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 (Achtouv 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

---

**Introduction**

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Three races of a green colonial microalga *Botryococcus braunii* are notable for their ability to synthesize hydrocarbons in addition to triacylglycerols (Achtouv 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

---

**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
Treouxiophyceae and tentatively named *Pseudo-
choriocysts ellipsoidea* also produces hydrocarbons (Satoh et al., 2010) of shorter carbon chains, such as heptadecene, heptadecane and eicosadienes as ma-
jor hydrocarbons. The hydrocarbon content in this or-
ganism 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 maxi-
mum 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 ob-
served (Ladygina et al., 2006), but the pathway for the
hydrocarbon synthesis has remained unclear until re-
cently. Schirmer et al. (2010) recently identified
two genes responsible for the hydrocarbon produc-
tion in cyanobacteria: an acyl-acyl carrier protein red-
ductase (PCC7942_orf1594 in *Synechococcus elong-
atus* 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 cyanobacte-
rial decarbonylase. In *B. braunii*, a cobalt-porphyrin-
containing enzyme that catalyzes decarbonylation of
aldehydes has been purified from the microsome frac-
tion, but the gene encoding the enzyme has not been
identified (Dennis and Kolattukudy, 1992). For the im-
provement of oil productivity in *P. ellipsoidea*, the iden-
tification and genetic manipulation of aldehyde decar-
bonylase 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 NaNO₃, 0.4 mM MgSO₄,
60 mM CaCl₂, 0.26 mM KH₂PO₄, 0.26 mM K₂HPO₄,
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 H₃BO₃, 150 mg L⁻¹ MnSO₄·5H₂O, 300 mg L⁻¹
ZnSO₄·7H₂O, 300 mg L⁻¹ CuSO₄·5H₂O, 70 mg L⁻¹
CoCl₂·6H₂O, 3 mg L⁻¹ Na₂MoO₄)] which was continu-
ously bubbled with air containing 1% (v/v) CO₂. G418
at a concentration of 100 μg ml⁻¹ was added if required.
MA5 plates were prepared by adding agar to the me-
dium to a final concentration of 1.5% (w/v).

**Construction of plasmids for transformation.** All
PCR reactions were done with KOD-Plus DNA poly-
merase (Toyobo, Osaka, Japan). For the cloning of
*nptII*, PCR was performed with the specific primer pair,
G418-F-Smal (5’-TCCCGGGATGGGATGGGCCATT
GAACAAGATG-3’, a Smal site is underlined) and
G418-R-XbaI (5’-CCTCTAGATCAGAAGAAC T C G T C A A
GAAGCGC-3’, an XbaI site is underlined), and the Eu-
karyotic 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 Smal and Xbal, and then inserted between the
Smal and Xbal 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, frag-
ment 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 ampli-
fied 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’-GGGATCCGGCCATTGAACA
AGAGGTG3’) and neo-SQ-R (5’-TCAGAGACTCTC
GAAAGGCGC-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 ex-
cised with EcoRI or KpnI, and cloned into the EcoRI or
KpnI site of pUC119. For the construction of pG418-
SI03 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 psI03 (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.

<table>
<thead>
<tr>
<th>Gene (plasmid)</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBIQUITIN1</td>
<td>Ubi5-ProF</td>
<td>cttgaacctagatctgctgc</td>
</tr>
<tr>
<td>(pG418-U1U and pG418-U1A)</td>
<td>Ubi5-ProR-neo</td>
<td>cttgtcaatggccagttcccaatcgcacctcgccacacacag</td>
</tr>
<tr>
<td>ARPI</td>
<td>Act5-ProF</td>
<td>ggccattctgctgagctgc</td>
</tr>
<tr>
<td>(pG418-A1U)</td>
<td>Act5-ProR-neo</td>
<td>cttgtcaatggccagttcccaatcgcacctcgccacacacag</td>
</tr>
<tr>
<td>UBIQUITIN2</td>
<td>Ubi3-ProF</td>
<td>gcacagcatgctgacagctgc</td>
</tr>
<tr>
<td>(pG418-U2U and pG418-U2A)</td>
<td>Ubi3-ProR-neo</td>
<td>cttgtcaatggccagttcccaatcgcacctcgccacacacag</td>
</tr>
<tr>
<td>TUBULIN1</td>
<td>Tub1-ProF</td>
<td>cttgtgagattccatccct</td>
</tr>
<tr>
<td>(pG418-T1U and pG418-T1A)</td>
<td>Tub1-ProR-neo</td>
<td>cttgtcaatggccagttcccaatcgcacctcgccacacacag</td>
</tr>
<tr>
<td>GAPDH1</td>
<td>gpdA1-proF</td>
<td>ccaccgcagctgacacagctg</td>
</tr>
<tr>
<td>(pG418-G1U)</td>
<td>gpdA1-proR-neo</td>
<td>cttgtcaatggccagttcccaatcgcacctcgccacacacag</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Gene (plasmid)</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBIQUITIN1</td>
<td>Ubi5-terF-neo</td>
<td>gccttcctgagcttctcctagataaagctcttcctgcccag</td>
</tr>
<tr>
<td>(pG418-U1U, -A1U, -U2U, -T1U, -G1U)</td>
<td>Ubi5-terR</td>
<td>cggatctgatgctgacacagctg</td>
</tr>
<tr>
<td>ACTIN1</td>
<td>Act1-terF-neo</td>
<td>gccttcctgagcttctcctagataaagctcttcctgcccag</td>
</tr>
<tr>
<td>(pG418-T1A, -T2A, -U2A, -U1A)</td>
<td>A1ter(1.5 kb)-R2</td>
<td>cggatctgatgctgacacagctg</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Gene (plasmid)</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBIQUITIN1 (pG418-U1U)</td>
<td>U5(pro)-F-EcoRI</td>
<td>acaGAATTCttgtctctcctcagcagcagccatcag</td>
</tr>
<tr>
<td>UBIQUITIN1 (pG418-U1A)</td>
<td>U5(pro)-F-KpnI</td>
<td>ccaGGTACCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>ARPI</td>
<td>A5(pro)-F-EcoRI</td>
<td>acagtgatgctgagttgccagtcag</td>
</tr>
<tr>
<td>(pG418-A1U)</td>
<td>A5(pro)-F-KpnI</td>
<td>ccaGGTACCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>UBIQUITIN2</td>
<td>U3(pro)-F-EcoRI</td>
<td>ccctGAATTCgagaatcgctgctgctg</td>
</tr>
<tr>
<td>(pG418-U2U)</td>
<td>U3(pro)-F-KpnI</td>
<td>ccctGAATTCgagaatcgctgctgctg</td>
</tr>
<tr>
<td>TUBULIN1</td>
<td>T1(pro)-F-EcoRI</td>
<td>gcaGAATTCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>(pG418-T1U)</td>
<td>T1(pro)-F-KpnI</td>
<td>gcaGAATTCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>TUBULIN1</td>
<td>T4(pro)-F-KpnI</td>
<td>gcaGAATTCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>(pG418-T2A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reverse primers

<table>
<thead>
<tr>
<th>Gene (plasmid)</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBIQUITIN1 (pG418-U1U, -A1U, -U2U, -T1U, -G1U)</td>
<td>U5(ter)-R-EcoRI</td>
<td>cagGAATTCttgctgctggagatgtgagctg</td>
</tr>
<tr>
<td>ACTIN1</td>
<td>A1ter(1.5 kb)-R-KpnI</td>
<td>accGGTACCggggtttgagacgctgag</td>
</tr>
</tbody>
</table>

Nucleotides in upper case indicate the EcoRI or KpnI sites.
Fragment 3 (the \textit{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).

\textit{Particle bombardment}. \textit{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.

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Plasmid & Primer & Sequence (5'-'3') \\
\hline
pG418-SI103 & SI103-F \& phspA-R\_G418-5' & gccgcttgaactagtggatc aatgccgatcccatctgaaatcttcagcaccgg cttatgcctcgccgtctgtatgtg aatggccgatccatgtttgcgggttgtgactgaaacg \\
pG418-HYG3 & Ptub-F \& Ptub-R\_G418-5' & cttatatgcttccggctcgtatgttg aatggccgatccatgtttgcgggttgtgactgaaacg \\
\hline
\end{tabular}
\caption{Primers prepared for fragment 1 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.}
\end{table}

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

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Plasmid & Primer & Sequence (5'-'3') \\
\hline
pG418-SI103 & G418-3\_'SI3'\_UTR-F \& SI3'-R & gacgagtttcctctg\textbf{gt}ggacgtgatggtgttggtg gaaacaaagctgggtacccgctttacaatagccc \\
pG418-HYG3 & G418-3\_'hyg3'\_UTR-F \& hyg-R & gacgagtttcctctg\textbf{gt}taaggatcccccgtcctgtaaat gtcctgcaaggccgattaagt \\
\hline
\end{tabular}
\caption{Primers prepared for fragment 2 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.}
\end{table}

Bold and underlined nucleotides indicate the stop codon of \textit{NPTII} and adapter sequences for the second PCR, respectively.

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Plasmid & Primer & Sequence (5'-'3') \\
\hline
pG418-SI103 & phspA-3\_'G418-F \& G418-R\_SI3'\_UTR & \textbf{gaagaattcgaaggcatggg}gatgccgcatggaacaagatg acaccatcaacctcctcaagaactcgtagaagaagcgc \\
pG418-HYG3 & Ptub-3\_'G418-F \& G418-R\_hyg3'\_UTR & tcaaaccccacacag\textbf{ggg}gatgcccctgcatggaacaagatg gagcggggatccattacaagaactcgtagaagaagcgc \\
\hline
\end{tabular}
\caption{Primers prepared for fragment 3 in the two-step PCR for construction of pG418-SI103 and pG418-HYG3.}
\end{table}

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

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Plasmid & Primer & Sequence (5'-'3') \\
\hline
pG418-SI103 & phspA-F\_SmaI \& SI3'\_UTR-R\_XbaI & tcCCC\textbf{GGGG}Gagctcgctgaggcttgaca ccT\textbf{CTAGA}cgcctaatagcccagcc \\
pG418-HYG3 & Ptub-F\_XbaI \& hyg-R\_HindIII & a\textbf{TCTAGA}cctctgctgagctagacactccag gcAAG\textbf{CTT}ggtacccgctttacaatagcc \\
\hline
\end{tabular}
\caption{Primers used for the second PCR or construction of pG418-SI103 and pG418-HYG3.}
\end{table}

Nucleotides in upper case indicate SmaI, XbaI, or HindIII site.
of 0.6 μm in diameter (Bio-Rad). After the incubation, those spots contained 1.8 to 5.8 × 10⁶ 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 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'-GATCGGCCATTGAACAAAGAT-3') and G418_R (5'-GCCATACCGTAAAGACACAGAG-3'), or Tub_Pro_F (5'-TATCTAGCGGGGTGTTGTTGA-3') and Act_Ter_R (5'-TCAACATCCACCTGCA-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 25 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 Transcripter 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 EcoRI, 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 mM 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.

**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,
#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 x 10⁶ cells were seeded. Thus, the transformation efficiencies ranged from 1.7 to 5.6 per 10⁷ 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 EcoRI, 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 EcoRI-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.

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
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.

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 UBQUITIN1 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 RBPCS2 promoters and RBPCS2 terminator function in P. ellipsoidea.
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.

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