Heterologous Gene Expression System Using the Cold-Inducible CnAFP Promoter in Chlamydomonas reinhardtii

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To increase the availability of microalgae as producers of valuable compounds, it is necessary to develop novel systems for gene expression regulation. Among the diverse expression systems available in microalgae, none are designed to induce expression by low temperature. In this study, we explored a cold-inducible system using the antifreeze protein (AFP) promoter from a polar diatom, Chaetoceros neogracile. A vector containing the CnAFP promoter (pCnAFP) was generated to regulate nuclear gene expression, and reporter genes (Gaussia luciferase (GLuc) and mVenus fluorescent protein (mVenus)) were successfully expressed in the model microalga, Chlamydomonas reinhardtii. In particular, under the control of pCnAFP, the expression of these genes was increased at low temperature, unlike pAR1, a promoter that is widely used for gene expression in C. reinhardtii. Promoter truncation assays showed that cold inducibility was still present even when pCnAFP was shortened to 600 bp, indicating the presence of a low-temperature response element between –600 and –477 bp. Our results show the availability of new heterologous gene expression systems with cold-inducible promoters and the possibility to find novel low-temperature response factors in microalgae. Through further improvement, this cold-inducible promoter could be used to develop more efficient expression tools.

Keywords: Cold-inducible expression system, antifreeze protein, truncated promoter, Chlamydomonas reinhardtii, Gaussia luciferase, fluorescent protein

Introduction

Microalgae are potential sustainable and natural feedstocks for future industries, with applications in the production of biodiesel, pharmaceuticals, and cosmetics [1-3]. Despite numerous attempts to optimize culture conditions and increase the productivity of valuable compounds [4, 5], the use of wild-type strains places limits on the variety of compounds that can be produced, and the productivity of these strains is also bounded [6]. Therefore, the improvement of production strains is required, and the development of novel methods to regulate gene expression has become an important consideration [1, 7].

Gene promoter regions are important for the initiation of gene expression. Several types of promoters have been developed to express heterologous genes in microalgae [8]. In Chlamydomonas reinhardtii, a model green microalga, promoter systems that regulate gene expression are particularly well developed and include the promoters of β-2-tubulin [9], HSP70A [10], RbcS2 [11], AR1 [10], and Lhcb-1 [12]. The AR1 promoter, which is a chimera of the HSP70A and RbcS2 promoters, has been used widely due to its high constitutive gene expression [10, 13]. However, the AR1 promoter cannot be used in expression systems where gene expression levels need to be modulated according to specific conditions. Unlike constitutive promoters, inducible promoters regulate gene expression when cells are exposed to specific conditions. In C. reinhardtii, the high-temperature–inducible promoter (HSP70A) and light-inducible promoter (LIP) are activated under heat and high light stress conditions, respectively [14, 15]. The HSP70A promoter is activated at 40°C, and the LIP promoter is activated by exposure to more than 500 μmol photons m–2 s–1.

Generally, high temperature caused by heat or high light stresses can affect the stability of protein structures and protein solubility. In the case of bacterial systems, cold-inducible promoters such as the cold-shock protein A (cspA) promoter are used to overexpress proteins at low temperature, thereby increasing protein solubility and stability [16]. However, cold-inducible promoters that can be used in C. reinhardtii have not yet been developed [17]. Recently, there have been attempts to produce useful recombinant proteins such as human growth factor, interferon β, or proinsulin using microalgal [18-20], so the development of cold-inducible expression systems may be necessary. Given its successful application in bacterial systems, the development of a vector system that...
uses a cold-inducible promoter might increase the efficiency of protein production in microalgae.

Antifreeze protein (AFP), which acts as antifreeze in some organisms, is an important defense mechanism against cold stress in organisms living in the polar regions [21, 22]. In our own previous research, the AFP sequence of polar diatom Chaetoceros neogracile was found using an expressed sequence tag database [23, 24]. In addition, its promoter sequence was obtained in the 5' upstream region of the AFP gene through inverse PCR [24, 25]. Interestingly, the protein expression of AFP was enhanced in response to temperature and light stresses [24–26]. Therefore, the promoter of CnAFP is thought to be useful for controlling heterologous gene expression in microalgae through temperature downshifts.

In this study, we aimed to develop a cold-inducible vector system using the AFP promoter of C. neogracile (pCnAFP) in C. reinhardtii. First, we obtained the promoter sequence from the genomic DNA of C. neogracile and constructed vectors containing either the Gaussia luciferase gene (GLuc) or the mVenus fluorescent protein gene (mVenus). Each reporter system was introduced into C. reinhardtii to confirm the regulation of expression by pCnAFP. Low-temperature responsiveness of these vectors was confirmed from the quantitative real-time PCR of GLuc and mVenus, and the luciferase assay of GLuc. Furthermore, we confirmed the minimum length of the cold-inducible promoter through a progressive truncation assay, and this work therefore supports the potential application of cold-inducible gene expression in C. reinhardtii.

Materials and Methods

Algal Strain and Standard Culture Conditions

The green microalga Chlamydomonas reinhardtii CC–4349 (cw15 mt+) was cultured in Tris-acetate-phosphate (TAP) [27] medium with shaking on an orbital shaker at 90 rpm under continuous white fluorescent light (75 ± 10 μmol photons/m² s⁻¹) at 25°C. To select transformants, cells were grown on solid TAP medium plates containing 1.5% agar with hygromycin B (25 μg/ml).

Isolation of the CnAFP Promoter and Prediction of Transcriptional Regulatory Elements

Based on the promoter region data on the CnAFP gene described in our previous paper [25], we obtained the promoter sequence through PCR from the genomic DNA of C. neogracile using a gene-specific primer set. The promoter fragment was cloned into the pBlunt-TOPO vector (MG Blunt TOPO Cloning Kit, Cancer Rop Co., Korea). The sequence of the cloned promoter region was verified by Sanger sequencing (Macrogen, Korea). The primers used for the isolation of the 1,225 bp CnAFP promoter and sequencing are listed in Table S1. In order to predict the transcriptional regulatory elements, the promoter sequence was investigated by using the following analysis tools: PLACE [28], PlantPAN [29], PlantCARE [30], and Softberry [31].

Vector Construction

We used the previously reported pChlamy3_GLuc as the vector backbone [32]. It was based on the pChlamy3 vector from the GeneArt Chlamydomonas Engineering Kit (Life Technologies, USA) and was designed to express the codon-optimized Gaussia luciferase (GLuc) gene according to the linked promoter sequence. As a selective marker of the pChlamy3 vector, a hygromycin resistance gene provides stable resistance against hygromycin B, a commonly used antibiotic [33]. Also, the GLuc was selected as a reporter protein to confirm the functionality of the constructed vector in C. reinhardtii [34]. The PCR products of the CnAFP promoter were inserted between the SpeI and KpnI sites of the MCS2 site. To express the fluorescent protein mVenus, the CnAFP promoter was cloned into the pChlamy3_mVenus vector by fragment replacement. The primers used to generate expression vector constructs are listed in Table S1.

Generation of Transgenic Chlamydomonas

Expression vectors were introduced into C. reinhardtii by electroporation following the protocol provided with the GeneArt Chlamydomonas Engineering Kit with slight modifications. The cells were grown to early exponential phase in TAP medium and 1 ml of culture (5 × 10⁵ cells) was harvested by centrifugation at 2,000 × g for 2 min. The cell pellet was resuspended in 0.25 ml of TAP medium containing 40 mM sucrose, to which vector DNA linearized by SpeI was added. The mixture was transferred into an electroporation cuvette (4 mm) and incubated for 5 min at room temperature. Electroporation was conducted with the selected parameters (600 V, 50 μF, and 200 Ω) using the Bio-Rad Gene Pulser X cell apparatus (Bio-Rad, USA). Immediately after electroporation, the cuvette was cooled on ice for 5 min. Then, the cells were transferred to 10 ml round-bottom tubes with up to 2 ml of TAP medium and incubated for more than 12 h at 25°C in the dark. After incubation, the cells were mixed with 2 ml of melted 0.5% agar cooled to 45°C, and then spread on solid TAP plates containing 25 μg/ml hygromycin. Putative transgenic cells formed colonies after 7–10 days and were tooth-picked to small volumes of liquid TAP medium. Insertion of transformed genes was confirmed by colony PCR according to a previous paper [15].

RNA Extraction and Quantitative Real-Time PCR

Transformants grown to mid-exponential phase under standard culture conditions were incubated in the dark for 6 h to remove the effects of other stress stimuli. Then, they were transferred to dark and low-temperature conditions (20°C, 10°C, or 0°C). After 2 h, 5 ml of low-temperature-treated cells was harvested and frozen to extract total RNA using the RNaseasy Plant Mini Kit (Qiagen, Germany). cDNA synthesis was performed using reverse transcription with 2x reverse transcription master premix (ELPIS Biotech, Korea). Synthesized cDNA was amplified using SYBR premix (Takara, Japan) and the Thermal Cycler Dice Real Time System TP 8200 (Takara).
The mRNA levels of GLuc and mVenus were compared between samples and normalized to that of the receptor of activated protein C kinase 1 (RACK1) gene. Results were analyzed by the ΔΔCt method. Sequences of primers used for quantitative real-time PCR are listed in Table S1.

**Luciferase Activity Assay**
Each transgenic *C. reinhardtii* sample in the mid-exponential phase was prepared with the same cell density based on absorbance (OD750nm = 1.0). The cells were incubated in the dark for 12 h to remove the effects of other stress stimuli, and then transferred to dark and low-temperature conditions (20°C, 10°C, or 0°C). After low-temperature treatment, cells were harvested by centrifugation at 13,000 × g for 2 min. Luciferase assays were performed using a Renilla Luciferase Assay Kit (Promega, USA) with a modified protocol [15]. Cell pellets were resuspended in 100 μl of lysis buffer and vortexed vigorously for 3 min. After centrifugation at 13,000 × g for 5 min (4°C), 90 μl of supernatant and 10 μl of 1× luciferase substrate were mixed in a new 1.5 ml tube. Immediately after mixing, the luminescence was measured using a GloMax 20/20 (Promega).

**Fluorescence Microscopy Analysis**
Representative transformants were cultivated in TAP medium and exposed to low temperature (10°C) as above. Live cells were dropped on a glass slide and covered with a coverslip. Fluorescence of mVenus was detected using green fluorescence under a Nikon Eclipse Ni fluorescence microscope (Nikon, Japan). Fluorescence detection wavelengths were 540 ± 20 nm with the YFP filter for mVenus and 630 ± 30 nm with the Texas RED filter for chloroplast auto-fluorescence.

**Results and Discussion**

**Response of the CnAFP Promoter Under Low-Temperature Conditions**
With the pCnAFP_GLuc vector (Fig. 1A), we transformed *C. reinhardtii* and isolated the putative transformants that formed colonies on a solid agar plate containing hygromycin B (25 μg/ml). We then used the collected colonies to perform PCR with diverse primer sets to verify vector insertion and confirm the presence of bands of expected sizes (Figs. 1B and 1C). This analysis confirmed that we had obtained the putative transformants with the pCnAFP_GLuc vector inserted into the genomic DNA of *C. reinhardtii*.

To determine the appropriate experimental methods and conditions to detect cold inducibility in transgenic *C. reinhardtii*, we conducted transcriptional analysis and enzyme activity assays. First, we observed the levels of...
GLuc expression and luciferase activity at several temperature conditions to determine the cold-inducible temperature of the pCnAFP vector. Exposure to 10°C induced the highest level of GLuc gene expression, with a 2.6-fold increase in comparison with 25°C (Fig. 2A). This pattern was similar to the result of the luminescence signal that indicates a readout for GLuc enzyme activity, in which the relative luminescence level increased about 1.5-fold relative to 25°C when the transformants were exposed to 10°C (Fig. 2B). On the basis of these results, we chose 10°C as the temperature for confirming the cold inducibility of the CnAFP promoter in further experiments.

Next, we examined time-dependent changes in the levels of GLuc mRNA expression and luminescence to choose the optimal sampling time point for assessing the cold inducibility of pCnAFP. The mRNA level gradually increased after a temperature shift to 10°C and was more than 2.6-fold the control level at 2 h (Fig. 2C). This transcriptional pattern controlled by pCnAFP in C. reinhardtii was similar to the results of previous studies [24, 25], suggesting the cold inducibility of pCnAFP in C. neogracile. The relative mRNA level of GLuc increased sharply at the 4 h and 8 h time points (Fig. 2C), but this increase of relative value was influenced by the expression level of reference gene (RACK1) decreasing 70% from the initial time point (Fig. S1). Therefore, it was confirmed that the 2 h time point is suitable for comparing cold inducibility in the mRNA expression of GLuc.

Meanwhile, the luminescence signal did not show cold inducibility in a short time (Fig. 2D). The luminescence signal gradually decreased until 4 h and then increased at 8 h and 12 h. Compared to 4 h and 12 h, the luminescence signal appears to increase 1.8-fold, but only 1.3-fold compared to 0 h and 12 h. We investigated the GLuc activity for up to 24 h after exposure at 10°C, but the luminescence level did not increase after 12 h (data not shown). As shown in Fig. 2, the increase of luminescence level occurred 6 h after the transcription level induction. This might be related to the time spent from transcription to translation. In addition, it is reported that short-term and long-term cold stresses affect C. reinhardtii in diverse aspects [35, 36]; particularly, they cause cell growth inhibition, membrane damage, and downregulation of some ribosome-related pathway. Therefore, cold stress may also affect protein synthesis, degradation, and secretion of GLuc in C. reinhardtii [37]. Consequently, the luciferase assay was not suitable for clear confirmation of cold inducibility in a short time, and therefore it was excluded from the following cold-inducibility test with truncated promoters.

**Comparison of the CnAFP Promoter with the ARI Promoter**

We assessed the strength of pCnAFP by comparing it to the ARI promoter (pARI), a well-known constitutive promoter for C. reinhardtii transformation. The sequences of both vectors were identical except for the promoter regions. GLuc expression driven by pCnAFP was 25% of the level of pARI (Fig. 3A). Like other heterologous promoters, pCnAFP has lower gene expression than pARI, a strong endogenous promoter of C. reinhardtii [38]. However, GLuc mRNA expression in the pCnAFP_GLuc transformants increased 2.6-fold at 10°C compared to...
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25°C, unlike that in pAR1_GLuc, which did not change (Fig. 3B). These results indicate that pCnAFP has low strength, but it can be used in a cold-inducible system unlike pAR1.

Progressive Truncation Assay of the CnAFP Promoter

To apply an expression vector system more effectively, vector optimization is necessary. Considering that pAR1 has high and constitutive expression levels in C. reinhardtii after being optimized [10], it might be possible to improve pCnAFP through sequence optimization. However, two low-temperature response element (LTRE) motifs (CCGAAA) that have been reported in the COR15a [39] and BN115 promoters [40, 41] of higher plants were predicted at the end of the 5′ region of pCnAFP (Table S2). Therefore, it was necessary to confirm cold inducibility in the presence of the LTRE regions. A 900 bp pCnAFP segment, which contained no LTRE regions, was cloned and investigated for low-temperature responsiveness. The level of GLuc expression driven by the 900 bp pCnAFP responded to low temperature similarly to that of the 1,225 bp pCnAFP segment (Fig. 4), although the fold increase of 900 bp pCnAFP was reduced from that of 1,225 bp pCnAFP. This result indicates that the cold inducibility of pCnAFP might be influenced by other transcriptional regulatory elements in addition to the LTRE motifs.

Next, we further truncated pCnAFP to determine the minimum length of the cold-inducible promoter. First, we made two variants of GLuc expression vectors; a 600 bp pCnAFP variant and a 300 bp pCnAFP variant. The 600 bp pCnAFP variant showed a significant increase in GLuc expression levels following low-temperature treatment, whereas the levels of transcription driven by the 300 bp variant remained unchanged at 10°C (Fig. 4). Since the gene expression driven by the 300 bp pCnAFP did not increase at 10°C, we expected that the unknown transcriptional regulatory elements related to cold/freezing conditions were located between –300 and –600 bp. However, low-temperature response elements were not predicted between –300 and –600 bp in the PLACE, PlantPAN, and PlantCARE databases (Table S2). To identify the unknown transcriptional regulatory elements in this region, the pCnAFP sequence was analyzed further with the TSSP (Recognition of human Pol II promoter...
region and start of transcription) tool using the RegSite Plant database on the Softberry web server [31]. Two regions similar to soybean embryo factor-4 binding sites (SEF4-BS: RTTTTR) were found at –497 bp and –547 bp of pCnAFP. In higher plants, SEF4-BS is a consensus sequence found in the promoter region of beta-conglycinin, which is involved in chilling stress response during soybean germination [42]. Thus, this region was expected to have transcriptional regulatory elements for cold inducibility.

Based on the predicted result, we made a GLuc expression variant that truncated the SEF4-BS-like region (477 bp pCnAFP), and the RNA expression in the transformants of 477 bp pCnAFP did not increase at 10°C (Fig. 4). This result showed that the SEF4-BS-like region could be involved in the cold inducibility of pCnAFP. However, the function of the SEF4-BS-like region and the factors interacting with it in microalgae are unknown, and further investigation is needed to verify that this region is the exact cold-inducible transcriptional regulatory

Fig. 5. Expression of pCnAFP_mVenus in C. reinhardtii under low-temperature conditions. (A) Vector backbone of pChlamy3_Empty_mVenus (left) does not have a promoter sequence in front of the mVenus fluorescent protein gene (mVenus). Vector backbone of pCnAFP_mVenus (right) with either 1,225 bp pCnAFP or 600 bp pCnAFP. (B and C) Confirmation of vector insertion in transformants by colony PCR with two primer sets (B, 467 bp for hygromycin resistance gene; C, 1,519 bp for the region connecting pCnAFP and mVenus). Lanes 1: plasmid positive for pCnAFP 1,225 bp_mVenus, lanes 2: plasmid positive for pCnAFP 600 bp_mVenus, lanes 3: wild-type C. reinhardtii, lanes 4–6: pCnAFP 1,225 bp_mVenus transformants, lanes 7–9: pCnAFP 600 bp_mVenus transformants. (D) Changes in the relative levels of mVenus mRNA in response to low temperature (10°C). Cold inducibility was confirmed by calculating mRNA levels, which are relative to those at 25°C. All experiments were conducted in more than triplicate. (E) Fluorescent images of transformants after low-temperature treatment (0h, 4h, and 8h). Merged images of mVenus (emission at 537–559 nm, excitation at 502–522 nm) and chlorophyll fluorescence (emission at 603–648 nm, excitation at 563–588 nm) are shown.
element in microalgae. Considering that gene expression in response to low temperature is regulated by the interaction of diverse transcriptional regulatory elements in higher plants [43], it is necessary to confirm which elements interact with SEF4/BS-like regions. Introducing longer vectors tends to be more difficult in comparison with shorter vectors, and 600 bp pCnAFP might be an effective cold-inducible promoter. Therefore, 600 bp pCnAFP was used for further comparative experiments as an optimized vector.

**Expression of Fluorescent Protein Using the CnAFP Promoter**

To verify the expression of diverse heterologous genes other than GLuc, we made another vector encoding mVenus, which is a widely used fluorescent protein, as a reporter protein in *C. reinhardtii* [44, 45]. We made two pCnAFP vectors (1,225 bp and 600 bp) and a pARI vector (positive control) through conjugation of the promoter–mVenus cassette to the pChlamy3 backbone (Fig. 5A). As with GLuc, we confirmed vector insertion in transformants selected from solid agar plates through colony PCR (Figs. 5B and 5C). The low-temperature responsiveness of the vectors was investigated by examining changes in mRNA levels and by fluorescence microscopy analysis. To determine mRNA levels, cells were collected at 0 h and 2 h after shifting from 25°C to 10°C. The patterns of changes in the relative expression levels of mVenus were clearly distinct between pCnAFPs and pARI (Fig. 5D). Similar with the GLuc results, the pCnAFP transformants showed an increase in gene expression over time, whereas the pARI transformants showed a decrease. However, the protein expression was not consistent with mRNA expression patterns. Although the fluorescent signal of the mVenus protein was detected in some cells of transformants, we could not confirm an increase of fluorescence according to the passage of time (Figs. 5E and S2). As in the case of GLuc, the mVenus protein synthesis in *C. reinhardtii* may have been affected by downregulation of some ribosome-related pathway at low-temperature conditions [36].

Moreover, protein synthesis regulated by pCnAFP can be affected by various other factors, such as mRNA stability at low temperature [46] and low promoter strength of pCnAFPs (Fig. 3A). Therefore, this cold-inducible promoter needs to be improved in terms of efficiency in consideration of these factors.

**Conclusion**

In this work, we made a new cold-inducible expression vector using the promoter sequence obtained from the polar diatom Chaetoceros neogracile. The cold-inducibility of this CnAFP promoter was confirmed by analysis of the mRNA expression of reporter genes (GLuc and mVenus) in *Chlamydomonas reinhardtii*. Consequently, our study demonstrates the availability of a cold-inducible heterologous promoter in *C. reinhardtii*, and also suggests a promising new transcriptional regulatory element responsive to low temperature. However, the strength and sensitivity of CnAFP promoter are not sufficient to regulate the protein expression in *C. reinhardtii*, therefore, further improvement is necessary through fusion with a stronger promoter or insertion of multiple key transcriptional regulatory elements.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

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