Invited Review:

Algal Genes Encoding Enzymes for Photosynthesis and Hydrocarbon Biosynthesis as Candidates for Genetic Engineering

Hidenobu Uchida1*, Ko Kato2, Kensaku Suzuki3, Akiho Yokota2, Shigeyuki Kawano4, Shigeki Matsunaga1 and Shigeru Okada1

1 Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113–8657, Japan
2 Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630–0101, Japan
3 Tohoku Agricultural Research Center, National Agriculture and Food Research Organization, Morioka, Iwate 020–0198, Japan
4 Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277–8562, Japan

Received March 31, 2016; accepted October 14, 2017

Summary In order to increase the biomass of photosynthetic organisms, upregulation of net CO2 fixation in daytime appears to be a promising approach. This can be attained by improving the turnover of chloroplast enzymes such as ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) or fructose bisphosphate phosphatase (FBPase). In a previous report, the genes for the large and small subunits of cyanobacterial Rubisco were coexpressed in tobacco with either with cyanobacterial chaperone for Rubisco or the carboxysomal CO2 concentrating mechanism (CCM) gene. This resulted in the generation of faster carboxylation of Rubisco and a similar gene expression approach may be adopted into algal hosts in the future. Expression of the cyanobacterial FBPase gene with protein targeting to the chloroplast in Euglena gracilis was reported to increase the wax ester content effectively. Though stable transformation of the oil producing alga Botryococcus braunii race B, is yet to be achieved, genes for key regulatory enzymes in 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, such as 1-deoxy-D-xylulose 5-phosphate synthase (DXS) or 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), might be possible targets for genetic modification in this alga. Challenging problems in the transformation of the microalgae, including C. reinhardtii, or those of the macroalgae are discussed herein for the design of transformation aiming at higher yield of biofuel production.

Key words Hydrocarbon, Isoprenoid, Triterpene, Squalene, Rubisco, Chloroplast gene manipulation.

We would like to describe algal metabolic pathways of photosynthetic carbon assimilation, isoprenoid and triterpene biosynthesis, genes encoding key regulatory enzymes, algal transformation and marine algae as transformation hosts. Discussion presented here might cast insight into comprehensive design of algal biofuel biotechnology from the viewpoints of photosynthesis and secondary metabolism.

Rubisco and photorespiration

Many organisms on the Earth utilize carbohydrates as their energy source. Carbohydrates are produced from CO2 and H2O by oxygen-evolving photosynthetic organisms. Figure 1 shows the biosynthesis schemes for Calvin cycle, photorespiratory pathway, glycolysis (gluconeogenesis) and MEP pathway. In the Calvin cycle Rubisco assimilates CO2 into RuBP (1) to produce two molecules of PGA (4). This chemical conversion, which is called carboxylation, is processed via two intermediates, ribulose bisphosphate enediol (2) and 2C3KABP (3). PGA (4) is then converted into GAP (5). Compound (5) is metabolized into FruPP (6), or SedPP (8). FruPP (6) is dephosphorylated into FruP (7) by FBPase. SedPP (8) is converted into SedP (9) by sedheptulose 1,7-bisphosphate phosphatase (SBPase). FBPase and SBPase are localized in the chloroplast stroma, and are activated in the light, as is Rubisco (Buchanan 1980). Steady state CO2 uptake in the light period for higher plants is limited by FBPase as well as Rubisco (Woodrow and Mott 1993). For these reasons, enhancement of the CO2 fixation ability of Rubisco or FBPase has been attempted by researchers.

The Rubisco enzyme from the proteobacterium Rodospirillum rubrum consists of only two large subunits
On the other hand, the Rubisco enzyme from cyanobacteria, eukaryotic green algae and higher plants consists of eight large and eight small subunits (L8S8, type I Rubisco). These facts mean the catalytically important sites for CO₂ fixation are located on the large subunits. It is also known that the proper folding of subunits with the aid of the chaperones, such as RbcX, is indispensable in type I Rubisco (Saschénbrecker et al. 2007).

The number of CO₂ molecules that can be fixed by Rubisco in one second is expressed as $k_{cat}$. $k_{cat}$ is the turnover number of a substrate which is catalyzed by the enzyme in one second. $k_{cat}$ means the $k_{cat}$ for a substrate, CO₂, of Rubisco. $k_{cat}$ values for Rubisco in a C3 plant such as tobacco are as low as 3.4 s⁻¹, while that of *R. rubrum* is 7.3 s⁻¹ (Sage 2002). Increasing the $k_{cat}$ of Rubisco through genetic modification would be expected in order to increase biomass production.

In addition to the carboxylation reaction, Rubisco also carries out an oxygenation reaction to incorporate O₂ into RuBP (1). The relative ratio of carboxylation as compared to oxygenation is referred to as $S_{rel}$, or $S_{C/O}$, and typical $S_{rel}$ values for a proteobacterial type II Rubisco is about 20, while those of type I Rubisco for cyanobacteria, green algae, and higher plants are 60, 80 and 100, respectively (Andrews and Lorimer 1987). These values ranging from cyanobacteria, via green alga, to higher plants, are in line with the chronological-
ly increased atmospheric O$_2$ concentrations on the Earth due to the appearance of O$_2$-evolving photosynthetic organisms. This atmospheric change would have caused increases in the $S_{at}$ values of these organisms. These things suggest that modifying algal Rubisco carboxylation active sites by the gene manipulation requires the evaluation of the side effect of oxygenation.

As a product of Rubisco oxygenation, 2-phosphoglycerate (10) is metabolized in the photorespiration pathway (Fig. 1) to be dephosphorylated into glycylate (11) by 2-phosphoglycerate phosphatase (PGPase). Glycylate (11) is metabolized into L-glycine (12). One molecule of CO$_2$ is released when two molecules of L-glycine (12) is converted into one molecule of L-serine (13). This release of CO$_2$ in the photorespiratory pathway reduces net daytime carbon fixation in photosynthetic organisms, and thus the oxygenation reaction of Rubisco or the photorespiration pathway in general should be minimized by genetic modification to optimize biomass production. Interestingly, mutants for PGPase cannot grow photosynthetically in ambient CO$_2$ because the substrate of PGPase, 2-phosphoglycerate (10), inhibits the activity of triosephosphate isomerase (TPI) during glycosylation (Fig. 1), and then is very toxic to the cells.

**Biosyntheses of isoprenoids and triterpenes**

Isoprenoids are stored as the primary and secondary metabolites, such as sterols, carotenoids, rubber, alkaloids, hydrocarbons, or moiety of chlorophylls. Isoprenoids possess unique molecular structure consisting of C$_5$ isoprene (2-methyl-1,3-butadiene) units (Fig. 1). Diterpenes (C$_{20}$), triterpenes (C$_{30}$, Fig. 2), and tetraterpenes (C$_{40}$) include 4, 6, and 8 C$_5$ isoprenoid units, respectively. C$_5$ isoprene units for use in isoprenoid biosynthesis are generated from glycolysis for eventual use in the MEP isoprenoid pathway (Fig. 1). In glycolysis, glucose (14) is converted into pyruvate (15) via FruP (7), FruPP (6), GAP (5) and PGA (4). GAP (5) is catalyzed into DHAP (16) by TPI. DHAP (16) is metabolized into glycerol (17). GAP (5) and pyruvate (15) are utilized for MEP pathway (Fig. 1) for isoprenoid biosynthesis.

In higher plants, C$_5$ IPP is synthesized by both the MVA pathway and the MEP pathway. On the other hand, green algae (Schwender et al. 2001), including *B. braunii* (Sato et al. 2003), possess only the MEP pathway. In contrast to the MVA pathway enzymes that are localized to the cytosol, MEP pathway enzymes are located in the chloroplast (Fig. 1). This reinforces the idea that genetic modification of algal isoprenoid enzymes in chloroplast would be important for increasing isoprenoid production.

In the MEP pathway, pyruvate (15) and GAP (5) are converted into DXP (19) by DXS. DXP (19) is converted into IPP (22) by 2-C-methyl-d-erythritol-4-phosphate reductase (DXR). IPP (22) is subsequently converted into DMAPP (23) by HDR. The enzyme catalyzing the initial step of the MEP pathway, DXS (Estévez et al. 2001) and the enzymes in the last two steps, HDR and DMAPP (23) are metabolized into IPP (22) and DMAPP (23) by HDR.

The enzyme catalyzing the initial step of the MEP pathway, DXS (Estévez et al. 2001) and the enzymes in the last two steps, HDR and DMAPP (23) are metabolized into IPP (22) and DMAPP (23) by HDR.

Algal Genes Encoding Enzymes for Photosynthesis and Hydrocarbon Biosynthesis as Candidates for Genetic Engineering
The gene duplication of triterpene biosynthesis enzymes in *B. braunii* appears to be unique among green algae whose genome has been investigated so far. Genome sequencing and contig assembling in *B. braunii* race B has been obtained (Browne et al. 2017). The genome size of this alga is 166.2 Mb (Weiss et al. 2010) and is much larger than that of a green alga *Medakamohakoo* (Kuroiwa et al. 2015, 2016). Though these two green algae are distantly related, comparison of the gene homologue number and their function between these organisms may cast insight into gene duplication, which might have result in functional differentiation of terpenoid biosynthesis in *B. braunii*.

Transcriptomic analysis is valuable for mining genes related with triterpene biosynthesis. Molnár et al. (2012) performed deep sequencing for transcriptomic analyses of *B. braunii* race B, Showa, assembling 1334609 reads into 46422 contigs, whose average length was 756bp. These contigs were registered in the GenBank Transcriptome Shotgun Assembly Sequence Database with the accession numbers of KA089548–KA133805 and KA659919–KA660048. Contigs related to terpe-

Fig. 2. Triterpene biosynthesis of green algae with and without hydrocarbon oil production ability. Squalene synthase (SS), presqualene diphosphate (26), squalene (27), squalene epoxidase (SQE), squalene 2,3-epoxide (28), squalene synthase-like proteins (SSLs), C30 botryococcene (29), C32 botryococcene (30), C34 botryococcene (31), triterpene methyltransferases (TMTs), dimethylsqualene (32) and tetramethylsqualene (33). Shades are as in Fig. 1. Note that *B. braunii* race B specifically possesses squalene synthase-like proteins, in addition to squalene synthase which is found in the other green algae that do not yield hydrocarbon oil. Molecules of triterpene compounds (27, 29, 30, 31, 32, 33) lack oxygen atoms and possess highly branched carbon backbones. These characteristics donate a higher heat value to *B. braunii* hydrocarbon oil than that of triacylglycerol oils synthesized in the other green algae, or diatoms.

other *B. braunii* oils.

The gene duplication of triterpene biosynthesis enzymes in *B. braunii* appears to be unique among green algae whose genome has been investigated so far. Genome sequencing and contig assembling in *B. braunii* race B has been obtained (Browne et al. 2017). The genome size of this alga is 166.2 Mb (Weiss et al. 2010) and is much larger than that of a green alga *Medakamohakoo* (Kuroiwa et al. 2015, 2016). Though these two green algae are distantly related, comparison of the gene homologue number and their function between these organisms may cast insight into gene duplication, which might have result in functional differentiation of terpenoid biosynthesis in *B. braunii*.

Transcriptomic analysis is valuable for mining genes related with triterpene biosynthesis. Molnár et al. (2012) performed deep sequencing for transcriptomic analyses of *B. braunii* race B, Showa, assembling 1334609 reads into 46422 contigs, whose average length was 756bp. These contigs were registered in the GenBank Transcriptome Shotgun Assembly Sequence Database with the accession numbers of KA089548–KA133805 and KA659919–KA660048. Contigs related to terpe-

Fig. 3. *Botryococcus braunii* race B colony. A colony of *B. braunii* race B Showa strain observed under a light microscope. One hundred milliliter of preculture was inoculated into 900 mL of modified Chul13 medium supplemented with vitamins and cultured for 6 weeks until the algal biomass reached to 2.3 g L\(^{-1}\) (dry weight). Culture conditions were as follows; 12 h:12 h light and dark regime at 25°C, bubbled with 3% CO\(_2\) and illuminated with a photon amount of 110 µmol m\(^{-2}\) s\(^{-1}\). Cells (arrows) in a colony were slightly pressed between a cover slip and the glass slide, and hydrocarbon oil droplets (arrowheads) were exuded from the intracellular matrix (*). Scale bar=10 µm.
noid biosyntheses were extracted from data and were subjected to reciprocal Blast searches using the non-redundant protein database in NCBI as comparators. B. braunii subisolate 779, race A was also subject to deep sequencing, giving rise to 27 million pair end reads, which were assembled into 61220 non-redundant contigs that are all more than 300 bp long (Fang et al. 2015). In order to know expression levels for these contigs, fragments per kilobase per million mapped reads (FPKM) were generated. Homologies of non-redundant contigs were checked by the BlastX suite against pool of ‘best’ proteins in six microalgal genomes of C. reinhardtii, Coccomyxa subellipsaidea C-169, Chlorella variabilis NC64A, Micromonas pusilla RCC299, Ostreococcus lucimarinus and Thalassiosira pseudonana CCMP 1335. Among 12292 best-hit contigs (Fang et al. 2015), 8888 and 3368 contigs were associated with at least one Gene Ontology (http://geneontology.org/) function and KEGG (http://www.genome.jp/kegg/) orthologue, respectively. Pioneer studies of deep sequencing for B. braunii, race B, BOT-22 (Ioki et al. 2012), and race A, BOT-88-2 (Baba et al. 2012) were reported. The former identified 27427 non-redundant contigs assembled from 209429 reads, and the latter revealed 29038 contigs assembled from 185936 reads. Transcriptome data mentioned above may be useful for mining the genes related with not only isoprenoids biosynthesis, but also photosynthesis, or cell division.

In B. braunii, derivatives of triterpenes, or carotenoids might be synthesized in the cell and exported into extracellular matrix (right panel in Fig. 2). Different localization of synthesis and storage of metabolites is one of the unique characters in this alga, and may be the reason why this organism accumulates a large amount of hydrocarbon compounds. Translocation of secondary metabolites into differentiated organ is also known in a petroleum plant Euphorbia tirucalli (Uchida et al. 2007b, 2009). When transformation system is established in either of these organisms, genes related with metabolites of transportation oil may be introduced into these organisms aiming at improving productivity of industrially important secondary metabolites.

Gene manipulation of rbcL, RBCS or PGP

Type I Rubisco in cyanobacteria, green alga and Magnoliophyta consists of eight large and eight small subunits. In eukaryotic green algae and higher plants, the genes encoding the large and small subunit proteins are found in the chloroplast and nucleus genomes, respectively, while prokaryotic cyanobacteria genome includes these two genes within a polycistrion.

In order to add two additional copies of rbcL to the chloroplast genome of C. reinhardtii, rbcL was ligated with an aadA cassette and introduced into each copy of the inverted repeat region of the C. reinhardtii genome (Uchida et al. 2005) using the particle gun. Since the original rbcL gene is located in a single copy region which flanks the inverted repeat regions, the transformant mentioned above is expected to possess three copies of rbcL in the chloroplast genome. In contrast to a single-copy nuclear genome, chloroplasts typically contain about 80 genome copies (Kuroiwa and Nakamura 1986). As shown in Southern blot analysis of this three-rbcL (3-rbcL) C. reinhardtii transformant contained only transformed genomes in the homoplasmic state and no endogenous copies of the genome (Uchida et al. 2005). In the 3-rbcL transformant synthesis and accumulation of rbcL transcripts was upregulated 2.3- and 1.4-fold more than those of the control using two different approaches to detect mRNA levels. In accordance with upregulation of rbcL transcript synthesis, the corresponding large subunit protein (LS) level increased 2.6-fold more than that of control (Uchida et al. 2005). However, the accumulation of LS in the 3-rbcL transformant was at most the same level of control. Interestingly, transcription of the RBCS gene also increased in 1.7-fold, which finally resulted in the almost equal level of small subunit protein (SS) accumulation of the control. These results suggest that upregulation of rbcL transcript synthesis, and/or its accumulation appears to positively affect expression of the RBCS gene. Synthesized LS appears to be associated with chaperons to form a high molecular weight protein complex in order to form an L8S8 Rubisco holoenzyme. For proper folding of eight LSs and eight SSs, equal moles of LSs and SSs may be required in the 3-rbcL transformant, and then accumulation of LS may be attenuated in accordance with lower levels of SS accumulation. This scenario was in line with almost the same density of immunogold particle numbers detected by anti-Rubisco antibody and similar levels of the pyrenoid diameter in 3-rbcL transformant, as compared with those of wild type (Uchida et al. 2007a).

In transgenic rice overexpressing of RBCS the total mRNA levels for RBCS and rbcL, and protein synthesis of RBCS and rbcL increased simultaneously (Suzuki and Makino 2012). This result was in line with the above-mentioned scheme hypothesizing that coordination of equal number of LS and SS is important for stoihometry of Rubisco holoenzyme.

The cyanobacterial rbc operon includes rbcL, rbcX and rbcS, positioned in this order. The RbcX protein promotes the production and assembly of Rubisco (Onizuka et al. 2004, Saschenbrecker et al. 2007). The CCM (Yamano et al. 2014, 2015) is indispensable for algal photosynthesis. The cyanobacterial CCM proteins Ms (CcmMs) are essential components for organizing the carboxysome including Rubisco and carbonic anhydrase (Long et al. 2010). In order to reinforce Rubisco carboxylase activity in tobacco, the endogenous rbcL was replaced with cyanobacterial rbcL-rbcX-rbsS cluster, and then either RbcX or Ccm35 from Synechococcus
elongatus was flanked downstream of the rbc cluster (Lin et al. 2014). This Rubisco transformant had higher rates of CO₂ fixation per unit of enzyme than the control. This result indicates that coexpression of chaperone genes or CCM protein gene is critically important in tobacco for overexpressing genes for Rubisco LS and SS, which are encoded on chloroplast and nuclear genomes, respectively.

Rubisco catalyzes not only carboxylation of RuBP (1), but also oxygenation of this compound (Fig. 2). Relative Rubisco specificity ratios, S₅₀ values, of the red algae Galdieria partita or Cyanidium caldarium, are about 2.5 times higher than that of spinach (Uemura et al. 1997). This may be because red algal Rubisco large subunit possesses a unique ‘Latch structure’ for loop 6, which plays an important role in the formation of the carboxylation reaction intermediate, 2C3KABP (3). Galdieria latch structure is formed by the hydrogen bond between V322 and Q386 and this structure is not found in green-like Rubisco. Point mutations aiming at the introduction of ‘Latch-like’ structure into the cyanobacterial Rubisco resulted in a 16% increase of S₅₀ (Ninomiya et al. 2008).

An amino acid substitution of S376T in the Anacystis nidulans Rubisco large subunit resulted in a decrease of oxygenase activity to 0.02% of wild type (Lee and McFadden 1992, see following text for the carboxylase activity). Aiming at a reduction of oxygenase activity (McFadden 1992, see following text for the carboxylase activity), A. nidulans Rubisco also lost carboxylation activity by Southern blot analysis in near future.

Substantial amounts of CO₂ are released when wild type plants are transferred into the dark. This is called photorespiration. Photorespiration is initiated by oxygenase reaction of Rubisco (Fig. 1). In order to prevent the loss of carbon for biomass accumulation, bypassing photorespiratory pathway is important (Xin et al. 2015). On the other hand, Somerville and Ogren (1979) isolated a photorespiration mutant in Arabidopsis thaliana. This mutant lacks enzymatic activity of PGPase, which catalyzes the conversion of the Rubisco oxygenation product 2-phosphoglycolate (10) into glycolate (11). 2-Phosphoglycolate (10) is finally metabolized to produce CO₂ and l-serine (13). Suzuki et al. (1990) isolated a high-CO₂ requiring mutant of C. reinhardtii that had the photosynthetic characteristics quite similar to those in Arabidopsis PGPase mutant (Somerville and Ogren 1979). PGPase activity in C. reinhardtii mutant (ppg-1) was substantially lower than that in the wild type. Nucleotide sequencing of the corresponding gene was performed in order to reveal the mutation for this mutant (Suzuki et al. 2005). Comparison of the ppg-1 sequence with that of wild type revealed the first nucleotide of the ppg-1 intron 1 possessed a point mutation from G to A, which disrupted the G/T splice donor site. This mutation caused the 54-bp 5’ region of ppg-1 intron1 to become a 17-residue peptide-coding sequence followed by a new stop codon. As a result, the ppg-1 nucleotide sequence encodes a 49-residue amino acid sequence, while that of wild type encodes a 330-residues of protein.

Since the null mutant of PGPase accumulates phosphoglycolate (10), which is a toxic compound, ppg-1 mutant cannot grow photoautotrophically (Suzuki et al. 1990). Suppressors recovering this negative growth have been identified (Suzuki 1992). The 7FR2N strain retained ppg-1 and its suppressor, and then possessed lowered photorespiration, higher affinity for CO₂ and improved growth under light (Suzuki et al. 1999). Suppressor gene alleviates the ppg-1 phenotype without recovering PGPase activity and locates in the different locus from that of ppg-1. Future study of overexpressing suppressor genes may contribute to alleviate loss of carbon assimilation. Cloning and characterization of this suppressor is now being performed.
Challenges in algal transformation

Though soluble proteins are mostly expressed in large amounts from transgenes integrated into the chloroplast genome, efficient expression of membrane-anchored proteins seems to be more difficult. Some cytochrome P450s are anchored to the cytoplasmic face of the endoplasmic reticulum and are involved in the biosyntheses of primary metabolites, such as carotenoid (Inoue 2004), chlorophyll (Christ et al. 2013), as well as sterols (Kajikawa et al. 2004). P450 enzymes require specific reductases for the reactivation of enzymatic activity in addition to a cofactor of NADPH. The gene of CY-P79A1, a ER localized cytochrome P450 enzyme which is involved in Dhurrin biosynthesis, was introduced into C. reinhardtii chloroplast genome and the encoded protein was expressed successfully (Gangl et al. 2015). This result suggests that the endogenous chloroplast redox protein in algal chloroplasts, such as ferredoxin, might help the function of P450 transgene expression. Future expression of other P450s may result in the production of more variety of secondary metabolites.

In higher plants, the MVA pathway contributes to production of C5 isoprenoids in addition to the MEP pathway. On the other hand, many green algae possess only the MEP pathway (Schwender et al. 2001). Some researchers believe introduction of MEV pathway enzyme genes into algal chloroplast genome should be tried for metabolic engineering for more isoprenoid production. Introducing a multigene construct including 6 MVA-pathway genes into the tobacco chloroplast genome resulted in the production of higher levels of mevalonate, carotenoids, squalene and sterols (Kumar et al. 2012). A Brassica napus cytoplasmic transformant expressing seven carotenoid biosynthesis enzyme genes that are arrayed tandemly produced 19- to 30-fold increased carotenoids (Fujisawa et al. 2009).

In order to upregulate photosynthetic capacity in Euglena gracilis, cyanobacterial gene for FBP/SBPase was introduced into nuclear genome by microprojectile bombardment (Ogawa et al. 2015). FBPase is one of the rate-limiting enzymes in the Calvin cycle and determines net CO₂ uptake under the light illumination. Cyanobacterial FBPase is bifunctional, possessing SBPase activity too. The corresponding gene, which was fused with a tobacco Rubisco small subunit transit-peptide encoding sequence, was introduced into the E. gracilis. The transformant obtained was cultured under a high light and high CO₂ conditions and then subsequently incubated in anaerobic conditions resulting in accumulation of 13- to 100-fold increased level of C₂₈ myristyl myristate (Ogawa et al. 2015).

For nuclear transformation of microalgae, particle gun bombardment, electroporation, grass beads agitation, or incubation with polyethylene glycol (PEG) methods have been adopted. For gene delivery into the nuclear genome and drug selection of stable transformants, the cell wall poses as a mechanical barrier that hinders penetration of introducing DNA and permeabilization of selection drugs into the cell. To overcome this problem, strains of Chlamydomonas lacking a cell wall have been used for methods such as electroporation, or grass bead agitation. In this context, technology for obtaining protoplast is still one of the most important techniques required in algal transformation. As shown in previous reports of micro- and macroalgae, the cell walls are degraded with endogenous enzyme(s) after two types of gametes are allowed to mate (Harris 1989, Kuroiwa et al. 1993, Uchida et al. 1996, Uchida and Hara 2001). Thus, the development of transformation protocol in these algae might require the prior removal of cell walls.

In the transformation of C. reinhardtii, the use of GC-rich drug-resistance gene (Harris 2009), codon-optimization of other marker genes (Fuhrmann et al. 1999), dual promoters (Harris 2009), or host cells suitable for foreign gene expression (Neupert et al. 2009, Uchida et al. 2012, Zhang et al. 2014) have contributed to the improved expression of transgenes. Since bacterial plasmid resistance protein (BLE) binds with DNA, the gene product from the chimeric transgene BLE::cGFP localizes to the nuclei and can often be segregated from chloroplast-derived background autofluorescence (Fuhrmann et al. 1999). Use of this chimeric gene has therefore may contribute to the establishment of transformation protocol in the other algae, which possess the chloroplast.

The expression of transgene in C. reinhardtii nuclear genome is often hindered by gene silencing, perhaps due to epigenetic regulation, and/or position effect. Artificial gene encoding B. braunii squalene synthase-like protein (BbSSL)-1 or BbSSL-3, whose codon usage was optimized for C. reinhardtii genome, was connected to a Chlamydomonas hybrid promoter, and introduced into UVM 4 mutant, which was mutagenized by UV-irradiation (Neupert et al. 2009), or DNA methyltransferase 1-null mutant, which was isolated from mutant pool (Zhang et al. 2014). However, transformants stably expressing these gene products were isolated in extremely low rates, perhaps due to gene silencing (Kong et al. 2015). This same tendency was also observed in the introduction of the C. reinhardtii gene for DXS, DXR, or SS into the C. reinhardtii nuclear genome (Kong et al. 2014).

Application of gene manipulation techniques established in model fresh water model alga, such as C. reinhardtii, to marine species could be a promising endeavor as well. This is because culture media using seawater is more commercially viable than media using fresh water. Single-celled swimmers, such as gametes of macroalgae, may be promising candidates for gene introduction (Suzuki et al. 2014, Endo et al. 2016).

Ulva, one of the most popular seaweeds found in the
coastal areas throughout most of the world, grows relatively faster than other macroalgae and can form huge biomass clumps, known as “green tide,” in highly eutrophicated coastal areas. In this macroalga, unicellular cells are recognized as haploid gamete (Uchida et al. 1996) or quadriflagellated swarmer (Hiraoka and Enomoto 1998). These previous works point to the potential of Ulva species as possible hosts for gene manipulation in order to achieve larger biomass production. Monostroma (Kuroiwa et al. 1993) may also be one of such hosts.

Population doubling time of B. braunii cultured in modified Ch13 medium is about one week. This is in contrast to doubling time of 12 h in C. reinhardtii cultured in TAP medium (Harris 1989). During longer cultivation of B. braunii colonies on the solidified medium supplemented with a selection drug may give rise to two different problems; degradation of drug and generation of spontaneously mutated drug-resistant colonies, which hinder mining of drug-resistant transformants. Possible reasons for these problems in B. braunii may be slow growth speed and rigid cell wall of this alga. Permeability of drugs through cell wall ensures the selection of stable transformants. B. braunii race B cells are connected with the extracellular matrix (ECM) containing fibrils and liquid hydrocarbons (Weiss et al. 2012), through which penetrations of DNA and permeabilization of selection drug are more difficult than ECM of C. reinhardtii (Harris 1989, Suzuki et al. 2000). In order to obtain protoplast of B. braunii, treatment of colonies with glycerol (Hou et al. 2014), or n-hexane (Suzuki et al. 2013) was performed to obtain single cells. However, viability of these cells appears to be lower after these treatments.

The values of $k_{\text{Pr}}^{\text{Pyr}}$ and $k_{\text{cat}}^{\text{GAP}}$ of B. braunii DXSs are 1.9–5.2 s$^{-1}$ and 2.0–6.0 s$^{-1}$ (Matsushima et al. 2012). These are as small as those in the carboxylation reaction of the C3 plant Rubisco (Sage 2002, Andrews and Whitney 2003), the rate-limiting enzyme in the Calvin cycle (Woodrow and Mott 1993). Affinities of BbDXSs for the substrates are also lower. In order to increase productivity of algal isoprenoid compounds, including hydrocarbons, or carotenoids, the turnover of BbDXSs should be upregulated.

Concluding remarks

In order to increase yield of the hydrocarbon oil in B. braunii race B, precursors of triterpene produced in MEP pathway are to be reinforced. Regulation of key regulatory enzyme genes in this pathway, DXS, HDS, and/or HDR would be important. Since photosynthetic CO$_2$ assimilation contributed to provide the precursors not only for triterpene hydrocarbons, but also for triacyl glycerols, reinforcement of genes for Rubisco and d-fructose 1,6-bisphosphate phosphatase in Calvin cycle can be promising for algal biomass increase. In a model green alga C. reinhardtii, chloroplast transformation appears to be more reliable than the nuclear transformation in the expression of genes for the enzymes in Calvin cycle or MEP pathway. This is because enzymes are located in chloroplast, and chloroplast genome transgenes appear to be less subjected to gene silencing. In order to establish transformation in B. braunii, its growth speed should be accelerated, and more efficient penetration of drugs through cell wall, or extracellular matrix should be guaranteed. Recent cloning and characterization of B. braunii triterpene biosynthesis genes, as well as accumulation of transcriptome data, may provide sufficient information for future genetic modification not only for B. braunii, but also for other organisms. Single-celled stage of seawater macroalgae, such as Ulva sp., might be promising target for stable transformation which is achieved in the cheaper costs since this alga increases very fast.

Acknowledgements

One of the authors (H.U.) would like to thank Drs. Tsureyoshi Kuroiwa, Naoki Sato and Haruko Kuroiwa from The University of Tokyo, Soichi Nakamura from University of Ryukyu, for their instructions. He also thanks Drs. Hisayoshi Nozaki, Hiroko Toyooka-Kawai, Masafumi Hirono, Hiroo Fukuda from The University of Tokyo, for allowing us to use experimental equipment. Dr. Peter Hegemann from Universität Regensburg for donating a plasmid pMF59, Drs. Timothy P. Devarenne from Texas M & A University, Masashi Suzuki and Victor Marco Emanuell N. Ferriols from The University of Tokyo, for reading the manuscript critically, Drs. Takeshi Ohama from Kochi University of Technology, Ken-Ichi Tomizawa and Kyoichi Isono from Research Institute of Innovative Technology for the Earth, Hideya Fukuzawa from Kyoto University, Megumi Iwano from Osaka University, Hirofumi Yamashita from Kyoto Prefectural University, for their continuous encouragements throughout the study. A part of the studies described here was supported by Research and Investigation Promotion Fund from the Japan Securities Scholarship Foundation in 2011 (10-069) donated to H.U.

References


Algal Genes Encoding Enzymes for Photosynthesis and Hydrocarbon Biosynthesis as Candidates for Genetic Engineering

2018

Algal Genes Encoding Enzymes for Photosynthesis and Hydrocarbon Biosynthesis as Candidates for Genetic Engineering


