Cytochemical characteristics of the released single cells from colonial oil-producing green alga *Botryococcus braunii* and expression cassettes for transformation

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ABSTRACT

The green colonial microalga, *Botryococcus braunii*, which widespreads in fresh and brackish water, is one of potential sources of biofuels due to its high productivity of hydrocarbon.

B. braunii is a promising candidate for fossil fuels substitute, however the growth is extremely slow, and oil production level are varied largely among strains. To accomplish the improved production of hydrocarbons at a reasonable price, application of molecular biological techniques for *B. braunii* is essential. However, accumulated hydrocarbon oils in the extracellular matrix hamper the biolistic transformation and stable selection of drug-resistant mutants. Extracellular oils also prevent the access of hydrolytic enzymes to prepare protoplasts. Therefore, single cells that are free of the oils must be extremely useful for genetic manipulation of this alga.

In this study, I described the detailed methodology to prepare single cells and the characteristics of the prepared single cells. Four different varieties of *B. braunii* were used in this work; *Showa* and *Sanshiro-5* are B race strains, while *UTEX572* and *Yamanaka* belong to A race. I tested for chemical reagents that would be useful in preparing a large number of vital single cells from colonial *B. braunii*. Among the 18 reagents assayed, glycerol and erythritol showed the highest potency for releasing single cells. Incubation in medium containing these reagents released 40%–50% single cells in 15 min. Fluorescent staining with Nile red revealed that except for the cap-like structures the released single cells were free of hydrocarbon oils. However, to maintain the prepared single cells in vital condition, they must be maintained at a high concentration ($>2\times10^7$ cells/mL); at low concentrations, they rapidly lost chlorophyll and get disrupted. In contrast to the above results obtained using *Showa* (B-race), single cells prepared from A-race varieties survived even at low cell concentrations. Those isolated single cells are supposed to be

useful material for molecular engineering, such as, cell fusion or genomic transformation. I made several nuclear transformation constructs that are composed of a domestic promoter, marker gene ORF and a terminator. As a marker gene, I used bacterial phlenomycin (*ble*), fused gene of *ble* and codon optimized GFP or GUS reporter gene. In addition to above, two constructs was made for *UTEX572* plastid transformation.

In this study, I also showed that a mesoporous metal oxide (TiO_2) with a density of approximately only one-tenth that of gold or tungsten have potency to be effective as a DNA carrier in biolistic bombardment of a rigid cell wall-containing alga. This new particle might be a good material with wide application prospects. Two peaks of gas pressures in the transformation ratio were detected irrespective of whether the particles were made of gold, tungsten, or TiO_2.

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CHAPTER 1 General introduction

1.1 Oil-producing micro-algal for sustainable energy supply

Vegetable oils for biodiesel feedstock include soy, sunflower, safflower, canola, and oil palm. However, millions hectares of fertile lands are necessary for the production of fuel to meet the current needs. Hence, finding of alternative feedstock that will not compete with food production is essential. One possible renewable energy sources is microalgal hydrocarbons. Microalgae are sunlight-driven cell factories that convert CO_2 to potential biofuels, foods, feeds and high-value bioactive compounds (Chisti Y, 2007). Recently, biofuel production from microalga as new sources of renewable energy has attracted attention. Many oil-producing green microalgal species have been isolated from fields, and their oil content has been detected, as shown in table 1.1.

Among oleaginous microalgae, colonial green alga *Botryococcus braunii* (*B. braunii*) is one of the most promising species because of its high oil content. *B. braunii* is classified into three biochemical races: A, B, and L, according to the types of primal hydrocarbon oils they produce (Metzger and Casadevall, 1991). The total lipid content is up to 86% of its dry weight (Brown *et al*., 1969; Largeau C *et al*., 1980), and the primal hydrocarbon produced by B race is named botryococcene (Metzger *et al*., 1983). Most of the botryococcene is stored in the colonial extracellular matrix (Weiss *et al*., 2010).

Table 1.1 Algal species and their typical oil content (Sheehan *et al.*, 1998)

Algal species	**Oil content (% dry weight)**
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crpthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunalielia primolecta	23
Isochryais sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschina sp.	45-47
Schiochytrium sp.	50-77
Tetraseknus sueica	15-23

1.2 Recent development of biofuel production from *B. braunii*

Except sufficient light, supply of CO_2 is another important factor to accelerate the growth of microalgae. Therefore, exhaust gas from an industrial process can be used to provide a CO_2-rich air for cultivation.

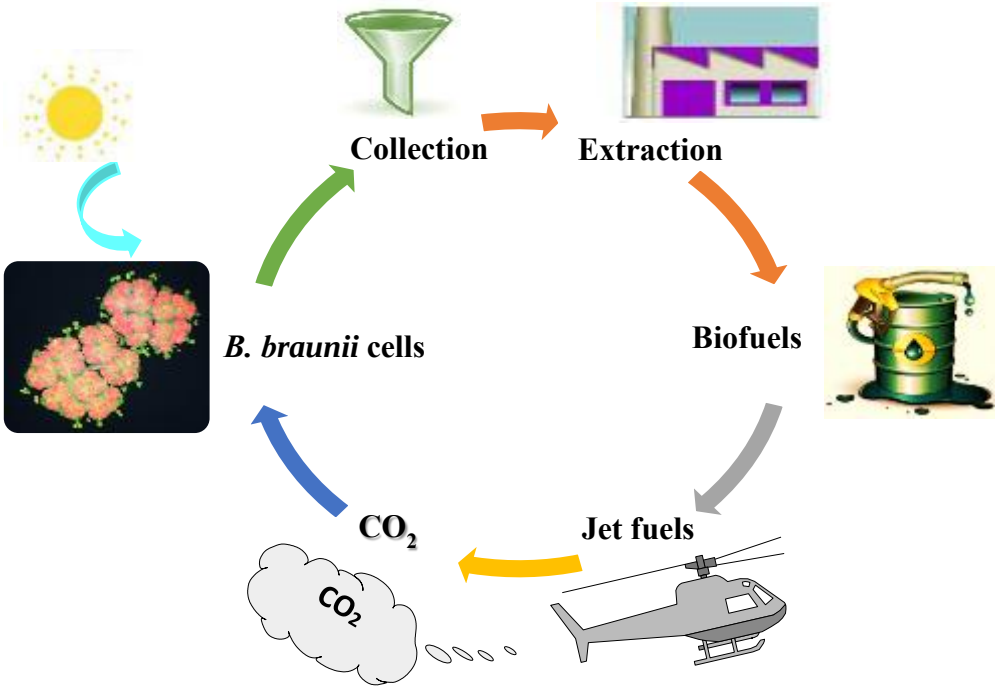


Figure 1.1 Lifecycle of *B. braunii* biofuels

B. braunii has been reported to convert 3% of the solar energy to hydrocarbons (Gudin *et al*., 1984).Recently, Japan economic newspaper reported that Japanese IHI Corp. successfully developed a technology for stable open culture of "Enomoto algae" which is a strain of *B. braunii*. IHI is planning to reduce the production cost of the oil to 100 yen per liter by 2020 to replace conventional petroleum (See figure 1.1).

1.3 Research purpose and outline

B. braunii is a prominent candidate for fossil fuels substitute, however, the growth of this species is extremely slow, and oil production level are dramatically different among strains (Niehaus *et al*., 2011; Eroglu *et al*., 2011). To accomplish production of the hydrocarbons at a reasonable price, application of molecular biological techniques for *B. braunii* is essential. However, successful transformation has been limited to be some algae such as *Chlamydomonas reinhardtii*, *Dunaliella salina* (Kindle *et al*., 1989, Tan *et al*., 2005), *Pseudochoricystis ellipsoidea* (Sousuke *et al*., 2012).

Four different strains of *B. braunii*, were used in this work, *Showa*, *Sanshiro-5*, *UTEX572*, and *Yamanaka*. They all have colony-formation, can be cultured in relative simple medium. This makes these organisms promising materials to analyze their basic characteristics and attributes of the prepared single cells.

In this study, I established the method of single cells isolation from A race and B race colonies, and searched optimal survival condition for released single cells. Single cells are supposed to be best useful for molecular engineering, such as, cell fusion or genomic transformation. Moreover, I made several transformation constructs of *B. braunii* that bear an antibiotic marker or reporter driven by an endogenous promoter. I also showed that a mesoporous metal oxide (TiO_2) with a density of approximately only one-tenth that of gold or tungsten have potency to be effective as a DNA carrier in biolistic bombardment of a rigid cell wall-containing alga. This new particle might be a good material with wide application prospect, for instance, transformation of *B. braunii* single cells. Two peaks of gas pressures in the transformation ratio were detected irrespective of whether the particles were made of gold, tungsten, or TiO_2. (The outline chart of this thesis is illustrated in figure 1.2)

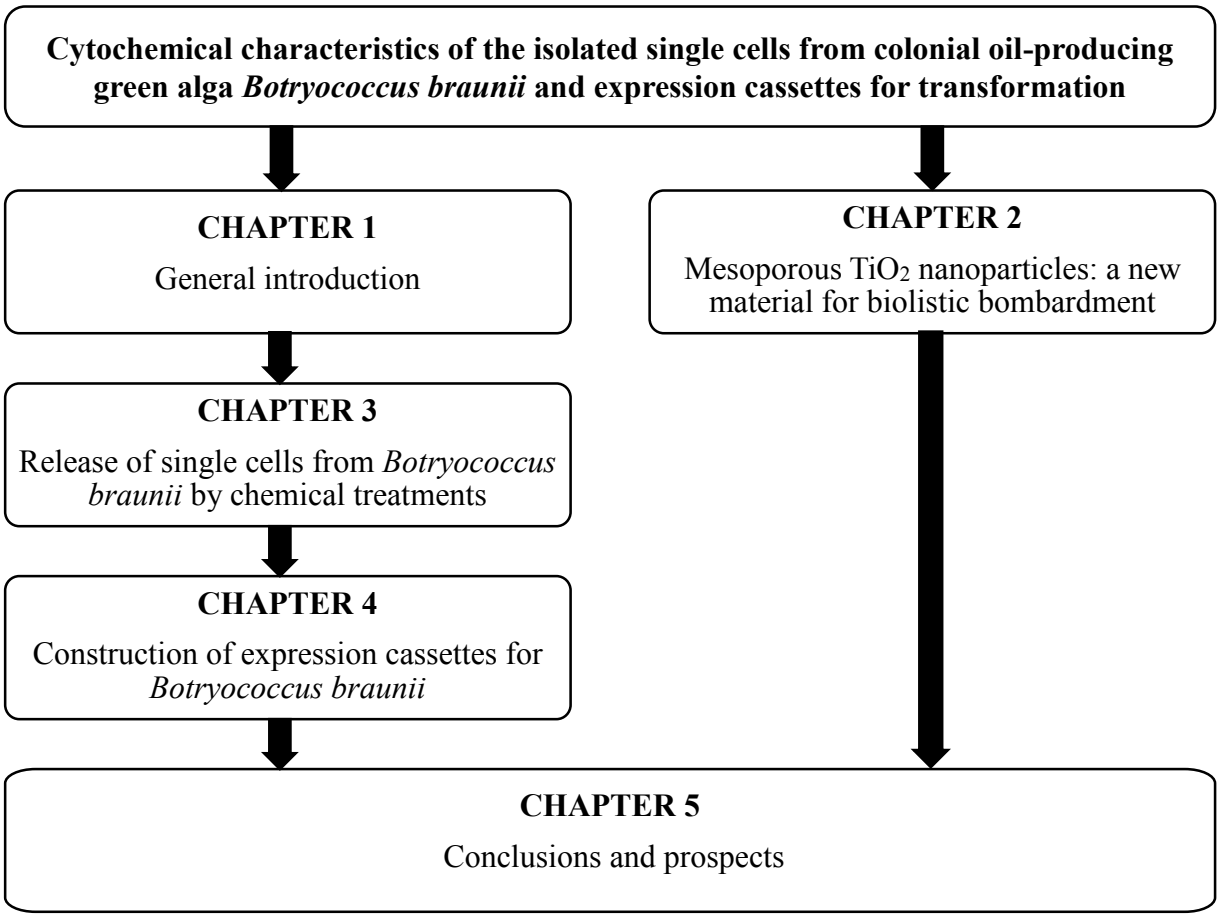


Figure 1.2 Outline chart of this thesis.

1.4 References

Chisti Y, (2007) Biodiesel from microalgae, Biotechnology Advances 25:294-306.

Brown AC, Knights BA, Conway E, (1969) Hydrocarbon content and its relationship to physiological state in the green alga *Botryococcus braunii*. Phytochemistry 8:543-547.

Largeau C, Casadevall E, Berkaloff C, and Dhamelincourt P, (1980) Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. Phytochemistry 19:1043-1051.

Metzger P, Casadevall E, (1983) Structure of 3 New Botrycoccenes Synthesized by a Strain of Botryococcus-Braunii Cultivated in the Lab. Tetrahedron Letters 24:4013-4016.

Weiss TL, Chun HJ, Okada S, Vitha S, and Holzenburg A, (2010) Raman spectroscopy analysis of botryococcene hydrocarbons from the green microalga *Botryococcus braunii*. J Biol Chem 285:32458-32466.

Metzger P, Casadevall E, (1991) Botryococcoid ethers, ether lipids from *Botryococcus braunii*. Phytochemistry 30:1439-1444.

Sheehan J, Dunahay T, Benemann J, and Roessler P, (1998) A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae, National Renewable Energy Laboratory, Golden, Colorado.

Gudin C, Bernard A, Chaumont DT, Thepnier C, and Hardy T, (1984) Direct conversion of solar energy into organic chemicals. World Biotech Rep 1:541.

Niehaus TD, Okada S, Devarenne TR, Watt DS, and Sviripa V, (2011) Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii*. PNAS 108:12260-12265.

Eroglu E, Okada S, Melis A, (2011) Hydrocarbon productivities in different *Botryococcus* strains: comparative methods in product quantification. J Appl Phycol 23:763-775.

Kindle KL, Schnell RA, Fernández E, and Lefebvre PA, (1989) Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 109:2589-2601.

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

Imamura S, Hagiwara D, Suzuki F, Kurano N, and Harayama S, (2012) Genetic transformation of *Pseudochoricystis ellipsoidea*, an aliphatic hydrocarbon-producing green alga. J Gen Appl Microbiol 58:1-10.

CHAPTER 2 Mesoporous TiO_2 nanoparticles: a new material for biolistic bombardment

2.1 Introduction

In the absence of detailed experiments, it has been assumed that particles for biolistic bombardment should be solid metals of high density. At a point of time, tungsten particles were the most popular materials for biolistic bombardment of algae; however, recently, gold particles have been preferred over tungsten particles. This is mainly because the surface of gold particles is much smoother than that of tungsten particles (Barbara *et al*., 2000). In general, the particle size is selected by considering the target cell size, whereas the He gas pressure required to deliver the particles into the cell is selected by considering the mechanical strength of the cell wall.

Various size-controlled TiO_2 nanoparticles can be prepared by varying the conditions of the chemical reaction with supercritical methanol (Wang and Kobiro, 2012). TiO_2 is an inert material in cells; the shape of the prepared TiO_2 particles is almost completely spherical, and their surface is quite smooth relative to that of tungsten particles (Wang and Kobiro, 2012). The calculated density of these particles is approximately 2 g/cm^3, which is approximately one-tenth the density of gold or tungsten (both approximately 19.3 g/cm^3).

2.2 Materials and Methods

2.2.1 DNA carrier particle:

Two different sizes of TiO_2 particles (particle A, 301 ± 160 nm; particle B, 264 ± 99 nm in diameter) (Wang and Kobiro, 2012) were tested in this study. For comparison, a 0.6-μm gold particle (165-2262, Bio-Rad, CA, USA) and a 0.7-μm tungsten particle (tungsten particle M-5, 165-2266, Bio-Rad) were also used.

2.2.2 Conditions for transformation:

I followed the instructions of Kindle (1998) to prepare particles onto which plasmid DNA was precipitated using spermidine. In total, 5 μg of the *pHyg3* plasmid (Berthold *et al*., 2002) in a supercoiled circular form, which carries *aphVII* conferring hygromycin resistance, was added to 0.6 μg of particles, and this was then divided into six aliquots for use in six individual biolistic shots. Transformants were selected on 1% TAP agar plates containing 20 μg/ml of hygromycin. Wild-type *Chlamydomonas reinhardtii* (Dangeard) CC-124 (Chlamydomonas Center, Duke University), a unicellular green alga of approximately 10-μm diameter and with a seven-layered cell wall consisting of hydroxyproline-rich glycoproteins (Harris, 1989), was used as a host. The membrane filter onto which approximately 1×10^7 cells of *C. reinhardtii* were collected was set at the second deck position of the PDS-1000/He (Bio-Rad).

2.2.3 Detection of the introduced DNA construct:

Successful introduction of the *pHyg3* plasmid into the host cells was confirmed by PCR amplification of a section of the plasmid. PCR was performed using a set of PCR primers (forward, 5′- AACTGGCGCAGTTCCTCT -3′ and reverse, 5′- TGCGGCATCAGAGCAGATT -3′) and an amplification program involving a step at 95°C for 5 min; 30 cycles each of 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s; and a final step at 72°C for 7 min on a Thermal Cycler 2720 (Applied Biosystems, CA, USA). DNA sequence of the PCR product was analyzed by direct sequencing based on the dideoxy chain termination method (Sanger *et al*., 1997).

2.3 Results and Discussion

I tested six different gas pressures, 650 psi (approximately 4.5 mPa), 1,100 psi (approximately 7.6 mPa), 1,300 psi (approximately 9.0 mPa), 1,550 psi (approximately 11 mPa), 1,800 psi

(approximately 12 mPa), and 2,000 psi (approximately 14 mPa), in combination with three types of particles made of gold, tungsten, or TiO_2. Two weeks after the bombardment, I counted the number of colonies. To confirm that the *pHyg3* plasmid had been introduced into the host, I randomly picked 10 independent hygromycin-resistant colonies that grew following the TiO_2 bombardment.

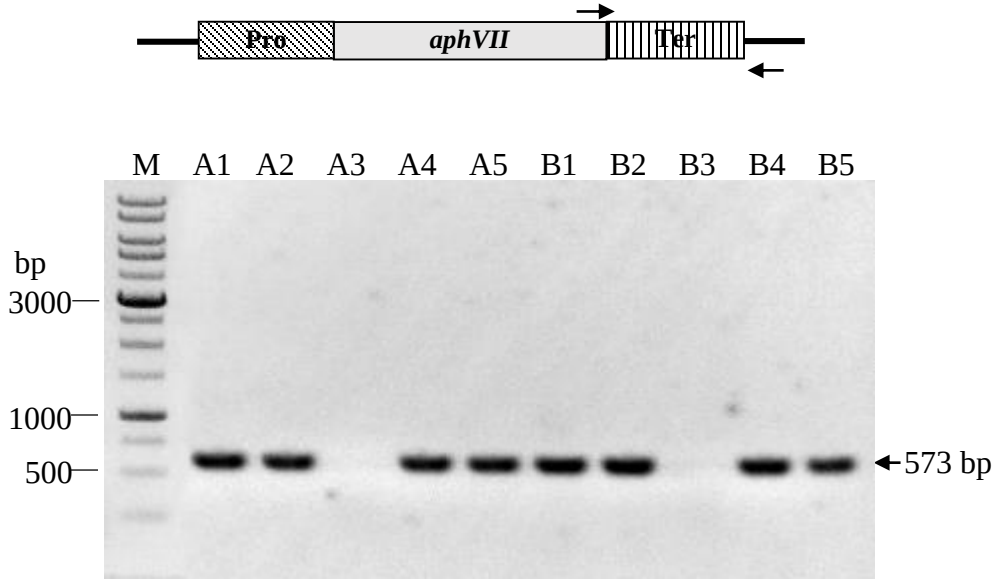


Figure 2.1 Confirmation of integration of transgene for TiO_2 particle transformed *C. reinhardtii* by genomic PCR. Primer set used to amplify transgene is indicated at the top schematic diagram. (M, 1-kb DNA ladder marker; Lane A1–A5, results of PCR of the transformants obtained using a TiO_2-A particle; Lane B1–B5, results of the PCR of the transformants obtained using a TiO_2-B particle. Arrowhead indicate that the expected size of PCR product is 573 bp.)

Total DNA isolated from five transformants generated using the TiO_2-A particle and from an additional five transformants generated using the TiO_2-B particle at 2,000 psi was analyzed by PCR. Eight of the 10 analyzed samples generated DNA fragment of the expected length (573 bp) (see Fig. 2.1). In addition, direct sequence analysis of the eight PCR products (A1, A2, A4, A5,

B1, B2, B4, and B5; see Fig. 2.1) showed that these amplicons had originated from the *pHyg3* plasmid. To our knowledge, this is the first report of successful transformation of an organism using a metal oxide particle for biolistic bombardment. Even the samples that did not generate any PCR fragment may actually have been transformants because it is known that the high genomic G+C content (approximately 65%) of *C. reinhardtii* results in poor amplification of the inserted DNA (Shrager *et al*., 2003).

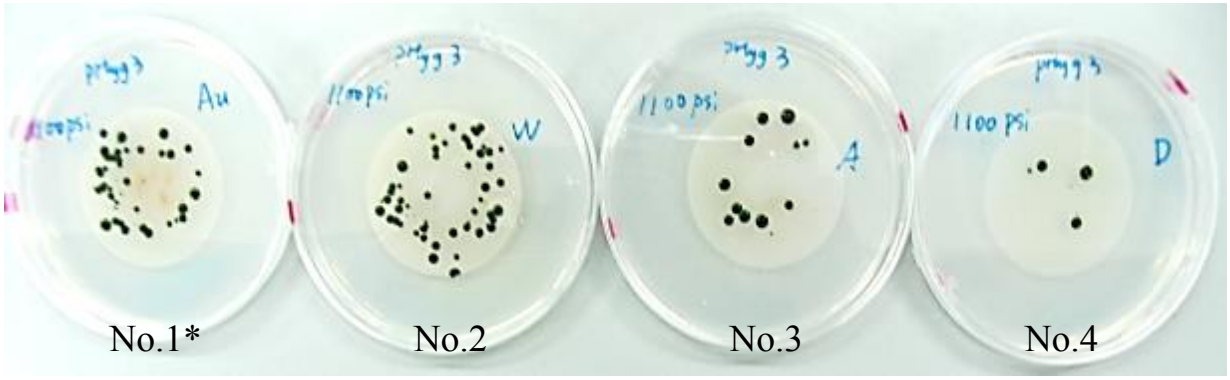


Figure 2.2 Representative results of transformation using gold, tungsten, and TiO_2-A and -B particles with 1,100 psi He-gas pressure.

*Note: No.1, gold particle (0.6-μm diameter); No.2, tungsten particle (0.7 μm); No.3, TiO_2-A particle (0.30 μm); No.4, TiO_2-B particle (0.26 μm).

Table 2.1 Transformation of *Chlamydomonas reinhardtii* using gold, tungsten, and TiO_2 particles.

Particle		**650 psi**	**1100 psi**	**1300 psi**	**1550 psi**	**1800 psi**	**2000 psi**
Gold (0.6µm)		88¶,68,142,74,82,150 (604)†	95,249,63,46,99,281 (833)	334,298,256,339,181,426 (1834)	29,13,40,27,42,29 (180)	51,62,29,52,70,45 (309)	272,163,77,202,84,122 (920)
M-5 tungsten (0.7µm)		1,0,0,0,0,0 (1)	27,34,79,68,11,63 (282)	45,27,12,14,28,21 (147)	13,2,5,4,3,1 (28)	4,38,10,28,1,14 (95)	17,6,19,1,21,29 (93)
TiO_2	A (0.30µm)	0,0,0,0,0,0 (0)	7,12,3,5,5,7 (39)	2,3,2,9,3,5 (24)	2,1,0,0,0,0 (3)	2,1,1,0,0,0 (4)	4,5,12,5,5,10 (41)
	B (0.26µm)	0,0,0,1,0,0 (1)	2,14,6,2,5,21 (50)	10,8,1,8,1,1 (29)	1,1,2,0,0,0 (4)	3,1,4,0,0,0 (8)	5,6,10,5,6,9 (41)

¶Number of colonies appeared by a biolistic shot.

†Sum of colonies appeared in six shots is shown in brackets.

The order of relative transformation efficiency was as follows: gold > tungsten > TiO_2 (Table 2.1 and also see Fig. 2.2). Among the pressures tested, the optimum pressures for gold and tungsten were 1,300 psi and 1,100 psi, respectively. TiO_2 performed similarly well at 1,100 and 2,000 psi, irrespective of the particle size (Table 2.1). Interestingly, a second optimum gas pressure was also detected for gold and tungsten particles. Gold particles showed a second peak of the transformation ratio at 2,000 psi besides the optimum pressure at 1,300 psi. Similarly, tungsten particles showed a second peak at 1,800 psi besides the optimum pressure at 1,100 psi (Table 2.1).

I assumed that the second peak of the transformation ratio in gas pressure is because of the aggregation of the particles as a result of the addition of plasmid DNA to the particles. The peak of the transformation ratio at high gas pressures probably indicates that this is the optimum gas pressure for nonaggregated particles. The peak at low gas pressures may be the optimum pressure for small aggregates. Aggregates of much larger sizes may not be useful for transformation because they can easily damage the target cells. This finding suggests that gold and tungsten particles easily form small aggregates because the highest transformation performance was observed at the lowest of the two gas pressures. In contrast, in the case of TiO_2, the ratio of nonaggregated particles and small aggregates must be similar because the transformation ratios did not differ substantially between the two high-performance gas pressures (Table 2.1).

I have previously shown that in the transformation of *C. reinhardtii* chloroplasts and mitochondria, a mixture of 100 and 600 nm gold particles performs better (approximately 1.5 times) than particles of a single size (Yamasaki *et al*., 2005). Considering this, not only different-sized particle mixtures of the same material but also mixtures of several particles composed of different materials may enhance the transformation ratio as well as mixtures of different-sized unimaterial particles.

Furthermore, as exemplified by TiO_2 particles, many metal compounds that have been not tested till date may have useful characteristics that have not exemplified using pure solid metals. Actually, a characteristic of TiO_2 particles is that they are less prone to form aggregates. This may even be the case for promising organisms for biofuel production, e.g., the hydrocarbon-accumulating colonial green alga *Botryococcus braunii* that possesses an extensive extracellular matrix (Banerjee *et al*., 2002).

2.4 References

Banerjee A, Sharma R, Chisti Y, and Banerjee UC, (2002) *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit Rev Biotec. 22:245-79.

Barbara RA, John EB, John D, (2000) Sub-micron gold particles are superior to larger particles for efficient Biolistic transformation of organelles and some cell types. *Bulletin No. 2015 US/EG*, Bio-Rad.

Berthold P, Schmitt R, and Mages W, (2002) An engineered *Streptomyces hygroscopicus aph 7"* gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. Protist 153:401-12.

Harris EH, (1989) The *Chlamydomonas* sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego, pp**.** 67-80.

Kindle KL, (1998) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Methods Enzymol 297:27-38.

Sanger F, Nicklen S, Coulson AR, (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci *(USA)* 74:5463-7.

Shrager J, Hauser C, Chang C, Harris E, Davies J, and McDermott J, (2003) *Chlamydomonas reinhardtii* genome project: A guide to the generation and use the cDNA information. Plant Physiology 131:401-8.

Wang P, and Kobiro K, (2012) Ultimately simple one-pot synthesis of spherical mesoporous TiO_2 nanoparticles in supercritical methanol. Chem Lett 41:264-6.

Yamasaki T, Kurokawa S, Watanabe K, Ikuta K, and Ohama T, (2005) Shared molecular characteristics of successfully transformed mitochondrial genomes in *Chlamydomonas reinhardtii*. Plant Mol Biol 58:515-27.

CHAPTER 3 Release of single cells from the colonial oil-producing alga *Botryococcus braunii* by chemical treatments

3.1 Introduction

Over the past two decades, interest in finding new sources of renewable energy has stimulated efforts to identify microalgae capable of generating large amounts of biofuel. The hydrocarbon oils of *Botryococcus braunii* (Chlorophyta, Trebouxiophyceae) are one of the most useful renewable sources of fossil fuel substitutes (Komárek and Marvan, 1992; Sawayama *et al*., 1995; Banerjee *et al*., 2002; Kita *et al*., 2010). This cosmopolitan fresh- to brackish-water green alga is classified into three biochemical races: A, B, and L, according to the types of primal hydrocarbon oils they produce (Metzger and Casadevall, 1991).

To accomplish the mass production of hydrocarbons at a reasonable price, application of molecular biological techniques for *B. braunii* are essential. However, accumulated hydrocarbon oils in the extracellular matrix hamper the biolistic transformation and selection of drug-resistant mutants. DNA fragments, which are noncovalently attached on the surface of biolistic bombardment particles, may get detached in the secreted oils before their delivery into the cell. Extracellular oils also prevent the access of enzymes added for cell wall digestion to prepare protoplasts for cell fusion. Single cells that are free of the oils would be extremely useful for genetic manipulation of this alga.

Preceding this study, successful isolation of single cells of *B. braunii* had been reported by the addition of glycerol (chemical race not mentioned) as a short proceeding of an annual meeting in Japan (Enomoto *et al.*, 2011). However, the detailed methodology and the characteristics of the prepared cells were not described. With this background, I searched for reagents useful in the

efficient release of single cells from *B. braunii* colonies, and analyzed the physical characteristics of the isolated single cells. This study was mainly performed using a B-race variety known as *Showa*. For comparative study, I used another B-race variety, *Sanshiro-5*, and two A-race varieties, *UTEX572* and *Yamanaka* (Okada *et al*., 1995).

3.2 Materials and methods

3.2.1 Strains and culture of B. braunii

Two A-race varieties, *UTEX572* (The Culture Collection of Algae, University of Texas at Austin) and *Yamanaka* (Okada *et al*., 1995), and two B-race varieties, *Showa* (University of California Berkeley Herbarium, accession no. UC 147504) (Nonomura, 1988) and *Sanshiro-5* (Okada *et al*., 1995), were used for comparative study. "Modified Chu 13 medium" (Grung *et al*., 1989) was used to culture the two B-race varieties and the *Yamanaka* variety of A-race. The A-race variety *UTEX572* was cultured in "modified Chu 10 medium" (Bold and Wynne, 1978). Conical Fernbach flasks capped with Styrofoam stoppers were used for liquid culture. They were maintained in an acrylic resin chamber filled with an approximately 0.8 % air–CO_2 mixture at 25°C under continuous white fluorescent illumination (50 μmol photons m−2s−1) without shaking.

3.2.2 Chemical reagents tested for the preparation of single cells

In total, I tested 18 reagents for their potency in releasing single cells: acetone (012-00343, Wako, Osaka, Japan), 2,3-butanediol (022-03242, Wako), diglycerol (627-82-7, TCI, Tokyo, Japan), dimethyl sulfoxide (043-07216, Wako), erythritol (056-00242, Wako), ethylene glycol (058-03965, Wako), glucose (041-00595, Wako), glycerol (072-00621, Wako), glycerol ethoxylate-co-propoxylate triol (51258-15-2, Sigma-Aldrich Japan), glyceryl guaiacolate (93-14-

1, Alfa Aesar, London, UK), mannitol (130-00855, Wako), n-hexane (085-00416, Wako), pentaerythriol (169-00585, Wako), sorbitol (098-03755, Wako), sucrose (196-00015, Wako), triglycerol (20411-31-8, Aldrich), Tween 20 (103168, ICN, California, USA), and xylitol (87-99-0, Sigma-Aldrich Japan, Tokyo, Japan). Each reagent, except acetone, mannitol, n-hexane, pentaerythritol, glyceryl guaiacolate, and Tween 20, was dissolved in modified Chu 13 medium to obtain a final concentration of 3.43 M; this is approximately equivalent to the concentration of 25% (v/v) glycerol. Tween 20 was dissolved in modified Chu 13 medium at a concentration of 25% (v/v). Saturated solutions of mannitol, pentaerythritol, and glyceryl guaiacolate were prepared because the solubility of these compounds is lower than 3.43 M. Absolute acetone and n-hexane were also used for single cell isolation.

3.2.3 Release of single cells

Experimental phase colonies were collected from 40 mL of culture by filtration using a 5 μm-nylon mesh. Colonies on the mesh were suspended in 10 mL of the appropriate medium (i.e., Chu 13 or Chu 10 containing a specific reagent) (see Table 3.1). Microscopic observation was conducted to estimate the ratio of single cells released from the colonies.

After treatment with a specific chemical reagent, released single cells and colonies were separated using a 5-μm nylon mesh: single cells passed through this mesh, while colonies were trapped. Single cells in the filtrate were precipitated by centrifugation (1,000 ×g for 5 min), and the cell pellet was suspended in 10 mL of Chu 13 (or Chu 10) and re-centrifuged to remove residual chemical reagents. Finally, precipitated single cells were suspended in an appropriate volume of medium to test their viability.

For small-scale preparation of single cells, released single cells were filtered through a 0.45-μm pore membrane filter, single cells in the membrane were washed three times with 10 mL of modified Chu 13 (or Chu 10) medium, and suspended in an appropriate volume of medium.

Table 3.1 Composition of Chu13 and Chu10 medium applied in this study

Component (mg/L)	Modified Chu13	Modified Chu10*
KNO_3	400	1000
$MgSO_4 \cdot 7H_2O$	100	25
$CaCl_2 \cdot 2H_2O$	54	−
$K_2HPO_4 \cdot 3H_2O$	52	10
FeNaEDTA	10	−
H_3BO_3	2.36	3.61
$MnSO_4 \cdot H_2O$	1.54	2.5
$ZnSO_4 \cdot 7H_2O$	0.22	0.26
$CoSO_4 \cdot 7H_2O$	0.09	−
$CuSO_4 \cdot 7H_2O$	0.08	0.08
$Na_2MoO_4 \cdot 2H_2O$	0.06	0.24
$Ca(NO_3)_2 \cdot 4H_2O$	−	40
Na_2CO_3	−	20
$Na_2SiO_3 \cdot 9H_2O$	−	25
$FeSO_4 \cdot 7H_2O$	−	2.75
$MnCl_2 \cdot 4H_2O$	−	0.3
$CoCl_2$	−	0.01
Na_2EDTA	−	3.75
Deionized water	1000 ml	1000 ml

* Sterilized vitamin mixtures was added to Chu10 after autoclave.

3.2.4 Measurement of ratio of chlorophyll-containing single cells to those without chlorophyll

I prepared single cells from experimental phase colonies using a 3.43 M glycerol treatment for 15 min, and analyzed the effect of cell concentration on vitality through measuring the ratio of chlorophyll-containing single cells on day six after establishing single-cell culture. Through microscopic observation, I distinguished the chlorophyll-containing cells from those without chlorophyll, and counted both types of cells using a hemacytometer. Moreover, the relationship between the ratio of single cells released and the concentration of chemical reagents was analyzed for glycerol, erythritol, glyceryl guaiacolate, xylitol, sorbitol, glucose, mannitol, and sucrose by incubating the isolated single cells in medium containing various concentrations (0.86 M, 1.72 M, and 3.4 M) of these reagents.

3.2.5 Preparation of crude hydrocarbons

Colonies of *Showa* (B-race) were harvested from 3-L cultures by filtration using 5-μm nylon mesh; each collected sample was then freeze-dried and weighed. Freeze-dried algal cells were extracted with acetone by sonication, and these were centrifuged at 1000 × g at room temperature for 5 min. I followed the methods described by Okada *et al*. (1997; 1998) and Tonegawa *et al*. (1998) to extract and purify the hydrocarbon oils.

3.2.6 Fluorescence observations

I stained hydrocarbons by adding 10 μl of Nile red (Sigma-Aldrich, Japan) stock solution (500 μg/mL in ethanol) to 1 mL of sample. I observed fluorescence within 30 min using an Olympus IX70 microscope equipped with an Olympus U-MWU2 filter unit.

To visualize cell walls, I added 10 µl of Fluorescent Brightener 28 (Sigma-Aldrich, Japan) (1 mg/mL in water) to 1 mL of isolated single cell suspension. I observed fluorescence after 15 min using an Olympus IX70 microscope equipped with a U-MWU2 filter unit.

3.3 Results

3.3.1 Effective chemical reagents for single cell release

The potency of each reagent for releasing single cells was evaluated by microscopic observation at 15 min after chemical treatment. Colonies prepared from exponential phase culture consistently produced about 30% more single cells than those from stationary phase culture across all potent reagents, irrespective of differences between races or varieties. Release of single cells began at the edges of the colonies (Figure 3.1, 3. 2), and the release of single cells slowed gradually to a halt after 30 min in the potent reagents. Results of the chemical treatments are summarized in Table 3.2. In *Showa* (B-race) and *Sanshiro-5* (B-race), glycerol and erythritol showed the highest potency among the 18 reagents tested. These two reagents released as much as 30%–50% of the cells in *Showa* and *Sanshiro-5* colonies within 15 min, while it was limited to 10%–20% in *Yamanaka* (A-race). Glyceryl guaiacolate was also very efficient reagent in the preparation of single cells for *Showa* (B-race) and *Sanshiro-5* (B-race), whereas it was not effective for *Yamanaka* (A-race). Unfortunately, this reagent was apparently harmful because colonies suspended in the medium changed color from green to brown in one hour. The ratio of single cells reached 20%–30% in treatments using sorbitol or xylitol for *Showa* (B-race) (Table 3.2). Glucose and mannitol treatments produced 10%–20% *Showa* single cells (B-race), while these reagents released almost no single cells in *Sanshiro-5* (B-race) and *Yamanaka* (A-race). Treatment with sucrose generated

very few *Showa* single cells, while it released no single cells in *Sanshiro-5* (B-race) and *Yamanaka* (A-race).

Because acetone is an efficient solvent for extracting botryococcene (Okada *et al*., 1997; Okada *et al*., 1998; Tonegawa *et al*., 1998), I tested its potency for releasing single cells. Acetone showed high potency for releasing single cells from *Showa* (B-race), *Sanshiro-5* (B-race), and *Yamanaka* (A-race), but not from *UTEX572* (A-race). The single cell ratio reached 20%–40% with absolute acetone treatment. However, acetone treatment disrupted almost all single cells released within 15 min, irrespective of the race. Therefore, irrespective of their high potency for releasing single cells, glyceryl guaiacolate and acetone are not optimal reagents for preparing vital single cells. *n*-hexane, another solvent tested, released only 2%–3% single cells from *Showa* (Table 3.2), but the single cells were not disrupted immediately.

For reagents identified as potent, I confirmed that there was a clear positive relationship between the reagent concentration and ratio of single cells released using *Showa* (Table 3.3). Whereas the single cell release ratio significantly decreased when the reagent concentration reduced to one half of the tested concentration, almost all reagents lost single cell release potential when one fourth of the test concentration was applied.

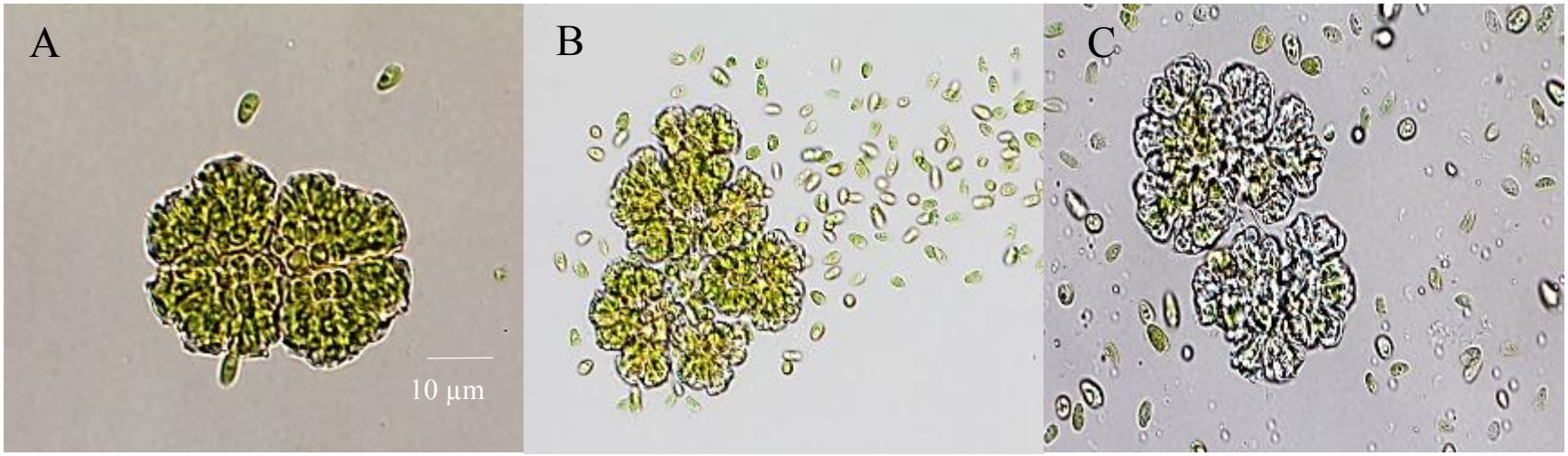


Figure 3.1 Process of single cell release following glycerol treatment

A: One minute after the addition of 3.43 M glycerol to a colony of *B. braunii* in an exponentially growing culture.

B: Ten minutes after the addition of glycerol.

C: Fifty minutes after the addition of glycerol.

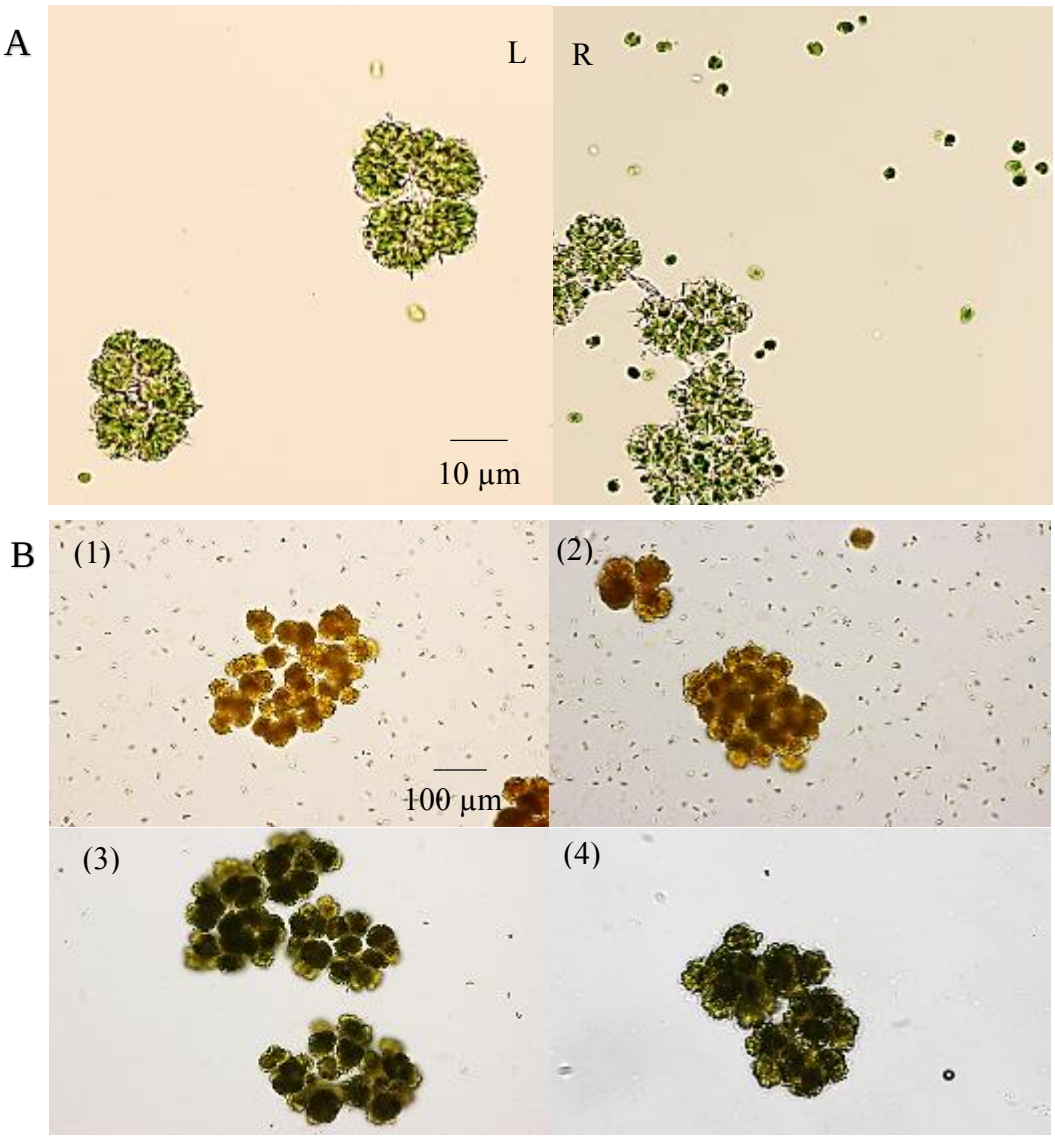


Figure 3.2 Release of single cells by treatment with chemical reagents

(A) Release of single cells from *Yamanaka* (A-race). L; Glyceryl guaiacolate-treated colonies. R; Erythritol-treated colonies.

(B) Release of single cells from *Sanshiro-5* (B-race). (1) Glyceryl guaiacolate-treated colonies. (2) Erythritol-treated colonies. (3) Sorbitol-treated colonies. (4) Xylitol-treated colonies.

Table 3.2 Comparison of released single cell ratios by chemical reagent treatments for *Botryococcus braunii* varieties

Reagents		**Glycerol (25%) ~3.43 mol/L**	**Erythritol ~3.43 mol/L**	**Acetone (Absolute)**	**Glyceryl guaiacolate ~0.25 mol/L**	**Sorbitol ~3.43 mol/L**	**Xylitol ~3.43 mol/L**	**Glucose ~3.43 mol/L**	**Mannitol ~1.0 mol/L**	***n*-hexane (Absolute)**
Potency for releasing single cells	***Showa* (B-race)**	+++++	+++++	++++	++++	+++	+++	++	++	+
	***Sanshiro-5* (B-race)**	++++	++++	+++	++++	+	+	+/–	+/–	–
	***Yamanaka* (A-race)**	++	++	+++	–	–	–	–	–	–
Reagents		Sucrose ~3.43 mol/L	2,3-Butanediol ~3.43 mol/L	Diglycerol ~3.43 mol/L	Dimethyl sulfoxide ~3.43 mol/L	Ethylene glycol ~3.43 mol/L	Glycerol ethoxylate-Co-Propoxylate triol ~3.43 mol/L	Pentaerythriol ~0.4 mol/L	Triglycerol ~3.43 mol/L	Tween 20 (25%) ~0.2 mol/L
Potency for releasing single cells	***Showa* (B-race)**	+	–	–	–	–	–	–	–	–
	***Sanshiro-5* (B- ace)**	–	–	–	–	–	–	–	N/A	N/A
	***Yamanaka* (A-race)**	–	–	–	–	–	–	–	–	–

–: no release of single cell; +/-: very limited number of single cells, less than 1%; +: 1%~10%; ++: 10%~20%; +++: 20%~30%; ++++: 30%~40%; +++++: 40%~50%; N/A: not applied.

Table 3.3 Relationship between the concentration of reagents and single cell releasing potency

Concentration of Reagent	Glycerol	Erythritol	Glyceryl guaiacolate	Xylitol	Sorbitol	Glucose	Mannitol	Sucrose
0.86 M	+	–	–*	–	–	–	–§	–
1.72 M	+++	++	+**	++	+	+	–§§	–
3.43 M	+++++	+++++	++++***	+++	+++	++	++§§§	+

Note: –: no release of single cell; +: 0%~10%; ++: 10%~20%; +++: 20%~30%; ++++: 30%~40%; +++++: 40%~50%; *, **, ***: 0.0625 M, 0.125 M, and 0.25 M, respectively (see text); §, §§, §§§: 0.25 M, 0.5 M, and 1.0 M, respectively.

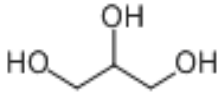
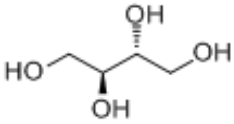
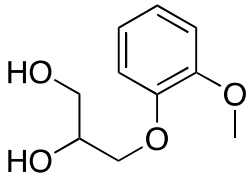
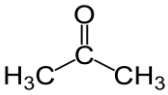
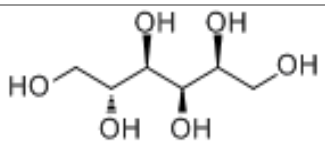
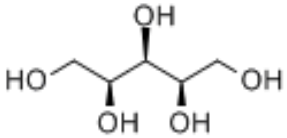
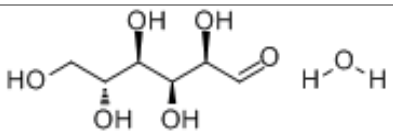
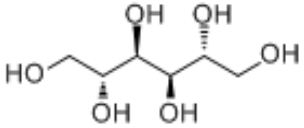
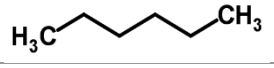
3.3.2 Characteristics of the reagents that showed potential to release single cells

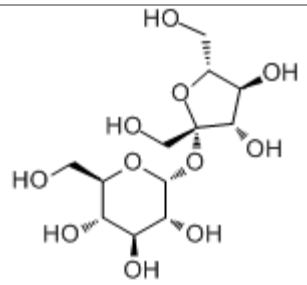
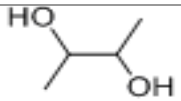
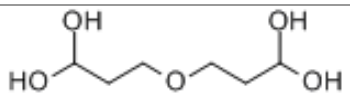
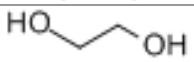
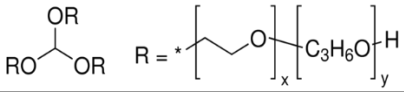
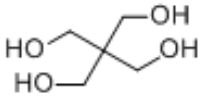
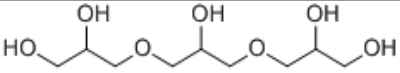
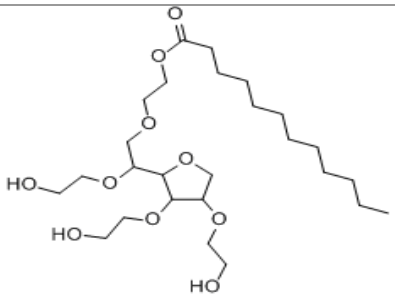
In this study, I tested reagents for potency in the preparation of large quantities of single cells from colonial *B. braunii*, considering that glycerol had already been reported to be a potent reagent (Enomoto *et al*., 2011). Glycerol is a polyalcohol; I therefore tested hydroxyl group (–OH)-containing reagents (the number of –OH groups are shown in brackets below): ethylene glycol (2), 2, 3-butanediol (2), glyceryl guaiacolate (2), diglycerol (4), meso-erythritol (4), pentaerythritol (4), xylitol (5), and triglycerol (5) (see Table 3.4 for structural formulae). I also tested glycerol ethoxylate-co-propoxylate triol because of its molecular configuration. Furthermore, I tested glucose, mannitol, sorbitol, and sucrose because in the process of reagent selection, xylitol was found to be a potent single-cell-inducing reagent. Two weak detergents, Tween 20 and dimethyl sulfoxide were also tested. For reagents with sufficient solubility, I prepared a 3.43 M solution of each reagent (corresponding to a 25% v/v concentration of glycerol) by dissolving them in Chu 13 medium. When the solubility of the reagent was below 3.43 M, I prepared a saturated solution.

Because acetone and n-hexane are often used as solvents to extract botryococcene, these reagents were also tested.

As many as 10 reagents showed potency (Table 3.2) for releasing *Showa* single cells (B-race). However, the 10 potent reagents did not share any apparent chemical similarities (Table 3.4).

Table 3.4 Summarize of chemical reagents used in this study.

Order	Chemical reagents	CAS No.	Structural formula	Molecular formula
1	Glycerol	56-81-5		$C_3H_8O_3$
2	meso- erythritol	149-32-6		$C_4H_{10}O_4$
3	Glyceryl guaiacolate (Guaiacol glyceryl ether)	93-14-1 12041-73-5 128707-44-8 1336-67-0		$C_{10}H_{14}O_4$
4	Acetone	016-00346 012-00343		C_3H_6O
5	Sorbitol	50-70-4		$C_6H_{14}O_6$
6	Xylitol	87-99-0 16277-71-7		$C_5H_{12}O_5$
7	D-glucose	5996-10-1		$C_6H_{14}O_7$
8	Mannitol	87-78-5		$C_6H_{14}O_6$
9	n-Hexane	085-00416		C_6H_{14}

10	Sucrose	57-50-1		$C_{12}H_{22}O_{11}$
11	2,3-butanediol	513-85-9		$C_4H_{10}O_2$
12	Diglycerol	59113-36-9 627-82-7		$C_6H_{14}O_5$
13	Dimethyl sulfoxide	67-68-5		C_2H_6OS
14	Ethylene glycol	107-21-1		$C_2H_6O_2$
15	Glycerol ethoxylate-Co-Propoxylate triol	51258-15-2		$C_8H_{22}O_7$
16	Pentaerythritol	115-77-5		$C_5H_{12}O_4$
17	Triglycerol	20411-31-8 56090-54-1		$C_9H_{20}O_7$
18	Tween 20	9005-64-5		$C_{18}H_{34}O_6.(C_2H_4O)_n$

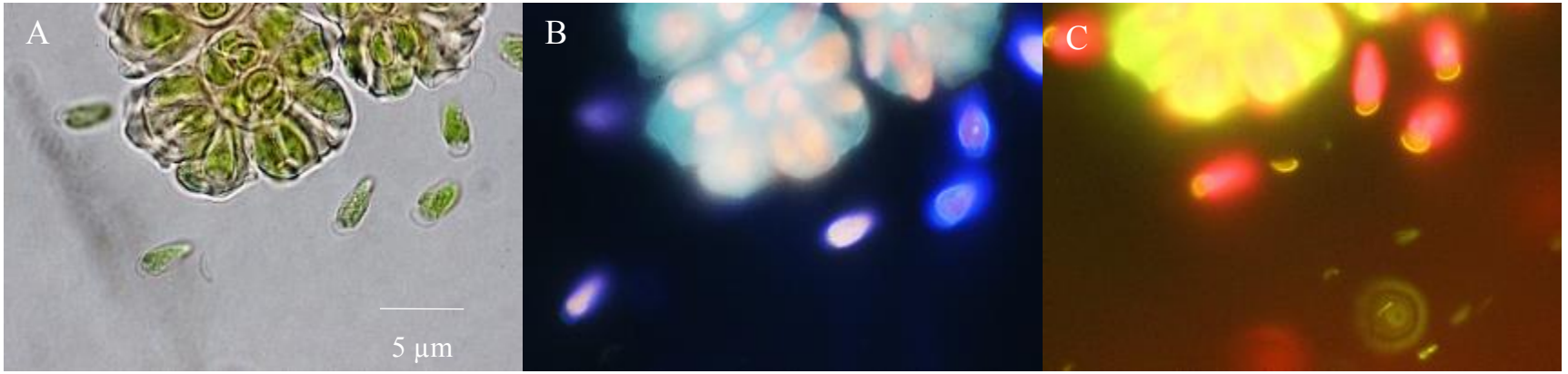


Figure 3.3 Characteristics of *Showa* single cells released by glycerol treatment

A: Normal view of *Showa* single cells.

B: *Showa* single cells stained with Fluorescent Brightener 28. The same targets shown in (**A**) were observed by switching the filter set.

C: *Showa* single cells stained by Nile red. The same targets shown in (**A**) were observed by switching the filter set.

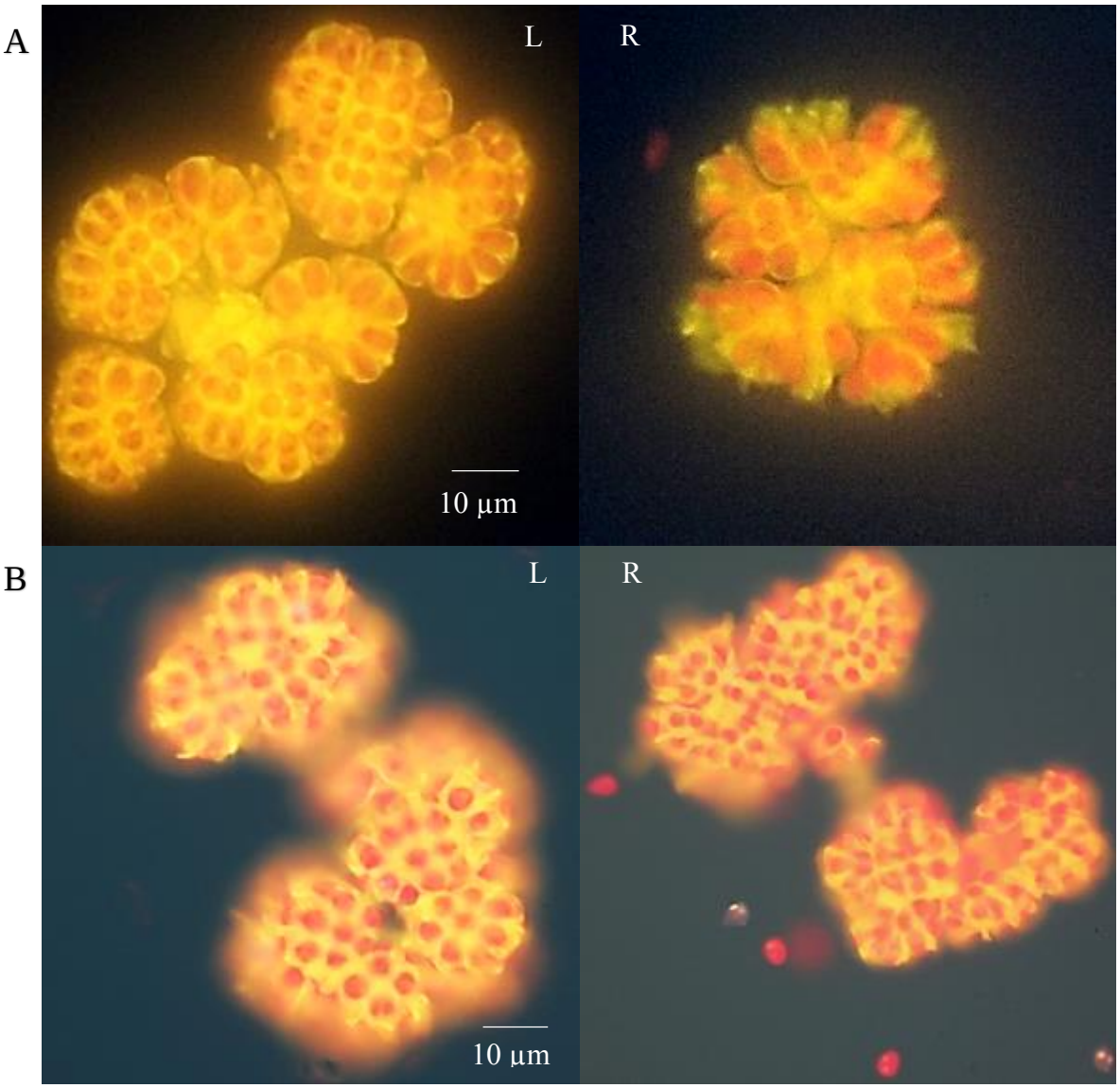


Figure 3.4. Amount of hydrocarbon oils remaining in the extracellular matrix after glycerol treatment to release single cells.

A: *Showa* colonies stained with Nile red. L; Control colonies of *Showa*. R; Glycerol-treated colonies of *Showa*.

B: *Yamanaka* colonies stained with Nile red. L; Control colonies of *Yamanaka*. R; Glycerol-treated colonies of *Yamanaka*.

3.3.3 Characteristics of released single cells

Through Fluorescent Brightener 28 staining, I confirmed the presence of a cell wall around single cells (data from *Showa* is shown in Figure 3.3 A and B). Except for the cap-like structures on the broad ends of single cells (see below), no Nile red fluorescence was observed around the cell wall of these single cells (Figure 3.3 C). Moreover, even after treatment with glycerol, the hydrocarbon content of the matrix was not apparently reduced (Figure 3.4). This suggests that glycerol is not an efficient solvent for the hydrocarbon oils located in the B-race matrix.

Immediate observation of the chemically released *Showa* single cells showed that they possessed cap-like structures on their broad ends (Figure 3.3 C). The cap-like structures were highly morphologically similar to the previously reported "shell" structure (Weiss *et al*., 2012) that was abundantly accumulated in the old culture medium of *Showa*. Therefore, the shell structure was assumed to be the excised segment of the retaining wall and its fibril sheath, which is the outermost barrier that prevents the dispersion of the secreted oils from the matrix.

Figure 3.5 Histochemical tests of the shells collected from the old *Showa* culture medium.

A: *Showa* shells collected from old medium.

B: *Showa* shells stained with Fluorescent Brightener 28.

C: *Showa* shells stained with Nile red.

Our histochemical tests of the shells collected from the old *Showa* culture medium revealed that they were not stained by Nile red or Brightener 28 (Figure 3.5), as reported by Weiss *et al*. (2012). However, the cap-like structures appeared by glycerol treatment were strongly stained by Nile red but not Brightener 28 (Figure 3.3 B and C). This result was also observed for another B-race variety, *Sanshiro-5*. With the exception of the difference in Nile red staining, the shape of the cap-like structure located at the top of the released single cell was exactly the same as that of the "shell" accumulated in the culture medium (Figure 3.5 A).

In contrast to the aforementioned observation, in the case of *Yamanaka* (A-race), the cap-like structures appeared to precede the release of single cells by glycerol treatment from the surface of the retaining wall. Subsequently, the cap-like structures and the single cells were separately slowly released into the medium. I collected the shell-like structures from the old culture medium of *Yamanaka* and B-race varieties. They were not stained by Nile red or Brightener 28, similar to *Showa* and *Sanshiro-5*.

In stark contrast to the above three cases, no single cells or shell-like structures of *UTEX572* were released at all by chemical treatments; therefore, I could not obtain any shell-like structures by centrifugation of the old culture medium of *UTEX572*.

3.3.4 Viability of prepared single cells

I first tested the growth of *Showa* single cells (B-race) on a PFE membrane filter placed on a 0.8% agar Chu 13 medium plate. Unexpectedly, greenish single cells on the membrane changed to white in two days, and after two months no single cell had formed a colony. However, colonial cells grew well on the filter (data not shown). Considering the above results, I undertook detailed experiments to discover conditions suited for the survival of *Showa* single cells.

I analyzed the effect of cell concentration on the vitality of single cells. *Showa* single cells (B-race) prepared using the glycerol treatment were incubated at several different concentrations: approximately 0.6×10^6 cells/mL, 1.5×10^6 cells/mL, 3×10^6 cells/mL, and 2×10^7 cells/mL in Chu 13 medium and kept under continuous light without shaking. I counted the number of chlorophyll-containing single cells on day six. Table 3 shows that when they were maintained at a concentration higher than 2×10^7 cells/mL in Chu 13 without any additions, approximately 70% *Showa* single cells contained chlorophyll on day six. In contrast, when maintained at a concentration lower than approximately 2×10^6 cells/mL, almost all single cells were disrupted or lost their chlorophyll by day six (Table 3.5). Thus, a high cell concentration seems to have a critical effect on the viability of single cells: *Showa* single cells (B-race) maintained at high concentration (>2×10^7 cells/mL) were gathered together to form aggregates (Figure 3.6 A), while the size of the aggregates were much smaller and looser for those kept at low concentration ($0.5–3.8\times10^6$ cells/mL) (Figure 3.6 B). The same phenomenon was observed for *Sanshiro-5* (B-race) (data not shown).

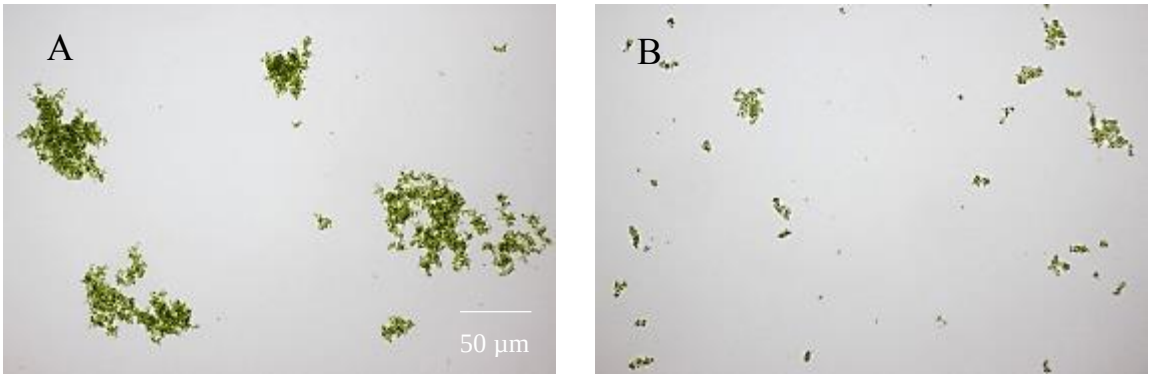


Figure 3.6 Aggregates of single cells

A: Aggregates of *Showa* single cells in Chu 13 medium at high cell concentration. Day three image of aggregates after the start of incubation of single cells.

B: Aggregates of *Showa* single cells in Chu 13 medium at low cell concentration. Day three image of aggregates after the start of incubation of single cells.

Table 3.5 Chlorophyll containing single cell ratio in various conditions

Number of single cells	Condition Medium			Sorbitol				Glycerol				Xylitol				NaCl			
	0%	50%	100%	0.1M	0.2M	0.4M	0.8M	0.1M	0.2M	0.4M	0.8M	0.1M	0.2M	0.4M	0.8M	0.1M	0.2M	0.4M	0.8M
(Exp.1) ×10^7	2.05	2.30	2.14	1.98	2.15	2.16	2.11	2.01	1.94	2.01	2.09	1.93	2.15	1.98	2.08	1.86	2.20	2.12	2.19
CC*	1.48	1.56	1.79	1.64	1.59	1.64	1.35	1.38	1.53	1.63	1.73	1.62	1.48	1.70	1.64	1.32	1.52	0.42	0
CCR$^{\#}$	72%	68%	84%	83%	74%	76%	64%	69%	79%	81%	83%	84%	69%	86%	79%	71%	69%	20%	0%
(Exp.2) ×10^7	2.05	2.35	1.89	2.11	2.01	2.08	1.91	1.96	1.96	2.02	1.93	1.98	2.14	1.68	1.90	1.69	1.99	1.79	1.88
CC*	1.29	2.00	1.33	1.78	1.68	1.82	1.62	1.44	1.30	1.71	1.54	1.64	1.92	1.49	1.57	0.99	1.35	0	0
CCR$^{\#}$	63%	85%	70%	84%	84%	88%	85%	73%	66%	85%	80%	83%	90%	89%	83%	59%	68%	0%	0%
(Exp. 3) ×10^7	2.48	2.35	1.94	1.89	1.95	1.98	1.89	2.16	1.60	1.96	1.92	1.93	1.66	1.92	1.82	1.64	2.00	1.74	1.73
CC*	1.89	1.76	1.70	1.56	1.82	1.48	1.78	1.64	1.45	1.68	1.52	1.84	1.59	1.67	1.74	1.11	0.94	0	0
CCR$^{\#}$	76%	75%	88%	83%	93%	75%	94%	76%	91%	86%	79%	95%	96%	87%	96%	68%	47%	0%	0%
(Exp. 4) ×10^6	0.69	0.64	0.54	0.52	0.61	0.67	0.52	0.64	0.62	0.62	0.70	0.73	0.59	0.63	0.50	0.51	0.51	0.51	0.49
CC*	0	0	0.005	0.08	0.09	0.13	0.08	0.01	0	0.006	0	0.04	0.08	0.13	0.07	0	0	0	0
CCR$^{\#}$	0%	0	<1%	15%	14%	20%	15%	2%	0%	1%	0%	6%	14%	20%	15%	0%	0%	0%	0%
(Exp. 5) ×10^6	1.54	1.51	1.55	1.54	1.53	1.50	1.59	1.51	1.53	1.58	1.50	1.51	1.56	1.57	1.50	1.51	1.54	1.53	1.50
CC*	0	0	0	0.19	0.24	0.20	0.04	0	0	0	0	0.03	0.05	0.08	0.01	0	0	0	0
CCR$^{\#}$	0%	0%	0%	12%	16%	13%	3%	0%	0%	0%	0%	2%	3%	5%	1%	0%	0%	0%	0%
(Exp. 6) ×10^6	2.56	3.44	3.72	3.44	3.64	2.80	3.84	2.40	3.48	3.08	3.60	2.88	3.04	3.64	3.04	3.20	3.12	3.24	3.72
CC*	0.06	0.04	0.16	0.56	0.75	0.82	0.39	0.19	0.22	0.4	0.14	0.51	0.44	0.74	0.57	0.03	0	0	0
CCR$^{\#}$	2%	1%	4%	16%	21%	29%	10%	8%	6%	13%	4%	18%	14%	20%	19%	1%	0%	0%	0%

CC*: number of single cells that contain chlorophyll on day six; CCR $^{\#}$: chlorophyll containing ratio on day six

Moreover, I tested the effect of various additives to Chu 13 medium on *Showa* single cells. Considering that osmotic pressure might be one of the factors affecting the survival rate of single cells (0.6 M mannitol is isotonic to the cytoplasm, Okada *et al*., unpublished data), various concentrations of glycerol, xylitol, sorbitol, and sodium chloride (NaCl), which are typically used in osmotic pressure adjustment, were selected for this study.

Addition of sorbitol or xylitol to low concentrations of cells (0.5–3.8×10^6 cells/mL) distinctly improved the ratio of chlorophyll-containing cells, to a maximum of 30% (Table 3.5), whereas the effect was more limited when the reagents were added to highly concentrated cell cultures. Addition of sorbitol enhanced cell aggregation, notably for low-concentration cell cultures, while the effect of xylitol was much weaker (data not shown). In contrast, xylitol and NaCl did not show such an effect nor induce aggregation of single cells grown at low cell concentration. The potential to induce aggregation seems to be related to the potential to increase viability of single cells.

In contrast to the results obtained for B-race varieties, *Yamanaka* (A-race) single cells prepared by glycerol treatment showed a high survival ratio. More than 60% of single cells contained chlorophyll on day six, regardless of whether they were maintained at high or low cell concentration (approximately 1×10^6 cells/mL) in Chu 13 medium without any additions.

Survival of single cells were also observed for the other A-race variety, *UTEX572*. None of the reagents tested were useful for releasing single cells of *UTEX572*. However, single cells were easily generated by vortexing with glass beads (0.5 mm diameter). Such mechanically generated single cells of *UTEX572*, maintained at low concentration (approximately 1×10^6 cells/mL), also showed a high survival ratio, similar to that observed in chemically isolated *Yamanaka* (A-race)

single cells. Such mechanically prepared single cells were used to evaluate the harmful effect of glycerol treatment as follows.

I treated the mechanically prepared *UTEX572* single cells with 3.43 M glycerol for 30 min to evaluate the influence of the chemical treatment. Even with such chemical treatment, the survival ratio of the *UTEX572* single cells on day six was not apparently different from the non-treated ones (data not shown). This shows that the rapid loss of chlorophyll observed in B-race single cells, which had been released by glycerol treatment, is not due to the harmful effects of the reagent.

3.4 Discussion

The main hydrocarbons produced by A-race *B. braunii* are alkadienes and alkatrienes (Templier *et al*. 1984; 1991; Metzger *et al*., 1985a; 1986), while that of B-race *B. braunii* is a triterpene known as botryococcene (Metzger *et al*., 1985b; 1987; 1988). Nile red is an extremely useful fluorescent lipophilic dye for staining botryococcene, alkadienes, alkatrienes, and other lipids. (Weiss *et al*., 2010a).

Showa and *Sanshiro-5*, which are varieties of the B-race, secrete large amounts of botryococcene (Figure 3.7 A, B) and their single colonies are embedded in an extracellular matrix composed of polymerized and liquid hydrocarbons (Blackburn 1936). *Yamanaka* (A-race) also secretes a substantial amount of oils into the colony matrix (Figure 3.7 C). However, *UTEX572* cells (A-race) secrete no or at most very limited amounts of oils (Figure 3.7 D). Cultures of *UTEX572* (A-race) and *Yamanaka* (A-race) contain a low ratio of naturally generated single cells, while no such single cells were observed in *Showa* (B-race) and *Sanshiro-5* (B-race). This implies A-race single cells inherently have the ability to survive without forming colonies.

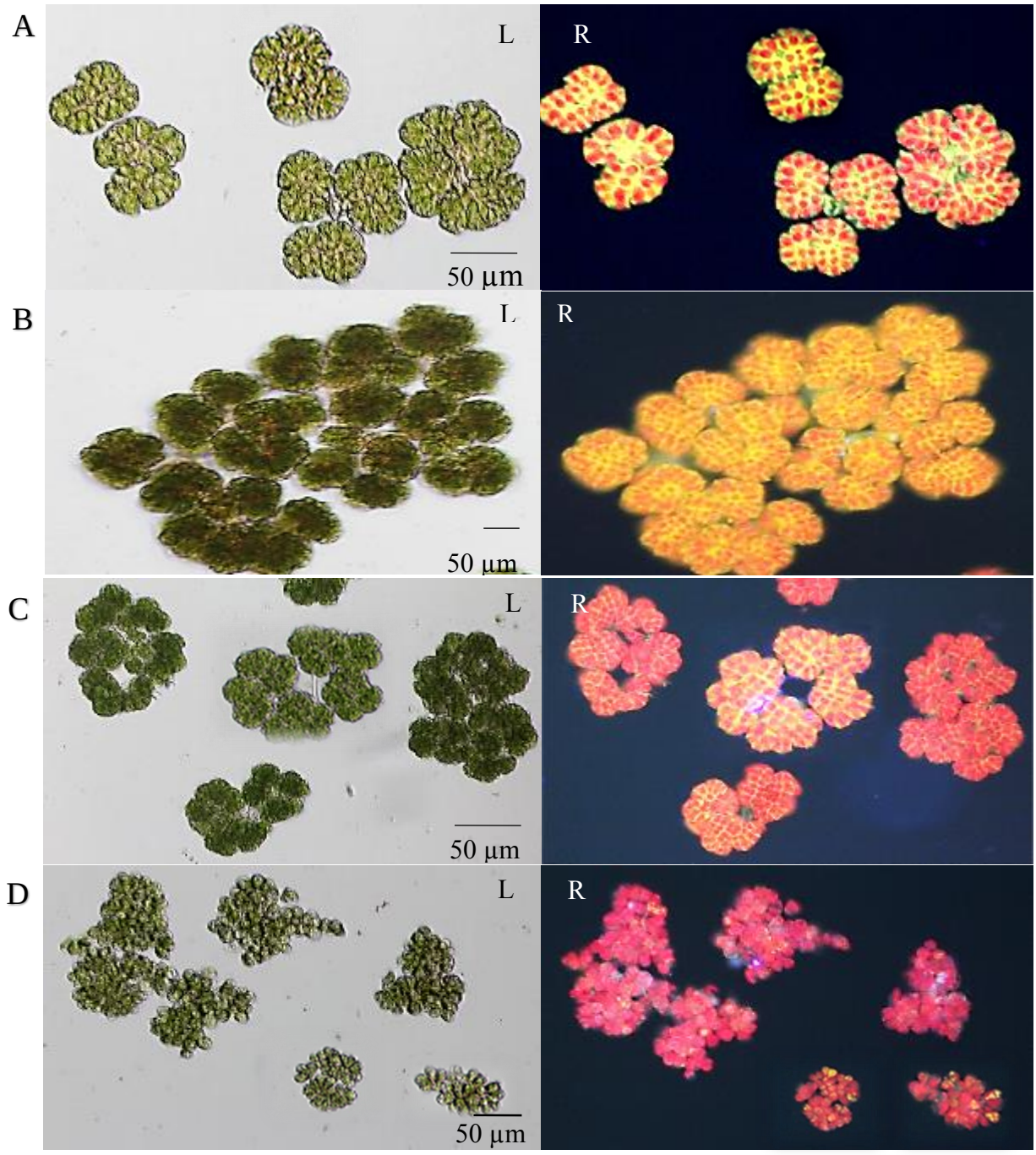


Figure 3.7 Colonies stained with Nile red.

A: *Showa* (B-race) strain stained with Nile red. **B:** *Sanshiro-5* (B-race) strain stained with Nile red. **C:** *Yamanaka* (A-race) strain stained with Nile red. **D:** *UTEX572* (A-race) strain stained with Nile red. L; Normal observation. R; Fluorescence observation.

Because it is known that glycerol is a potent releaser of single cells (Enomoto *et al*., 2011), I assessed 18 reagents, including glycerol, in this study. Among those tested, 10 reagents (including acetone and n-hexane) demonstrated the potential to release *Showa* single cells (Table 1). However, I were unable to identify any common chemical characteristics that were shared by these potent reagents. Most of the potent reagents useful for *Showa* were also useful for *Yamanaka*, which produced alkadienes, but not botryococcene, as the major hydrocarbon products. Thus, these findings suggest that single cell release was probably not due to organic chemical reactions between the added reagents and the hydrocarbon oils.

Microscopic observation of the single cell release process suggested that they were mechanically forced out of the extracellular matrix. The mode of action of release was apparently the same, irrespective of differences in reagents and varieties. I suspect that the mechanical force may be a hydraulic pressure that was generated by the increased volume of the extracellular matrix, which was caused by the dissolution of chemical reagents into the matrix hydrocarbons. It is possible that the volume of the extracellular matrix was increased due to the resolved chemical reagents in the matrix hydrocarbon oils. I hypothesize that the hydraulic force caused by the aforementioned mechanism must push the extracellular matrix hydrocarbon oils into the retaining wall and also the embedded cells to outside of the matrix, thereby leading to the breakdown of the retaining wall. This model is illustrated in Figure 3.8.

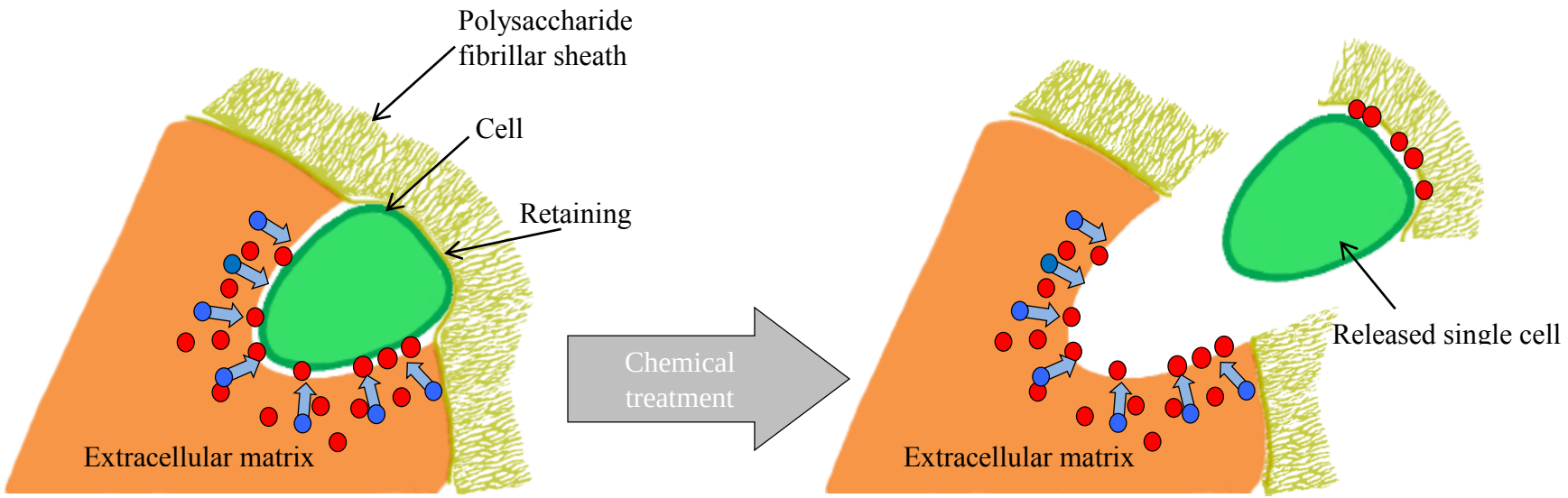


Figure 3.8 Single cell release model

Single cell release model of *Showa* by chemical reagent treatment. The hydraulic pressure caused by the dissolution of chemical reagents pushes the hydrocarbon oils into the retaining wall, which pushes the cells out of the matrix. Red and blue circles indicate hydrocarbon oils and chemical reagents, respectively

It is likely that the naturally accumulated shells in the old culture medium are generated via the enlargement of colonies and their separation into sub-colonies. In such cases, the fragmentation of the retaining wall probably occurs very slowly and without the accompanying strong hydraulic pressure that pushes the matrix hydrocarbon oils into the retaining wall. This might be the reason why the naturally accumulated shells contained no hydrocarbon oils, whereas the chemically induced shells of the B-race varieties did.

Because they were different from *Showa*, the cap-like structures of *Yamanaka* were not stained by Nile red (Figure 3.9). I speculated that in the case of *Yamanaka*, the retaining wall might break very easily without the need for the hydrocarbon oils being pushed into the matrix. This might be the reason why the cap-like structures of *Yamanaka* were not stained by Nile red. In support of this hypothesis, the appearance of the cap-like structure of *Yamanaka* preceded the single cell release (Figure 3.9 A). Unexpectedly, I found no shell-like structures in the old culture medium of *UTEX572* (data not shown). This might simply be due to the fact that *UTEX572* lacks a retaining wall because it secretes no hydrocarbon oils. This might be the related to the finding that no chemical reagent was potent enough to release the single cells of *UTEX572*.

The observed phenomenon that exponentially growing colonies release significantly larger number of single cells than stationary colonies seems to be related to the release mechanism. It is possible that the retaining wall, which must be broken through cell release, is much thicker in stationary colonies than in colonies in the exponential growth phase. The thicker retaining wall may effectively reduce the number of single cells released from stationary phase colonies. This easily explains why the single cell release ratio was consistently higher in exponentially growing colonies.

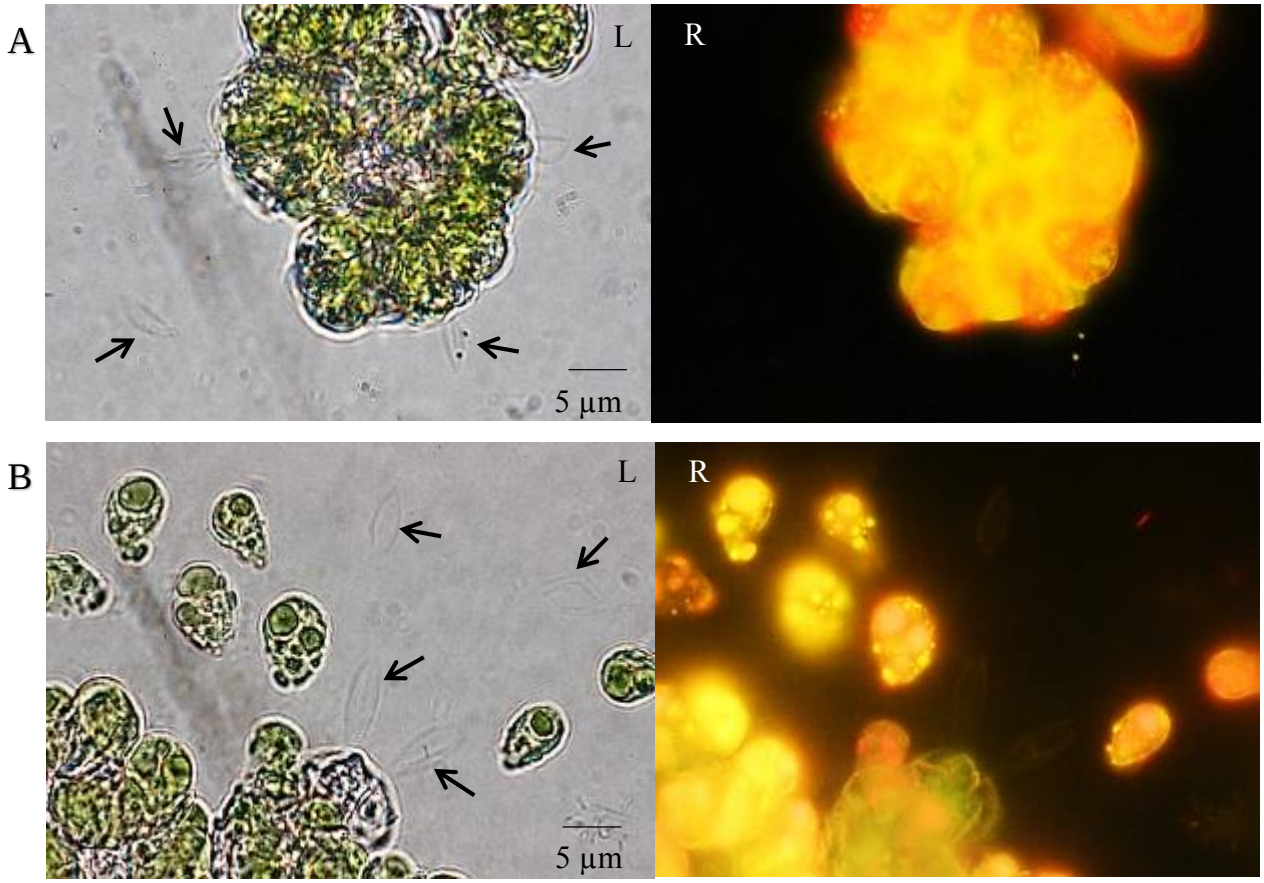


Figure 3.9 Release of single cells and the breakdown of the retaining wall in *Yamanaka* (A-race).

A: Breakdown of retaining wall before single cell release. This image was taken immediately after glycerol treatment.

B: Released single cells and cap-like structures. This image was taken 8 min after glycerol treatment.

L, Normal observation; R, Fluorescence observation. Arrow shows the cap-like structures.

It is unclear why single cells of B-race *B. braunii* maintained at low concentration easily lose their viability and die in a short amount of time. Additions to the medium that induce aggregation of single cells showed positive effects on the survival ratio (Table 3.3). The death of these single cells may be triggered by a decrease in the concentration of some hormone-like substance located in the oily extracellular matrix. This implies that formation of aggregates may prevent the diffusion of a hormone-like substance secreted from single cells.

A similar phenomenon has been reported in the isolated protoplasts of land plants. A high concentration is also essential for the survival of these protoplasts (Power *et al*., 1976). In tobacco, the protoplast concentration must be $> 5\times10^3$ cells/mL for survival; at lower concentrations, the protoplasts fail to divide (Evans and Cocking, 1977). This implies that land plants and *B. braunii* B-race cells share a common mechanism or similar substance.

Considering that colonies of *Showa* contain typical land plant hormones (analyzed concentration of them in *Showa* are shown below), such as abscisic acid (1 ppm), cytokinin (1 ppm), gibberellin (5 ppm), and jasmonic acid (3 ppm) (Ohama *et al*. unpublished results), I attempted to culture single cells at low concentrations (4×10^6 cells/mL) in medium containing these four land plant hormones at the aforementioned concentrations. However, these land plant hormones did not enhance the survival ratio of *Showa* single cells, nor did they induce formation of solid aggregates (data not shown). This implies the putative substance that is essential for survival of single cells may be different from these land plant hormones or another specific concentration of them is essential. Related to the above, Ikehata *et al*. (2011) published a brief report indicating that "conditioned culture medium," which is used culture medium prepared by removing colonies by centrifugation, enhanced the viability of single cells kept in a small vial. This also supports the existence of hormone-like substances.

Another possibility is that loss of the hydrocarbon oils surrounding the cell surface in the matrix causes cell mortality. To test this postulate, I added several drops of prepared crude *Showa* hydrocarbon oils to the single cells held on a membrane filters; however, this treatment had no positive effect on the survival of single cells on the membrane (data not shown). This implies that loss of the surrounding hydrocarbons is not the direct cause of cell mortality, or that the effective substance was lost during the preparation of the crude hydrocarbon oils. Therefore, the characteristics of the substance essential for the survival of *Showa* single cells remain unknown.

Judging from the 4′, 6-Diamidine-2-phenylindole dihydrochloride (DAPI) stained observations, cultures of *Showa* and *UTEX572* were axenic, while those of *Sanshiro-5* and *Yamanaka* were contaminated with bacteria. Therefore, the effects of existing bacteria on our results are unclear.

A molecular phylogenetic study of *B. braunii* showed that the three chemical races share a common ancestor (Senousy *et al*., 2004; Weiss *et al*., 2010b). Therefore, most of the observed differences in physiological characteristics among varieties must be the result of independent evolution after their divergence.

3.5 References

Banerjee A, Sharma R, Chisti Y, Banerjee UC, (2002) *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol 22: 245-279.

Blackburn KB, (1936) *Botryococcus* and the algal coals. Part I. A reinvestigation of the alga *Botryococcus braunii* Kützing. Trans Roy Soc Edinb 58: 841-853.

Bold HC, Wynne MJ, (1978) Introduction to the algae: structure and reproduction. In: Cultivation of Algae in the laboratory. Prentice-Hall INC, Englewood Cliffs, New Jersey, pp 571-578.

Evans PK, Cocking EC, (1977) Isolated plant protoplasts. In: Plant tissue and cell culture Street, H.E. (ed.). Blackwell, Oxford, pp 103-135.

Grung M, Metzger P, Liaaen-Jensen S, (1989) Primary and secondary carotenoids in two races of the green alga *Botryococcus braunii*. Biochem Syst Ecol 17: 263-269.

Ikehata F, Yamawaki Y, Matsusaki T, Kamei C, Enomoto Y, and Enomoto T, (2011) Development of methods for single cell separation from tight *Botryococcus braunii* colony. Japan Institute of Energy 216-217.

Kita K, Okada S, Sekino H, Imou K, Yokoyama S, and Amano T, (2010) Thermal pre-treatment of wet microalgae harvest for efficient hydrocarbon recovery. Appl Energ 87: 2420-2423.

Komárek J, Marvan P, (1992) Morphological differences in natural populations of the genus *Botryococcus* (Chlorophyceae). Arch Protistenk 141: 65-100.

Metzger P, Berkaloff C, Casadevall E, and Coute A, (1985a) Alkadiene-and botryococcene-producing races of wild strains of *Botryococcus braunii*. Phytochemistry 24: 2305-2312.

Metzger P, Casadevall E, Pouet MJ, and Pouet Y, (1985b) Structures of some botryococcenes: branched hydrocarbons from the B-race of the green alga *Botryococcus braunii*. Phytochemistry 24: 2995-3002.

Metzger P, Templier J, Largeau C, and Casadevall E, (1986) An *n*-alkatriene and some *n*-alkadienes from the A race of the green alga *Botryococcus braunii*. Phytochemistry 25: 1869-1872.

Metzger P, David M, Casadevall E, (1987) Biosynthesis of triterpenoid hydrocarbons in the B-race of the green alga *Botryococcus braunii*. Sites of production and nature of the methylating agent. Phytochemistry 26: 129-134.

Metzger P, Casadevall E, Coute A, (1988) Botryococcene distribution in strains of the green alga *Botryococcus braunii*. Phytochemistry 27: 1383-1388.

Metzger P, Casadevall E, (1991) Botryococcoid ethers, ether lipids from *Botryococcus braunii*. Phytochemistry 30: 1439-1444.

Nonomura AM, (1988) *Botryococcus braunii var*. *Showa* (Chlorophyceae) from Berkeley, California, United States of America. Jpn J Phycol 36: 285-291.

Okada S, Murakami M, Yamagichi K, (1995) Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii*. J Appl Phycol 7: 555-559.

Okada S, Murakami M, Yamagichi K, (1997) Hydrocarbon production by the Yayoi, a new strain of the green microalga *Botryococcus braunii*. Appl Biochem Biotechnol 67: 79-86.

Okada S, Murakami M, Yamagichi K, (1998) Characterization of hydrocarbons from the Yayoi strain of the green microalga *Botryococcus braunii*. Phytochem Anal 8: 198-203.

Power JB, Frearson EM, George D, Evans PK, Berry SF, Hayward C, and Cocking EC, (1976) The isolation, culture and regeneration of leaf protoplasts in the genus Petunia. Plant Sci Lett 7: 51-55.

Sawayama S, Inoue S, Dote Y, and Yokoyama SY, (1995) Carbon dioxide fixation and oil production through microalga. Energ Convers Manage 36: 729-731.

Senousy HH, Beakes GW, Hack E, (2004) Phylogenetic placement of *Botryococcus braunii* (Trebouxiophyceae) and *Botryococcus sudeticus* isolate UTEX 2629 (Chlorophyceae). J Phycol 40: 412-423.

Templier J, Largeau C, Casadevall E, (1984) Mechanism of non-isoprenoid hydrocarbon biosynthesis in *Botryococcus braunii*. Phytochemistry 23: 1017-1028.

Templier J, Largeau C, Casadevall E, (1991) Biosynthesis of *n*-alkatrienes in *Botryococcus braunii*. Phytochemistry 30: 2209-2215.

Tonegawa I, Okada S, Murakami M, and Yamaguchi K, (1998) Pigment composition of the green microalga *Botryococcus braunii* Kawaguchi-1. Fish Sci 64: 305-308.

Weiss TL, Chun HJ, Okada S, Vitha S, Holzenburg A, Laane J, and Devarenne TP, (2010a) Raman Spectroscopy Analysis of Botryococcene Hydrocarbons from the Green Microalga *Botryococcus braunii*. J Biol Chem 285: 32458-32466.

Weiss TL, Johnston JS, Fujisawa K, Sumimoto K, Okada S, Chappell J, and Devarenne TP, (2010b) Phylogenetic Placement, Genome Size, and GC Content of the Liquid-Hydrocarbon-Producing Green Microalga *Botryococcus Braunii* Strain Berkeley (*Showa*) (Chlorophyta). J Phycol 46: 534-540.

Weiss TL, Roth R, Goodson C, Vitha S, Black I, Azadi P, Rusch J, Holzenburg A, Devarenne TP, and Goodenough U, (2012) Colony Organization in the Green Alga *Botryococcus braunii* (Race B) Is Specified by a Complex Extracellular Matrix. Eukaryot Cell 11: 1424-1440.

CHAPTER 4 Expression cassettes for transformation of *B. braunii*

4.1 Introduction

The potential of microalgal biofuels as alternative energy sources has attracted worldwide attention (Scott *et al*., 2010; Yoshida *et al*., 2012). Among oleaginous microalgae, *Botryococcus braunii* is considered to be one of the most promising feedstock for the production of microalgal biofuels because it's prominent potential to synthesize and accumulate lipids and hydrocarbons (Metzger and Largeau, 2005; Watanabe and Tanabe, 2013). However, productivity is restricted markedly by its slow growth rate. Application of molecular breeding techniques is essential to improve this alga for the production of biofuels at reasonable price. Genetic engineering (e.g. transformation) is still in infancy stage for many algal species including *B. braunii*. Transformation has been proved to be feasible in some chlorophyte algae such as *Chlamydomonas reinhardtii*, *Dunaliella salina* (Kindle *et al*., 1989, Tan *et al*., 2005), as well as hydrocarbon-producing microalgae, such as *Pseudochoricystis ellipsoidea* (Sousuke *et al*., 2012). To date no successful transformation of *B. braunii* has been reported. This chapter includes the preliminary work for transformation of *B. braunii*, and construction of expression cassettes for it.

Interestingly, *UTEX572*, one of the A race strains, has attractive trails, such as occurrence of natural individual cells, no oil secretion, very limited amount of extracellular matrix. These characteristics make this organism a propitious object for genetic engineering.

In this study, phleomycin resistance (*ble*) gene (Fuhrmann *et al*., 1999) and green fluorescent protein (*gfp*) gene were chosen as transformation selection markers because of the sensitivity of *B. braunii* to Zeocin and limited green autofluorescence in the cells. Additionally, codon optimized *BbGUS* gene was also used as a reporter gene. To drive the exogenous genes, strong and

constitutive promoters are preferred. However, unfortunately, only partial genomic sequence of *Showa* is available, while for *UTEX572* strain, very limited genomic sequence data is available even from NCBI database. Therefore, 5 Gb sequence data for *UTEX572* was determined using MiSeq Next-Generation Sequencing (NGS) apparatus as a co-operative work with Filgen Inc. (Nagoya, Japan). Obtained and public sequence data were utilized to elucidate appropriate promoter region sequences. Totally six kinds of expression cassettes were generated for nuclear transformation of *Showa* and *UTEX572* strain.

In addition to them, two transformation cassettes were built for *UTEX572* chloroplast transformation.

4.2 Cloning of elements for construction of *B. braunii* transformation DNA cassettes

4.2.1 Constructs for nuclear transformation of B. braunii Showa strain

4.2.1.1 Culture of *Showa* cells and genomic DNA isolation

Condition for culture of *Showa* cells are described in Chapter 2. Isolation of genomic DNA from *Showa* cells was carried out following the CTAB method (Sambrook and Russell, 2001).

4.2.1.2 Strategies for transgenic DNA construction

5'-UTR sequence of *Showa rbcS*, *SQS*, *PsaD* genes were obtained by BLAST search of the combined data. Using the primers synthesized on the information, these 5'-UTR regions were amplified from *Showa* genome DNA with Ex Taq DNA polymerase (Takara, Tokyo, Japan). *Ble* gene was selected as transformation marker due to the sensitivity of *Showa* against Zeocin. The ORF was amplified from a plasmid that containing the entire *ble* gene. It is reported that *C.*

reinhardtii rbcS2 terminator properly functions even in other algal species (Imamura *et al*., 2012), since, *ble*:Cr-*rbcS*-Ter fragment was amplified from plasmid pSP124S, and used as an element for *Showa rbcS* and *SQS* constructs, while endogenous 3'-UTER of *PasD* gene was employed for the *PsaD* construct. To assemble the expression cassette elements, unique restriction sites located at the ends of PCR fragments were utilized. Entire region of the constructs were sequenced to confirm the correctness of intended sequences (Macrogene, Tokyo, Japan). Resultant plasmids are shown in Figure 4.1.

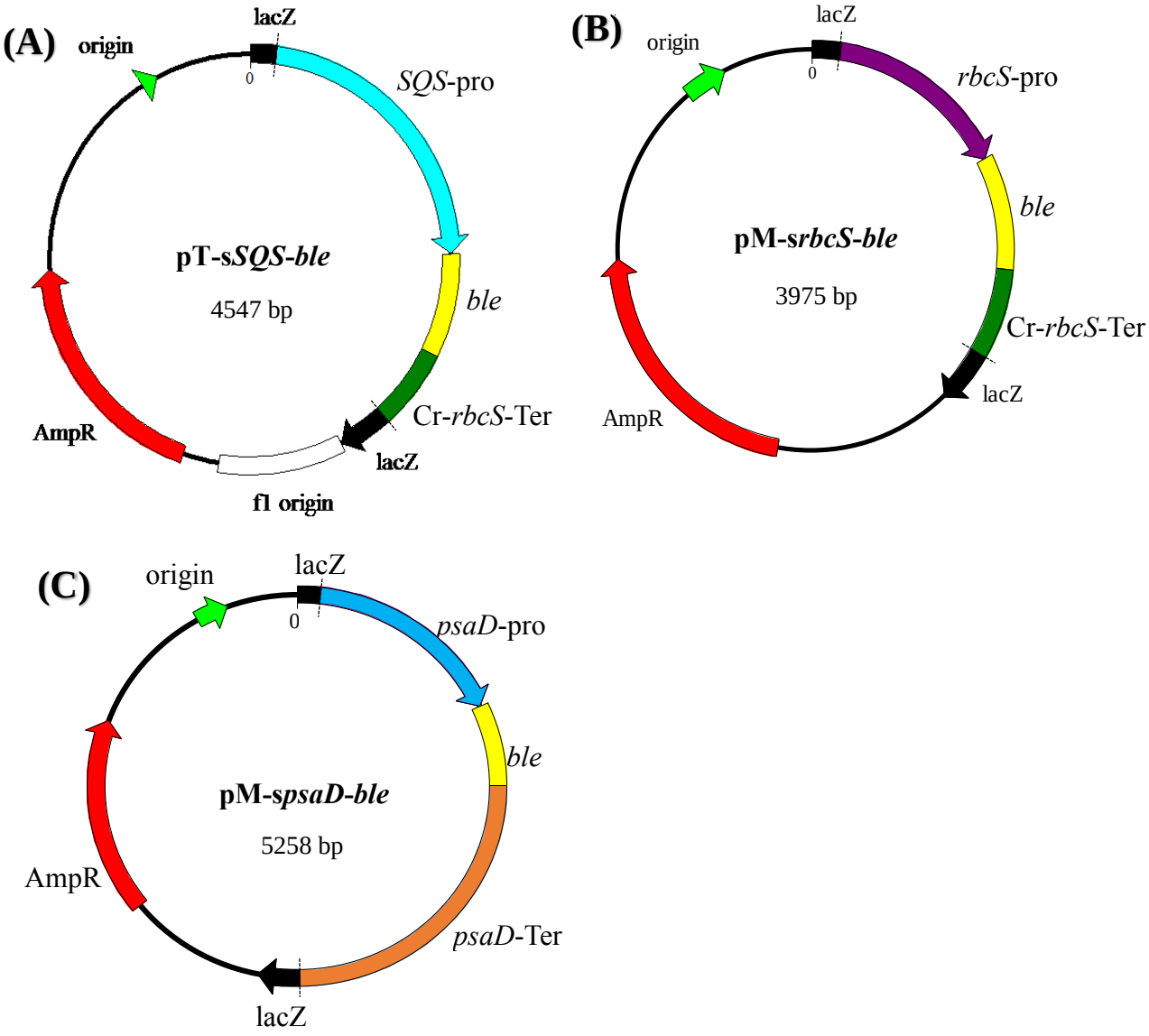


Figure 4.1 Generated constructs for *Showa* nuclear transformation.

The 5'-untranslated regions of *Showa SQS*, *rbcS*, and *pasD* genes that are expected to contain promoters are shown in cyan, purple and blue color, respectively. The 3'-untranslated regions of *Chlamydomonas rbcS* gene and *Showa pasD* gene that work as terminators are indicated in green and orange color, respectively. The marker gene *ble* is shown in yellow.

4.2.2 Constructs for nuclear transformation of B. braunii UTEX572 strain

4.2.2.1 Acquirement of necessary sequence information for transgenic constructs

Highly expressing endogenous promoters are essential to drive marker or reporter genes in transgenic constructs. However, only limited genomic sequence was available for *UTEX572* strain. Therefore, 5 Gb genomic sequencing data of *UTEX572* was obtained using MiSeq (Illumine) based on Next-Generation Sequencing (NGS) method. Straight Walk method (Tsuchiya *et al*., 2009) and RESDA-PCR (González-Ballester *et al*., 2005) method were applied to extend the short 5'-UTR region to include the promoter regions.

4.2.2.2 Strategies for construct making of nuclear transformation

To date, there is no successful report on transformation of *B. braunii* yet. *Crgfp* gene encoding the *Chlamydomonas*-codon usage adapted green fluorescent protein (GFP) ORF was expected to be a good reporter gene, considering the similarity of their GC contents (approximately 65% for *Chlamydomonas* and 60% for *UTEX572* strain). Therefore, *ble::Crgfp* fused ORF in the plasmid pMF59 was used to amplify the part by PCR. Moreover, specific localization of the product in nucleus makes the detection of GFP green fluorescence easy (Fuhrmann *et al*., 1999; Rasala *et al*., 2012). Additionally, codon optimized synthetic *BbGUS* ORF was also used as reporter to observe possible transient expression in the transformants.

The whole cassette of *ble::Crgfp* was amplified from pMF59 (Fuhrmann *et al*., 1999), while reporter gene *BbGUS* was amplified from vector that harboring the synthesized *GUS* by PCR using KOD FX Neo polymerase (Toyobo, Osaka, Japan), then cloned into an entry vector pDONR221 (see Figure 4.2) via BP reaction following the protocol of MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Tokyo, Japan),

1.3 kb, 1.2 kb, and 1.3 kb-long 5'-UTR regions for *Actin*, *LhcB*, and *psaD* genes; 620 bp, 540 bp, and 420 bp-long 3'-UTR regions for these genes were extracted from the obtained genomic sequence data of *UTEX572*. PCR products corresponding to these regions were used as promoter and terminator elements for transgenic constructs. They were cloned into the entry vectors pDONR41 and pDONR23 (Figure 4.2), respectively via BP reaction following instruction of Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Tokyo, Japan). Finally, six plasmids were constructed via LR reaction of Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Tokyo, Japan) (Figure 4.2).

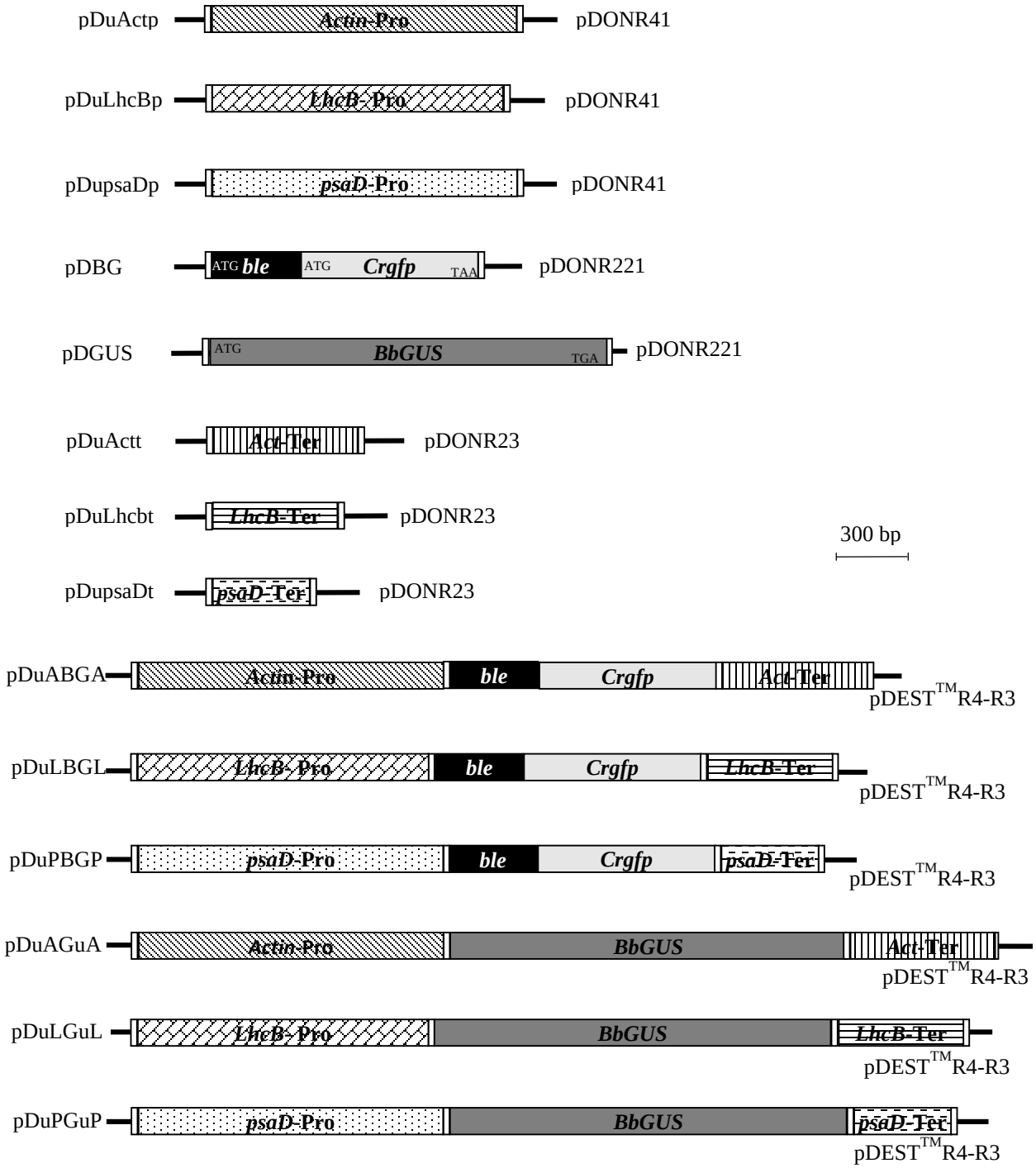


Figure 4.2 Generated constructs for *UTEX572* nuclear transformation.

Start codons (ATG) and stop codons (TAA/TGA) are indicated in pDBG and pDGUS. Open boxes represent att sites for BP/LR recombination. The gateway pDESTTMR4-R3 was used for destination vector for the cloning of three-fragment recombination.

4.2.2.3 Constructs for chloroplast transformation of B. braunii UTEX572 strain

Based on the assembled MiSeq sequence data and the extended sequence data by the straight walk method, two long chloroplast genomic sequence data were obtained, which are approximately 10 kb long. One contained the *rbcL* gene sequence, while another had *tufA* gene sequence.

Codon optimized *aphVIII* (*BbaphVIII*), which confers paromomycin resistance, was synthesized and served as a marker gene of plastid transformation constructs. In order to ensure that transcription could start regularly, the *BbaphVIII* fragment was inserted at five codons downstream of the start codon of *rbcL* gene or *rpl19* gene. For the homologous recombination, 1.5 kb-1.0 kb PCR fragments generated from *rbcL* or *rpl19* gene were used to sandwich the *BbaphVIII* ORF (Figure 4.3).

In this experiment, splicing by overlap extension PCR (SOE) method (Horton *et al*., 1989) was employed to connect three independent fragments. The resulting fragments were restricted with *BamHI* and *XbaI*, and then cloned into *BamHI* / *XbaI* site of pMD20 destination vector to create plastid transformation plasmids.

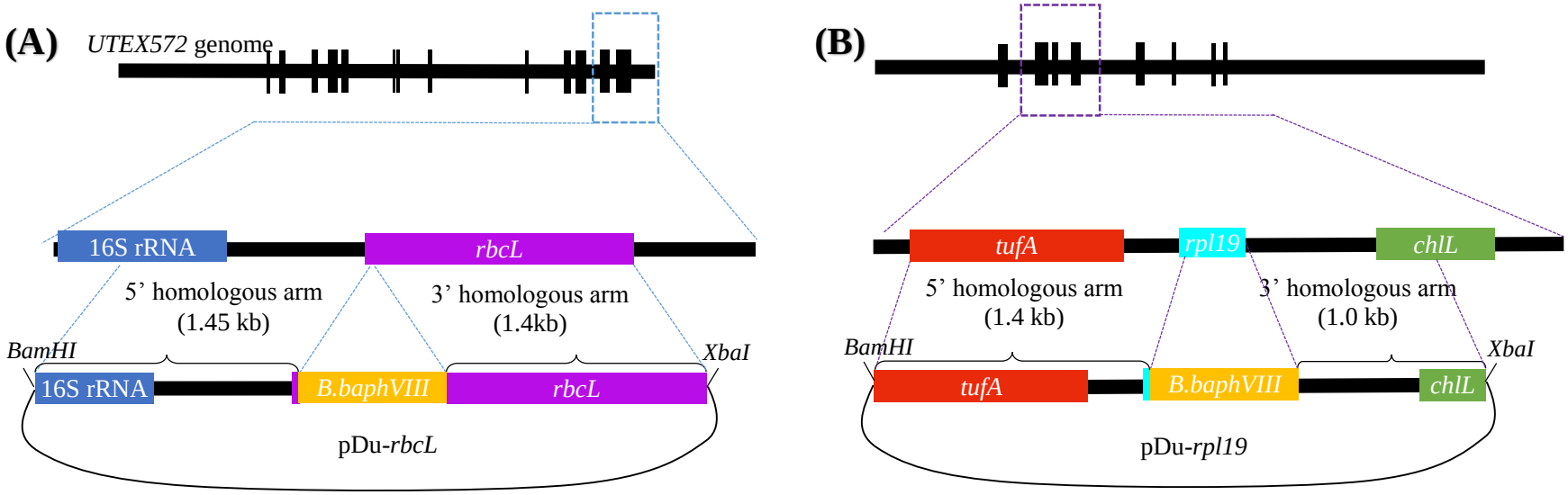


Figure 4.3 Generated constructs for UTEX572 chloroplast transformation.

Bold backbone indicate *UTEX572* plastid genomic sequence. *BbahpVIII* ORF was flanked by 1.0 kb or 1.5 kb-long left and right homologous arms for homologous recombination.

4.3 Sequence data of all expression cassettes used in this study

Sequence data of all expression cassettes used in this study shown in Table 4.1.

Table 4.1 Sequence data of all expression cassettes used in this study

Transgenic plasmid	Sequence data of expression cassette
pT-sSQS-ble	cagggacgcggaacctttctatttgtactgctggtgcagtacttacctcggggcttcccctctactgtccctatttcgtttcacccaaggaaat cctcttttggccttttggtttccgctaccccacacccccagaggaatcctggacgccagttcatagatttacagaatctgaaatattcacttttttc cttctaagaatcctttggtcgctctattacaggtttagtaatgtgtacacgtgggggggaaaccttccagggatatacaggactaaaaaggat taaacgaccaaccaaggggccggaacaaaagcaggtgcggcccttcttcctcacaaaaatatcctacactcttgggcgggaaatcgcct gcaaattcaaatgaagcttcccctgtgcctgtttgccacggctgcctgtcgtacccccttcgacttgtctacgaagcgtggtgaggagtcgta aaatctgcccggcgattgcccgtgcgatacaatctgcctaaagattgccctcaaattctgtcacatttcacggtctgccccagtcacctcagt tggaaatagggcatgcgcacagagatctttgagaatttcgggacgcaaaggcctgtacacgacatttatttgttgggggggggtgctatgaa tcctaaaatttttcgagattctcgtggtttgggtttggtgtgaattctcagtcggaagttctcactcatggctgtgaatgatggacgcgtttatcg gactagggttggtgccgatcattcgactgtcaatgactgaatggggtggtacttcagaaccaggcttcccgccatttgcccaaacccacgc tcgtgcaacagcaacaagtcctctgcgtcaggcaaaacgtccgtttgtatggcttgcgcttgaaagctggtggggataaacgtcaaaaga aagaagctctgttcggggttcacgggtgtcgtttagtactttcccctacgacatcgtcagccttggctcatcgcaatccaaccaacc**ATG** ACCAAGCTGACCAGCGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGG TCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTC GCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGT GGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTAC GCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCAT GACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCC GGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGAC**TAA**ccgacgtcgacccactctagaggatc cccgctccgtgtaaatggaggcgctcgttgatctgagccttgccccctgacgaacggcggtggatggaagatactgctctcaagtgctga agcggtagcttagctccccgtttcgtgctgatcagtctttttcaacacgtaaaaagcggaggagttttgcaattttgttggttgtaacgatcctc cgttgattttggcctctttctccatgggcgggctgggcgtatttgaagcgggtacc
pM-srbcS-ble	agggcgcgatatatagtttactaacagaagtgcgccggcgcagtatgatgtccgtataataaaggggtggaatctctgcatttggattagac cctacgggtgcgagtaatcttccttcctttccttcctcctacgggcccgtgcgagtgcaaactacaaaatcgcatgctgcacgcgccgggt ggtgctgactggtcaatcgcattgtgatcccttgcagatccctcgagattgtatgcagatcgtagcttggggtcgctcacgtgacggggctc cgagggcgcagaggaaccgccgactatgaaaagcgtcttctaccgtgagcactgattggtcgagcatgcgtacggccgccagaattaa gggttgaaatcccggaacccgttccatcatggaaatctctggtacggaatcgtgctcaggtggcatcacgaggcaccagacatcgtaatt ggccacagccagattatggcctgcctatggatcaggcagataagcttaagcttgcttatctgccggctactagacacctgagagcgcctg caaatatcaaagttagaaattctcttcagctttgatattacttgcaaaaacaggttacc**ATG**ACCAAGCTGACCAGCGCC GTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCG GCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACG ACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTG GCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCG TGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCA GCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTC GTGGCCGAGGAGCAGGAC**TAA**ccgacgtcgacccactctagaggatccccgctccgtgtaaatggaggcgctcgtt gatctgagccttgccccctgacgaacggcggtggatggaagatactgctctcaagtgctgaagcggtagcttagctccccgtttcgtgctg atcagtctttttcaacacgtaaaaagcggaggagttttgcaattttgttggttgtaacgatcctccgttgattttggcctctttctccatgggcgg gctgggcgtatttgaagcgggtacc

pM-*spsaD-ble*	tggttcttgtgaaaattttcacgtgtctcttcaatggaacatatatcacaaatacttcgtcgcacaccacatacgccacagtcgacaggttata gcagttcgctgcacctgtgtcaacgaacgccatccaccgatgctggctgccataccgagttatgcaagaatcatatacccatgattgtgcc cacttgctttcgtgctggtactccctgaaatactggtattcaaggacgcctgcttcggtgtaagcgtataattgcttaatgagcgggacagaa ctgttgtgatcgaaaacataaaatttagaggcccccaattttctgtgatattctatccattcgaggacatccaatggctggtcttttacgatgag gcacatagctagatatcccggatccgtccattgaacgtcctctggaaccgcaattgattgcactaccaaactttcaggaggtattggtagac gaccccagtcaacgcagagactacttagtaaaagcagcgcgacaccgcagcagaaaacggggcataagcacttggtcgttacgcaac ccatcggtcgactcgaaagcttatgttcaacctacattatggttgagcaacgttgtaggcatcggatacttcctaggttgcttctggtacggg aagttttgtggaatgattaccaaggaggctgctgatatttttgggggatggtaagttggcagtacaagtgttgattgtacgttcgttaccgggc tcacgtaacggagaagatcccttatctgcaaaaggctcacctgttccggattggttgaccctgcgcaatcaagtcaggctattttgcagagc ctgcggccactctcatctttcgagtgaaa**ATG**GCCAAGCTGACCAGCGCCGTTCCGGTGCTCACCGC GCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGG ACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATC AGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGC GCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCG GGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAG TTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGG AC**TAA**gagttggcaaccccggacgccggattgggcagaacgtgaacccagttcaggtgcgcttgattttatcaaacggggagttat gcatgcttcaggtgattacgttgttcacaacgcggcatgacatttcacagtgcttcttgtattctccacgagttaacatggcgtctagttcaaac ctatcagatagttgggggttggaactcccgaagctttgggttggtaggtaggcagggagcttcttcatccaaagaagtctctcaaatgtgaa tttaaggagctttttcaagatttcaaaagcagggattgtagttgtatttcctcggtgtctactgtttgggcgtgttggggtgctgttcagctgcgg gctatccagtctggggtttctgaggccattgttgtggaaaatcaggatatggtgccgacaggggtcctatgtgccgcggcataaatttgtgt gtgacctgtcgtataccctatggttttcctccccagtttgtatgtgccacagcttgtactcgaaatgcgtttgaccatctgttttggtggaggtatt ccggccgaggaaaaggcttcctaatgttactctcacgggtcacttgcccaaatttgcaagtccggtgatctctttgttaggcaagtcagaatc agcatttctttgcaatttcctttcgttgtttgacagcttgtttgggcttgcagatcaagttcaccggcaaggttggaactttcgacatctaaagga gcccctacttttacagcagcgatgttgagtccatgaaacacggtatgtggcaggactgaccgcggcaattgccctcgttcaagcaatggg actgcagtctagggagcccactagctgcattcctagtggcctagtatggcaagggagtggttgctgctgcagctttcctgctcctttgctgtg aggagggtgtgcgtgtggcagtgttgcaaggttgtgtggcaccatgatttctccatacttcagggggctttacagcagcagaaggcgattt aaacatttcttttgattcttcctcctgcgctagtgttggccgtgtatcgcagtgtaggccccttcatgtgcataggggaacagtatgggctag attcctggttaccagacattctgtaatgaaggttttgagtacgtcactgcgttcgtcctttgatggtctcgtggcaattctcccttggcaatttata agttgtgtgacttttcatattgtggacaccacttgtaactctgtcagatctctgaatcggatccccgggtaccgagctcga

pDuABGA	tcgaagctaggactcatgacggcgtctgacgatgtattacatagggtttacggtgtcttacggtgcaacatcggggtacagaccgactatta gaggcctgccctaccgttcggccaatgttaatgaggcaacgacccctccccctggccgcgctctttttgccctcgagcaactgagcaaaat ccattggcccaaccttccccaatggttcttgtccgcgcgcgtgtcggtgcctttctagctgggtttgctgtcgcgggtggcctggccttgtat cagctccgccaggacgtgtgggaaagccaccgtgtgctggctgcccaggtaacccttgtctgccgccttgcagtttgaaggctgtcagaa ccgagcgatttttttggtcgcgctgggcttccttggcagttgtacctattgcatgcttgctgcaccttcgttggcctcccgggtgcagataggtc tcctgtgggtgctgccttccttctggcctttgtatgtcaagtagtttgaaaagtctttcagcgttggcggtcttggcaggcctgctgtcagccgt tggtgggtgccgctctgcttttgcttttgcatccgttgttttcgtgcttgcgcttggtgtggtcccagctgcttgtgtaggtggttgtgatccttcg gtgtcaccggccgatgcggccgtctgtcaagttagtgaaccgcaagcctgtcgacatgccgtgtaacgttcagctaggccaccctggtgg gtgctgttgaagtgagtcgttcggatgtgtaccggcttccttgctgggtgcggtcgtgacgcagagacggaccgacttccttccgataagg tatccgcatatgcgggccgaggacaagggcttcaatgatgtttttttcgcaagttcaggaagtcagctgtctttgcagttggagagcgtggat cgggcccttgaggagcgcgtcagccagttggaggctgccgtaggagaaaagcagcactgaaagccttatcttccaggtggggcagcat ggactgatttgaatggccatgctgtgcggaagtgcttacctcatggcaaccttcgtggttttcttgtacgacatgcagactcccttcccctcc agtcttaggtgtctggctggtcagttgcggtagtgccctgggtccagtatatgagcaagcactgagcatccctcttcaaacacggagttgta taaacgtgcctttgtccccagtcagaatctgaggattgaaacagcatgtgcacttgttgggaagggccatagattttgtgctctgcttgcagg tgaatcacc<u>CAAGTTTGTACAAAAAAGCAGGCT</u>**ATG**GCCAAGCTGACCAGCGCCGTTCC GGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTC GGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGT GACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCT GGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTC CACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCG TGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGC CGAGGAGCAGGACggaggaggaATGACCATGATTACGCCAAGCTTGCATGCCTGCAGG TCGACTCTAGAGGATCCCCCCAAGGGCGAGGAGCTGTTCACCGGTGTGGTCCCCAT CCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAGTTCTCCGTCTCCGGCGAG GGTGAGGGTGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG GCAAGCTGCCCGTGCCCTGGCCCACCCTGGTCACCACCCTGACCTACGGTGTGCAG TGCTTCTCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCAT GCCCGAGGGCTACGTGCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTAC AAGACCCGCGCCGAGGTCAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGCCACAAGCTGGAGTA CAACTACAACTCCCACAACGTGTACATCATGGCCGACAAGCAGAAGAACGGCATC AAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCTCCGTGCAGCTGGCCG ACCACTACCAGCAGAACACCCCCATCGGCGATGGCCCCGTGCTGCTGCCCGACAAC CACTACCTGTCCATCCAGTCCGCCCTGTCCAAGGACCCCAACGAGAAGCGCGACCA CATGGTCCTGCTGGAGTTCGTCACCGCTGCCGGCATCACCCACGGCATGGACGAGC TGTACAAG**TAA**<u>ACCCAGCTTTCTTGTACAAAGTGG</u>acgggctggagctggtttggcagtccgagctgg ggaggggctgtttggtcgaagaagccaggatgggggtttctaggcaggttcttgcattgtctggctattgtgggcaggcagtgtgtttggtg gtgagctggtaggctggcagttgtgatgtggtttggtgcagggatgttctatctaggtgtgatgttttctcctgctggagttcagagaaatataga ggctgatatctgcatgccaattcaggatatatgacttatgtgagctgtctcactgccagtaggtttaggcatgtccaacttgtttgtgcatgtgg gactctgccacaccgaactgtggaaggctcgtgtccttggggtgtgccggtccgtgaaaccgcagaagcggcttcaggtaaggaggcc aggttctagtgatgcatattgtccctaatgggcgacaaatctttgggaggttctgagatgcagttgggagattccatgatgcaaggatgggc aggacgatgtggtgtgggttggaatcctgcagactgacggtgtctgtttgtcgtatgggaattcggatggctgacccaggcatgcaggttc tctgtaactgtgtggaggggt

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Note: Start codons (ATG) and stop codons (TAA/TGA) are indicated in uppercase characters with bold. Uppercase characters with underline represent att sites for BP/LR recombination. The ORF region of marker gene and reporter genes are shown in uppercase.

4.4 References

Fuhrmann M, Oertel W, Hegemann P, (1999) A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. Plant J 19: 353-361.

González-Ballester D, de Montaigu A, Galván A, and Fernández E, 2005 Restriction enzyme site-directed amplification PCR: a tool to identify regions flanking a marker DNA. Analytical biochemistry 340: 330-335.

Horton RM, Hunt HD, Ho SN, Pullen JK, and Pease LR, (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68.

Imamura S, Hagiwara D, Suzuki F, Kurano N, and Harayama S, (2012) Genetic transformation of *Pseudochoricystis ellipsoidea*, an aliphatic hydrocarbon-producing green alga. J. Gen. Appl Microbiol 58: 1-10.

Kindle KL, Schnell RA, Fernández E, and Lefebvre PA, (1989) Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 109: 2589-2601.

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

Rasala BA, Lee PA, Shen Z, Briggs SP, Mendez M, and Mayfield SP, (2012) Robust expression and secretion of xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. PLOS ONE 7: e43349.

Sambrook J, and Russell DW, (2001). Molecular Cloning: A laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, and Smith AG, (2010) Biodiesel from algae: challenges and prospects. Curr Opin Biotechnol 21: 277-286.

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

Tsuchiya T, Kameya N, and Nakamura I, (2009) Straight walk: a modified method of ligation-mediated genome walking for plant species with large genomes. Analytical biochemistry 388: 158-160.

Watanabe MM, Tanabe Y, (2013) Biology and industrial potential of *Botryococcus braunii*. In: Richmond A, Hu Q (eds) Handbook of microalgal culture, 2nd edn. Wiley-Blackwell, Oxford, pp 369-387.

Yoshida M, Tanabe Y, Yonezawa N, and Watanabe MM, (2012) Energy innovation potential of oleaginous microalgae. Biofuels 3: 761-781.

CHAPTER 5 Conclusions and prospects

5.1 Conclusions

First, I showed TiO_2 nanoparticles are useful as DNA carrier for biolistic bombardment. Two peaks of optimized gas pressures in the transformation ratio were detected irrespective of whether the particles were made of gold, tungsten, or TiO_2.

Second, I established the method to prepare single cells from colonial *B. braunii*: *Showa*, *Sanshiro-5*, and *Yamanaka* strains. Eight chemical reagents were found to have potency for releasing single cells from *Showa* strain. In addition, I made a model for the single cell release by chemical reagent treatment. Moreover, I closely characterized the prepared single cells via staining.

Lastly, I generated DNA constructs to transform *B. braunii* nuclear and chloroplasts.

5.2 Prospects

Isolated single cells are supposed to be useful material for molecular engineering, such as cell fusion or genomic transformation. NEPA21 apparatus is a unique electroporator that can achieve high transformation efficiency without cell-wall removal. Using this apparatus, two successful paper for transformation of algae have been published. Therefore, electroporation of single cells with NEPA21 must be worth challenging. Additionally, TiO_2 might be a useful material to transform wide range of algal species including *Botryococcus*.

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ACHIEVEMENT

List of Publications

1. **L Hou**, P Wang, F Kong, H Park, K Kobiro, and T Ohama. 2013. Mesoporous TiO_2 nanoparticles: A new material for biolistic bombardment. *Phycological Research*, 61: 58–60.

2. **L Hou**, H Park, S Okada, and T Ohama. 2013. Release of single cells from the colonial oil-producing alga *Botryococcus braunii* by chemical treatments. *Protoplasma*, 251(1): 191-199.

3. U Resnati, **L Hou**, S Okada, and T Ohama. 2014. High yield cocktail to prepare protoplasts from oil-producing green alga *Botryococcus braunii* variety *UTEX572*. **Submitted** to *Protoplasma*.

4. F Kong, T Yamasaki, K Sari, **L Hou**, X Li, N Ivanova, S Okada, T Ohama. 2014. Robust expression of heterologous genes by selection marker fusion system in improved *Chlamydomonas* strains. **Submitted** to *JBB*.

International conference

1. The 9th Asia-Pacific Marine Biotechnology Conference (APMBC) (2012, Kochi, Japan, **Oral**)

2. The 90th Anniversary Meeting on Biotechnology for Green Growth (2012, Kobe, Japan, attend)

3. The 4th International Symposium of Frontier Technology (ISFT) (2013, Shenyang, China, **Oral**)

Patent

ボツリオコッカスブラウニーの単細胞単離方法及び単細胞培養方法 WO2013121509 A1

Awards

1. 2013.3.18 KUT Academic Research Award 高知工科大学学術研究奨励賞
2. 2014.3.20 KUT Academic Research Award 高知工科大学学術研究奨励賞