FEBS Open Bio 3 (2013) 11–15

journal homepage: www.elsevier.com/locate/febsopenbio

Establishment of the reporter system for a thylakoid-lacking cyanobacterium, *Gloeobacter violaceus* PCC 7421

Mie Araki, Yuichiro Shimada[1], Mamoru Mimuro, Tohru Tsuchiya[*]

Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 20 September 2012
Received in revised form 4 November 2012
Accepted 10 November 2012

Keywords:
Cyanobacteria
Luciferase
RSF1010
Transformation
Gloeobacter violaceus PCC 7421

ABSTRACT

***Gloeobacter violaceus* PCC 7421 is considered, by molecular phylogenetic analyses, to be an early-branching cyanobacterium within the cyanobacterial clade. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks thylakoid membranes. There is only one report on the development of a transformation system for *G. violaceus* [H. Guo, X. Xu, Prog. Nat. Sci. 14 (2004) 31–35] and further studies using the system have not been reported. In the present study, we succeeded in introducing an expression vector (pKUT1121) derived from a broad-host-range plasmid, RSF1010, into *G. violaceus* by conjugation. The frequency of transformation of our system is significantly higher than that described in the previous report. In addition, luciferase heterologously expressed in *G. violaceus* functioned as a reporter. The established system will promote the molecular genetic studies on *G. violaceus*.**

1. Introduction

Cyanobacteria are considered to be the first oxygenic photosynthetic organisms that emerged about 2.7 billion years ago. Most of the genes that are responsible for photosynthesis are widely conserved from cyanobacteria to eukaryotic photosynthetic organisms, this conservation is a convincing evidence of the endosymbiotic acquirement of eukaryotic chloroplast from a cyanobacterium. This high conservation has prevented us from understanding of the evolution of photosynthetic mechanisms from the primordial one. Therefore, cyanobacteria diverged from early stage of the cyanobacterial evolution may be helpful in studying the evolution of photosynthetic mechanisms, because such cyanobacteria are expected to retain a part of primordial properties that had been lost during the evolution of other major cyanobacteria. However, "primordial cyanobacteria" that retain a part of primordial properties rarely exist nowadays.

Gloeobacter violaceus PCC 7421 (hereafter referred to as *G. violaceus*) is a unicellular cyanobacterium, and is considered to be an early-branching cyanobacterium within the cyanobacterial clade, by the molecular phylogenetic analyses [1–4]. Almost all oxygenic photosynthetic organisms form the internal membranes called thylakoid membranes, which are the site for the light reaction of photosynthesis. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks the thylakoid membranes [5]. This unique property has been found only in this organism. Accordingly, both the photosystems and the respiratory chain in *G. violaceus* are localized at the cytoplasmic membrane. This indicates that photosynthetic activity per cell in *G. violaceus* is much lower than those in other cyanobacteria and eukaryotic photosynthetic organisms. For these unique characteristics, the complete genome of *G. violaceus* was sequenced in 2003 [6]. The genome sequence revealed that a part of the genes that are responsible for photosynthesis was not found in *G. violaceus*, whereas those genes are highly conserved among other oxygenic photosynthetic organisms [6]. Therefore, in recent years, protein complexes that are responsible for photosynthesis (e.g. photosystem I and phycobilisome) in *G. violaceus* were biochemically analyzed based on the genome information [7–11]. These recent results partly solved unique features previously reported [12–14]. Recently, it was reported that both the photosynthetic and respiratory complexes were concentrated at the respective domains, which may have specialized functions, in the cytoplasmic membrane of *G. violaceus* [15]. Moreover, the comparison of state transitions between *G. violaceus* and *Synechocystis* sp. PCC 6803 showed the commonalities and differences [16]. *G. violaceus* exhibited state transitions and non-photochemical fluorescence quenching like *Synechocystis* sp. PCC 6803 [16]. In *G. violaceus*, the structure of phycobilisome was quite different from other cyanobacterial phycobilisomes [12]. Nevertheless, orange carotenoid protein that binds to phycobilisome was also correlated with blue-light-induced heat dissipation in *G. violaceus*, like *Synechocystis* sp. PCC 6803 [16]. These results suggest that *G. violaceus* is an ideal organism for investigating the evolution of photosynthetic system by comparison of other

Abbreviations: CBB, Coomassie Brilliant Blue; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Sm, streptomycin

[1] Present address: Institute of Industrial Science, The University of Tokyo, Tokyo 153-8505, Japan.

[*] Corresponding author. Address: Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-nihonmatsu-cho, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: +81 75 753 6575; fax: +81 75 753 7909.

E-mail address: tsuchiya.toru.8e@kyoto-u.ac.jp (T. Tsuchiya).

2211-5463
http://dx.doi.org/10.1016/j.fob.2012.11.003

cyanobacteria.

Molecular genetics, such as the production and analysis of mutants, is a preferable method to analyze the function of individual genes in *G. violaceus*. Unfortunately, molecular genetic analysis cannot be applied to *G. violaceus* because of the lack of a highly-reproducible transformation system for this organism. Only one report on the development of a transformation system for *G. violaceus* has been published to date [17]. However, there is no subsequent paper that describes the functional expression of the foreign genes in *G. violaceus* using the system. In the present study, we re-examined the transformation system reported previously, and developed a highly-reproducible transformation system for *G. violaceus*. We succeeded in introducing an expression vector derived from a broad-host-range plasmid into *G. violaceus* by conjugal gene transfer. Using this system, we introduced a luciferase gene into *G. violaceus*, and the resultant transformant exhibited significant luciferase activity.

2. Materials and methods

2.1. Culture of G. violaceus

G. violaceus was grown photoautotrophically in BG11 medium [18] under the continuous white light (10 μmol photons m^{-2} s^{-1}) at 25 °C, and air was supplied via an air filter (Millex-FG, Millipore, Massachusetts, USA). For transformants, 10 μg ml^{-1} streptomycin (Sm) was added to the medium. BG11 agar medium containing 1 mM TES–NaOH (pH 8.2) was used for solid culture.

2.2. Construction of plasmids and transformation of G. violaceus

We used a plasmid vector pKUT1121 [19], which was constructed from a broad-host-range plasmid RSF1010 [20], to establish a transformation system for *G. violaceus*. The coding region of firefly luciferase gene (*luc*) was amplified by polymerase chain reaction (PCR) using pGL3-Basic vector (Promega, Wisconsin, USA) as a template. The PCR product containing *luc* gene with additional restriction sites for *Nde*I and *Xho*I at the 5′- and 3′-ends, respectively, was amplified using the following primers: 5′-GGGCATATGGAAGACGCCAAAAACAT-3′, 5′-GCGGAAAGATCGCCGTGTAACTCGAGAAA-3′. After the PCR product was subcloned into pZErO-2 (Invitrogen, California, USA), the sequence of cloned *luc* gene was confirmed by sequencing. The *luc* gene was excised from the plasmid by *Nde*I and *Xho*I treatment, and subcloned into pKUT1121 to yield pKUT-*luc*.

Transformation was performed by diparental mating basically according to the method of Elhai and Wolk [21]. First, a conjugative helper plasmid, pRK2013 [22], was introduced into *Escherichia coli* XL1-Blue MRF′ (Agilent Technologies, California, USA). Subsequently, the expression vector (pKUT1121 or pKUT-*luc*) was introduced into XL1-Blue MRF′ (pRK2013). Equal amounts of resultant transformant cells and *G. violaceus* cells were mixed, and then aliquots of the mixture were spotted onto nitrocellulose membrane on a BG11 agar medium. Following a 48 h incubation under the light of 5 μmol photons m^{-2} s^{-1}, the membrane was transferred onto BG11 agar medium containing 5 μg ml^{-1} Sm. Streptomycin-resistant colonies appeared after several months, and each colony was finally cultured in BG11 liquid medium containing 10 μg ml^{-1} Sm. Total DNA was prepared from *G. violaceus* cells using hexadecyl-trimethyl-ammonium bromide [23]. The presence of marker gene in the total DNA was checked by PCR.

2.3. SDS–PAGE and Western blotting

Total protein of *G. violaceus* cells was prepared by the following procedure. *G. violaceus* was resuspended with a buffer (20 mM MES–NaOH (pH 6.5), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM NaCl, 0.6 M betaine). The suspended cells were disrupted by repeated agitation with glass beads (ϕ = 0.1 mm) at 4 °C. After the debris was removed by centrifugation (2000 × *g*, 5 min, 4 °C), Triton X-100 was added to the supernatant at the final concentration of 1% to solubilize the membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [24] using 12% (w/v) of polyacrylamide gel. Total proteins (10 μg) were loaded on each lane of a gel, and the gel was stained with Coomassie Brilliant Blue (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, New Jersey, USA). Western blotting and chemiluminescence detection were performed according to the manufacturer's instructions. Anti-luciferase antibody (Luciferase (251-550), Santa Cruz Biotechnology, California, USA) was used as a primary antibody. After the treatment with secondary antibody (Anti-Rabbit IgG, Jackson Immuno Research Europe, Suffolk, UK), luciferase was detected by chemiluminescence (ECL Plus Western Blotting Detection System, GE Healthcare) with a luminescent image analyzer (LAS-3000 UV mini, Fujifilm, Tokyo Japan).

2.4. Luciferase assay

The concentration of *G. violaceus* cells was adjusted to 1.0 × 10^7 cells ml^{-1} with BG11 medium. After the cells were adapted to darkness for 5 min, background luminescence was measured with a luminometer (GloMax™ 20/20n Luminometer, Promega). Then, luciferin (Beetle Luciferin, Promega) was added to the cells at the final concentration of 100 μM, and the luminescence derived from luciferase reaction was measured.

3. Results

3.1. Antibiotic susceptibility of G. violaceus

First, we tried to culture *G. violaceus* at 28 °C under the light of 20 μmol photons m^{-2} s^{-1} according to Guo and Xu [17], however, cells were not able to survive. Therefore, we applied our routine culture conditions to further study. We examined the antibiotic susceptibility of *G. violaceus* for the use of antibiotic resistance genes as marker genes of transformant. We tested gentamicin, hygromycin, spectinomycin and zeocin in addition to antibiotics used in Guo and Xu [17]. Table 1 summarizes the result of antibiotic susceptibility test of wild type *G. violaceus*. Three antibiotics showed same susceptibility as described in Guo and Xu [17], however, the others showed different susceptibility (for details, see Section 4). Three of nine antibiotics, erythromycin, Sm and spectinomycin exhibited antibiotic activity against *G. violaceus* within the range of 1–50 μg ml^{-1}. For these three antibiotics, we also checked the antibiotic activity against *G. violaceus* on the agar medium. *G. violaceus* cells adjusted to the concentration of 1.0 × 10^3 to 1.0 × 10^9 cells ml^{-1} were spotted onto nitrocellulose membrane on BG11 agar medium including each antibiotic. As a result, Sm was the most effective for killing cells at lower concentration (5 μg ml^{-1}). Therefore, we chose Sm resistance gene (*aadA*) as a marker gene for the screening of transformant.

3.2. Development of transformation system for G. violaceus

Because *G. violaceus* was sensitive to Sm (Table 1), we tried to introduce a broad-host-range plasmid derived expression vector, pKUT1121 [19] that possesses Sm resistance gene cassette, by conjugal gene transfer. After the treatment of exconjugants with Sm, Sm resistant colonies appeared (Fig. 1A) . In contrast, no colony was formed in the spot of negative control (Fig. 1B). The frequency of transformation of *G. violaceus* was approximately 1.2 × 10^{-4} per recipient cell for pKUT1121. Total DNA prepared from the Sm resistant strain and wild type were used as template of PCR (Fig. 2) to confirm successful introduction of the plasmid. As a marker gene,