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Short communication

High-efficiency transformation of the chlorarachniophyte *Amorphochlora amoebiformis* by electroporation

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ABSTRACT

Chlorarachniophytes are marine unicellular algae that acquired a complex plastid through an endosymbiosis with a green alga. As such, they have attracted attention especially from evolutionary biologists as a model for studying organellogenesis. A transient transformation system by particle bombardment was previously established for an amoeboid species of chlorarachniophytes, *Amorphochlora amoebiformis*, and this system has been utilized for expressing fluorescent protein tags, such as GFP. However, the transformation efficiency of this approach is likely too low to generate stable transformants, limiting its utility. Recently, we succeeded in transfecting *A. amoebiformis* cells by electroporation, and here we optimized electroporation conditions to achieve high transformation efficiencies. Interestingly, successful conditions for *A. amoebiformis* were similar to those for mammalian cells, despite their distant relationship. The maximum transformation efficiency was calculated to be over 1000 per 1×10^6 cells, which is 1000-fold higher compared to the previous bombardment system. Furthermore, we succeeded in generating a stably transformed line by culturing isolated transformants. We believe that this genetic tool will contribute to our understanding of cell biology in chlorarachniophyte algae and provide insights into the evolution of secondary endosymbioses.

1. Introduction

Currently, abundant molecular sequence data are available for diverse microalgae. Dozens of nuclear genomes have been sequenced in representative species of major algal groups (chlorophytes, rhodophytes, glaucophytes, ochrophytes, haptophytes, dinoflagellates, chromerids, cryptophytes, and chlorarachniophytes) [1], and hundreds of algal transcriptomes have been generated by the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) [2]. In contrast to molecular data, effective genetic tools are only available for several model organisms [3,4]. Therefore, the development of genetic transformation systems for diverse microalgae is necessary for cell biological studies, as well as bioengineering applications, such as the production of high-value end products.

Chlorarachniophytes are marine unicellular algae that evolved through a secondary endosymbiosis between a cercozoan protist and a green alga [5]. Their complex plastids still harbor a relict nucleus of the green algal endosymbiont, termed the nucleomorph [6]. Therefore, chlorarachniophytes likely represent an intermediate stage of secondary endosymbiosis. To date, 15 species have been described in the phylum Chlorarachniophyta [7]. The nuclear genome was sequenced from *Bigelowiella natans* [8], and MMETSP transcriptomes were generated for 9 species of chlorarachniophytes [2]. However, genetic transformation systems are available only in a single species, *Amorphochlora amoebiformis*. In 2008, we reported a transient transformation system for the amoeboid species *A. amoebiformis* (formerly *Lotharella amoebiformis*), using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA) [9]. We succeeded in expressing green fluorescent protein (GFP) using the native promoter and terminator of a RuBisCO small subunit gene. However, the transformation efficiency was quite low (approximately 10 per 10^7 cells) [9], and stably transformed cells have never been established. This is probably due to insufficiency of transformation because the frequency that transient transformants became stable was estimated to be only 2–5% in the plant *Nicotiana tabacum* [10].

Genetic transformation systems have been reported for several groups of marine microalgae, with the most successful systems in diatoms. The first nuclear transformation of the pennate diatom,

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Phaeodactylum tricornutum, was developed using a Biolistic Particle Delivery System [11]. A decade later, convenient transformation methods by electroporation were developed for the same species [12,13]. To date, transformation systems have been established in several species of diatoms, such as *Thalassiosira pseudonana* [14], *Cylindrotheca fusiformis* [15], and *Chaetoceros gracilis* [16], using particle bombardment or electroporation. These studies indicate that electroporation generally provides a higher transformation efficiency compared to particle bombardment. Recently, we showed that electroporation is potentially also an effective transformation method for *A. amoebiformis* [4]. Here, we optimized the electroporation conditions to achieve a high transformation efficiency of over 1000 per 1×10^6 cells which was approximately 1000-fold higher compared to the previous bombardment method. This degree of transformation efficiency was sufficient to generate stable transformants, and we show that a stably transformed line can be established by culturing isolated transformants without antibiotic selection.

2. Materials and methods

2.1. Culture conditions

Amorphochlora amoebiformis (CCMP2058) cells were cultured at 20 °C under white illumination (50–80 μmol photons·m^{-2}·s^{-1}) on a 14:10 h light:dark cycle in 500 mL Erlenmeyer flasks containing 200 mL ESM medium [17]. The amoeboid cells were allowed to adhere to the bottom of the flask. After decanting the medium, cells from stationary phase cultures (1–2 weeks old) were resuspended in 5 mL medium by gentle pipetting, and then 1 mL of resuspended cells was transferred to a flask with 200 mL fresh medium. After 5–7 days of growth, the cells reached a density of ~4×10^7 cells/flask. These cells were gently resuspended in a small volume of medium and used in the following experiments.

2.2. Electroporation

Plasmid DNA, pLaRbcS182 + GFP [18], was propagated in the *Escherichia coli* strain DH5α and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). The concentration of plasmid DNA was quantified by a NanoPhotometer NP80 (Implen, Munich, Germany), and adjusted to 5 μg/μL with distilled water. A total of 5×10^6 cells of *A. amoebiformis* were collected by centrifugation at 2000*g* for 5 s in a 1.5 mL microtube, and resuspended in 100 or 200 μL of 1 M mannitol solution, GeneArt MAX Efficiency Transformation Reagent for Algae (Life Technologies, Carlsbad, CA), or Gene Pulser electroporation buffer (Bio-Rad), with 1 to 50 μg of the plasmid DNA or 0.1 mg fluorescein isothiocyanate (FITC)-dextran (wt 70,000, Sigma-Aldrich, St. Louis, MO). The cell solution was transferred into an electroporation cuvette with 0.2 or 0.4 cm gap (Bio-Rad). Electroporation was carried out using a Gene Pulser Xcell Electroporation System (Bio-Rad) set to the following parameters: for 1 M mannitol solution, an exponential decay pulse at 200–1500 V, 50 μF capacitance, 500 Ohm resistance in a 0.2 cm cuvette (100 μL); for GeneArt MAX Efficiency Transformation Reagent, an exponential decay pulse at 500 V, 50 μF capacitance, 800 Ohm resistance in a 0.4 cm cuvette (200 μL); for Gene Pulser electroporation buffer, an exponential decay pulse at 100–150 V, 500 μF capacitance, infinity resistance in a 0.2 cm cuvette (100 μL) or one to three square wave pulse(s) of 25 msec at 100–150 V in a 0.2 cm cuvette (100 μL). Immediately after electroporation, 0.9 mL fresh ESM medium was added to the cuvette. An appropriate volume of electroporated cells was spread on a glass-bottom 6-well/24-well plate (Iwaki, Tokyo, Japan) containing ESM medium. After 24 h of incubation, cells were observed under an Olympus IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP74 color camera. GFP/FITC fluorescence and chlorophyll autofluorescence were detected with appropriate filter sets (excitation 470–495 nm and emission 510–550 nm; excitation 460–495 nm and emission 510 nm long pass). The number of GFP-expressing cells was counted in each well to calculate the transformation efficiency. The cell viability after electroporation was estimated by counting the number of living cells with a hemocytometer; the viability of 0 V treatment was set to 100%.

2.3. Antibiotic sensitivity

Approximately 1000 *A. amoebiformis* cells were inoculated into 24-well plastic plates containing 1 mL ESM medium. The cell growth was tested in the presence of different concentrations of zeocin (InvivoGen, San Diego, CA; up to 1000 μg/mL), hygromycin B (Nacalai, Kyoto, Japan; up to 1000 μg/mL), puromycin (Wako, Osaka, Japan; up to 200 μg/mL), and bleomycin (LKT Labs, St. Paul, MN; up to 200 μg/mL) for two weeks. We observed the cells under an inverted microscope every 2 days.

2.4. Western blot analysis

Total proteins were obtained from 1×10^6 *A. amoebiformis* cells by resuspending in 200 μL Laemmli sample buffer (Bio-Rad). After heating at 95 °C for 5 min, 20 μL cell lysates were electrophoresed in an Any kD Mini-PROTEAN TGX gel (Bio-Rad), and separated proteins were blotted to a PVDF membrane using a Trans-Blot Turbo Transfer System (Bio-Rad) at 2.5 A, 25 V for 3 min. Immunoblotting was performed using an iBind Western System (Life Technologies) with an anti-GFP monoclonal antibody (JL-8; Takara, Shiga, Japan) diluted 1:1000, and an anti-mouse IgG horseradish peroxidase (HRP)-linked secondary antibody (NA931; GE Healthcare, Buckinghamshire, UK) diluted 1:10,000. Signals were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and a ChemiDoc XRS Plus (Bio-Rad).

2.5. Southern blot analysis

Total genomic DNA was extracted from the wild-type and AaGPY1 (*A. amoebiformis* with green pyrenoids 1) cells, using the Cetyl trimethylammonium bromide (CTAB) method [19]. After digestion with *Eco*RI (pLaRbcS182 + GFP has an *Eco*RI site behind the *egfp* gene), 10 μg of genomic DNA and 5 ng of pLaRbcS182 + GFP were electrophoresed on a 0.8% and 1.0% agarose gels, respectively, and then blotted to Nytran SPC nylon membranes using a TurboBlotter System (GE Healthcare). The PCR-amplified fragment of 720 bp encoding GFP was used as a hybridization probe. Probe labeling and hybridization were performed using an AlkPhos Direct Labeling and Detection System with CDP-*Star* (GE Healthcare) according to manufacturer's instructions. Chemiluminescent signals were detected by a ChemiDoc XRS Plus (Bio-Rad) with an exposure time of 30 min.

3. Results and discussion

3.1. Optimization of electroporation conditions for A. amoebiformis

To roughly determine conditions for introducing foreign DNA into *A. amoebiformis* cells by electroporation, FITC-dextran was used as an analog of DNA. We examined several different parameters with an exponential decay and square wave pulses under different buffers, 1 M mannitol solution, GeneArt MAX Efficiency Transformation Reagent for Algae (Life Technologies), and Gene Pulser electroporation buffer (Bio-Rad). After electroporation, cells were observed under a fluorescence microscope. We detected uptake of FITC-dextran in a moderate number of living cells when the cells were treated with a square wave pulse at low voltages in Gene Pulser electroporation buffer (Bio-Rad). We next transfected the cells with the circular plasmid DNA, pLaRbcS182 + GFP [18], that encodes the fusion protein of a RuBisCO small subunit and GFP. Cells transformed with this plasmid showed GFP fluorescence in pyrenoids as several green foci [18], and their unique localization made

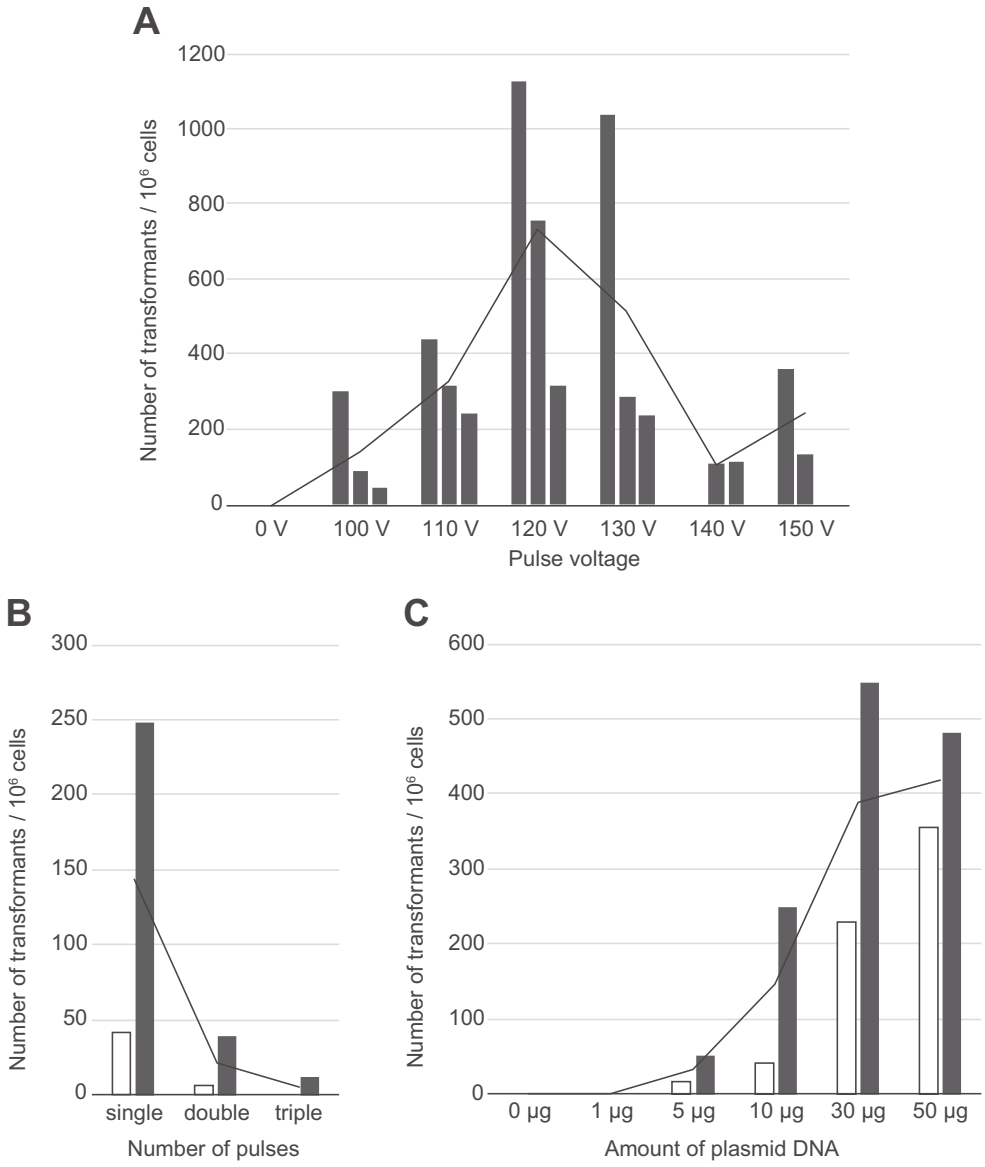

Fig. 1. Transformation efficiency of *Amorphochlora amoebiformis* under various electroporation conditions (A) Transformation efficiencies when 5×10^6 cells were electroporated with 50 μg plasmid DNA under six different voltages between 100 and 150 V. Bars represent biological replicates using different batches of cultures, and the black line shows the average transformation efficiency among the two or three replicates (two replicates for 140 V and 150 V). (B) Transformation efficiencies when 5×10^6 cells were electroporated with 10 μg plasmid DNA by single, double, or triple 25 msec pulses at 120 V. Two bars represent biological replicates using the same batch of cultures. (C) Transformation efficiencies when 5×10^6 cells were electroporated with different amounts of plasmid DNA between 0 and 50 μg by a 25 msec pulse at 120 V. Two bars represent biological replicates using the same batch of cultures. The white and black bars show the first and second experiments, respectively.

it easier to distinguish transformants from non-specific fluorescence under a microscope.

To optimize electroporation conditions, we first examined six different voltages between 100 and 150 V with a 25 msec square wave pulse, and then calculated transformation efficiency by counting the number of GFP-expressing cells. Electroporation at 120 V resulted in the highest transformation efficiencies between 300 and 1100 per 10^6 cells (Fig. 1A). The cell viability was calculated to be 5–13% in this condition. There was more than a 3-fold difference in transformation efficiency among the three replications (Fig. 1A). This instability may be caused by minor differences of our experimental manipulation and cell culture batches, because all three experiments were performed using the same instruments. We next investigated effects of changes in pulse number and DNA concentration. There were no improvements in transformation efficiency when multiple pulses (double or triple 25 msec pulses with 0.5 s interval) were applied instead of a single pulse (Fig. 1B). When five different concentrations (1, 5, 10, 30, and 50 μg/cuvette) of plasmid DNA were tested, high amounts of plasmid DNA resulted in a higher transformation efficiency compared to low amounts (Fig. 1C). We repeated this experiment twice under the same conditions with the same culture at the same time, but the transformation efficiencies were significantly different; the second experiment showed up to 6-fold higher transformation efficiency than the first one in all cases (Fig. 1C). Intriguingly, the first electroporation was carried out immediately after resuspension of cells in the electroporation buffer with plasmid DNA, while the second experiment had a few minutes between the resuspension and the electroporation due to some wait time for the first electroporation to finish. We suspect that this time lag possibly contributed to increase the transformation efficiency.

Taken together, the optimized electroporation conditions for *A. amoebiformis* are as follows. Approximately 5×10^6 cells are resuspended in 100 μL Gene Pulser electroporation buffer with 30 to 50 μg plasmid DNA. After a few minutes incubation, the cells are electroporated by a single 25 msec square wave pulse at 120 V using a 0.2 cm cuvette. Unexpectedly, these electroporation conditions are similar to mammalian protocols that are pre-set in the Gene Pulser Xcell Electroporation System, and Gene Pulser electroporation buffer is intended for mammalian cells. Previous studies on marine microalgae, such as *Ostreococcus tauri* [20], *Nannochloropsis* sp. [21], and *P. tricornutum* [12,13], show these species are likely to prefer high voltage pulses and high resistance buffers in electroporation. Although chlorarachniophytes are also marine algae, we obtained no positive results when *A. amoebiformis* cells were treated with a high voltage from 200 to 1500 V in 1 M mannitol solution.

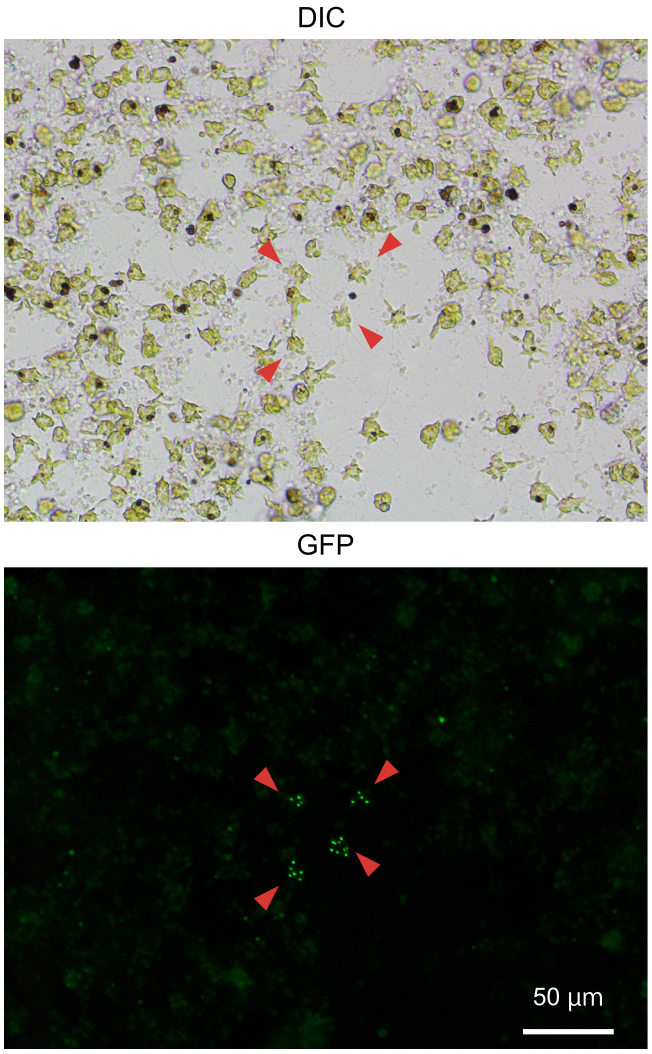

Fig. 2. Candidates of stably transformed cells. Arrowheads indicate the GFP-expressing daughter cells derived from a transformed cell. The pictures were taken a week after electroporation.

3.2. Stably transformed line of *A. amoebiformis*

To establish stably transformed lines, GFP-expressing *A. amoebiformis* cells were manually sorted and cultured. A week after electroporation, we confirmed that some of the transformed cells had divided, maintaining their GFP signals (Fig. 2). These cells were assumed to have successfully passed the introduced *gfp* gene to their daughter cells. Therefore, we manually isolated such cells using a glass micropipette and transferred them into a 24-well plate. A week after isolation, more than 50 cells showing GFP fluorescence were observed in several wells. Through a second round of isolation to remove contamination of bacteria and wild-type cells, we finally established a stably transformed line, named AaGPY1 (*A. amoebiformis* with green pyrenoids 1), that has been maintained for over a year by subculturing at 2 to 3 week intervals (Fig. 3A). To verify expression of the GFP fusion protein in AaGPY1, in addition to fluorescence observation, we performed Western blot analysis with an anti-GFP antibody. A strong band around 40 kDa was detected in the lane of AaGPY1 (Fig. 3B), which is consistent with the calculated molecular weight of the mature form of the GFP fusion protein (41.1 kDa). The identity of a weaker band around 27 kDa is unknown (Fig. 3B). To demonstrate genomic integration of the introduced gene, Southern blot analysis was carried out using a specific probe against the *gfp* gene and total genomic DNA digested with *Eco*RI. To confirm the specificity of the probe, we also used the introduced plasmid DNA (pLaRbcS182+GFP) digested with *Eco*RI. A single band was detected in AaGPY1, and the band size at approximately 9 kbp was higher than the size of the plasmid (5.4 kbp) (Fig. 3C), suggesting that one copy of the *gfp* gene was integrated into the nuclear genome.

Although it has yet to be attempted, fluorescence activated cell sorting (FACS) would enable the establishment of stably transformed lines more easily and extensively, compared to manual isolation. Furthermore, antibiotic selection systems are also useful for effective screening of stable transformants. To identify suitable selectable markers for *A. amoebiformis*, we tested its sensitivity to four antibiotics, zeocin, hygromycin B, puromycin, and bleomycin. Since *A. amoebiformis* cannot grow on solid medium, we used liquid medium. Cells were cultured in 24-well plates containing different concentrations (0 to 1000 μg/mL) of antibiotics for two weeks. The growth was completely inhibited by 500 μg/mL of zeocin, 200 μg/mL of hygromycin B, 200 μg/mL of puromycin, and 50 μg/mL of bleomycin (Fig. 3D). Considering the cost efficiency, the *hph* gene encoding a hygromycin B phosphotransferase is a likely candidate selectable maker.

4. Conclusions

Here we established a high-efficiency transformation method for the chlorarachniophyte *Amorphochlora amoebiformis* using an electroporation system that improved transformation efficiency up to 1000-fold compared to the previous particle bombardment system. This method is not only efficient and reproducible, but easy to conduct. Its accessibility will allow less specialized laboratories to transform *A. amoebiformis*, establishing the species as a model for investigating plastid function, evolution, and cell biology in chlorarachniophytes. Using this method we succeeded in generating a stably transformed line by manually isolating GFP expressing cells. Coupling these advances with the development of an effective screening system for transformants, for example using antibiotics and selectable marker genes, will be a goal for future studies. The stably transformed line, named AaGPY1, showing green fluorescence in their pyrenoids will be useful for investigating pyrenoid biogenesis as in a previous study of the model green alga *Chlamydomonas reinhardtii* [22].

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable.

CRediT authorship contribution statement

Kodai Fukuda: Investigation, Formal analysis, Writing - review & editing. **Elizabeth C. Cooney:** Investigation, Formal analysis, Writing - review & editing. **Nicholas A.T. Irwin:** Investigation, Formal analysis, Writing - review & editing. **Patrick J. Keeling:** Conceptualization, Writing - review & editing. **Yoshihisa Hirakawa:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

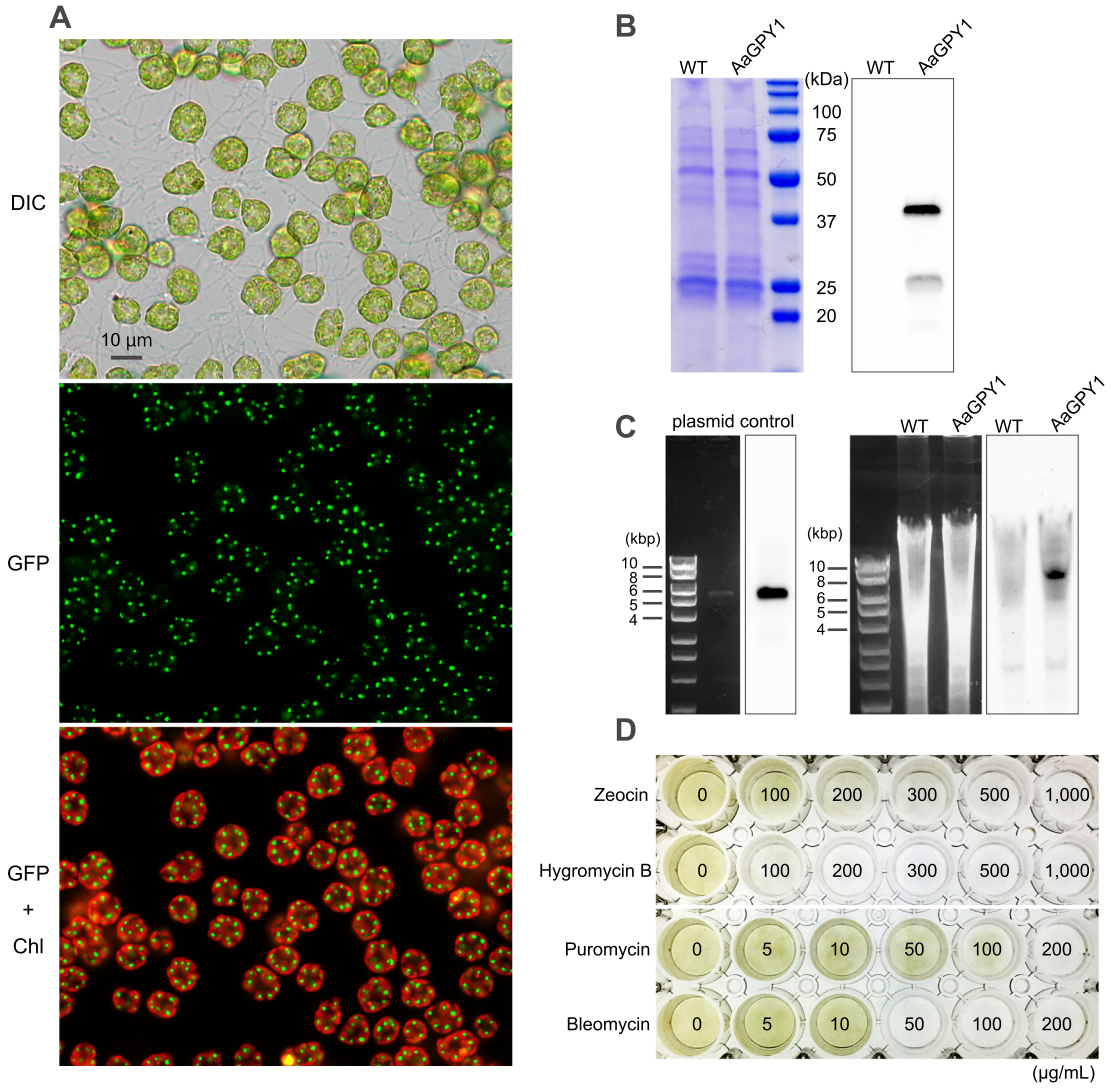

Fig. 3. Stably transformed cells of *Amorphochlora amoebiformis* (A) Micrographs of the stably transformed line, AaGPY1, showing GFP (green) and chlorophyll autofluorescence (red). The top image shows differential interference contrast (DIC). (B) The left image shows a Coomassie-stained gel loaded with proteins extracted from the wild-type (WT) and AaGPY1 cells, and the right image is the result of Western blot analysis with an anti-GFP antibody. (C) Southern blot analysis with a specific probe against the *gfp* gene. Total DNA extracted from WT and AaGPY1 cells and the plasmid pLaRbcS182 + GFP (positive control) were digested with *Eco*RI and loaded on a 0.8% and 1.0% agarose gel, respectively. The images with a black background show DNA and a molecular marker (HyperLadder I, Bioline) stained with SYBR Safe (Life Technologies), and the images with white background represent the hybridization signals. (D) Antibiotic sensitivity tests for wild-type *A. amoebiformis* using four antibiotics (zeocin, hygromycin B, puromycin, and bleomycin). The image of the well-plate was taken after two weeks of cultivation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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