J. Gen. Appl. Microbiol., **58**, 1–10 (2012)

Full Paper

Genetic transformation of *Pseudochoricystis ellipsoidea*, an aliphatic hydrocarbon-producing green alga

Sousuke Imamura,[1,*] Daisuke Hagiwara,[1,2] Fumi Suzuki,[1] Norihide Kurano,[2] and Shigeaki Harayama[1]

[1] *Department of Biological Sciences, Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo 112–8551, Japan*
[2] *Research Laboratories, DENSO CORPORATION, Nisshin, Aichi 470–0111, Japan*

(Received July 2, 2011; Accepted September 1, 2011)

Pseudochoricystis ellipsoidea **is a recently isolated unicellular green alga, which is classified within the family Trebouxiophyceae. This alga has a unique ability to synthesize and accumulate intracellularly a significant amount of aliphatic hydrocarbons. To elucidate molecular mechanisms of the hydrocarbon production in this organism, the development of genetic methods including DNA transformation methods are important. Towards the goal, we constructed several plasmids in which neomycin phosphotransferase II-encoding G418-resistant gene (*nptII*) is flanked by a *P. ellipsoidea*-derived promoter and terminator. These plasmids were introduced into *P. ellipsoidea* cells through particle-gun bombardment, and transformants were screened among G418-resistant cells by PCR amplification of plasmid-borne genes. Southern blot analysis demonstrated that the exogenous DNA was integrated into the genome of the transformants. Furthermore, the expression of *nptII* was confirmed at the transcript and protein levels by RT-PCR and immunoblot analyses, respectively. These results clearly indicated that a genetic transformation system was successfully established for *P. ellipsoidea*.**

Key Words——biodiesel; green alga; hydrocarbon; particle-gun bombardment; *Pseudochoricystis ellipsoidea*; transformation

Introduction

Biodiesel is generally synthesized from biological sources such as vegetable oils and animal fats (Juan, 2011; Karmakar, 2010). Recently, microalgae have been considered to be a possible biodiesel source because of their rapid growth rates and high lipid contents: maximum growth rates higher than one division per day are common (Nielsen, 2006), and lipid contents of some strains exceed 70% of their total dry weight (Chisti, 2007; Metting, 1996). Lipids in the microalgae are largely composed of long-chain triacylglycerols that can be converted to biodiesel by chemical transesterification of their fatty acid moiety (Scott et al., 2010).

Three races of a green colonial microalga *Botryococcus braunii* are notable for their ability to synthesize hydrocarbons in addition to triacylglycerols (Achitouv et al., 2004; Metzger and Largeau, 2005; Metzger et al., 1990). Many studies using various *B. braunii* strains have been conducted to enhance their growth rates and hydrocarbon productivity by controlling nutritional or cultural conditions (e.g., temperature, pH and salinity) to channel the metabolic flux of photosynthetic fixed carbon into hydrocarbon biosynthesis (Courchesne et al., 2009).

A green alga that is classified within the family

* Address reprint requests to: Dr. Sousuke Imamura, Chemical Resources Laboratory, Tokyo Institute of Technology, Midori-ku, Yokohama 226–8503, Japan.
Tel: +81–45–924–5859 Fax: +81–45–924–5859
E-mail: simamura@res.titech.ac.jp

Trebouxiophyceae and tentatively named *Pseudochoricystis ellipsoidea* also produces hydrocarbons (Satoh et al., 2010) of shorter carbon chains, such as heptadecene, heptadecane and eicosadienes as major hydrocarbons. The hydrocarbon content in this organism increases more than 10 times under nitrogen starvation conditions, and becomes approximately 9% of dry weight. The triglyceride content also increases to 30% of dry weight (Satoh et al., 2010). The maximum growth rate of *P. ellipsoidea* (3.46 g dry weight L^{-1} d^{-1}) is much faster than that of *B. braunii* (1.05 g dry weight L^{-1} d^{-1}; Kurano et al., unpublished data). Therefore, *P. ellipsoidea* is regarded as a potential source of renewable diesel fuel.

Biological production of hydrocarbon is widely observed (Ladygina et al., 2006), but the pathway for the hydrocarbon synthesis has remained unclear until recently. Schirmer et al. (2010) recently identified two genes responsible for the hydrocarbon production in cyanobacteria: an acyl-acyl carrier protein reductase (PCC7942_orf1594 in *Synechococcus elongatus* PCC7942) and an aldehyde decarbonylase (PCC7942_orf1593). Our blast search indicated that no homologue of the aldehyde decarbonylase was found in plant lineages including green algae implying the independent evolutional origin of the cyanobacterial decarbonylase. In *B. braunii*, a cobalt-porphyrin-containing enzyme that catalyzes decarbonylation of aldehydes has been purified from the microsome fraction, but the gene encoding the enzyme has not been identified (Dennis and Kolattukudy, 1992). For the improvement of oil productivity in *P. ellipsoidea*, the identification and genetic manipulation of aldehyde decarbonylase in this organism are crucial steps; and the establishment of a genetic transformation method is the first step towards this goal. Here, we report, for the first time, genetic transformation of *P. ellipsoidea* using a particle bombardment method.

Materials and Methods

Strain and growth conditions. *P. ellipsoidea* was isolated from a hot spring as a unicellular green alga that can produce hydrocarbons as well as triglycerides (Satoh et al., 2010). *P. ellipsoidea* was grown at 25°C under continuous fluorescent light (80 μmol m^{-2} s^{-1}) in liquid MA5 medium [18 mM $NaNO_3$, 0.4 mM $MgSO_4$, 60 nM $CaCl_2$, 0.26 mM KH_2PO_4, 0.26 mM K_2HPO_4, 20 mM HEPES-KOH (pH 7.0), 0.4% (v/v) Fe solution (3 g L^{-1} citric acid, 4.9 g L^{-1} ammonium iron citrate, 0.5 g L^{-1} EDTA), 0.1% (v/v) trace element solution (70 mg L^{-1} H_3BO_3, 150 mg L^{-1} $MnSO_4{\cdot}5H_2O$, 300 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 300 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 70 mg L^{-1} $CoCl_2{\cdot}6H_2O$, 3 mg L^{-1} Na_2MoO_4)] which was continuously bubbled with air containing 1% (v/v) CO_2. G418 at a concentration of 100 μg ml^{-1} was added if required. MA5 plates were prepared by adding agar to the medium to a final concentration of 1.5% (w/v).

Construction of plasmids for transformation. All PCR reactions were done with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). For the cloning of *nptII*, PCR was performed with the specific primer pair, G418-F-SmaI (5′-TCCCCGGGATGGGATCGGCCATTGAACAAGATG-3′, a *Sma*I site is underlined) and G418-R-XbaI (5′-CCTCTAGATCAGAAGAACTCGTCAAGAAGGCG-3′, an *Xba*I site is underlined), and the Eukaryotic Neomycin Selection Cassette (FRT-PGK-gb2-neo-FRT; Gene Bridges GmbH, Dresden, Germany) containing the entire *nptII* sequence as a template DNA. The resulting amplified fragment was restricted with *Sma*I and *Xba*I, and then inserted between the *Sma*I and *Xba*I sites of pUC119 to create pUC-G418. For the construction of each of nine plasmids (pG418-U1U, -A1U, -U2U, -T1U, -G1U, -T1A, -T2A, -U2A, and -U1A), we conducted a two-step PCR as described previously (Imamura et al., 2009). In the first PCR, fragment 1 (1.0, 1.5 or 2.0 kb-long upstream region of the relevant gene) was amplified by PCR with primer sets shown in Table 1, and *P. ellipsoidea* genomic DNA as a template; fragment 2 (1.0 or 1.5 kb-long downstream region of the *TUBULIN1* or *ACTIN1* gene) was amplified by PCR with primer sets shown in Table 2, and *P. ellipsoidea* genomic DNA as a template; fragment 3 (0.8 kb of the *nptII* gene) was amplified by PCR with the primers neo-SQ-F (5′-GGGATCGGCCATTGAACAAGATGG-3′) and neo-SQ-R (5′-TCAGAAGAACTCGTCAAGAAGGCG-3′) and pUC-G418 as a template. The second PCR was performed with the primer sets shown in Table 3 using fragments 1, 2 and 3 as the template DNAs. The resulting DNA fragments were excised with *Eco*RI or *Kpn*I, and cloned into the *Eco*RI or *Kpn*I site of pUC119. For the construction of pG418-SI103 and pG418-HYG3, we also performed two-step PCR as described above except that the primer sets shown in Tables 4 and 5 were used, and the promoter (fragment 1) and terminator (fragment 2) regions were amplified with pSI103 (Sizova et al., 2001) and pHyg3 (Berthold et al., 2002), respectively, as templates.

Table 1. Primers prepared for fragment 1 in the two-step PCR.

Gene (plasmid)	Primer	Sequence (5′–3′)
UBIQUITIN1 (pG418-U1U and pG418-U1A)	Ubi5-ProF	cttgagctagcagtgatactctgc
	Ubi5-ProR-neo	cttgttcaatggccgatcc***cat***tccacctctgccacaacag
ARP1 (pG418-A1U)	Act5-ProF	ggcattctcgttggcactgc
	Act5-ProR-neo	cttgttcaatggccgatcc***cat***accgaccacagctac
UBIQUITIN2 (pG418-U2U and pG418-U2A)	Ubi3-ProF	gacaggtgcttgacaggtgc
	Ubi3-ProR-neo	cttgttcaatggccgatcc***cat***aaaaagcagtctgataagc
TUBULIN1 (pG418-T1U and pG418-T1A)	Tub1-ProF	ctgtgcgattggatgtctcc
	Tub1-ProR-neo	cttgttcaatggccgatcc***cat***cttgaacgacttccct
GAPDH1 (pG418-G1U)	gpdA1-proF	ccatgatgaagacttgtgcgcctgtg
	gpdA1-proR-neo	cttgttcaatggccgatcc***cat***ggtcaataccagtaggaatg
TUBULIN2 (pG418-T2A)	Tub4-ProF	ggtgcttccacaggctatgg
	Tub4-ProR-neo	cttgttcaatggccgatcc***cat***ccttgataattttctacatc

Nucleotides in bold italics and underlined nucleotides indicate the initiation codon of *NPTII* and adapter sequences for the second PCR, respectively.

Table 2. Primers prepared for fragment 2 in the two-step PCR.

Gene (plasmid)	Primer	Sequence (5′–3′)
UBIQUITIN1 (pG418-U1U, -A1U, -U2U, -T1U, -G1U)	Ubi5-terF-neo	gccttcttgacgagttcttc**tga**tcaaatgcacttcctgcgg
	Ubi5-terR	cgatgtacaaaagcgcaaactgacc
ACTIN1 (pG418-T1A, -T2A, -U2A, -U1A)	Act1-terF-neo	gccttcttgacgagttcttc**tga**ggtgagcctgaaacag
	A1ter(1.5 kb)-R2	gcggttgttgagttgagcactgg

Bold and underlined nucleotides indicate the stop codon of *NPTII* and adapter sequences for the second PCR, respectively.

Table 3. Primers used for the second PCR.

Gene (plasmid)	Primer	Sequence (5′–3′)
Forward primers		
UBIQUITIN1 (pG418-U1U)	U5(pro)-F-EcoRI	acaGAATTCtgtcctctcctccacagccatcagc
UBIQUITIN1 (pG418-U1A)	U5(pro)-F-KpnI	ccaGGTACCtggccgcagcatttctgtcc
ARP1 (pG418-A1U)	A5(pro)-F-EcoRI	acaggtacctgtgaggtgGAATTCcagc
UBIQUITIN2 (pG418-U2U)	U3(pro)-F-EcoRI	cctGAATTCagggaacttgcagcttctc
UBIQUITIN2 (pG418-U2A)	U3(pro)-F-KpnI	cctGGTACCagggaacttgcagcttctc
TUBULIN1 (pG418-T1U)	T1(pro)-F-EcoRI	gcaGAATTCtgcatggcattcaacaagg
TUBULIN1 (pG418-T1A)	T1(pro)-F-KpnI	gcaGGTACCtgcatggcattcaacaagg
GAPDH1 (pG418-G1U)	gpdA(pro)-F-EcoRI	gctGAATTCagcaaatgcgtcatcatttttgg
TUBULIN2 (pG418-T2A)	T4(pro)-F-KpnI	cccGGTACCgggtgtagatgtgagagc
Reverse primers		
UBIQUITIN1 (pG418-U1U, -A1U, -U2U, -T1U, -G1U)	U5(ter)-R-EcoRI	cagGAATTCctgcgatgcgtttggtggtactcc
ACTIN1 (pG418-T1A, -T2A, -U2A, -U1A)	A1ter(1.5 kb)-R-KpnI	accGGTACCggttgttgagttgagcactggac

Nucleotides in upper case indicate the *Eco*RI or *Kpn*I sites.

Fragment 3 (the *nptII* gene) for pG418-SI103 and pG418-HYG3 was amplified with the primer set shown in Table 6, and pUC-G418 as a template. The primers used in the second PCR are shown in Table 7. The constructed plasmids were sequenced with an ABI PRIZM 310 genetic analyzer to verify their sequences (Applied Biosystems, Foster, CA, USA).

Particle bombardment. *P. ellipsoidea* cells at stationary growth phase were diluted into 0.15 ml MA5 medium to OD_{750} = 0.75 and then spotted onto the central area of a MF-Millipore membrane filter (Millipore, Bedford, MA, USA), which was placed on an MA5 plate. The cells were incubated at 25°C for 4 days under continuous fluorescent light at 50 μmol m^{-2} s^{-1} and subjected to particle bombardment (PDS-1000/He, Bio-Rad, Hercules, CA, USA) using gold particles

Table 4. Primers prepared for fragment 1 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.

Plasmid	Primer	Sequence (5′–3′)
pG418-SI103	SI103-F	gccgctctagaactagtggatc
	phspA-R_G418-5′	aatggccgatcc***cat***gcttcgaaattcttcagcaccgg
pG418-HYG3	Ptub-F	ctttatgcttccggctcgtatgttg
	Ptub-R_G418-5′	aatggccgatcc***cat***gtttgcgggttgtgactgaaacg

Nucleotides in bold italics and underlined nucleotides indicate the initiation codon of *NPTII* and adapter sequences for the second PCR, respectively.

Table 5. Primers prepared for fragment 2 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.

Plasmid	Primer	Sequence (5′–3′)
pG418-SI103	G418-3′_SI3′UTR-F	gacgagttcttc**tga**gggacctgatggtgttggtg
	SI3′-R	gaacaaaagctgggtacccgcttcaaatacgcc
pG418-HYG3	G418-3′_hyg3′UTR-F	gacgagttcttc**tga**taaggatccccgctccgtgtaaat
	hyg-R	gtgctgcaaggcgattaagttg

Bold and underlined nucleotides indicate the stop codon of *NPTII* and adapter sequences for the second PCR, respectively.

Table 6. Primers prepared for fragment 3 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.

Plasmid	Primer	Sequence (5′–3′)
pG418-SI103	phspA-3′_G418-F	gaagaatttcgaagc***atg***gatcggccattgaacaagatg
	G418-R_SI3′UTR	acaccatcaggtccc**tca**gaagaactcgtcaagaaggcg
pG418-HYG3	Ptub-3′_G418-F	tcacaacccgcaaac***atg***gatcggccattgaacaagatg
	G418-R_hyg3′UTR	gagcggggatcctta**tca**gaagaactcgtcaagaaggcg

Nucleotides in bold italics and bold indicate the initiation and stop codon of *NPTII* gene, respectively. Underlined nucleotides indicate the adapter sequences for the second PCR.

Table 7. Primers used for the second PCR or construction of pG418-SI103 and pG418-HYG3.

Plasmid	Primer	Sequence (5′–3′)
pG418-SI103	phspA-F-SmaI	tcCCCGGGgagctcgctgaggcttgaca
	SI3′UTR-R-XbaI	ccTCTAGAcgcttcaaatacgcccagcc
pG418-HYG3	Ptub-F-XbaI	acTCTAGActttcttgcgctatgacacttccag
	hyg-R-HindIII	gcAAGCTTggtacccgcttcaaatacgc

Nucleotides in upper case indicate *Sma*I, *Xba*I, or *Hind*III site.

of 0.6 μm in diameter (Bio-Rad). After the incubation, those spots contained 1.8 to 5.8×10^6 cells. A 0.8 μg aliquot of circular plasmid DNA and 0.5 mg gold particles were used per trial. Plasmid DNA was absorbed to particles according to the protocol for the PDS-1000/He Particle Delivery System (Bio-Rad). The cells spotted on a MF-Millipore membrane filter on a MA5 plate were placed under the stopping screen at a distance of 6 cm and bombarded in a vacuum pressure at 125 mm Hg using a helium pressure at 900, 1,100, 1,350 or 1,550 psi to accelerate particles. After bombardment, the membrane was transferred onto an MA5 plate containing 1% (w/v) glucose, and incubated at 25°C under dark conditions for 3 days before the cells were suspended in 1 ml MA5 medium containing G418 at a final concentration of 50 μg ml^{-1}. The suspension was spread on a MA5 plate containing G418 at a final concentration of 100 μg ml^{-1}, and G418-resistant cells were isolated.

Preparation of genomic DNA and PCR analysis. *P. ellipsoidea* cells were disrupted by grinding in liquid nitrogen with a mortar and pestle, and transferred to a 1.5 ml microcentrifuge tube. The cells were suspended in TEN buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA). After phenol/chloroform/isoamyl alcohol extraction and 2-propanol precipitation, samples were treated with RNase A, and the genomic DNA was recovered by ethanol precipitation following phenol/chloroform/isoamyl alcohol and chloroform treatments. Genomic PCR was performed with isolated 0.1 μg genomic DNA as a template and the primer sets G418_F (5′-GATCGGCCATTGAACAAGAT-3′) and G418_R (5′-GCGATACCGTAAAGCACGAG-3′), or Tub_Pro_F (5′-TATCTAGCGGGGTTGGTTGA-3′) and Act_Ter_R (5′-TCCAAACATCCACCTGTCAA-3′), and 5 μl GoTaq Master Mix (Promega, Madison, WI, USA). Amplifications were performed by incubating reaction mixtures at 95°C for 2 min prior to 30 cycles of 15 s at 95°C followed by 20 s at 52.5°C and 60 s at 72°C. Assays with no template were examined for every experiment as a negative control.

RT-PCR analysis. Total RNA was prepared by Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and purified with an RNeasy Mini Kit column (QIAGEN, Hilden, Germany). The purified RNA (1.5 μg) was transcribed into cDNA employing the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) as described by the manufacturer. PCR was performed as for genomic PCR analysis described above with slight modifications. The conditions of the PCR were as follows: 95°C for 2 min prior to 25 cycles of 15 s at 95°C followed by 20 s at 52.5°C and 60 s at 72°C. Assays with samples that were prepared without reverse transcriptase in the cDNA synthesis reaction and with no template were examined for every experiment as a negative control.

Southern blot analysis. *P. ellipsoidea* genomic DNA (3 μg) was digested with *Eco*RI, separated on 1% (w/v) agarose gel and blotted onto a Biodyne Plus nylon membrane (Pall Corporation, Ann Arbor, MI, USA) by a standard capillary transfer method using 20× SSC as a transfer buffer (1× SSC is 15 mM sodium citrate, 0.15 M NaCl, pH 7.0). The blotted filter was cross-linked in an ultraviolet cross-linker CL-1000 (UVP, Upland, CA, USA). A specific probe was synthesized using a DIG-dNTP labeling kit (Roche Applied Science) with the primer set G418_F and G418_R. Hybridization and signal detection were performed as described previously (Imamura et al., 2010).

Immunoblot analysis. *P. ellipsoidea* cells (50 ml) were harvested by centrifugation (3,000×*g*, 4°C, 5 min) and stored at −80°C until use. The frozen cells were thawed on ice and resuspended in 3 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol) containing Complete Mini, EDTA-free, protease inhibitor mixture (Roche Applied Science). These cells were broken by passage through a French press cell at 18,000 psi. Cell extracts were centrifuged (15,000×*g*, 4°C, 10 min) and the supernatants were used as soluble total protein fractions in this study. The total protein (8 μg) was separated by 10% SDS-PAGE and electrotransferred onto Immobilon-P membranes (Millipore), and reacted with the polyclonal anti-NPTII antibody as described previously (Imamura et al., 2003). The NPTII antibody was purchased from Millipore.

Results

Construction of plasmids that are used for genetic transformation

For the establishment of a genetic transformation system for *P. ellipsoidea*, it is essential to identify selectable marker genes and selection conditions. Then, the sensitivity of *P. ellipsoidea* to various drugs used for genetic selection was examined on MA5-based agar plates. Results indicated that G418 (50 μg ml^{-1})

effectively prevented the growth of this alga, while chloramphenicol, spectinomycin, hygromycin, paromomycin and kanamycin, of which the final concentration ranged from 50 to 100 μg ml^{-1}, did not strongly affect its growth (data not shown). Therefore, a G418-resistant gene encoding neomycin phosphotransferase II (*nptII*) was used as a selection marker gene in this study.

Other important elements for the plasmid construction are promoters and terminators that direct the transcription and termination, respectively, of the marker gene. We used the upstream regions of six *P. ellipsoidea* genes, *UBIQUITIN1, UBIQUITIN2, actin-related protein 1* (*ARP1*), *TUBULIN1*, *TUBULIN2* and *GAPDH1*, to provide promoter function, and the downstream regions of two genes, *UBIQUITIN1* and *ACTIN1,* to provide terminator function; these genes have been shown to be expressed constitutively at high levels in *P. ellipsoidea* (our unpublished data). One of the upstream regions was fused to the 5′-end of the *nptII* gene while one of the downstream regions was fused to the 3′-end of the *nptII* gene to construct nine plasmids (pG418-U1U, -A1U, -U2U, -T1U, -G1U, -T1A, -T2A, -U2A, and -U1A) as shown in Fig. 1. These plasmids were used for the subsequent genetic transformation experiments.

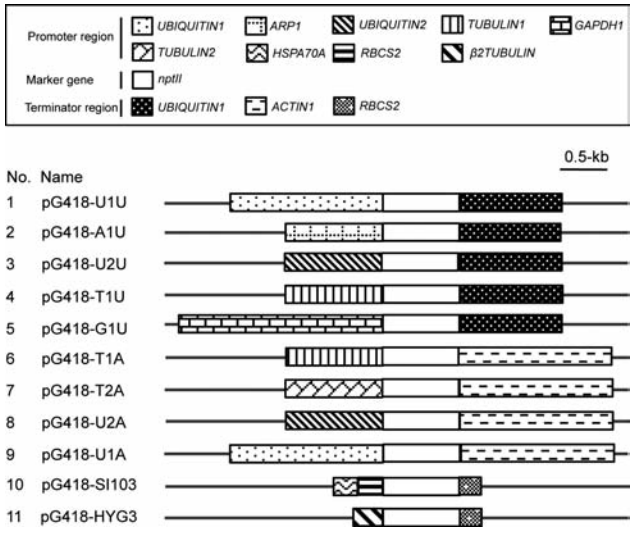

Fig. 1. Construction of plasmids used for transformation.

Eleven plasmids consisted of the following elements: an upstream (promoter) region, the *nptII* gene and a downstream (terminator) region. Patterned rectangles and white rectangles denote the promoter or terminator region and the *nptII* gene, respectively. The promoters of *HSPA70A*, *RBCS2*, and *β2TUBULIN*, and the terminator of *RBCS2* are derived from *C. reinhardtii*. Other promoter and terminator regions are derived from *P. ellipsoidea*.

Exogenous DNA introduction into P. ellipsoidea cells

In *Chlamydomonas reinhardtii* and other green algae, electroporation is a useful tool to introduce exogenous DNA into cells partially deprived of their cell wall (Coll, 2006; Liu et al., 2006; Maruyama et al., 1994). Therefore, we first attempted to introduce the plasmids listed in Fig. 1 into protoplasts of *P. ellipsoidea*. However, transformable protoplasts are not yet successfully prepared in our hands (data not shown). We then conducted particle bombardment experiments, as this method can successfully introduce exogenous DNA into cells with a rigid cell wall (Coll, 2006; Teng et al., 2002). We tested various pressure conditions, and G418-resistant colonies developed on selective plates were examined by genomic PCR with *nptII*-specific primers to determine whether the *nptII* DNA was introduced in the cells. A summary of the analyses is given in Fig. 2A and 2B. Under each of the four pressure conditions (900, 1,100, 1,350, and 1,550 psi), we obtained two to five G418-resistant colonies, and one positive clone among them, in which the *nptII* fragment was amplified (lanes 2−5). Interestingly, these positive clones were obtained only when pG418-T1A (No. 6 plasmid) was used (900 psi, named #6-30; 1,100 psi,

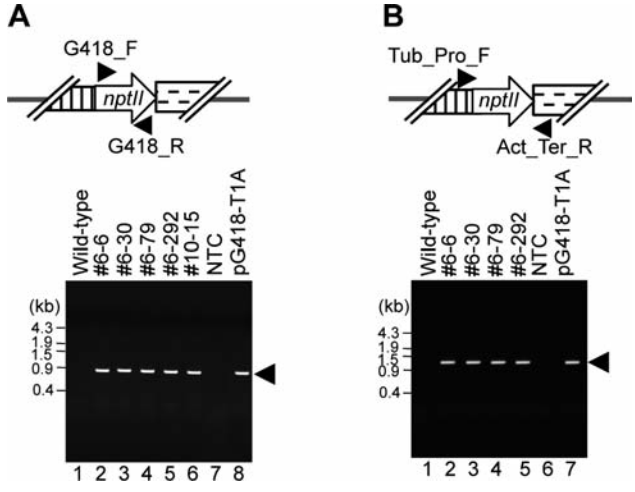

Fig. 2. Selection of transformants by genomic PCR analysis.

The genomic DNA was analyzed by PCR with the primer sets G418-F/G418-R (A) or Tub_Pro_F/Act_Ter_R (B). Each primer position is indicated at the top. Patterned rectangles denote the same as in Fig. 1. The PCR products were resolved by 1.0% (w/v) agarose gel electrophoresis. Arrowheads indicate the expected positions of the PCR product (0.8 kb in panel A and 1.2 kb in panel B). The positions of a molecular size marker are indicated as kilobase pairs at the left. NTC, no template control.

#6-6; 1,350 psi, #6-79; 1,550 psi, #6-292). These results indicated that plasmid pG418-T1A was introduced into the *P. ellipsoidea* cells. In the particle bombardment experiments, each transformant was obtained from plates where 1.8 to 5.8×10^6 cells were seeded. Thus, the transformation efficiencies ranged from 1.7 to 5.6 per 10^7 cells.

Integration of the exogenous DNA into the genome

To analyze whether the positive clones were generated by the integration of the marker gene into the genome, total DNAs were isolated from the cells, digested with *Eco*RI, and subjected to Southern blot analysis with a DIG-labeled probe, which specifically hybridizes to the *nptII* gene. The results are shown in Fig. 3. The hybridization signals were detected in the *Eco*RI-cleaved genomic DNAs from the #6-6, -30, -79, and -292 clones (panel B, lanes 4–7), but not from the wild-type strain (lane 3). These signals were of different sizes, none of which matched the size of pG418-T1A (lane 1). These observations clearly indicated that the plasmid was integrated at different positions on the host genome.

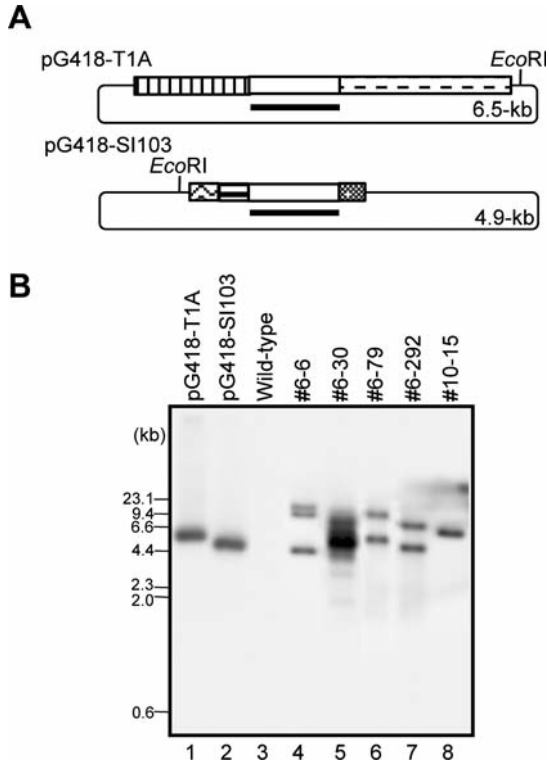

Fig. 3. Southern blot analysis of DNA prepared from transformants.

Total DNAs were isolated from wild-type, #6-6, -30, -79, -292, and #10-15 strains and were digested with *Eco*RI, resolved by 1.0% agarose gel electrophoresis, and hybridized with the *nptII* gene probe. Structure of the pG418-T1A and pG418-SI103 plasmids and position of the *nptII* gene probe (bold line) are indicated at the top. Patterned rectangles and white rectangles denote the same as in Fig. 1. A single band of 6.5 or 4.9 kb was observed with either plasmid pG418-T1A or pG418-SI103. One to three bands of different sizes were observed with the genomic DNA of transformant cells. No bands were observed for the DNA from wild-type cells. Positions of a molecular size marker are indicated as kilobase pairs at the left.

nptII expression in the cells

We then examined *nptII* expression in the transformants at transcript level by RT-PCR of *nptII* transcripts from their total RNAs. As shown in Fig. 4, amplified products of the expected size (0.8 kb) were detected in the transformants (lanes 3, 5, 7, and 9), but not in the wild-type strain (lane 1). To examine the existence of NPTII protein, we conducted immunoblot analysis using a polyclonal anti-NPTII antibody. Based on the amino acid sequence of NPTII, the molecular size was expected to be about 29 kDa. In all the total protein

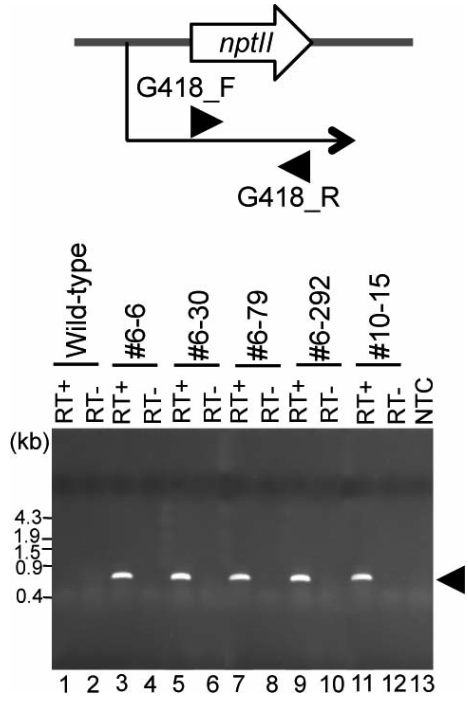

Fig. 4. Confirmation of *nptII* expression in the transformants by RT-PCR analysis.

RT-PCR amplification of cDNA derived from wild-type or transformant cells was achieved using the *nptII*-specific primer pair. RT+, cDNA synthesis with reverse transcriptase; RT−, cDNA synthesis without reverse transcriptase. The arrowhead indicates the expected positions of the PCR product (0.8 kb). Others are the same as in Fig. 2.

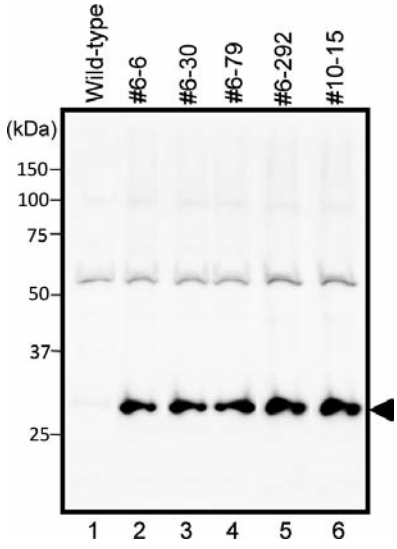

Fig. 5. Immunoblot analysis for NPTII.

Aliquots of total protein (each 8 μg) prepared from wild-type or transformant cells were separated by 10% SDS-PAGE and analyzed by immunoblotting with a polyclonal anti-NPTII antibody. Positions of a molecular size marker are indicated in kDa at the left. The arrowhead indicates the expected position of NPTII protein (29 kDa).

samples prepared from the transformants, signals coinciding with the molecular size were detected (Fig. 5, lanes 2–5), which was not observed in the sample from the wild-type strain (lane 1). Weak signals of about 55 kDa in size would be a result of non-specific cross-reaction, as the signals were observed in all the samples examined. These results clearly indicated that *NTPII* expression correctly occurred in the transformant cells.

Promoters derived from Chlamydomonas reinhardtii function in P. ellipsoidea cells

Chlamydomonas reinhardtii is a model organism for the study of plants (Harris, 2001), and several plasmids have been constructed for genetic transformation. To investigate whether promoters and terminators derived from *C. reinhardtii* function in *P. ellipsoidea*, we carried out transformation experiments with two of the *Chlamydomonas* plasmids, pSI103 (Sizova et al., 2001) and pHyg3 (Berthold et al., 2002). pSI103 encodes the *aphIII* gene, which confers resistance to the antibiotic paromomycin and is controlled by the *HSP70A* and *RBCS2* tandem promoters and the single *RBCS2* terminator. pHyg3 encodes the *aph7″* gene, which confers hygromycin resistance and is controlled by the promoter and terminator of *RBCS2*. Although hygromycin and paromomycin are often used to select transformants in *C. reinhardtii*, *P. ellipsoidea* was, as described above, resistant to both antibiotics. Hence, we replaced the *aphIII* gene in pSI103 and the *aph7″* gene in pHyg3 with the *nptII* gene (see MATERIALS AND METHODS), to construct pG418-SI103 and pG418-HYG3, respectively (Fig. 1). We then performed bombardment experiments at 900 psi using these two plasmids, and obtained one positive clone (named #10-15) with pG418-SI103 (Fig. 2A, lane 6). For the #10-15 clone, Southern blot, RT-PCR, and immunoblot analyses were performed as for #6-6, -30, -79, -292 clones. The Southern blot analysis indicated that pG418-SI103 was integrated in the *P. ellipsoidea* genome, as the size of signal with DNA prepared from the #10-15 clone was not identical to that with pG418-SI103 (Fig. 3B, lanes 2 and 8). The RT-PCR and immunoblot analyses revealed *nptII* expression at transcript and protein levels in the #10-15 clone (Fig. 4, lane 11; Fig. 5, lane 6). These results strongly suggest that the *C. reinhardtii HSP70A* and/or *RBCS2* promoters and *RBCS2* terminator function in *P. ellipsoidea*.

Discussion

In the present study, we successfully established a genetic transformation system for *P. ellipsoidea* for the first time. Interestingly, all transformants were obtained only with pG418-T1A and pG418-SI103 (Figs. 2–5). Although pG418-T1U has the same promoter region as pG418-T1A, the terminator region is different between them suggesting that the 1.0 kb *UBIQUITIN1* in pG418 -T1U is less effective in establishing stable transformants. By the same reasoning, pG418-T2A, pG418-U1A and pG418-U2A harbor the same terminator region as pG418-T1A without yielding transformants, suggesting that the promoter regions used in pG418-T2A, pG418-U1A and pG418-U2A are less effective than that in pG418-T1A. In addition, we showed that *C. reinhardtii HSP70A* and/or *RBCS2* promoters could drive *nptII* gene expression in *P. ellipsoidea*. Such information will be useful for future construction of plasmids to be used for genetic transformation of *P. ellipsoidea*. The promoters that drive expression of the *nptII* gene in the transformants are derived from the nuclear genome of *P. ellipsoidea* or *C. reinhardtii*, implying that the exogenous plasmids were integrated into the nuclear genome.

The transformation efficiencies established in this

study ranged from 1.7 to 5.6 per 10^7 cells. In other microalgae, the efficiencies by particle bombardment varied from <1 to 250 per 10^7 cells (Coll, 2006). Since the type and size of particles and the growth phase of cells affect the transformation efficiency (Coll, 2006; Sun et al., 2005; Tan et al., 2005), it would be possible to increase the transformation efficiency in *P. ellipsoidea* after optimizing several parameters.

This paper is the first report of genetic transformation in hydrocarbon-producing green algae. The results in this study enabled us to use various genetic techniques including overexpression or RNA interference-mediated silencing of specific genes in *P. ellipsoidea*. Based on this technology, the mechanism of hydrocarbon production, which is an attractive and important subject in *P. ellipsoidea*, can be examined genetically in near future.

Acknowledgments

We thank Drs. M. Hanaoka and M. Kato for their technical advice. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) from the Ministry of Economy, Trade and Industry of Japan (to N.K.) and a Grant-in-Aid for Young Scientists (A) (22681010) (to S.I.)

References

Achitouv, E., Metzger, P., Rager, M. N., and Largeau, C. (2004) C_{31}–C_{34} methylated squalenes from a Bolivian strain of *Botryococcus braunii*. *Phytochemistry*, **65**, 3159–3165.

Berthold, P., Schmitt, R., and Mages, W. (2002) An engineered *Streptomyces hygroscopicus aph7* gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. *Protist*, **153**, 401–412.

Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnol. Adv*., **25**, 294–306.

Coll, J. M. (2006) Methodologies for transferring DNA into eukaryotic microalgae. *Span. J. Agric. Res*., **4**, 316–330.

Courchesne, N. M., Parisien, A., Wang, B., and Lan, C. Q. (2009) Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. *J. Biotechnol*., **20**, 31–41.

Dennis, M. and Kolattukudy, P. E. (1992) A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. *Proc. Natl. Acad. Sci. USA*, **15**, 5306–5310.

Harris, E. H. (2001) *Chlamydomonas* as a model organism. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., **52**, 363–406.

Imamura, S., Kanesaki, Y., Ohnuma, M., Inouye, T., Sekine, Y., Fujiwara, T., Kuroiwa, T., and Tanaka, K. (2009) R2R3-type MYB transcription factor, CmMYB1, is a central nitrogen assimilation regulator in *Cyanidioschyzon merolae*. *Proc. Natl. Acad. Sci. USA*, **106**, 12548–12553.

Imamura, S., Terashita, M., Ohnuma, M., Maruyama, S., Minoda, A., Weber, P. M., Inouye, T., Sekine, Y., Fujita, Y., Omata, T., and Tanaka, K. (2010) Nitrate assimilatory genes and their transcriptional regulation in a unicellular red alga *Cyanidioschyzon merolae*: Genetic evidence for nitrite reduction by a sulfite reductase-like enzyme. *Plant Cell Physiol*., **51**, 707–717.

Imamura, S., Yoshihara, S., Nakano, S., Shiozaki, N., Yamada, A., Tanaka, K., Takahashi, H., Asayama, M., and Shirai, M. (2003) Purification, characterization, and gene expression of all sigma factors of RNA polymerase in a cyanobacterium. *J. Mol. Biol*., **325**, 857–872.

Juan, J. C., Kartika, D. A., Wu, T. Y., and Hin, T. Y. (2011) Biodiesel production from jatropha oil by catalytic and non-catalytic approaches: An overview. *Bioresour. Technol*., **102**, 452–460.

Karmakar, A., Karmakar, S., and Mukherjee, S. (2010) Properties of various plants and animals feedstocks for biodiesel production. *Bioresour. Technol*., **101**, 7201–7110.

Ladygina, N., Dedyukhina, E. G., and Vainshtein, M. B. (2006) A review on microbial synthesis of hydrocarbons. *Process Biochem*., **41**, 1001–1014.

Liu, S., Liu, C., Huang, X., Chai, Y., and Cong, B. (2006) Optimization of parameters for isolation of protoplasts from the Antarctic sea ice alga *Chlamydomonas* sp. ICE-L. *J. Appl. Phycol*., **18**, 783–786.

Maruyama, M., Horáková, I., Honda, H., Xing, X., Shiragami, N., and Unno, H. (1994) Introduction of foreign DNA into *Chlorella saccharophila* by electroporation. *Biotechnol. Tech.,* **8**, 821–826.

Metting, F. B. (1996) Biodiversity and application of microalgae. *J. Ind. Microbiol*., **17**, 477–489.

Metzger, P., Allard, B., Casadevall, E., Berkaloff, C., and Coute, A. (1990) Structure and chemistry of a new chemicalrace of *Botryococcus braunii* that produces lycopadiene, a tetraterpenoid hydrocarbon. *J. Phycol*., **26**, 258–266.

Metzger, P. and Largeau, C. (2005) *Botryococcus braunii*: A rich source for hydrocarbons and related ether lipids. *Appl. Microbiol. Biotechnol*., **66**, 486–496.

Nielsen, S. L. (2006) Size-dependent growth rates in eukaryotic and prokaryotic algae exemplified by green algae and cyanobacteria: Comparisons between unicells and colonial growth forms. *J. Plankton Res*., **28**, 489–498.

Satoh, A., Kato, M., Yamato, K., Ishibashi, M., Sekiguchi, H., Kurano, N., and Miyachi, S. (2010) Characterization of the lipid accumulation in a new microalgal species, *Pseudochoricystis ellipsoidea* (*Trebouxiophyceae*). *J. Jpn. Inst. Energ*., **89**, 909–913.

Schirmer, A., Rude, M. A., Li, X., Popova, E., and del Cardayre, S. B. (2010) Microbial biosynthesis of alkanes. *Science*, **329**, 559–562.

Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, C. J., Lea-Smith, D. J., and Smith, A. G. (2010) Biodiesel from

algae: Challenges and prospects. *Curr. Opin. Biotechnol*., **21**, 277–286.

Sizova, I., Fuhrmann, M., and Hegemann, P. (2001) A *Streptomyces rimosus aphVIII* gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. *Gene*, **277**, 221–229.

Sun, Y., Yang, Z., Gao, X., Li, Q., Zhang, Q., and Xu, Z. (2005) Expression of foreign genes in *Dunaliella* by electroporation. *Mol. Biotechnol*., **30**, 185–192.

Tan, C., Qin, S., Zhang, Q., Jiang, P., and Zhao, F. (2005) Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *J. Microbiol*., **43**, 361–365.

Teng, C., Qin, S., Liu, J., Yu, D., Liang, C., and Tseng, C. (2002) Transient expression of *lacZ* in bombarded unicellular green alga *Haematococcus pluvialis*. *J. Appl. Phycol*., **14**, 495–500.