Development of efficient genetic-transformation- and genome-editing systems, and the isolation of a CRISPR/Cas9-mediated high-oil mutant in the unicellular green alga Parachlorella kessleri strain NIES-2152

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Abstract

Background

*Parachlorella kessleri* strain NIES-2152, a unicellular green alga, has the characteristics of rapid growth and high lipid productivity; thus this strain has been considered to be a candidate for use in biofuel production. However, the commercialization of biofuels requires significant cost reduction, and strain improvements may be needed to achieve this objective.

Results

In the first part of this study, we developed a genetic transformation method applicable to *P. kessleri* as follows. During the cell cycle of *P. kessleri*, daughter cells develop within the mother cell wall. Therefore, *P. kessleri* cells are encompassed by two cell-wall layers: a daughter cell wall and a mother cell wall, during most of its cell cycle except for a brief period after the cleavage of the mother cell wall. We found that cells just hatched out of the mother cell wall exhibited high efficiency in taking up DNA through electroporation. After optimizing the electroporation conditions, we achieved a transformation efficiency of 3.2 to 4.6 × 10^{-5} transformants per input cell.

Previously, we had isolated a mutant of *P. kessleri* named strain PK4 that accumulated higher concentrations of lipids than the wild-type strain. Resequencing of the PK4 genome had identified three genes with potential defects associated with the high lipid phenotype. In the second part of this study, we delivered Cas9-gRNA ribonucleoproteins (RNPs) targeting each of the three genes into *P. kessleri* cells using the same protocol as the genetic transformation, and successfully disrupted these three genes separately. The disruptants of one gene encoding a plastidic ATP/ADP translocase exhibited >30% higher lipid productivity than that of the wild-type strain under diurnal conditions.

Conclusions

We established an effective gene-editing method applicable to *P. kessleri*. Using this method, we succeeded in creating strains showing significantly higher lipid productivity than the wild-type strain, especially under diurnal rhythms.

Background

*Parachlorella kessleri* is a unicellular alga belonging to the family Chlorellaceae in the class Trebouxiophyceae, with a haploid life cycle characterized by autospore formation [1]. This species exhibits a relatively rapid photosynthetic growth rate and accumulates a high level of lipids under stress conditions [2, 3], making it a top contender for biodiesel production. Furthermore, *P. kessleri* serves as an excellent feedstock for high-value products, including carotenoids exhibiting antioxidant activities [4] and extracellular polysaccharides (EPS) exhibiting antitumor and immunomodulatory activities, as well as remarkable heavy metal sorption ability [5–7]. Moreover, this organism demonstrates efficient nitrogen
and phosphorous removal from wastewater [8, 9]. Due to these impressive features, *P. kessleri* has garnered considerable interest across various industrial sectors.

*P. kessleri*, formerly known as *Chlorella kessleri* [1, 10, 11], has been utilized as food supplement for many years, earning it recognition for its safety to human health [12, 13]. Furthermore, this organism is deemed suitable for outdoor mass cultivation for the production of biofuel, food, feed, other high-value products, and wastewater treatment.

Despite the immense potential of *P. kessleri* for the industrial applications, the production costs associated with raw materials from this alga are still too high to be competitive with existing materials. Consequently, significant cost reduction in manufacturing biomass- and value-added products is needed. One of the methods to realize it is strain improvement, achieved through molecular breeding. A draft genome sequence of *P. kessleri*, crucial information for the molecular breeding, has been determined, and major metabolic pathways have been annotated [14, 15; GenBank assembly accession number: GCA_001598975]. Furthermore, interesting mutants have been isolated through mutagenesis by heavy-ion-beam irradiation [16, 17]. Still, genetic transformation techniques applicable to *P. kessleri* are in their infancy. So far, two methods for the transformation of *P. kessleri* have been reported: chloroplast genome transformation using biolistic bombardment [18] and *Agrobacterium*-mediated nuclear genome transformation [19]. Electroporation, a technique for the introduction of polynucleotides, proteins, and other macromolecules into algal cells [20–24], shows promise in this context. In the present study, we established an effective electroporation-based genetic transformation method applicable to *P. kessleri*. Furthermore, we succeeded in precise disruption of target genes using CRISPR/Cas9-mediated gene editing.

**Results and discussion**

**Synchronization of cultures of strain NIES-2152**

In our previous study for the development of a genetic transformation system applicable to autospore-forming green alga *Coccomyxa*, we found that young daughter cells, released from cleaved mother cell walls, are highly competent to receive transforming DNA [24]. *P. kessleri* strain NIES-2152 (hereinafter referred to as "strain NIES-2152" or "the wild-type strain") is also an autospore-forming unicellular green alga, and it has been reported that the cell wall thickness is thinnest in daughter cells immediately after their release from their mother cell walls [1]. To enrich such daughter cells, cells of strain NIES-2152 were grown under a (16 h light)/(8 h dark) cycle (L/D 16:8 cycle) for 3–6 days to synchronize nuclear and cellular division. Light-microscopic observation of the cells revealed that the cell population with smaller cell sizes increased with increased dark period. The percentage of cells with a cross-sectional area lower than 20 µm² reached maximum during the cycle between 8-h dark (= 0-h light) and 1-h light (Fig. 1). Subsequently, cell sizes increased with an increased light period. This observation indicated that the cleavage of the mother-cell wall, followed by the release of young daughter cells, terminated around the end of the dark period.
Construction of plasmids for genetic transformation

In our studies to transform *Coccomyxa* strains, two drug-resistant genes, the neomycin/G418-resistant gene (*neo*) [25] and the bleomycin/Zeocin®-resistant gene (*ble*) [21], have successfully been used as selection markers for generating transformants.

To express these drug-resistant genes in strain NIES-2152, effective promoters and terminators are required to construct expression constructs for the selection marker genes. For this purpose, highly expressed genes in strain NIES-2152 were selected from our preliminary transcriptome data, and their 5'-untranslated region (UTR) and the 3'-UTR were deduced to identify putative promoter- and terminator regions. Consequently, the heat shock protein 90-family gene (*HSP90*) and the plastidic ATP/ADP translocase 1 gene (*AATP1*) were chosen as they possess promoter- and terminator regions that could be confined within 1-kb regions. Additionally, the promoter/terminator regions from the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene 4 (*RBCS4*) were used because the promoters and terminators of *RBCS* from various green algae including *Chlamydomonas reinhardtii* [26–31], *Coccomyxa* sp. [32], and *Dunaliella salina* [33], were successfully used for expressing transgenes. The promoter and terminator regions of these three genes were PCR-amplified, and fused to the coding sequences of the *ble, neo*, or codon-optimized *neo* genes.

Genetic transformation of strain NIES-2152 by electroporation

When the *neo*- or codon-optimized *neo*-expression constructs were introduced in strain NIES-2152, the G418-resistance level of the transformants were 25 µg ml\(^{-1}\). However, at this G418 concentration, the emergence of spontaneous mutants resistant to G418 was observed at a frequency higher than \(10^{-6}\) per input cells. Therefore, only *ble*-expression constructs shown in Fig. S1 were utilized in subsequent studies.

We first investigated the electroporation conditions that yield the highest number of Zeocin®-resistant (*Zeo*\(^{r}\)) transformants of strain NIES-2152 after introduction of the expression construct of *ble* consisting of the promoter and terminator sequence of HSP90 (*bleHH*). No spontaneous *Zeo*\(^{r}\) mutants of strain NIES-2152 were observed when \(10^7\) cells were spread on agar plates containing 35 µg ml\(^{-1}\) Zeocin®. Therefore, *Zeo*\(^{r}\) transformants were screened on agar plates containing 35 µg ml\(^{-1}\) Zeocin®.

The electroporation process was facilitated using the ELEPO21 electroporator, which delivers two types of electric pulses called the poring pulse (Pp) and the transfer pulse (Tp) (https://www.nepagene.jp/e_products_nepagene_0029.html). The electric-field strength and the pulse-width of Pp were changed between 1,500 and 2,500 V cm\(^{-1}\), and between 2.5 and 15 ms, respectively. For Tp, the electric-field strengths were changed between 100 and 500 V cm\(^{-1}\), while the pulse width and number of pulses were fixed at 50 ms and 5 pulses, respectively. The cells of strain NIES-2152 were cultivated in BG-11 medium under an L/D 16:8 cycle and harvested 2 h after the beginning of the light
period, when OD\textsubscript{750} ranged between 0.2 and 2. A total of 114 electroporation runs were conducted using eight independent cultures under various combinations of electric-field strength and pulse widths. The presence of the \textit{ble} coding sequence in the genomes of Zeo\textsuperscript{r} colonies isolated from 13 independent electroporation samples were examined by PCR. All 116 Zeo\textsuperscript{r} transformants were positive in the \textit{ble} PCR (Fig. S2). Accordingly, genetic transformation frequencies were calculated by dividing the number of Zeo\textsuperscript{r} colonies on the selection plates by the number of input cells.

The transformation frequencies at different electric-field strengths of Pp were shown in Fig. 2a. The results demonstrated that the highest transformation efficiency was achieved when the electric-field strength and pulse-width of Pp were set at 2,500 V cm\textsuperscript{−1} and 15 ms, respectively. To determine optimum Tp conditions, the electric-field strength and pulse-width of Pp were fixed at 2,000 V cm\textsuperscript{−1} and 9 ms, respectively, while three electric-field strengths of Tp at 100, 250, or 500 V cm\textsuperscript{−1} were tested (Fig. 2b). The results demonstrated that an electric-field strength of Tp at 250 V cm\textsuperscript{−1} achieved the highest transformation efficiency.

The effect of growth stage on transformation efficiency was also examined as follows. Cells were harvested at OD\textsubscript{750} of 0.5 or 1.2, and electroporation was conducted with Pp electric-field strength of 2,500 V cm\textsuperscript{−1}, Pp pulse-width of 9 ms, and Tp electric-field strength of 100 V cm\textsuperscript{−1}. The transformation efficiency of cells harvested at OD\textsubscript{750} of 0.5 was 1.4 ± 0.6 × 10\textsuperscript{−5} transformants per input cells, while that harvested at OD\textsubscript{750} of 1.2 was 5.2 ± 0.4 × 10\textsuperscript{−6} transformants per input cell. Under other electroporation conditions as well, cells harvested at OD\textsubscript{750} below 1 showed higher transformation efficiency than those harvested at OD\textsubscript{750} above 1. This let us to conclude that the transformation efficiency is higher in cells harvested at OD\textsubscript{750} less than 1. The highest genetic transformation efficiency, ranging from 3.2 to 4.6 × 10\textsuperscript{−5} transformants per input cell, was obtained under the following conditions: Pp electric-field strength of 2,500 V cm\textsuperscript{−1}, Pp pulse-width of 15 ms, Tp electric-field strength of 250 V cm\textsuperscript{−1}, and harvesting cell density less than OD\textsubscript{750} of 1.

When cells grown to OD\textsubscript{750} between 0.2 and 0.5 were harvested at 0, 1, and 2 h after the beginning of the light period, and electroporated with Pp at 2,500 V cm\textsuperscript{−1} for 15 ms, and Tp at 250 V cm\textsuperscript{−1}, the transformation efficiencies were 3.8 ± 0.1 × 10\textsuperscript{−5}, 2.3 ± 0.8 × 10\textsuperscript{−5}, and 4.0 ± 0.6 × 10\textsuperscript{−5} transformants per input cells, respectively. Thus, no statistical difference was observed between the transformation efficiencies of cells harvested at different time points between 0 and 2 h after the onset of light period. Similar results were obtained under other electroporation conditions.

We also conducted 30 independent electroporation using cells grown under continuous light, i.e., without synchronization. The average of transformation efficiency was only 3.5 ± 3.8 × 10\textsuperscript{−7} transformants per input cells, which was two orders of magnitude smaller than that obtained with synchronized cells. Therefore, synchronization was found to be crucial for efficient genetic transformation in strain NIES-2152, and potentially in other green algae belonging to Trebouxiophyceae.
Finally, we compared the transformation efficiencies of bleHH with those of other two ble expression constructs, bleAA consisting of the promoter- and the terminator sequences of AATP1 that flank the ble coding sequence, and bleRR consisting of the promoter- and terminator sequences of RBCS4 that flank the ble coding sequence, using the optimum electroporation conditions described above. With each of these constructs, 10 electroporation experiments were carried out. The transformation efficiencies with bleHH, bleRR, and bleAA were $2.5 \pm 1.4 \times 10^{-5}$, $2.8 \pm 0.6 \times 10^{-5}$, and $2.0 \pm 1.0 \times 10^{-6}$ transformants per input cells, respectively. The size of bleAA is almost the same as that of the bleHH, and smaller than bleRR; therefore, the low transformation efficiency of bleAA should not be due to its size.

**Gene knockout by CRISPR/Cas9**

In our previous study, we isolated a mutant named strain PK4 from strain NIES-2152, which exhibits higher levels of lipid accumulation, using mutagenesis by heavy-ion beam irradiation [16, 17]. Whole genome resequencing of strain PK4 identified mutations in three genes, 8741_t (GenBank accession number LC42335), 9067_t (LC424334), and 9934_t (LC424333) [14, 17]. Therefore, CRISPR/Cas9 editing of these genes individually would help identifying the causative mutation responsible for the high lipid accumulation.

The 9934_t gene was deduced to code for a protein containing the C2 domain (Fig. 3a). This domain is known to be involved in calcium-dependent membrane targeting [34]. Thus, this gene is named CDMT1. Subsequently, several CRISPR RNAs (crRNAs) targeting the C2 domain (Table S2, Fig. 3a) were designed, and three crRNAs were selected for editing CDMT1 (Table S2). Since the genome editing frequency in Coccomyxa, belonging to the same class as strain NIES-2152 (Trebouxiophyceae), was very low, below $10^{-4}$ per input cells [21], co-delivering the bleHH DNA with Cas9- RNP was adopted in this study, and genome-edited clones were identified among Zeo$^r$ transformants. DNA was isolated from each of Zeo$^r$ colonies, and clones containing a bleHH insertion in the crRNA recognition sequence were screened by PCR using gene-specific primer sets, CDMT1_F and CDMT1_R (Table S1). When 7, 13, and 48 Zeo$^r$ colonies obtained with CDMT1_1, CDMT1_2, and CDMT1_3 guide RNA (gRNA), respectively, were examined, bleHH insertions were detected only in the CDMT1_3 target (target 3) site. Two independently isolated clones, CR24 and CR26, each carrying a bleHH insertion at the target 3 site were selected for further analyses. DNA sequencing of the target 3 region of strain CR24 revealed a truncated bleHH fragment with a 3-bp deletion ($\Delta$3) and a 99-bp deletion ($\Delta$99) at the 5- and 3-sides of the ble coding sequence, respectively, which was inserted at the position 3-bp upstream of the protospacer adjacent motif (PAM) site where Cas9 recognizes and cleaves DNA (Fig. 3c). On the other hand, the target site of strain CR26 cleaved 3-bp upstream of the PAM site acquired 1-bp insertions at both ends of the cleavage site, between which two copies of bleHH in an inverted orientation were integrated (Fig. 3c).

The volumetric biomass yields and lipid contents of CDMT1-knockout strains were analyzed. Strain NIES-2152, strain PK4, and the two CDMT1-knockout strains, CR24 and CR26, were grown in 1/5 strength urea-phosphate (1/5 UP) medium for 14 days under continuous light. The growth yields of the CDMT1-knockout strains, determined as cell-mass dry weight per liter of culture, were almost equal to the wild-
type strain, whereas that of strain PK4 was significantly lower than those of other strains (Fig. 3d). The lipid contents of the CDMT1-knockout strains at days 7, 11, and 14 were significantly lower than that of strain PK4, and almost equal to the wild-type strain (Fig. 3e). From these results, it was concluded that the CDMT1 mutation was not the cause of higher lipid accumulation in strain PK4.

Next, we disrupted the 8741_t gene. The 8741_t gene consists of duplicated coding sequences of endo-1,4-β-mannanase, and its gene product carries two conserved cellulase domains at its C-terminal half (Fig. 4a). We named this gene DMAN1 (duplicated mannanases 1). We designed crRNAs targeting three different sequences in the cellulase domain (Table S2, Fig. 4a). gRNA containing each crRNA was then conjugated with Cas9 protein, and the RNP complex and the bleHH DNA fragment were delivered into cells of strain NIES-2152. DNA was isolated from 40, 40, and 37 Zeo’ colonies isolated after introduction of the DMAN1_1, DMAN1_2, and DMAN1_3 gRNA, respectively, and clones containing a bleHH insertion in the crRNA recognition sequence were screened by PCR using the primer set, DMAN1_F and DMAN1_R (Table S1). From most of the clones, the 1,492-bp-long DNA corresponding the PCR product from the wild-type sequence was amplified, except three clones from which longer DNA fragments of about 3-kb were amplified in addition to the 1,492-bp-long DNA fragment (Fig. S3). This result suggested that these three clones were not pure, but consisted of two types of populations, one carrying a bleHH insertion in the target site, and the other not carrying bleHH insertion in the target site. Therefore, these clones were further purified through several rounds of single colony isolation, and pure clones carrying a bleHH insertion in the target site were recovered from two of the three clones. The two clones were named strains CR189 and CR193, and their genomic DNA around the crRNA recognition sequence were sequenced. The DNA sequence in strains CR189 indicated that the target sequence had been cleaved 3-bp upstream of the PAM sequence into which bleHH with a 1-bp deletion (Δ1) at both sides and a 1-bp (G) insertion at one side was integrated (Fig. 4c). On the other hand, in the genome of strain CR193, one copy of bleHH with a 1-bp deletion (Δ1) at one side and a 2-bp (GG) insertion at the other side was integrated at the cleavage site (Fig. 4c).

Next, we analyzed the effects of the knockout of DMAN1 on the volumetric biomass yield and the lipid content. Strain NIES-2152, strain PK4, and two knockout strains of DMAN1, namely strains CR189 and CR193, were grown in 1/5 UP medium for 14 days under continuous light conditions. The growth yields of the two knockout strains at days 11 and 14 were significantly lower than that of the wild-type strain, and slightly higher than that of strain PK4 (Fig. 4d).

The lipid contents in the two knockout strains at days 7, 11, and 14 were significantly lower than that of strain PK4, and almost the same as that of the wild-type strain (Fig. 4e). These results indicated that the mutation in the DMAN1 was not the cause of high lipid phenotype of strain PK4, but one of the causes of the low growth yield of strain PK4.

The product of the 9067_t gene exhibited similarities to plastidic ATP/ADP translocases (AATPs) from Arabidopsis thaliana. Therefore, the gene was designated as AATPL1 (ATTP-like 1). AATP is also called plastidic ATP/ADP antiporter or plastidic nucleotide transporter (NNT). The AATP family proteins are
found in many plants and algae. They are localized to the inner membrane of the plastid envelope and catalyze the import of ATP in the plastid coupled with the export of ADP from the plastid. AATPs thus provide the plastid stroma with ATP, which is required for many anabolic processes [35]. The AATP family generally comprises 12 transmembrane helices [36–38] (Fig. 5a). In the protein sequence of AATPL1, 12 transmembrane helices were predicted using the Deep TMHMM program [39], with 6 helices located in the N-terminal half and another 6 helices located in the C-terminal half. These two regions were designated as the conserved domains of AATPs (IPR004667) in the InterPro database. We designed three crRNAs, namely AATPL1_1, AATPL1_2, and AATPL1_3, each targeting different sequences in the N-terminal IPR004667 domain (Fig. 5a, Table S2). Each of these crRNAs was hybridized with tracrRNA and then conjugated with Cas9 protein to be electroporatically introduced into cells of strain NIES-2152 along with the bleHH DNA fragment. Forty-five clones were randomly selected from Zeo\(^r\) transformants obtained with each crRNA for genomic DNA isolation, and those carrying a bleHH insertion in the crRNA recognition sequence were screened by PCR using the primer set, AATPL1_F and AATPL1_R (Table S1). We successfully obtained knocked-in clones with all gRNA. Two independent clones, CR12 and CR97, were then selected for further analyses. The DNA sequencing of the regions around the AATPL1_1 and AATPL1_3 target sites in the genomes of strains CR12 and CR97, respectively, revealed that their genomes have been cleaved 3-bp upstream of the PAM sequence followed by the insertion of bleHH. In strain CR12, two copies of bleHH were inserted in tandem, with a 13-bp deletion (Δ13) at one end. The target site was also rearranged after Cas9 cleavage: at one end, an 8-bp deletion (Δ8) plus a 36-bp insertion (ins36) were observed, while at the opposite end, a 1-bp insertion (C) occurred. (Fig. 5c). In strain CR97, two copies of bleHH were inserted in inverted repeats with a 1-bp deletion (Δ1) at one end. The target site was also rearranged after Cas9 cleavage with a 1-bp insertion (T) at one end, and a 2-bp insertion (GT) at the opposite end (Fig. 5c).

Next, we analyzed the effects of the knockout of AATPL1 on the volumetric biomass yield and the lipid content. Strain NIES-2152, strain PK4, and the two knockout strains of AATPL1, strains CR12, and CR97, were grown in 1/5 UP medium for 14 days under continuous light condition.

The lipid contents in the two AATPL1-knockout strains at days 11 and 14 were almost the same as that of strain PK4 and significantly higher than that of the wild-type strain (Fig. 5e). This result indicates that the mutation in AATPL1 was the cause of high-lipid phenotype observed in strain PK4. On the other hand, the growth yields of two knockout strains were similar to that of the wild-type strain but significantly higher than that of strain PK4 (Fig. 5d). Thus, two phenotypes of strain PK4, namely higher lipid content and lower growth yield than those of the wild-type strain, were caused by separate mutations. The volumetric lipid yield of the wild-type strain or its derivatives was calculated by multiplying volumetric biomass yield by lipid content. Those of the AATPL1-knockout strains were significantly higher than those of strain PK4 and slightly higher than that of the wild-type strain (Fig. 5f). The starch contents of strain PK4 and the AATPL1-knockout strains (CR12 and CR97) were lower than that of the wild-strain (Fig. 5g).
A. thaliana possesses two genes for AATPs, At1g80300 and At1g15500, encoding AATP1 and AATP2, respectively. The loss of the function of ATTP1 marginally affected the plant development. Similarly, mutants of A. thaliana defective in AAT2 grew normally under long-day conditions. However, under short-day conditions at low light, their growth was severely impaired [40]. Thus, in A. thaliana, nocturnal ATP import into the plastid seems to be required for proper anabolic metabolism and normal plant development.

Bearing in mind of the results of the A. thaliana AAT2 mutants, strain NIES-2152, strain PK4, and the two AATPL1-knockout mutants (strains CR12 and CR97) were grown in 1/5 UP medium for 14 days under short-day conditions with an L/D 10:14 h cycle. Under the reduced day length from 24 h to 10 h, the growth yields at day 14 of strain NIES-2152 decreased from 4.9 g l$^{-1}$ to 3 g l$^{-1}$. The growth yields of other strains also decreased similarly: 2.8 g l$^{-1}$ for strain PK4, and 3.2 g l$^{-1}$ for the two AATPL1-knockout strains (Fig. 6a). Rather unexpectedly, very similar growth yields were observed when these strains were cultured under different L/D cycles, namely 16:8 h-, 12:12 h-, and 10:14 h cycles (Supplementary Fig. 4, 5). These results clearly demonstrate that the mutation in AATPL1 did not affect the growth of the host under short-day conditions. Accordingly, the phenotypes of the AATPL1 mutants were different from those of the A. thaliana AAT2 mutants, suggesting that different AATPs can have distinct functions in plants and algae.

The lipid contents of strain NIES-2152 and its derivatives defective in AATPL1, strains PK4, CR12, and CR97, at day 14 were almost 50% (w/w) or more when they were grown under continuous light. However, their lipid contents decreased as day length decreased. Under all light conditions examined, the lipids contents of strains PK4, CR12, and CR97 were always higher than that in the wild-type strain. Lipid productivity (g l$^{-1}$ day$^{-1}$) is an important index for economical feasibility of biodiesel production. In AATPL1-knockout strains, these values were $0.23 \pm 0.01$, $0.15 \pm 0.01$, $0.12 \pm 0.01$, and $0.11 \pm 0.01$ under day lengths of 24, 16, 12, and 8 h, respectively, which were 0%, 43%, 22%, and 29% higher than those of the wild-type strain. Thus, the AATPL1-knockout strains may be promising candidates for outdoor production of biofuels.

Below, two-letter species abbreviations, At and Pk, are used as prefixes in gene and protein names of A. thaliana and P. kessleri origins, respectively. In the genome of strain NIES-2152, two other genes encoding AATPs were found in addition to PkAATPL1. One of them is the above-mentioned PkATTP1 (GenBank accession number OR344785) whose promoter and terminator regions were used for the bleAA construction, while the other was named PkAATPL2 (GenBank accession number OR344784). The subcellular location of the PkAATP1, PkAATPL1, and PkAATPL2 predicted by DeepLoc-2.0 (https://services.healthtech.dtu.dk/service.php?DeepLoc) was plastidic (Table S3). The amino acid sequence of PkAATP1 showed 68% and 69% identities with those of AtAATP1 and AtAATP2, respectively, while the amino acid sequence of PkAATPL1 and PkAATPL2 showed less than 37% identity with those of AtAATP1 and AtAATP2. A similarity search of the PkAATPL1 sequence against the UniProt database (https://www.uniprot.org/proteomes/) revealed that proteins highly similar to PkAATPL1 were distributed only in green algae (Table S8). Search in the UniProt database also showed that green algae have more
than three different ATTPs, similarly to strain NIES-2152. Then, the phylogenetic relationships among ATTPs from \textit{A. thaliana}, strain NIES-2152, and nine green algae listed in Table S9 were deduced using the maximum likelihood method (Fig. S6). In this analysis, ATTP sequences translated from the truncated gene sequences (A0A0D2NFA4, A0A0D2MTH2, and A0A7J7Q1S0) and a glutathione S-transferase fusion protein (E1Z4I7) (Table S8) were not included. The analysis showed that AATPs were divided into three clades: AtATTP1 and PkAATP1 belonged to clade 3, PkAATPL1 belonged to clade 2, and PkAATPL2 belonged to clade 1. The molecular phylogenetic analysis of the amino acid sequence of the N-terminal region of truncated gene A0A7J7Q1S0 from \textit{Scenedesmus} sp. strain NREL 46B-D3 showed that it belonged to clade 2, thus all the green algae except \textit{Chlorella sorokiniana} strain UTEX 1602 had plastidic ATTP belonging to clade 2. These results suggested that plastidic ATTP belonging to clade 2 distributed only in green algae and has some function specific to green algae.

Although the mechanisms that increase lipid contents of the \textit{PkAATPL1}-knockout mutant remained unknown, we concluded that the high-lipid phenotype of strain PK4 was due to the \textit{PkAATPL1} mutation. We also revealed that the slower growth of strain PK4 compared to the wild-type strain was not caused by the \textit{PkAATPL1} mutation, but by the mutation in \textit{PkDMAN1}.

**Conclusions**

In this study, we established an electroporation-mediated genetic transformation method applicable to \textit{P. kessleri}. The genetic transformation frequency achieved by the method was 3.2 to 4.6 × 10^{-5} transformants per input cell. Using this method, we have succeeded in delivering Cas9-gRNA RNP into \textit{P. kessleri} cells and disrupting target genes. We disrupted three genes one by one, which might cause the high-lipid phenotype of strain PK4, and identified that the inactivation of \textit{PkAATPL1}, encoding a plastidic AATP, resulted in the high-lipid phenotype. Consequently, we succeeded in creating strains showing significantly higher lipid productivity than the wild-type strain, especially under diurnal rhythms. The gene knockout frequency by CRISPR/Cas9 was very low, being less than 3.3 × 10^{-6} per input cell. We are currently working to improve the selection method without using exogenous marker genes.

**Methods**

**Algal strains and culture conditions**

\textit{P. kessleri} strain NIES-2152 was obtained from the Microbial Culture Collection, National Institute for Environmental Studies (NIES) (Tsukuba, Japan). Strain NIES-2152 and its derivatives were cultured in 120-ml test tubes containing 50-ml BG-11 medium \cite{41} under continuous illumination with daylight fluorescent tubes (40W FL40S • FR • P, Panasonic, Japan) at 100 µmol m^{-2} s^{-1} in a plant-growth chamber (type #CLE-303, TOMY, Japan). To induce lipid biosynthesis under nitrogen-depleted conditions, cells were grown in 1/5 strength urea-phosphate (1/5 UP) medium \cite{42}. These cultures were bubbled with 1% (v/v) CO_{2} at 25°C. To prepare agar plates, media were solidified with 1.5% (w/v) agar (Bacto Agar, BD Difco, USA). The plates thus prepared were inoculated with strain NIES-2152 or its derivatives and were
incubated in a plant-growth chamber. When necessary, Zeocin® at 35 µg ml\(^{-1}\) was included in the agar plates.

**Cell size measurement**

One-ml cell suspension was sampled from cultures of strain NIES-2152 grown in BG-11 medium and centrifuged at 2,000 g for 5 min at room temperature. Subsequently, cells in the pellet were fixed in 1 ml of BG-11 medium containing 1% (v/v) formaldehyde. The fixed cells were observed by light-microscopy (BX51; Olympus, Japan). The microscopic images were recorded with a CCD camera, and cell sizes were determined using the ImageJ software [43].

**Construction of plasmid**

For the isolation of genomic DNA, cells of strain NIES-2152 in 50-ml of culture were collected in a 1.5-ml screw-cap microtube (WATSON #1392 – 200, Japan) and frozen in liquid nitrogen. The frozen cells were then disrupted using pre-chilled metal crusher (TAITEC Corporation, Saitama, Japan). The disrupted cells were suspended in TE buffer, and DNA was isolated from the lysate with phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. The DNA fraction was further purified with RNase A treatment, the second phenol/chloroform/isoamyl alcohol extraction, and the second ethanol precipitation.

PCR reactions were carried out in 50 µl of PCR reaction buffer (Takara, Japan) containing an appropriate amount of DNA template, 10 pmol of the forward and reverse primers listed in Table S1, and 25 µl of PrimeSTAR Max DNA polymerase Premix (2 ×), according to the manufacturer's protocols (Takara, Japan). PCR products were purified with NucleoSpin gel and PCR clean-up Kit (Takara, Japan).

The pbleHH plasmid (NCBI/DDBJ accession number LC775354), carrying an expression construct of ble consisting of the promoter- and terminator sequences of *HSP90* that flank the ble coding sequence, was constructed as follows. First, the 0.4-kb fragment comprising the ble coding sequence was PCR-amplified using the Hsp_ble_F- and Hsp_ble_R primers and 10 ng of pble-PeEGFP-KE1E DNA [21] as the template. In parallel, the 0.5-kb fragment comprising the *HSP90* promoter was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the HSP90P_F- and HSP90P_R primers; similarly, the 1.1-kb fragment comprising the *HSP90* terminator was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the HSP90T_F- and HSP90T_R primers. These three DNA fragments were assembled into a single fragment by PCR using 10 ng each of the three DNA fragments as the templates and the HSP90P_F- and HSP90T_R primers. The resulting 1.7-kb DNA fragment was purified using the NucleoSpin Gel and PCR clean-up kit (Takara, Japan), digested using EcoRV and EcoRI, and cloned into the EcoRV and EcoRI sites of pBluescript II sk (+).

The pbleAA plasmid (NCBI/DDBJ accession number LC775355), carrying the ble expression construct consisting of the promoter- and the terminator sequences of *AATP1* that flank the ble coding sequence, was constructed as follows. The 0.4-kb fragment comprising the ble coding sequence was PCR-amplified using 10 ng of the pbleHH DNA as the template and ble_F- and ble_R primers. The 0.4-kb fragment
comprising the promoter sequence of \textit{AATP1} was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the AATP1P\textsubscript{F} and AATP1P\textsubscript{R} primers. The 0.8-kb fragment comprising the terminator sequences of \textit{AATP1} was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the AATP1T\textsubscript{F} and AATP1T\textsubscript{R} primers. These three DNA fragments were assembled into a single DNA fragment by PCR using 10 ng each of the three fragments as the templates and the AATP1P\textsubscript{F} and AATP1T\textsubscript{R} primers. The resulting 1.6-kb DNA fragment was purified using the NucleoSpin Gel and PCR clean-up kit and cloned into the HincII site of pUC118.

The pbleRR plasmid (NCBI/DDBJ accession number LC775356), carrying the \textit{ble} expression construct consisting of the promoter- and terminator sequences of \textit{RBCS4} that flank the \textit{ble} coding sequence, was constructed as follows. The 0.4-kb fragment of the \textit{ble} coding sequence was PCR-amplified using 10 ng of pbleHH DNA as the template and the ble\textsubscript{F} and ble\textsubscript{R} primers. The 0.9-kb DNA fragment comprising the promoter sequence of \textit{RBCS4} was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the RBCS4P\textsubscript{F} and RBCS4P\textsubscript{R} primers. The 0.9-kb DNA fragment comprising the terminator sequence of \textit{RBCS4} was PCR-amplified using 0.1 µg of the genomic DNA of strain NIES-2152 as the template and the RBCS4T\textsubscript{F} and RBCS4T\textsubscript{R} primers. These three DNA fragments were assembled into a single fragment by PCR using 10 ng each of the three fragments as the templates and the RBCS4P\textsubscript{F} and RBCS4T\textsubscript{R} primers. The resulting 2.1-kb DNA fragment was purified using the NucleoSpin Gel and PCR clean-up kit, and cloned into the HincII site of pUC118.

The sequences of the pbleHH, pbleAA, and pbleRR plasmids (Supplementary Fig. 1) thus constructed were verified by Sanger sequencing on both strands. For the genetic transformation, a DNA fragment containing a \textit{ble} expression construct in one of the three plasmids was prepared by PCR-amplification using 10 ng of the pbleHH-, pbleAA-, or pbleRR DNA as a template and an appropriate PCR-primer set shown in Table S1. The PCR products were purified with the NucleoSpin Gel and PCR clean-up kit and used for genetic transformation.

**Delivery of RNP and/or DNA in cells of strain NIES-2152 using electroporation**

Cells were precultured under an L/D 16:8 cycle at a light intensity of 100 µmol m\textsuperscript{–2} s\textsuperscript{–1} in BG-11 medium for 3–4 days until the cell density reached OD\textsubscript{750} of 1.0. The preculture was diluted to OD\textsubscript{750} of 0.0001–0.00005 in 50 ml of fresh BG-11 medium, and cells were grown under the same conditions as those in the preculture. On the fifth day, 2 h after the start of a light period, cells were harvested by centrifugation at 2,900 \textit{g} for 5 min, washed twice with 5 mM 2-morpholinoethanesulfonic acid buffer (pH 5.5), and suspended in Max Efficiency\textsuperscript{®} Transformation Reagent for Alga (Thermo Fisher Scientific, USA) supplemented with 1% (w/v) glucose to cell densities of 1.0 × 10\textsuperscript{9} cells ml\textsuperscript{–1}. The cell suspension was kept on ice prior to electroporation.

To deliver only DNA, 1 µg of a DNA fragment carrying a \textit{ble} expression construct (bleHH) was added to 30 µl of the cell suspension kept on ice, and the mixture was transferred to a 2-mm-gapped electroporation
cuvette (EC-002S, Nepa Gene, Japan) to incubate at 16 °C for 2 min. The cuvette was placed into an electroporator (ELEPO21, Nepa Gene, Japan), and its electrode impedance was checked to be higher than 4 kΩ. A single poring pulse (Pp) was applied at 1,500–2,500 V cm⁻¹ with a pulse duration of 2.5–15 ms and a pulse interval of 50 ms. Immediately after the poring pulses, five transfer pulses (Tps) of alternative polarities (+ and −) were applied at 100–500 V cm⁻¹ with a 50 ms pulse duration and a 50 ms pulse interval.

CRISPR/Cas9-mediated genome editing was performed as described previously [21] with some modifications. CRISPR RNAs (crRNAs) were designed using the CRISPRdirect software [44], and listed in Table S2. Potential off-target sites for each of the designed crRNAs were searched in the genome of strain NIES-2152 for 12-bp-long sequences adjacent to the PAM sequence that perfectly matches with the 3′-end sequence of the designed crRNAs. If the number of the potential off-target sites for a crRNA was larger than one, this crRNA was not used for gene editing experiments. crRNAs and trans-activating crRNA (tracrRNA) were chemically synthesized by FASMAC (Japan). Three μl of crRNA (200 pmol μl⁻¹) and 3 μl of tracrRNA (200 pmol μl⁻¹) were mixed in a screw-cap microtube and incubated at 95°C for 5 min followed by slow cooling to hybridize crRNA and tracrRNA. Ten μl of Cas9 nuclease (15 μg μl⁻¹) (Nippon Gene, Japan) was diluted 50-fold with RNase-free water, then the volume of the protein solution was reduced below 10 μl with an Amicon Ultra spin filter (100 kDa, Milipore, USA), into which RNA-free water was added to the final volume of 60 μl. Thus, the total salt concentrations of the Cas9 nuclease solution were below 1 mM at which arcing during electroporation was not observed. Two μl of hybridized crRNA-tracrRNA (200 pmol each) and 4 μl of Cas9 protein (10 μg) were mixed and incubated for at least 30 min to form RNP complexes. The RNP complexes thus formed were delivered into cells of strain NIES-2152 as follows. Thirty μl of cell suspension containing 3 × 10⁷ cells prepared as described above, 6 μl of the RNP solution, and 1 μg of the bleHH DNA fragment were mixed in a 2-mm gap electroporation cuvette, and electroporation was done as described above, except that a single Pp at 2,500 V cm⁻¹ with a 15-ms pulse duration followed by five Tp of alternative polarity at 250 V cm⁻¹ with a 50-ms pulse duration and 50 ms pulse interval were applied.

After electroporation, cells were incubated on ice for 10 min, transferred in 1 ml of BG-11 medium containing 55-mM glucose, and incubated under dim light for recovery. In the case when only DNA of a ble expression construct was electroporated, cells were incubated at 25 °C for 24 h with gentle shaking under dim light. When both an RNP and DNA of a ble expression construct were electroporated, cells were incubated for 6 h at 37 °C, a temperature at which Cas9 nuclease shows optimum nuclease activity [45]. The incubation temperature was lowered to 25°C, and the incubation was continued for a further 18 h with shaking. The cells were then spread on a BG-11 agar plate containing 35 μg ml⁻¹ of Zeocin® and incubated in an incubator containing 1% (v/v) CO₂ at 25 °C under continuous light at 100 μmol m⁻² s⁻¹.

Several single colonies grown on the plates were randomly selected for PCR analyses to detect the ble coding sequence. The templates for such PCR were prepared as follows: cells were picked up from each single colony grown on the plates, suspended in 50 μl of TE buffer containing 6% (w/v) Chelex-100
(BioRad, USA), and disrupted by boiling for 10 min. Cell debris was removed by centrifugation, and PCR was performed using the supernatant as a template. For the detection of the ble coding sequence, the ble_F- and ble_R primers (Table S1) were used. For the detection of the ble coding sequence inserted at CRISPR/Cas9 target sites, PCR was performed using the primer sets that amplify DNA fragments encompassing CRISPR/Cas9 target sites (Table S2). PCR products with sizes corresponding to the ble-inserted sequence were selected for Sanger sequencing to determine insertion loci.

**Measurement of lipid content**

The lipid content of the lyophilized cells was determined using a benchtop, low-resolution pulsed NMR instrument (model MQC; Oxford Instruments, UK) following the International Standard Organization (ISO) 10565 protocol [46]. Olive oil was used as a standard [47–49]. Volumetric lipid yield (mg lipid l⁻¹) was calculated by multiplying volumetric biomass yield (mg dry weight of cells per liter of culture) by the lipid content.

**Measurement of starch content**

Starch content in cells was determined using the reducing-sugar quantification method with dinitrosalicylic acid (DNS) reagent [50]. The DNS reagent was prepared by dissolving 5 g of DNS and 150 g of potassium sodium tartrate in 400 ml of 0.2 N NaOH at 80°C, and the volume was then adjusted to 500 ml with distilled water. A known dry weight of lyophilized cells was transferred in a screw-cap microtube (SSIbio #2330-00, USA) containing 1.0 ml of 80% ethanol and 1.0–1.5 g of Zilconia/Silica beads (φ = 0.1 mm, BioSpec, USA). The microtube was vigorously agitated using a bead beating grinder (FastPrep, MP Biomedicals, USA) set at a speed of 6.0 m s⁻¹ for 1 min to homogenize the cells. The homogenized cells were then centrifuged to remove the supernatant containing soluble sugars, and the pellet was suspended in 1 ml of MilliQ water and 50 µl of 2 M sodium acetate. Heat-stable alpha-amylase (A3306, Sigma-Aldrich, USA) was added at an amount greater than 200 units to the solution, and the mixture was incubated at 80°C for 1 h. After centrifugation, 100 µl of the supernatant was mixed with an equal volume of DNS reagent, and heated at 100°C for 20 min. Absorbance at 550 nm was then measured. The concentration of starch was calculated from a calibration curve prepared with known quantities of soluble starch (Nacalai Tesque, Japan) that underwent hydrolysis with the heat-stable amylase.

**Declarations**

**Competing interests**

SH has received a research grant from Algal Bio Co., Ltd. YK, ST, TT, and SH have a patent pending for Chuo University and Algal Bio Co. Ltd. However, all the authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

**Authors’ contributions**
YK and SH conceived the study and designed the experiments. SO, KI, TT, and SK performed genetic analysis. ST optimized the culture conditions for synchronization. YK performed genetic transformation and genome-editing experiments, and analyzed and interpreted the data. YK and SH wrote the article. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

No applicable.

**Consent for publication**

All authors agree to publication.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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**References**


**Figures**

![Cell size distribution graphs](image)

**Fig. 1**
Figure 1

Changes in size of cells of strain NIES-2152 after transitioning from dark to light periods.

Cells of strain NIES-2152 were cultured in BG-11 medium under the L/D 16:8 cycle. Samples were taken at different time points during the 3rd dark period and 4th light period. The horizontal axis represents the cell area (μm²) determined using the ImageJ software, and the vertical axis represents the frequency of appearance.
Figure 2

Transformation efficiency of strain NIES-2152.

a. Transformation efficiencies at different electric-field strengths with various pulse durations of Pp. The electric-field strength, number of pulses, and pulse duration of Tp were fixed at 100 V cm\(^{-1}\), 5 pulses, and 50 ms, respectively. b. Effect of electric-field strength of transfer pulses on transformation efficiency. The
electric-field strength and pulse duration of Pp were fixed at 2,000 V cm⁻¹ and 9 ms, respectively. The transformation efficiency was calculated by dividing the number of Zeocin-resistant colonies by the number of input cells (3.0 × 10⁷).

**Figure 3**

**Construction and characterization of CDMT1-knockout strains.**
a. Structure of *CDMT1*. Black boxes represent exons, thin lines represent introns, and red arrows represent target sites of crRNAs. The yellow bar underneath the gene indicates a conserved domain in this gene. b. PCR detection of a bleHH insertion into a crRNA target site. M: molecular size markers (λ-EcoT14 I digest), PCR product amplified with DNA from W: strain NIES-2152, CR24: strain CR24, CR26: strain CR26, N: no DNA. c. The target sequence of CDMT1_3 crRNA and the corresponding sequences in the knockout strains, CR24 and CR26. Each broad orange arrow represents one unit length of the bleHH fragment. A D symbol followed by a number indicates a deletion of bases with this number at one end of the bleHH fragment. Inserted nucleotide(s) are shown in red. d. e. Cells of the wild-type strain, strain PK4, and *CDMT1*-knockout strains were grown in 1/5 UP medium under continuous light for 4 to 14 days under the described conditions. d. Volumetric biomass yield [cell mass dry weight per liter of culture (g l$^{-1}$)]. e. Lipid content in percentage of dry weight biomass (w/w). Bars represent standard deviations of data from three independent samples. Statistical significance of differences between the values of strain PK4 and those of other strains was tested by Student’s t-test (two-tailed), and the results are shown as asterisks. Single asterisks indicate P-values between 0.01 and 0.05, and double asterisks indicate P < 0.01.
Construction and characterization of *DMAN1*-knockout strains.

**a.** Structure of the *DMAN1*. Black boxes represent exons, thin lines represent introns, and red arrows represent target sites of crRNAs. Two yellow bars underneath the gene indicate conserved domains in this gene. **b.** PCR detection of a bleHH insertion. The primer set DMAN1_F and DMAN1_R2 (Table S1) was
used to detect bleHH insertion into a crRNA target site. M: molecular size markers (λ-EcoT14 I digest), PCR product amplified with DNA from W: strain NIES-2152, CR189: strain CR189, CR193: strain CR193, N: no DNA. c. The target sequence of DMAN1_2 crRNA and the corresponding sequences in the knockout strains, CR189 and CR193. Each of broad orange arrow represents one unit length of the bleHH fragment. A D symbol followed by a number indicates a deletion of base(s) with this number at one end of the bleHH fragment. Inserted nucleotide(s) are shown in red. d, e. Cells of the wild-type strain, strain PK4, and CDMT1-knockout strains were grown in 1/5 UP medium under continuous light for 4 to 14 days under the described conditions. d. Volumetric biomass yield [cell mass dry weight per liter of culture (g l$^{-1}$)]. e. Lipid content in percentage of dry weight biomass (w/w). Bars represent standard deviations of data from three independent samples. Statistical significance of differences between the values of strain PK4 and those of other strains was tested by Student's t-test (two-tailed), and the results are shown as asterisks. Single asterisks indicate P-values between 0.01 and 0.05, and double asterisks indicate P < 0.01.
Construction and characterization of \textit{AATPL1}-knockout strains.

\textbf{a.} Structure of \textit{AATPL1}. Black boxes represent exons, thin lines represent introns, and red arrows represent target sites of crRNAs. The yellow bars underneath the gene indicate conserved domains in this gene. \textbf{b.} PCR detection of a bleHH insertion into crRNA target sites. M: molecular size markers (λ-EcoT14 I
digest), PCR product amplified with DNA from W: strain NIES-2152, CR12: strain CR12, CR97: strain CR97, N: no DNA. c. The target sequences of AATPL1_1- and AATPL1_3 crRNAs and the corresponding sequences in the knockout strains, CR12 and CR7. Each broad orange arrow represents one unit length of the bleHH fragment. A D symbol followed by a number indicates a deletion of base(s) with this number at one end of the bleHH fragment. Inserted nucleotide(s) are shown in red. At one side of the cleavage site generated by AATPL1_1 crRNA, an 8-bp deletion (D8) plus a 36-bp insertion (ins36) occurred. d. e. Cells of the wild-type strain, strain PK4, and AATPL1-knockout strains were grown in 1/5 UP medium under continuous light for 4 to 14 days under the described conditions. d. Volumetric biomass yield [cell mass dry weight per liter of culture (g l\(^{-1}\))]. e. Lipid content in percentage of dry weight biomass (w/w). f. Volumetric lipid yield [volumetric biomass yield × lipid content]. g. Starch content in percentage of dry weight biomass (w/w). Bars represent standard deviations of the data from three independent samples. Statistical significance of differences between the values of strain PK4 and those of other strains or between the wild-type strain and other strains were tested by Student’s t-test (two-tailed), and the results are shown in Table S4.
Figure 6

Growth and contents of lipid and starch in the wild-type strain, strain PK4, and AATPL1-knockout strains under the L/D 10:14 cycle.

a. Volumetric biomass yield [cell mass dry weight per liter of culture (g l\(^{-1}\))]. b. Lipid content in percentage of dry weight biomass (w/w). c. Volumetric lipid yield (g l\(^{-1}\)). d. Starch content in percentage of dry
weight biomass (w/w). Bars represent standard deviations of the data from three independent samples. Statistical significance of differences between the values of strain PK4 and those of other strains or between the values of the wild-type and those of other strains was tested by Student's t-test (two-tailed), and the results are shown in Table S5.

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