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Activity of Early Gene Promoters from a Korean *Chlorella* Virus Isolate in Transformed *Chlorella* Algae

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Abstract As a unicellular green alga that possesses many of the metabolic pathways present in higher plants, *Chlorella* offers many advantages for expression of heterologous proteins. Since strong and constitutive promoters are necessary for efficient expression in heterologous expression systems, the development of such promoters for use in the *Chlorella* system was the aim of this study. Proteins encoded by the early genes of algal viruses are expressed before viral replication, probably by the host transcriptional machinery, and the promoters of these genes might be useful for heterologous expression in *Chlorella*. In this study, putative promoter regions of DNA polymerase, ATP-dependent DNA ligase, and chitinase genes were amplified from eight Korean *Chlorella* virus isolates by using primer sets designed based on the sequence of the genome of PBCV-1, the prototype of the *Phycodnaviridae*. These putative promoter regions were found to contain several *cis*-acting elements for transcription factors, including the TATA, CAAT, NTBBF1, GATA, and CCAAT boxes. The amplified promoter regions were placed into *Chlorella* transformation vectors containing a green fluorescence protein (GFP) reporter gene and the *Sh ble* gene for phleomycin resistance. *C. vulgaris* protoplasts were transformed and then selected with phleomycin. The GFP fluorescence intensities of cells transformed with chitinase, DNA polymerase, and DNA ligase gene promoter-GFP fusion constructs were 101.5, 100.8, and 95.8%, respectively, of that of CaMV 35S-GFP-transformed *Chlorella* cells. These results demonstrate that these viral promoters are active in transformed *Chlorella*.

Key words: *Chlorella*, *Chlorella* virus, *Chlorella* transformation, early gene promoter

Chlorella, a eukaryotic microalga that has been widely used in aquaculture, is potentially an attractive system for overexpression of heterologous proteins for several reasons. As a unicellular green alga possessing many of the metabolic pathways present in higher plants, some complex, posttranslationally modified proteins may be expressed in *Chlorella* in their biologically active forms. Furthermore, *Chlorella* requires only limited amounts of sunlight and carbon dioxide as energy sources, and thus can be easily and economically cultured on a large scale in a pool using sunlight. It also grows relatively fast, dividing 2–9 times per day, depending on the light intensity and temperature [31]. These characteristics provide a rationale for use of *Chlorella* as a bioreactor for heterologous protein expression. Indeed, a transformed *Chlorella* system has recently been used to successfully express a fish growth hormone in its biologically active form [15]. In this report, we describe the isolation of strong, constitutive promoters suitable for use in a *Chlorella* expression system.

The viruses that infect *Chlorella* have large double-stranded DNA genomes of about 330–380 kb. The viral genome encodes many useful proteins, including restriction/modification enzymes, topoisomerase, chitinase, and hyaluronan synthase [5, 17, 32, 43, 47]. Many promoters with potential for use in heterologous protein expression systems are also present on the viral genome. For example, the upstream region of the viral adenine methyltransferase gene functions as a strong promoter in both plants and bacteria [21].

An essential consideration in choosing target promoters is their temporal specificity. Transcription of *Chlorella* viruses can be divided into early and late stages. Early stage genes include immediate early genes and genes that are expressed in host cells as early as 5–10 min post-inoculation. Some early genes of PBCV-1, a related virus, have been found to encode proteins with significant homology to transcription factors, mRNA-processing proteins, DNA helicases, mRNA capping enzymes, factors influencing translational aminoacyl-tRNA synthetases, ribosomal proteins, and enzymes of unknown function [23, 30, 44].

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Because the early genes of algal viruses usually encode proteins required for viral replication, which are probably transcribed by the host transcription system, the promoters of early genes are likely to be recognized by the host transcriptional machinery. The promoters of almost all of the immediate early genes of PBCV-1 contain a typical TATA-box and the common 5'-ATGACAA-3'-element, which may be recognized by the host RNA polymerase and transcription factors [13]. In contrast, late transcription begins 60–90 min post-inoculation, after initiation of viral replication. Late genes generally encode structural proteins.

When the complete genome of PBCV-1, a prototypical *Phycodnaviridae* virus, was analyzed, no DNA-dependent RNA polymerase was found [35]. Combined with the temporal regulation of early and late gene expression, this result suggests that the host RNA polymerase transcribes the late viral genes after modification by viral proteins. In fact, several transcription factor genes have been identified in the PBCV-1 genome [36].

A DNA polymerase, an ATP-dependent DNA ligase, and a chitinase have been identified as the early genes that are essential for infection and DNA replication of algal viruses [35]. In this study, the promoter regions of these genes from Korean isolates of *Chlorella* viruses were amplified and characterized. In addition, the promoters isolated from *Chlorella* virus strain HS-1 were tested for their activity in transformed *Chlorella* using a green fluorescence protein (GFP) reporter gene. The activities of these promoters were compared with the activity of the 35S promoter of cauliflower mosaic virus (CaMV). The latter promoter, which was used to express fish growth hormone in *Chlorella*, is currently the most commonly used promoter in plant transformation systems.

Materials and Methods

Virus Culture and Purification

Chlorella strain NC64A was cultured in modified Bold's basal medium (MBBM) as previously described [4, 40]. *Chlorella* virus strains KH-1, KH-2, SS-1, SS-2, HS-1, YK, YK-1, and YK-2 were previously isolated from fresh water in Korea [2]. For infection, a 100-ml culture of actively growing *Chlorella* NC64A was inoculated with virus at a multiplicity of infection of 0.01–0.001. Cells were incubated until completely lysed, and the lysate was centrifuged in a Sorvall GS-3 rotor at 5,000 rpm for 5 min at 4°C. Triton X-100 was added to the supernatant fraction to a final concentration of 0.1%, and the mixture was stirred for 20 min at 4°C. Virus particles were then pelleted by centrifugation 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°C). The virus band was collected from the 30–40% interface and then pelleted for 3 h at 27,000 rpm in a T-880 rotor. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8 [40].

Isolation and Analysis of Viral Genomic DNA

Isolated virus (400 μl) was mixed with 60 μl of 10× TEN buffer (100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 M NaCl), 60 μl of 1% Na-sarcosyl, 0.6 μl of 60% (w/w) CsCl, and a trace amount of ethidium bromide. The mixture was heated at 75°C for 15 min and then loaded onto a pre-formed 40–60% (w/w) CsCl gradient. The mixture was centrifuged in a Sorvall TH-641 rotor at 35,000 rpm for 18 h at 25°C [37], the DNA band collected, and the ethidium bromide removed by butanol extraction. The DNA was precipitated with ethanol, dried, and resuspended in 1× TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

PCR Amplification of *Chlorella* Virus Early Gene Promoters

The upstream regions of the viral DNA polymerase, DNA ligase, and chitinase genes were amplified by PCR with primers designed based on the published PBCV-1 sequences. The resulting DNA fragments were about 420 bp in length. Amplification was carried out in 50 μl of PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing 2.5 mM dNTPs, 4 μl of Taq DNA polymerase (5 U/μl), and 100 pmol each of the forward and reverse primers. PCR was performed with an initial denaturation step at 94°C for 10 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min), and extension (72°C for 1 min), followed by a final extension (72°C for 7 min). PCR products were analyzed by electrophoresis in a 1.2% agarose gel and visualized with UV light.

Cloning and Sequencing of Amplified Promoters

The amplified PCR products were eluted from the agarose gel using a gel extraction kit (Bioneer, Korea) and cloned into the pGEM-T Easy vector (Promega, U.S.A.) for confirmation by DNA sequencing. XL1-Blue *E. coli* cells were transformed with the ligated DNA, and clones containing the insert were screened with X-gal and isopropylthio-β-D-galactoside. White (positive) colonies were inoculated into LB broth containing ampicillin (100 μg/ml), and plasmid DNA was extracted by the alkaline lysis method.

The plasmid DNA was digested with EcoRI and electrophoresed on a 1% agarose gel. Plasmids of the appropriate size were purified using a plasmid purification kit (Bioneer) and used as templates for sequencing by PCR. The sequencing reaction mixtures consisted of 1,000 ng of template DNA, 4 pmol of T7 primer or SP6 primer for the early gene promoters, and 2 μl of terminator-ready reaction mix. PCR was performed with 25 cycles of denaturation (96°C for 10 sec), annealing (5°C for 7 sec), and extension (60°C for 4 min) in a Mastercycler 9600 (Eppendorf). The sequencing reaction products were precipitated with

ethanol and dissolved in 25 μl of template suppressing reagent (Perkin Elmer). They were then denatured for 2 min at 98°C, cooled on ice, and analyzed with an ABI PRISM™ 310 Analyzer (Perkin Elmer).

Construction of *Chlorella* Transformation Vectors

Chlorella transformation vectors containing the putative viral promoters were constructed [20, 25] by replacing the CaMV 35S promoter sequence in pMinGFP [15] with the amplified viral promoter sequences. The pMinGFP vector contains a green fluorescent protein (GFP) reporter gene, a replication origin for both *E. coli* and *Agrobacterium* (*ori*V), the *npt* kanamycin resistance gene, the *trf a* gene for replication support, the right and left borders of T-DNA for integration, and the *Sh ble* gene for selection of transformants with phleomycin. The early gene promoters were excised from the pGEM-T Easy vector derivatives and cloned into the HindIII-BamHI site of pMinGFP to replace the CaMV 35S promoter. The resulting transformation vectors containing the DNA polymerase, DNA ligase, and chitinase gene promoters were designated pPGFP, pLGFP, and pCGFP, respectively (Fig. 1).

Chlorella Culture

Chlorella vulgaris (Strain #KMCC FC-001) was provided by the Korean Marine Microalgae Culture Center of Pukyong National University. Cells were cultured without shaking in Guillard F/2 medium in which sea water was replaced with fresh water (DW F/2) [7] containing chloramphenicol and streptomycin at 50 μg/ml. Cells were inoculated at an initial concentration of 1×10^6 cells/ml and cultured under fluorescent lamp (3,000 lux) at 25°C with 18:6 h light:dark cycles.

Chlorella Protoplast Preparation

Cells were harvested for protoplast formation when they reached $1-2\times10^8$ cells/ml, usually 8–9 days after inoculation, by centrifugation of 50 μl of culture for 5 min at 3,000 ×*g*. Cells were washed once with 25 mM phosphate buffer (pH 6.0) and suspended in 5 μl of the same buffer containing 0.6 M sorbitol, 0.6 M mannitol, 4% (w/v) cellulase (Calbiochem, U.S.A.), 2% (w/v) Macerase (Calbiochem), and 1% (w/v) pectinase (Sigma). The cell suspension was then incubated at 25°C for 16 h in the dark with gentle shaking.

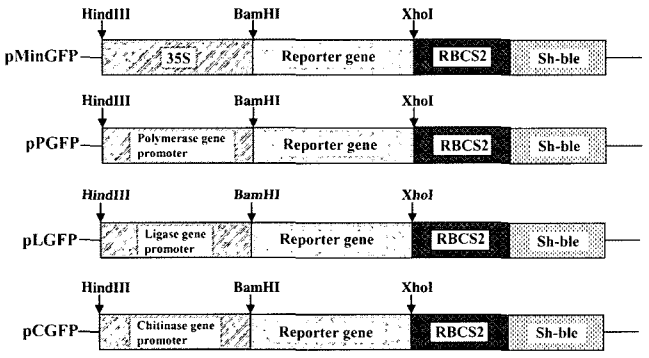

Fig. 1. Plasmid constructs for *Chlorella* transformation.
Early gene promoters from *Chlorella* virus HS-1 were fused to a green fluorescent protein (GFP) reporter gene. The phleomycin resistance gene *Sh ble* was used for selection.

Chlorella Transformation

Protoplasts were centrifuged at 400 ×*g* for 5 min, and the supernatant fraction was decanted. The protoplasts were gently resuspended in 5 μl of DW F/2 medium containing 0.6 M each of sorbitol and mannitol and pelleted by centrifugation at 400 ×*g* for 5 min. The pellet was suspended in 1 μl of a solution containing 0.6 M sorbitol, 0.6 M mannitol, and 0.05 M $CaCl_2$. Protoplasts in 0.4 μl (10^7-10^8 cells) were placed into a new microcentrifuge tube, and 5 μl of vector DNA was added with 25 μl of calf thymus DNA as a carrier (Sigma). After 15-min incubation at room temperature, 200 μl of PNC [0.8 M NaCl, 0.05 M $CaCl_2$, 40% PEG 4000 (Sigma)] was added with gentle mixing. The mixture was incubated for 30 min at room temperature, and then 0.6 μl of DW F/2 medium containing 0.6 M sorbitol, 0.6 M mannitol, 1% yeast extract, and 1% glucose was added. The cells were incubated at 25°C for 12 h in the dark [18]. The transformed cells were transferred to fresh DW F/2 containing 1 μg/ml phleomycin and cultured under a fluorescent lamp (3,000 lux) at 25°C with a 18:6 h light:dark cycle. After 8 days of culture, cells were transferred to fresh medium containing the same concentration of phleomycin.

Assay for GFP Gene Expression in Transformed *Chlorella*

Transformed cells were monitored for GFP expression every 2 days using a fluorescence microscope (Olympus BH2-RFL-T3) with a WB filter. Fourteen days after the initial transfer of cell wall-regenerated protoplasts to DW F/2 medium containing phleomycin, transformed and non-transformed *Chlorella* cells were harvested. Cells were counted five times using a hemacytometer chamber and centrifuged at 400 ×*g* for 5 min. The supernatant was decanted, and the cell pellet was washed once with phosphate buffer (pH 6.0) and resuspended to a final cell count of 3.0×10^6 cells/ml in the same buffer.

GFP expression levels in the resuspended cells were measured using a fluorescence spectrometer (Perkin Elmer LB-500). Fluorescence was measured using 1 ml of cells and 5 samples from each preparation. Preliminary absorbance spectra of non-transformed and transformed cells were obtained to determine the excitation wavelength. Emission spectra were obtained with an excitation wavelength of 375 nm, and GFP intensity values at an emission wavelength of 435 nm were taken for comparison.

RESULTS

Amplification of *Chlorella* Virus Early Gene Promoters

PCR was used to amplify the putative promoter regions of the DNA polymerase, DNA ligase, and chitinase genes from eight *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 fragments of about 420 bp in length. As shown in Fig. 2, all three putative promoter regions yielded DNA products of the expected size from some, but not all, of the eight strains. The DNA polymerase gene promoter region was amplified from five strains (KH-2, SS-1, SS-2, HS-1, and YK), and the promoter regions of the DNA ligase and chitinase genes were amplified from four strains (KH-1, HS-1, YK, and YK-1). Only PBCV-1 and the HS-1 and YK *Chlorella* virus strains yielded PCR products for all three early gene promoters.

Cloning and Sequencing of the Three Early Gene Promoters

When the amplified promoter regions were cloned into the pGEM T-Easy vector and sequenced, they were found to have high sequence similarity to the corresponding regions of the PBCV-1 genome. The DNA polymerase gene promoter sequences from KH-2, HS-1, and YK exhibited 99.8, 96.2, and 95.5% sequence homology, respectively, to that from PBCV-1. For the DNA ligase gene, promoter sequences from KH-1, HS-1, and YK were identical to the sequence of the PBCV-1 promoter, whereas the sequence from KH-2 differed at just one out of 419 nucleotides. The chitinase gene promoter sequences from KH-1, HS-1, and YK were identical to the corresponding region of PBCV-1, whereas YK-1 differed at just one out of 418 nucleotides.

Because the sequences of the three early gene promoters from HS-1 exhibited the highest overall homology to PBCV-1, the HS-1 sequences were selected for use in construction of *Chlorella* transformation vectors. The plant *cis*-acting regulatory DNA elements (PLACE) database was used to identify conserved sequences and transcription factor binding sites (Figs. 3–5). Many transcription factor binding sites were identified, including TATA, CAAT, NTBBF1, GATA, and CCAAT boxes. Two TATA boxes, three CAAT boxes, and five GATA boxes were identified in the DNA polymerase gene promoter (Fig. 3). One CCAAT box, three TATA boxes, five CAAT boxes, and four GATA boxes were identified in the DNA ligase gene promoter (Fig. 4), and one CCAAT box, one NTBBF1 box, four TATA boxes, five CAAT boxes, and four GATA boxes were identified in the chitinase gene promoter (Fig. 5). The three HS-1 early gene promoters did not exhibit any sequence homology to each other (data not shown) besides the TATA, CAAT, and GATA boxes common to all three promoters.

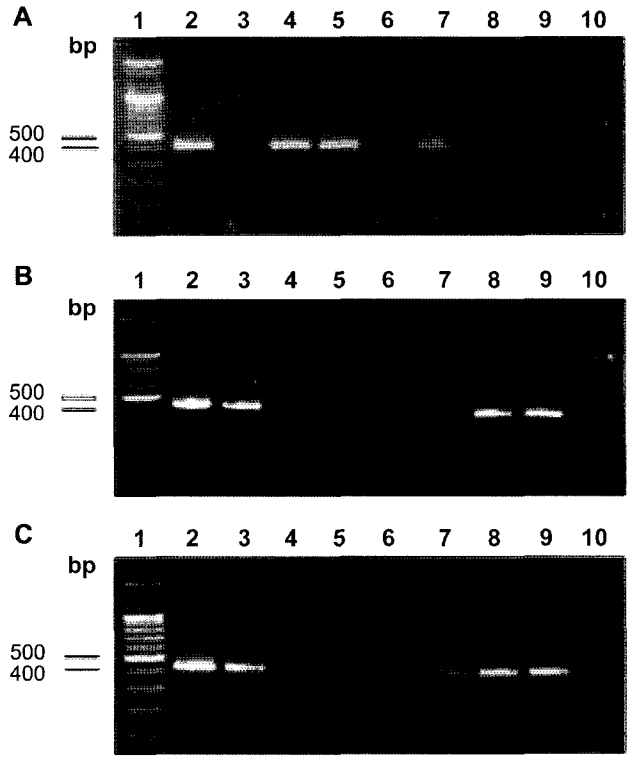

Fig. 2. Amplification of putative promoters of *Chlorella* virus early genes.

The promoters of the early genes for (**A**) DNA polymerase, (**B**) DNA ligase, and (**C**) chitinase were amplified from eight *Chlorella* virus strains and PBCV-1 by PCR, and were subjected to agarose gel electrophoresis. Lane 1, 100-bp DNA ladder; 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.

Fig. 3. The promoter region of the DNA polymerase gene from *Chlorella* virus HS-1.

The AT-rich region is shown as a dotted box, and the locations of conserved boxes are indicated. AAGCTT (HindIII) and GGATCC (BamHI) sites used for cloning are underlined, and the location of the initiation codon (ATG) is indicated. The orientations of the conserved motifs are indicated by (+) and (−).

Fig. 4. The promoter region of the DNA ligase gene from *Chlorella* virus HS-1.
The AT-rich region is shown as a dotted box, and the locations of conserved boxes are indicated. HindIII and BamHI sites used for cloning are underlined, and the location of the initiation codon (ATG) is indicated. The orientations of the conserved motifs are indicated by (+) and (−).

Fig. 5. Analysis of the promoter region of the *Chlorella* virus HS-1 chitinase gene.
The AT-rich region is shown as a dotted box, and the locations of conserved boxes are indicated. HindIII and BamHI sites used for cloning are underlined, and the location of the initiation codon (ATG) is indicated. The orientations of the conserved motifs are indicated by (+) and (−).

Transformation of Protoplasts with Early Gene Promoter/GFP Fusion Vectors

Protoplasts were transformed with pMinGFP derivatives containing the early gene promoters using polyethylene glycol. After cell wall-regenerated protoplasts were initially transferred to DW F/2 medium containing phleomycin, expression of GFP in the transformed *Chlorella* was analyzed by fluorescence microscopy. As shown in Fig. 6, non-transformed *Chlorella* cells were red with autofluorescence, but most of cells from the transformed *Chlorella* displayed the green color typical of GFP, indicating that they were transformed cells.

Measurement of GFP Gene Expression Levels

GFP expression levels were measured using fluorescence spectroscopy (Perkin Elmer LB-500) 14 days after the initial transfer of cell wall-regenerated protoplasts to DW F/2 medium containing phleomycin. The spectra of non-transformed and transformed cells exhibited absorption and emission maxima at 375 and 435 nm, respectively. Therefore, GFP intensities of each sample were measured at these wavelengths. As shown in Fig. 7A, non-transformed *Chlorella* cells exhibited weak fluorescence in the same wavelength range as that of transformed cells, indicating autofluorescence. Fluorescence spectra of *Chlorella* cells transformed with GFP constructs, which were fused to either the CaMV 35S promoter or the HS-1 early gene promoters were rather similar.

GFP fluorescence intensities were also used to compare the activities of the early gene promoters with each other. Each sample was assayed five times, and measurements

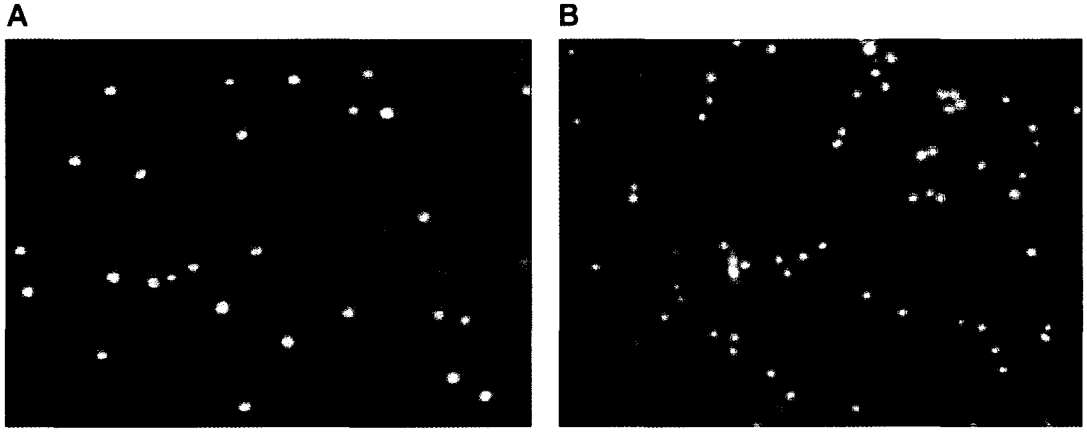

Fig. 6. Expression of GFP in transformed *Chlorella vulgaris*, as shown by fluorescence microscopy.
A. Non-transformed *Chlorella*. **B**. *Chlorella* cells transformed with vectors containing early gene promoter-GFP fusion constructs.

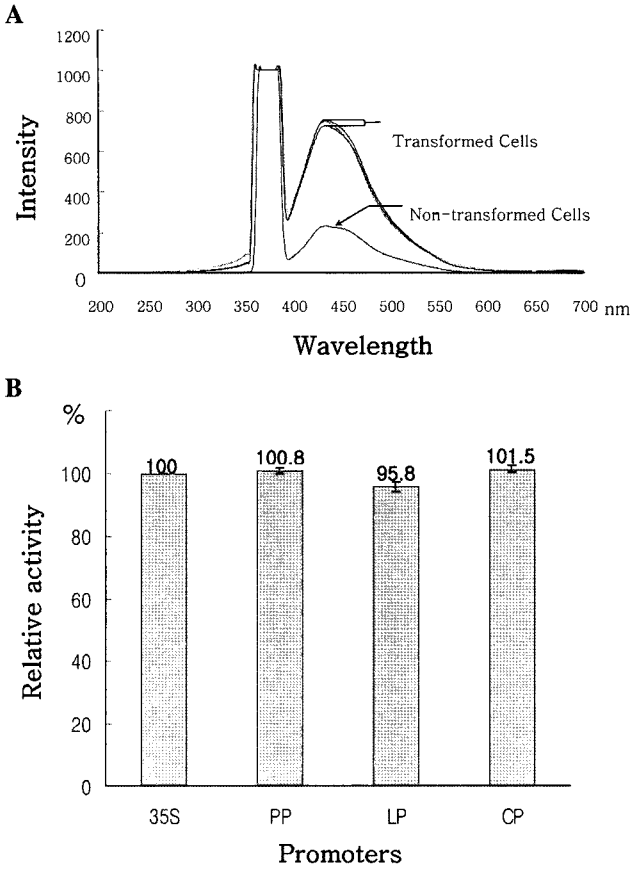

Fig. 7. Expression of the GFP reporter gene in transformed *Chlorella* cells as determined by fluorescence spectroscopy.
Constructs contained a GFP reporter gene fused to early gene promoters from *Chlorella* virus HS-1. **A.** Emission spectra of transformed and non-transformed cells were obtained at an excitation wavelength of 375 nm. **B.** Relative GFP fluorescence of *Chlorella* cells transformed with different promoter-GFP constructs. GFP intensities are shown relative to the values from a CaMV 35S-GFP fusion (35S) with their standard deviation. The other fused promoters were from the DNA polymerase gene (PP), the DNA ligase gene (LP), and the chitinase gene (CP).

were corrected for autofluorescence. For each construct, intensities from transformed cells were calculated relative to the intensity from CaMV 35S-GFP-transformed cells, and the mean relative intensities for each construct were obtained.

Although no significant differences in GFP expression were observed for the various constructs (Fig. 7B), the chitinase and DNA polymerase gene promoters were slightly more active (101.5 and 100.8%, respectively) than the CaMV 35S control (100%). The DNA ligase gene promoter was slightly less active, with a relative intensity of 95.8%.

Discussion

Chlorella offers many advantages as an expression system for heterologous proteins. However, development of this expression system has been hampered by the lack of appropriate transformation vectors. Recently, flounder growth hormone was expressed in transformed *Chlorella* [15]. The transformed *Chlorella* promoted the growth of juvenile flounder when the protein was indirectly provided to small fish through zooplanktons, demonstrating that the expressed protein retained its native biological activity.

Several aspects of the *Chlorella* system have yet to be optimized for commercial production of therapeutic proteins. In particular, strong constitutive promoters are needed. Although the CaMV 35S promoter has proven successful for transformation of microalgae and subsequent expression [22, 45], promoters native to *Chlorella* or *Chlorella* virus would be preferable for expression in *Chlorella*. The *Chlorella* viruses have large double-stranded DNA genomes of about 330–380 kb that encode many genes and promoters that are, or may be, useful for heterologous protein expression. For example, the upstream region of the *Chlorella* virus adenine methyltransferase gene functions as a strong promoter in both plants and bacteria [21].

The early and late *Chlorella* virus genes encode proteins involved in viral replication and in formation of virus particles, respectively. The early genes of PBCV-1 are expressed in host cells as early as 5–10 min post-inoculation [30]. Since PBCV-1 does not encode its own DNA-dependent RNA polymerase, the host polymerase probably transcribes the immediate early genes [35]. Therefore, in the present study, promoters from three early genes of *Chlorella* virus were amplified using specifically designed primer sets, cloned, and tested for their activity in transformed *Chlorella*. These genes encode a DNA polymerase and an ATP-dependent DNA ligase that are essential for viral replication, and a chitinase that is involved in host cell wall degradation [35].

Some of the eight *Chlorella* virus strains used as templates for amplification did not yield amplified products, probably due to sequence differences between the target promoters and the corresponding regions of the PBCV-1 genome, which was used in designing primer sets. This supposition is supported by restriction analysis of the Korean *Chlorella* virus genomes, which yield different band patterns, suggesting sequence variation [2]. Furthermore, the amino acid sequences of ORF A478L from *Chlorella* virus strains SS-1 and PBCV-1 are only 51% homologous [2]. Alternatively, some of the target genes may be absent in those virus strains that did not yield amplified products. Although the DNA polymerase and ATP-dependent DNA ligase genes are essential for viral replication, some *Chlorella* virus strains are known to lack the chitinase gene [35, 42].

All of the successfully amplified early gene promoters showed extremely high sequence homology to their corresponding regions on the PBCV-1 genome. The promoters from strain HS-1 exhibited the highest overall sequence

homology to those of PBCV-1. Therefore, the HS-1 promoters were selected for use in constructing *Chlorella* transformation vectors for promoter activity assays.

A typical TATA box, which defines a transcription initiation site 20–30 bp downstream, and the common element 5'-ATGACAA-3' have been identified in the promoter regions of almost all immediate early genes, where they may be important in promoter recognition by the host RNA polymerase and transcription factors [13]. The early gene promoter regions from HS-1 were analyzed for these and other positive or negative regulatory elements, some of which are characterized as enhancers or silencers [33], using the plant *cis*-acting regulatory DNA elements (PLACE) database. In the promoters of the HS-1 DNA polymerase, DNA ligase, and chitinase genes, 2, 3, and 4 TATA boxes were found, respectively. The 5'-ATGACAA-3' sequence element was not found in any of the three HS-1 early gene promoters. Several other binding sites for transcription factors were found, including CAAT, NTBBF1, GATA, and CCAAT boxes (Figs. 3–5). The NTBBF1 motif found in the chitinase gene promoter is a candidate binding site for Dof proteins, which have been found to be responsible for tissue-specific expression of the *rolB* oncogene in tobacco [1]. The CCAAT box is a Myb-binding site that has been reported to be involved in tissue-specific expression of the *GUS* gene in transgenic tobacco [46]; it is expressed exclusively in meristematic tissue and conductive tissue associated with vascular bundles. The presence of many known transcription factor binding sites suggests that some of these transcription factors or related proteins are also present in *Chlorella*. Further study will be required to identify the factors present.

Promoter activities were assessed by measuring the fluorescence intensity of GFP expressed in transformed cells. As shown in Fig. 7A, non-transformed *Chlorella* cells exhibited autofluorescence. Owing to this autofluorescence and the low level of emission at 488 nm, which is the fixed detection wavelength of most fluorescence-activated cell sorters (FACS), intensity was measured using a fluorescence microscope. Fluorescence measurements were made after transformed *Chlorella* cells were selected in the presence of 1 μg/ml phleomycin for 14 days, and fluorescence values were corrected for autofluorescence by subtraction of the fluorescence of an equivalent number of non-transformed cells. As shown in Fig. 7B, promoter activities determined by this method were 101.5, 100.8, and 95.8% for the HS-1 chitinase, DNA polymerase, and DNA ligase genes, respectively, relative to the CaMV 35S promoter (100%). Because the same number of cells were used for the measure of fluorescence values, and previous genomic Southern blot analysis of transformed *Chlorella* cells showed that one copy of introduced DNA is present in the genomic DNA of these cells [15], the difference in fluorescence values are probably due to the relative activity of the promoters.

The slight variations in promoter activity were not due to variations in length, since the lengths of the cloned promoter regions were the same for all three promoters. Differences in nucleotide sequence were likely to have caused much of the variability. As shown in Fig. 5, the chitinase gene promoter region contains many transcription factor binding sites, including an NTBBF1 motif that is not present in the other promoters. Overlapping, parallel, or antiparallel ORFs may also play a role in activity. The cloned chitinase gene (A181/182R) promoter region of 418 bp contains a small ORF (A180R) of 326 bp with a part of the putative promoter, but whether any transcript is made for this ORF is not known. Serial deletion constructs could be used to assess the influence of this upstream AT-rich region. The promoter regions for the DNA polymerase gene (A185R) and the DNA ligase gene (A544R) overlap with putative promoter regions of ORFs A184L and A543L, respectively. These ORFs are antiparallel to the polymerase and ligase genes, respectively, and their influence remains to be elucidated. Despite the lack of detailed knowledge about factors affecting promoter activity, the present results clearly demonstrate that *Chlorella* virus promoters are active in transformed *Chlorella*. These promoters should prove useful for expression of heterologous proteins in this attractive system.

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