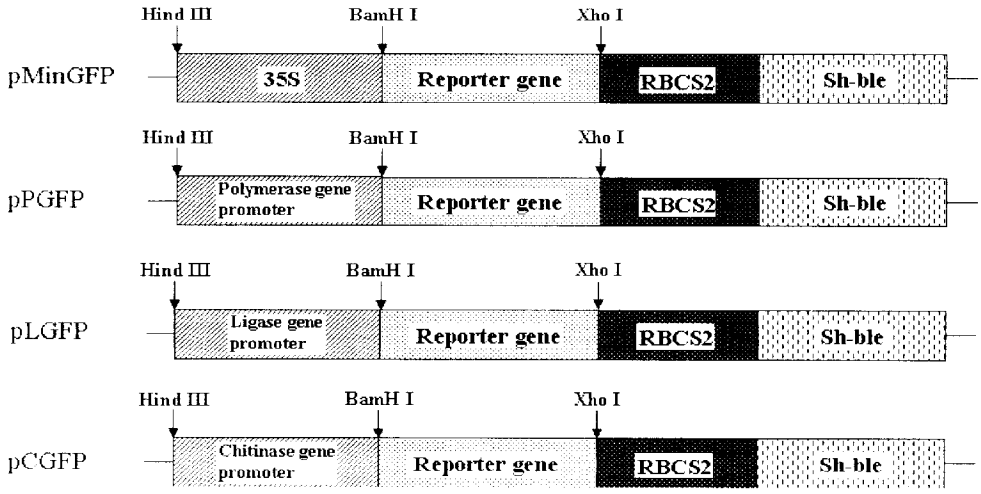


Figure 2. Plasmid constructs for *Chlorella* transformation. Early gene promoters from *Chlorella* virus HS-1 were fused to the green fluorescent protein (GFP) gene and the phleomycin resistance Sh ble gene for selection.

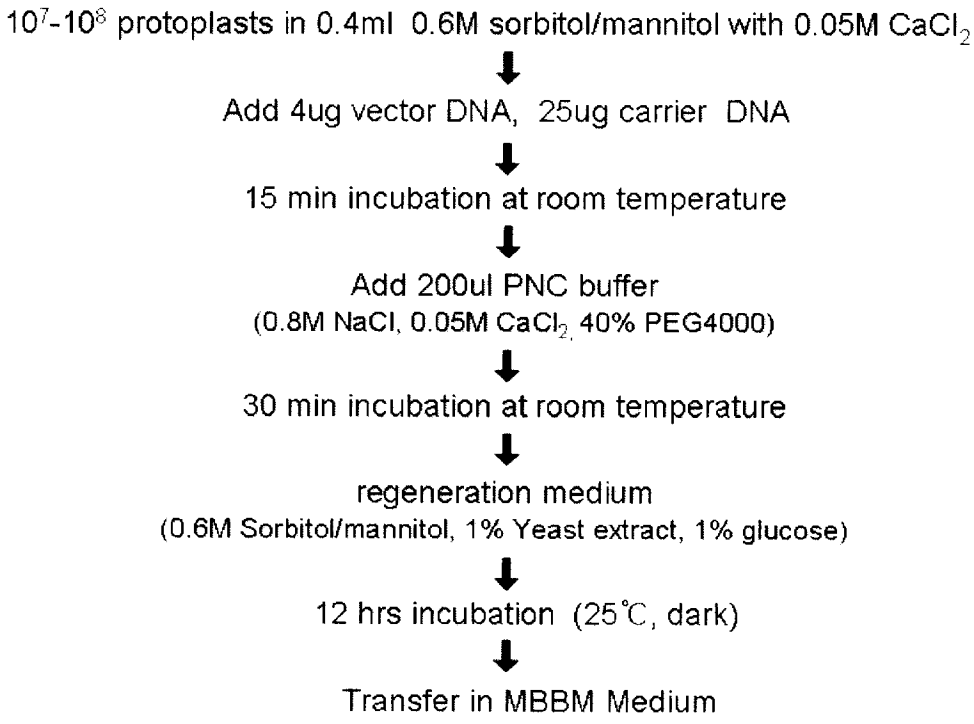


Figure 3. General procedures of microalgae transformation.

Observation of the GFP gene expressed in transformed *Chlorella*

Transformed cells were monitored for GFP expression every 2 days using a fluorescence microscope (Olympus BH2-RFL-T3) using WB filter. After 14 days culture from the initial transfer of cell wall regenerated protoplasts to D.W. F/2 medium containing phleomycin, GFP expression level was measured using a fluorescence spectrometer (Perkin Elmer LB-500). Transformed and non-transformed *Chlorella* cells were prepared for 3.0×10^6 cells/㎖ as follow; *Chlorella* cells were first counted using a hemocytometer chamber for five times. Cell culture containing enough cells was 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 were taken for comparison.

RESULTS

PCR amplification of *Chlorella* virus early gene promoters

Putative promoter region of three early genes, DNA polymerase, DNA ligase and chitinase gene, were amplified by PCR from 8 *Chlorella* virus strains (KH-1, KH-2, SS-1, SS-2, HS-1, YK, YK-1 and YK-2) isolated in Korea. The PCR primers were designed to amplify DNA fragment of about 420 bp. As shown in Fig. 4, DNA products of expected size were detected from PCR reactions for each promoter region, but not from all of the 8 strains used for PCR. In case of the DNA polymerase gene, the putative promoter regions were amplified from 5 of 8 *Chlorella* viruses, KH-2, SS-1, SS-2, HS-1 and YK. In case of the DNA ligase gene and the chitinase gene, the promoter regions were amplified from 4 of 8 *Chlorella* viruses, KH-1, HS-1, YK and YK-1. The results are summarized in Table 2. PCR products of the promoter regions of three genes were obtained only from PBCV-1, HS-1 and YK.

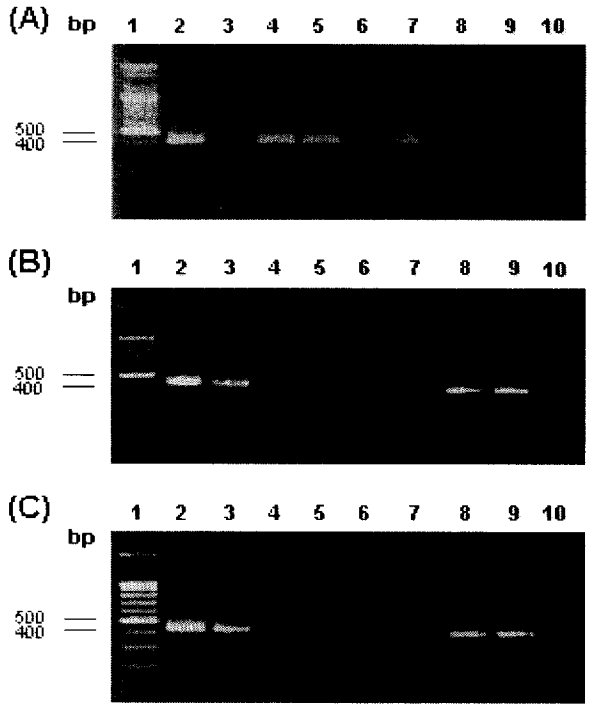


Figure 4. PCR amplification of *Chlorella* virus early gene promoters. PCR products of DNA polymerase promoter (A), DNA ligase promoter (B) and chitinase promoter (C) in 8 *Chlorella* viruses. Lane 1, 100 bp size marker; Lane 2, PBCV-1; Lane 3, KH-1; Lane 4, KH-2; Lane 5, SS-1; Lane 6, SS-2; Lane 7, HS-1; Lane 8, YK; Lane 9, YK-1; Lane 10, YK-2

Table 2. Results of PCR amplification of early gene promoters from 8 *Chlorella* viruses isolated in Korea.

	PBCV-1	KH-1	KH-2	SS-1	SS-2	HS-1	YK	YK-1	YK-2
Polymerase gene promoter	+	-	+	+	+	+	+	-	-
Ligase gene promoter	+	+	-	-	-	+	+	+	-
Chitinase gene promoter	+	+	-	-	-	+	+	+	-

+: promoter regions were amplified, -: not amplified, the promoters from HS-1 were used for *Chlorella* transformation vector construction.

Cloning and sequencing of the three early gene promoters

These PCR products were cloned into pGEM T-Easy vector and their sequences were determined. Sequence analysis of these early gene promoters showed high sequence homology to the corresponding regions of PBCV-1 genome. Fig. 5-7 shows the cloned promoter regions of *Chlorella* viruses isolated in Korea and those of PBCV-1. In case of the polymerase gene promoter, KH-2, HS-1 and YK showed 99.8%, 96.2% and 95.5% sequence homology to that of PBCV-1, respectively (Fig. 5). The DNA ligase gene promoter regions of KH-1, HS-1 and YK have 100% sequence homology to that of PBCV-1 but the same region of KH-2 was different in one nucleotide out of 419 nt (Fig. 6). In case of the chitinase gene promoter, KH-1, HS-1 and YK were identical to the corresponding region of PBCV-1 but YK-1 was different in one nucleotide out of 418 nt (Fig. 7). One of the *Chlorella* viruses, HS-1 showed the highest sequence homology to the corresponding regions of PBCV-1 in the three early gene promoters, and promoters from this virus stain were selected to construct *Chlorella* transformation vectors for promoter assay. The early gene promoters of HS-1 were analyzed using plant cis-acting regulatory DNA elements (PLACE) database to search for conserved sequences and binding sites of transcriptional factors (Fig. 8-10). Many transcriptional factor binding sites were identified in early gene promoters, typically TATA box, CAAT box, NTBBF1 box, GATA box and CCAAT. In the DNA polymerase gene promoter, 2 TATA boxes, 3 CAAT boxes and 5 GATA boxes were identified (Fig. 8). In the DNA ligase gene promoter, one CCAAT box, 3 TATA boxes, 5 CAAT boxes and 4 GATA boxes were found (Fig. 9). In the chitinase gene promoter, one CCAAT box and NTBBF1 box, 4 TATA boxes, 5 CAAT boxes and 4 GATA boxes were identified (Fig. 10). Three early gene promoters from HS-1 did not show any sequence homology to each others (data not shown) except for the TATA box, CAAT box and GATA box that identified

from all three promoters.

Figure 5. Comparison of putative promoter regions of the DNA polymerase gene of *Chlorella* viruses isolated in Korea and that of PBCV-1. The asterisks indicate nucleotides identical in all of the viruses.

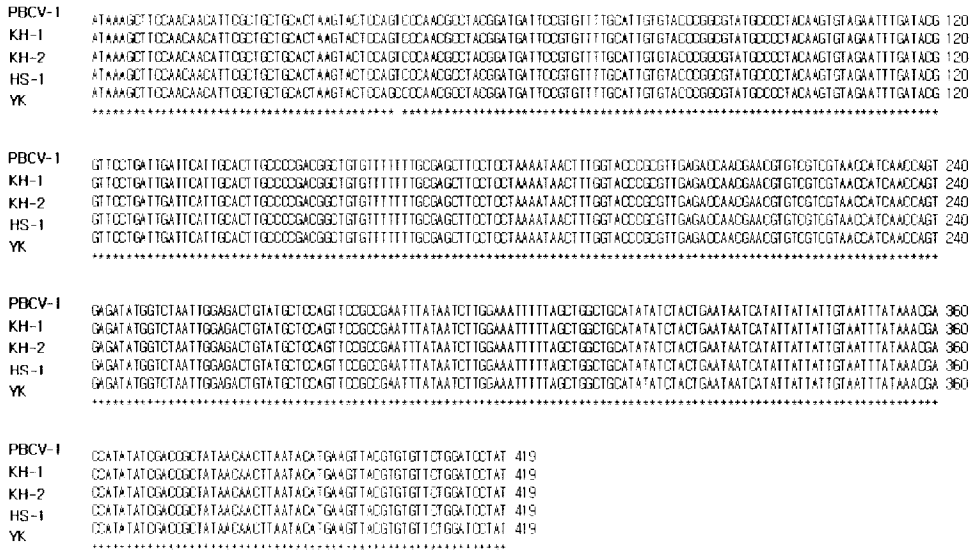


Figure 6. Comparison of putative promoter regions of the DNA ligase gene of *Chlorella* viruses isolated in Korea and that of PBCV-1. The asterisks indicate nucleotides identical in all of the viruses.

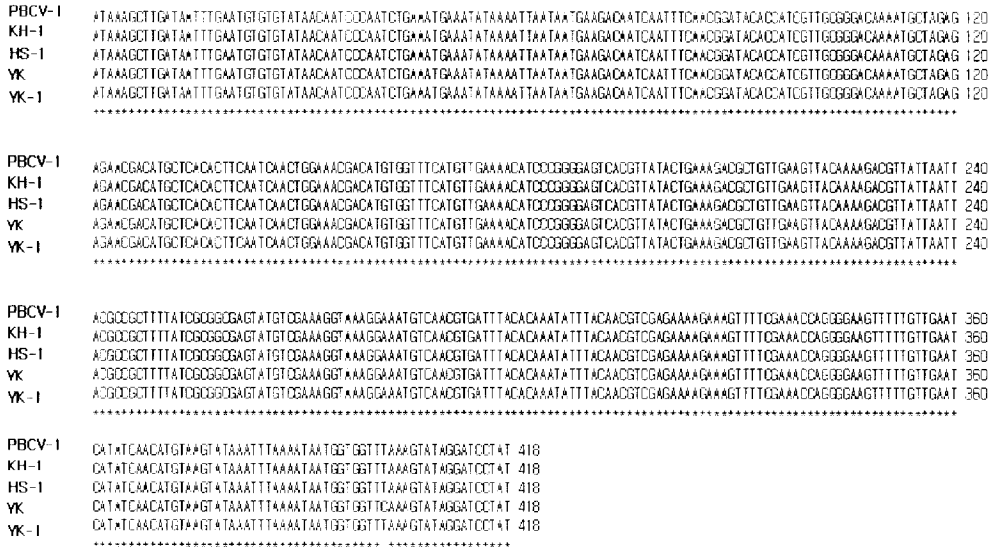


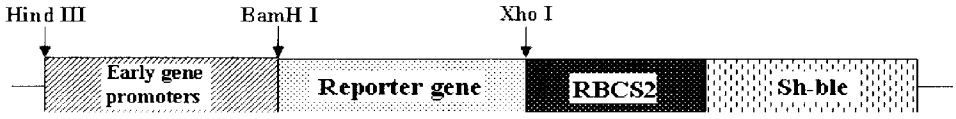
Figure 7. Comparison of putative promoter regions of the chitinase gene of *Chlorella* viruses isolated in Korea and that of PBCV-1. The asterisks indicate nucleotides identical in all of the viruses.

Figure 8. Analysis of the DNA polymerase gene promoter region of *Chlorella* virus HS-1. The AT rich region is shown as a doted box with conserved boxes indicated. AAGCTT (Hind III) and GGATCC (BamH I) sites used for cloning are underlined and the location of the initiation codon (ATG) is indicated. (+) and (-) of conserved motifs indicates orientation of the boxes.

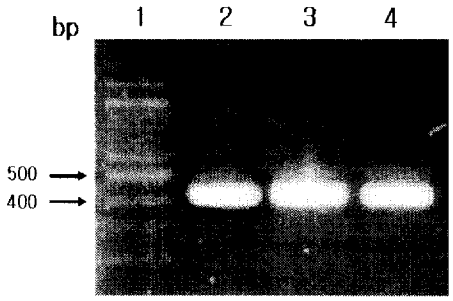
Figure 9. Analysis of the DNA ligase gene promoter region of *Chlorella* virus HS-1. Location of conserved boxes indicated. AAGCTT (Hind III) and GGATCC (BamH I) sites used for cloning are underlined and the location of the initiation codon (ATG) is indicated. (+) and (-) of conserved motifs indicates orientation of the boxes.

Figure 10. Analysis of the chitinase gene promoter region of *Chlorella* virus HS-1. The AT rich region is shown as a doted box with conserved boxes indicated. AAGCTT (Hind III) and GGATCC (BamH I) sites used for cloning are underlined and the location of the initiation codon (ATG) is indicated. (+) and (-) of conserved motifs indicates orientation of the boxes.

(A)



(B)



(C)

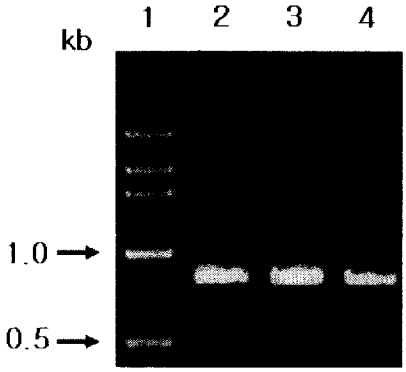


Figure 11. Structure of *Chlorella* transformation vector (A) and confirmation of the constructed vectors by PCR of the promoter regions (B) and the GFP gene (C). (B) Lane 1, 100 bp DNA ladder; lane 2, polymerase gene promoter lane 3, ligase gene promoter; lane 4, chitinase gene promoter (C) Lane 1, 1kb DNA ladder; Lane2, GFP in vector fused polymerase gene promoter; Lane 3, GFP in vector fused ligase gene promoter; Lane 4, GFP in vector fused chitinase gene promoter.

Chlorella protoplast formation

Protoplast formation was confirmed by calcofluor white staining. The untreated cells exclusively showed blue fluorescence under fluorescent microscope (Fig. 12). However, over 90% of enzyme treated cells were red, which indicated removal of cellulose component from the cell wall to which calcofluor white bind. After regeneration of the protoplast, the cells showed blue again. Thus, the enzyme mixture and reaction conditions were used in the following transformation experiments.

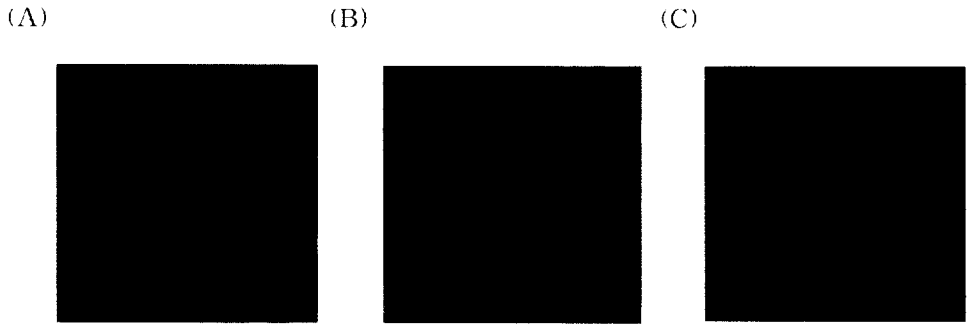


Figure 12. Detection of protoplast formation and cell wall regeneration by calcofluor white staining. (A) Non-transformed *Chlorella* (B) *Chlorella* cells treated with enzyme mixture for 12 hrs (C) After cell wall regeneration for 16 hrs.

Transformation of protoplasts with vectors fused the early gene promoters and the GFP gene

Protoplasts prepared by above method were transformed with vector containing early gene promoters in the pMinGFP by using polyethylene glycol. After initial transfer of cell wall regenerated protoplasts to D.W. F/2 medium containing phleomycine, observed the GFP gene expressed in the transformed *Chlorella* using a fluorescence microscope. As shown in Fig. 13, before the selection of transformants cells with phleomycin, *Chlorella* cells showing typical green color of GFP gene and non-transformed red colored cells were both present. In contrast, there was no green colored cell in the culture of non-transformed *Chlorella* cells. The cell population containing both non-transformed and transformed cells were grown for 2 weeks in culture medium containing 1ug/ml phleomycin for the selection of transformed cells. As shown in Fig. 14, there was no red colored cell in this population, which indicated complete selection of transformed *Chlorella* cells.

Measurement of the GFP gene expression levels

The GFP gene expression levels were measured using of fluorescence spectrometer (Perkin Elmer LB-500) after 14 days culture from the initial transfer of cell wall regenerated protoplasts to D.W. F/2 medium containing phleomycin of 1㎍/㎖. Absorption and emission spectrum of non-transformed and transformed cells were obtained first, which showed maximum absorption and emission at 375 nm and 435 nm, respectively. Therefore, GFP intensity of each sample was measured at this condition. As shown in Fig. 15, fluorescence spectrum of non-transformed *Chlorella* cells showed weak fluoresce in the same wave length range as transformed cell, which indicates autofluorescence of non-transformed

Chlorella cells. Fluorescence spectrums of *Chlorella* cells transformed with constructs containing the GFP gene fused to 35S promoter and promoters from *Chlorella* virus HS-1 were very similar.

The GFP gene expression intensity in *Chlorella* cells were measured to compare each promoter activity. Five groups, which represent 5 repeats for each sample, containing *Chlorella* cells transformed with each construct and non-transformed cells were prepared and their GFP intensity was measured at fixed excitation wave length of 375 nm and emission wave length of 435 nm. GFP intensity of non-transformed cells in each group was subtracted from the measurement to compensate the autofluorescence. The relative intensity from cells transformed with each construct were calculated using the intensity from cells transformed with 35S-GFP construct (100%) in each group, and the mean relative intensities for each construct were obtained.

Although there was no significant difference among the constructs as shown in Fig. 16, the chitinase gene promoter (101.5%) and the DNA polymerase gene promoter (100.8%) showed higher promoter activity than the CaMV 35S promoter (100%). However, the DNA ligase gene promoter showed lower promoter activity (95.8%) than CaMV 35S promoter.

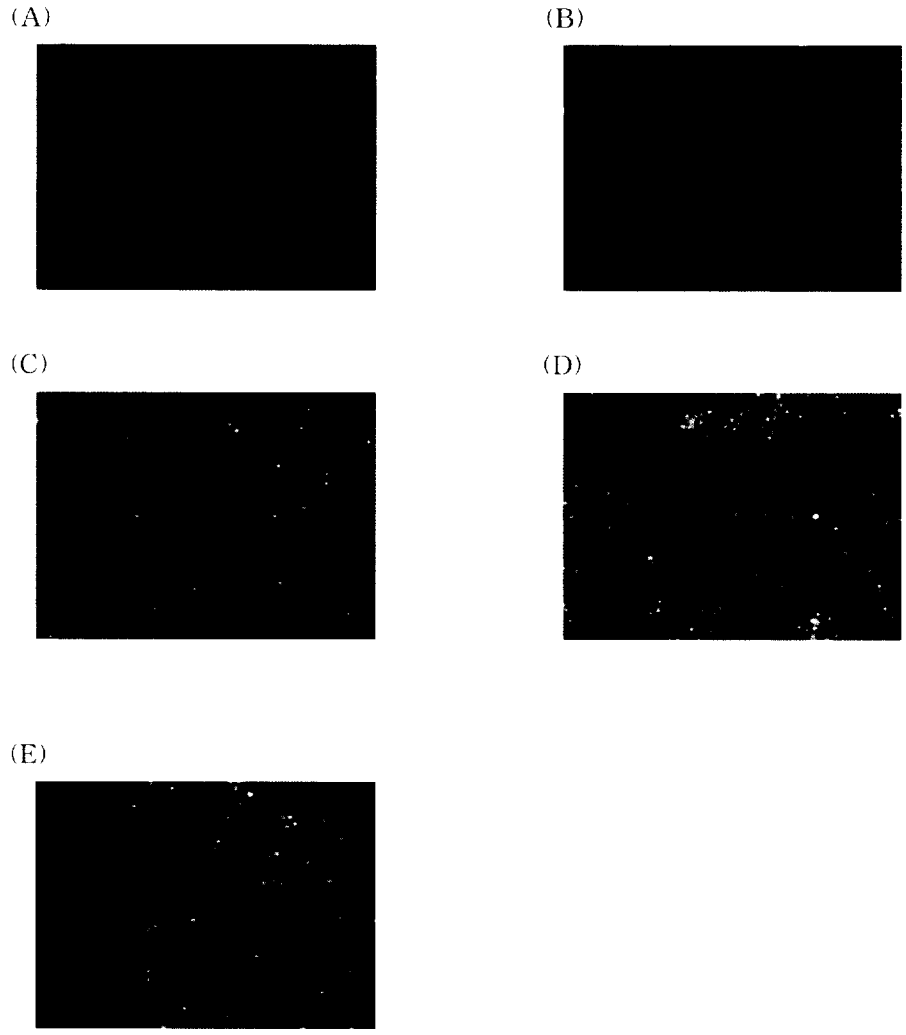


Figure 13. Expression of the GFP gene in transformed *Chlorella vulgaris*.
A; non-transformed *Chlorella*, B - E; *Chlorella* cells transformed with vectors containing CaMV 35S-GFP fusion, the DNA polymerase gene promoter-GFP fusion, the DNA ligase gene promoter-GFP fusion, the chitinase gene promoter-GFP fusion constructs, respectively.

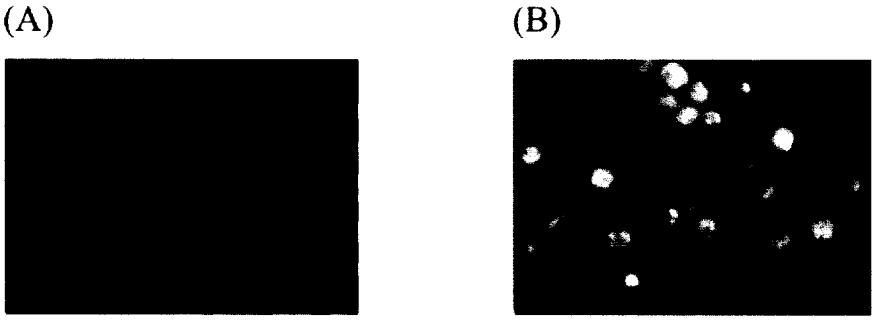


Figure 14. Transformed *Chlorella* cells after selection with phleomycin. (A) Non-transformed *C. vulgaris*, (B) Transformed *C. vulgaris* after selection with phleomycin for 14 days.

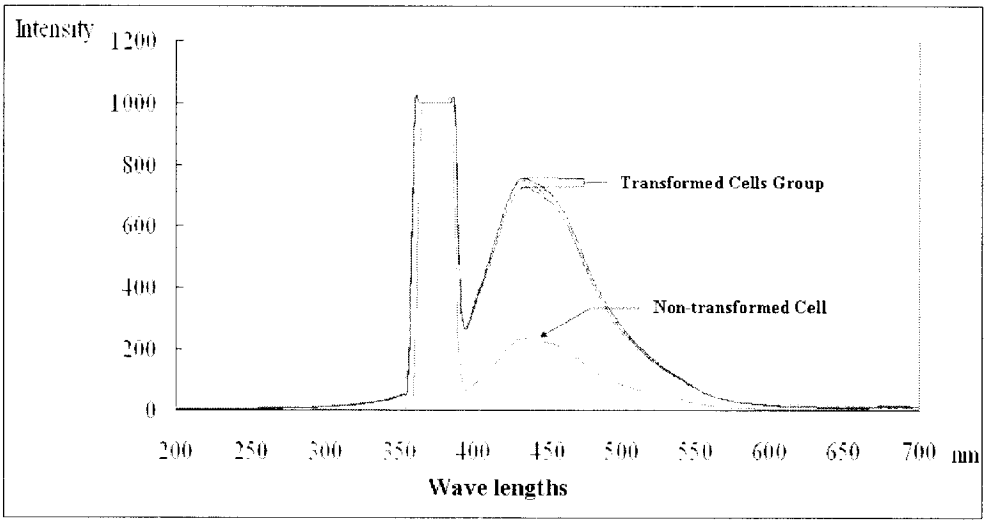


Figure 15. Fluorescence spectrum of *Chlorella* cells transformed with constructs containing the GFP gene fused to promoters from *Chlorella* virus HS-1. The emission spectrums were obtained at the excitation wave length of 375 nm.

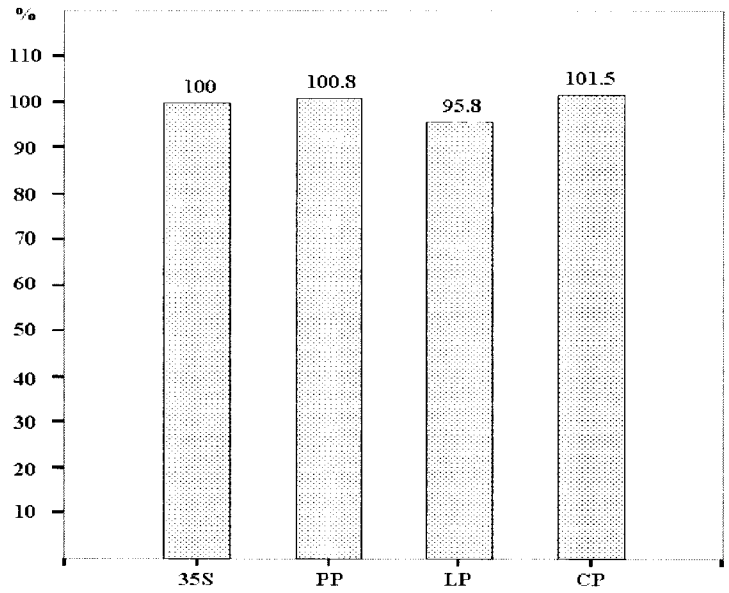


Figure 16. Comparison of the GFP expression in *Chlorella* cells transformed with constructs containing *Chlorella* early gene promoters-GFP gene fusion. The relative GFP intensities was obtained by comparing that values from CaMV 35S-GFP fusion (35S 100%). The fused promoters are indicated. PP, the DNA polymerase gene promoter; LP, the DNA ligase gene promoter; CP, the chitinase gene promoter.

Discussion

Chlorella has many advantages as a new system for foreign protein expression in many aspects. However, the development of this organism as an expression system has been hampered by the lacks of appropriate transformation vector. Recently, the flounder growth hormone has been expressed in transformed *Chlorella* (Kim *et al.*, 2002). The transformed *Chlorella* promoted the growth of juvenile flounder when the protein was indirectly provided to small fish through zooplanktons, which indicated that the expressed has biological activity.

There are several thing to be improved for the application of this system for the commercial production of proteins of pharmaceutical importance, and one of them is development of strong and constitutive promoters. The CaMV 35S promoter has been used in the transformation of microalgae and has been proven to work in them (Mitra *et al.*, 1994; Ying *et al.*, 2001). However, promoters from either *Chlorella* or *Chlorella* virus are desirable because they will use same RNA polymerase and other transcriptional factors in the transformed cell.

Chlorella viruses are large double-stranded DNA virus of about 330-380 kb and contain many useful genes and promoters that can be used in heterologous protein expression. For example, the upstream region of *Chlorella* virus adenine methyltransferase gene functions as a strong promoter both in plants and bacteria (Mitra *et al.*, 1994).

Genes encoded by viruses can be divided into early and late genes which encode proteins involved in virus replication and formation of virus particles, respectively. Early genes of PBCV-1 are expressed in host cells as early as 5-10 min p.i., or immediate early (Schuster *et al.*, 1996). It is known that PBCV-1 does not encode its own DNA dependent RNA polymerase and probably use the enzyme from host (Van Etten, 2003). Therefore, promoters from three early genes of *Chlorella* virus, a DNA polymerase, an ATP-dependent DNA ligase and a

chitinase gene that have been known to be essential for the DNA replication (Van Etten, 2003), were cloned and tested for their activity in transformed *Chlorella*.

Each early gene promoters were amplified using a primer designed specifically, but promoters were amplified not from all of the 8 *Chlorella* virus strains (Table 2). There are two possibilities for this result. One possibility is sequence variation among the viruses. The primers were designed based on the published sequence of PBCV-1. Although they are related viruses, the sequences where the primers are located may not identical, and the target DNA could not be amplified. In fact, restriction enzyme digestion of genomic DNAs purified from *Chlorella* viruses isolated in Korea showed different band pattern, which suggested sequence variation (Cho *et al.*, 2002). Also, amino acid sequence comparison of the A478L ORF from *Chlorella* virus SS-1 and PBCV-1 showed only 51% homology (Cho *et al.*, 2002). The other possibility is the absence of the specific genes in some virus strains. It is know that the chitinase gene does not present in some *Chlorella* virus strains (Van Etten, 2003; Van Etten *et al.*, 2002), although the DNA polymerase and the ATP-dependent DNA ligase genes is know to be essential for virus replication.

Sequence analysis of these early gene promoters showed high sequence homology to the corresponding regions of PBCV-1 genome. Fig. 5-7 shows the cloned promoter regions of *Chlorella* viruses isolated in Korea and those of PBCV-1. In case of the DNA polymerase gene promoter, KH-2, HS-1 and YK showed 99.8%, 96.2% and 95.5% sequence homology to that of PBCV-1, respectively (Fig. 5). The DNA ligase gene promoter regions of KH-1, HS-1 and YK have 100% sequence homology to that of PBCV-1 but the same region of KH-2 was different in one nucleotide out of 419 nt (Fig. 6). In case of the chitinase gene promoter, KH-1, HS-1 and YK were identical to the corresponding region of PBCV-1 but YK-1 was different in one nucleotide out of 418 nt (Fig. 7). One of the *Chlorella* viruses, HS-1 showed the highest sequence homology to

the corresponding regions of PBCV-1 in the three early gene promoters, and promoters from this virus stain were selected to construct *Chlorella* transformation vectors for promoter assay.

A typical TATA box and a common element 5′-ATGACAA-3′ have been identified in the promoter region of almost all of the immediate early genes, which may be involved in the recognition of the promoter by host RNA polymerase and transcription factors (Kawasaki *et al.*, 2004). TATA box is a initiator element as well as well-defined transcription initiation site about 20 to 30 bp downstream to TATA element. In the upstream region, several gene promoters were found to contain positive or negative regulatory elements, some of which were characterized as enhancers or silencers (Tyagi A.K., 2001).

The early gene promoters of HS-1 were analyzed using plant cis-acting regulatory DNA elements (PLACE) database. The results showed that 2 TATA boxes were identified in the DNA polymerase gene promoter, 3 TATA boxes were identified in the DNA ligase gene promoter and 4 TATA boxes were found in the chitinase gene promoter but 5′-ATGACAA-3′ sequence element was not found in all three early gene promoters. In addition to TATA box, binding sites for many transcriptional factors including CAAT box, NTBBF1 box, GATA box and CCAAT box were also present (Fig. 8-10). The NTBBF1 motif found in the chitinase gene promoter is a candidate of binding site for the Dof proteins, which has been found to be responsible for tissue-specific expression of the rolB oncogene in tobacco (Baumann *et al.*, 1999). The CCAAT box is a Myb-binding site and has been reported to be involved in tissue-specific expression of GUS gene in transgenic tobacco (expressed exclusively in meristematic tissue and conductive tissue associated with vascular bundles) (Wissenbach *et al.*, 1993). The presence of many binding site for the transcriptional factors suggests that some of these proteins or related proteins are also present in the *Chlorella* cells, and further analysis for these factors are necessary.

The activity of the promoters were analyzed by the intensity of GFP from transformed cells. As shown in Fig. 15, non-transformed *Chlorella* cells showed autofluorescence. Because of the autofluorescence and low emission level at 488 nm at which the emission spectrum of most fluorescence activated cell sorters (FACS) are fixed, the intensity were measured using a fluorescence microscope. The autofluorescence of non-transformed cells were subtracted from the fluorescence from same number *Chlorella* cells transformed with each construct and selected in the presence of 1ug/ml pheomycin for 14 days. As shown in Fig. 16, promoter activities determined by the fluorescence were 101.5%, 100.8%, 95.8% for the chitinase, the DNA polymerase and the DNA ligase gene, respectively compared to that of CaMV 35S promoter (100%).

Because the size of cloned promoter region was same for the three promoters, there can be several factors affecting the activity of the promoter. One and the most important factor would be sequence of the promoter regions. As shown in Fig. 10, the chitinase gene promoter region contains many sites for transcriptional factors including a NTBBF1 motif that does not present in other promoters. The other possibility is the overlapping of ORF in same or different orientation. The cloned chitinase gene (A181/182R) promoter region of 418 bp contains a small ORF (A180R) of 326 bp and part of a putative promoter for this ORF. It is not clear at this moment whether any transcript is made for the ORF A180R. The influence of this upstream AT rich region could be determined with serial deletion constructs for this region. In contrast, the promoter regions for the DNA polymerase gene (A185R) and the DNA ligase gene (A544R) overlap with putative promoter regions of ORF in opposite direction, A184L and A543L, respectively. The influence of these overlapping promoters running opposite orientation need to elucidated further.

Although the factors affecting the promoter activity have not been clearly analyzed, the present results show that promoters from *Chlorella* virus are active

in transformed *Chlorella*, which could be used in the expression of heterologous proteins in this attactive system.

국문 초록

*Chlorella*는 고등 식물과 물질 대사 경로를 공유할 수 있는 단세포 녹색 조류로, 이들은 외래의 단백질을 발현하기 위한 system으로써 많은 장점을 가지고 있다. 최근 형질 전환된 *Chlorella*로부터 생물학적으로 활성을 가지는 여러 성장 호르몬을 발현하는데 성공하였으며, 이는 이 system이 외래의 단백질을 발현할 수 있는 생물 반응기로서 이용이 가능하다는 것을 제시한다. 이종의 발현 system에서 강력하면서도 지속적인 발현을 유도할 수 있는 프로모터는 목적하는 단백질의 효율적인 발현을 위한 필수 요소이며, 이러한 새로운 프로모터의 개발이 이 실험을 수행하는 목적이다.

*Phycodnaviridae*에 속하는 대표적인 바이러스인 PBCV-1이 encoding하고 있는 유전자는 그것의 발현 시작 시간에 따라 초기 발현 유전자와 후기 발현 유전자로 나뉘어 질 수 있다. 초기 발현 유전자는 바이러스의 복제에 필요한 단백질을 encoding하고 있고 숙주의 전사 기관에 의하여 바이러스가 복제되기 이전에 발현하며, 이러한 이유로 *Chlorella*의 형질전환 벡터를 구축함에 있어 이들 유전자의 프로모터들이 이용될 수 있는 가능성을 제공한다.

국내에서 분리한 8 종의 *Chlorella* 바이러스로부터 DNA polymerase, ATP-dependent DNA ligase 그리고 chitinase 유전자의 프로모터로 예상되는 부분을 PBCV-1 게놈의 서열을 바탕으로 하여 제작한 primer set들을 이용하여 증폭하였다. 이들 초기 발현 유전자 프로모터들의 서열을 분석한 결과 PBCV-1 게놈의 서열과 상당히 일치하였다. 프로모터로 예상되는 이 부분들을 분석을 통하여 TATA box, CAAT box, NTBBF1 box, 그리고 CCAAT box를 포함하여 전사에 영향을 미칠 것으로 보이는 cis-acting element가 많이 존재한다는 것을 확인하였다.

Chlorella 바이러스 HS-1으로부터의 세 가지 프로모터 부분을 녹색 형광 단백질(GFP)의 상부에 결합하여 *Chlorella* 형질전환 벡터를 구축하고 이들을 *Chlorella vulgaris*의 원형질체에 형질전환 하였다. 형광 현미경을 통하여 형질전환된 *Chlorella* 내에서 GFP가 발현한 것을 확인하였다. 형질전환된 세포들은 phleomycin이 함유된 배지에서 배양하여 분리하고 각각의 다른 construct로 형질전환한 세포들을 동일한 수만큼 준비하여 세포 내에서 발현한 GFP의 발현 정도를 측정하여 비교하였다.

CaMV 35S-GFP fusion construct로 형질전환한 *Chlorella* 로부터의 GFP의 발현 정도를 100%로 하였을 때, chitinase, polymerase, ligase gene promoter-GFP constructs에서의 GFP의 발현 정도는 각각 차례대로 101.5%, 100.8%, 95.8%로 나타났다.

이상과 같은 결과를 통하여 *Chlorella* 바이러스의 초기 발현 유전자 프로모터들은 형질전환된 *Chlorella* 내에서 활성을 가지며, *Chlorella* 바이러스는 많은 중요한 유전자를 비롯하여 프로모터들을 제공할 수 있는 유용한 sources임을 보여 주었다.

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지금껏 부모님과 같은 마음으로 사랑과 걱정을 아끼지 않으시고 오늘 여기까지 올 수 있도록 처음 소중한 이 길을 열어주신 최태진 교수님께 진심으로 감사드리며, 부족한 논문을 세심하게 읽어주시고 끝까지 관심을 갖고 지도해주신 이원재 교수님과 김균도 교수님께도 감사드립니다. 대학원 과정동안 이 결실의 밑거름을 만들어주신 김진상 교수님, 이명숙 교수님, 송영환 교수님, 김영태 교수님께도 머리 숙여 감사의 말씀을 드립니다.

짧은 시간이었지만 함께하는 동안 어려움이 있을 때면 큰 힘이 되어준 은영 언니, 밝은 모습과 매사에 최선을 다하는 모습 보여준 현향 언니, 언제나 한결같은 마음으로 항상 따뜻한 웃음 지으며 의지가 되어준 종오 선배, 실험실 생활의 기초를 가르쳐 준 현화 언니와 지연 언니, 실험실의 사소한 부분까지도 세심하게 신경쓰고 특히, 힘든 시간 잘 이겨낼 수 있도록 이끌어준 홍묵 선배와 변거로움 마다않고 끝까지 실험을 도와주신 영욱 선배께도 감사드립니다. 어려움이 있을 때마다 웃음을 잃지 않도록 해준 유쾌한 경규 선배와 재정선배, 친구처럼 가장 가까운 곳에서 함께하며 언제나 용기를 북돋아주고 다독여준 선미언니, 온갖 궂은일 마다않고 뒤에서 묵묵히 큰 도움을 준 성우선배, 동기이지만 많이 신경써주지 못하고 늘 힘든 일 혼자 하게한 다영에게도 고마움을 전합니다. 그리고 마이리스 실험실과 인연을 맺은 처음부터 지금까지 동고동락한 혜진이... 동기란 이름만으로도 힘이 되어준 혜신이에게 고마움과 미안한 마음을 함께 전합니다. 함께한 시간동안 항상 나를 위로하고 웃게 했던 정현이, 한국생활이 낯설고 어렵지만 잘 해내고 있는 Tham, 그리고 귀여운 우리 막내들, 미나, 지은, 기욱이는 앞으로 더 열심히 최선을 다해서 마이리스 실험실의 발전과 함께 큰 사람이 되기를 바랍니다. 대학원 생활동안 많은 조언과 도움을 주신 각 실험실의 선배님들과 후배들의 관심과 배려는 오래도록 기억할 것이며, 특히 대성 선배와 용배 선배에 고맙습니다.

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