Expression of botryococcene synthesis related genes in a unicellular green alga *Chlamydomonas reihardtii*

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GENERAL INTRODUCTION

1-Deoxy-D-xylulose-5-phosphatesynthase (DXS1) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1) proteins that are expected to be key enzymes for isoprene synthesis in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, while squalene synthase (SQS) protein that catalyzes the first enzymatic step in the central isoprenoid pathway during sterol and triterpenoid biosynthesis. cDNAs for these enzymes were introduced into various *Chlamydomonas reinhardtii* strains including mutant strain [UV-mutated (UVM) 4] and UVM11 strains, which were reported to have a high potency for expressing transgenes in the nuclear genome. The results showed that *DXS1*, *DXR1*, and *SQS* cassettes were not readily overexpressed in the wild-type strains at levels where the products were easily detectable by western blotting using a monoclonal antibody. In contrast, western blot-positive SQS cassette transformants were frequently detected in the UVM4 and UVM11 strains, i.e., at an approximately 4.5 times higher frequency than that in the CC-124 wild-type strain. Moreover, in these strains highly SQS accumulating transformants were detected with 2.2-folds frequently than that in CC-124. Position effect against the integrated expression cassettes was obviously detected not only in the wild-type but also in UVM strains. This suggests that the epigenetic repression mechanism of transgenic genes is not completely knocked out, even in the UVM strains. Thus, further improved *Chlamydomonas* strains are required to easily generate transformants that are expressing the aimed gene robustly.

It was reported that squalene synthase-like (*SSL*) genes are belonging to a family of squalene synthase gene, which encode for key enzymes of botryococcene synthesis in *Botryococcus braunii*. *SSL* genes were also heterologously expressed in

various *Chlamydomonas* strains. To improve the expression, codon usage of the open reading frame (ORF) was optimized to match that of the *C. reinhardtii* nuclear encoded genes. In addition to that, the ble2A expression system was utilized in combined with the UVM4 strain to overcome the poor expression. It resulted in that *SSL-3* was more efficiently expressed in the UVM4 strain, i.e., PCR-positive ratio was approximately 1.5-folds higher and western blot-positive frequency was at an approximately 5-folds higher than that in the CC-1690 strain, respectively. These results show that the strategy, ble2A nuclear expression vector system combined with UVM4 host strain is the most efficient system for heterologous expression of cDNAs in *Chlamydomonas*.

CHAPTER 1: Expression levels of domestic cDNA expression cassettes integrated in the nuclear genomes of various *Chlamydomonas reinhardtii*

1.1 Abstract

We attempted to overexpress three types of expression cassettes, each of which contained a different open reading frame (ORF) of domestic *Chlamydomonas* cDNAs. Each ORF was driven by a strong artificial hybrid promoter. We used two kinds of wild-type *Chlamydomonas* strains (i.e., CC-124 and CC-125) and two mutant strains [i.e., UV-mutated (UVM) 4 and UVM11] that have been reported to have a high potency for expressing nondomestic nuclear transgenes. We found that the 1-deoxy-D-xylulose-5-phosphatesynthase (DXS1), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1), and squalene synthase (SQS) cassettes were not easily overexpressed in the wild-type strains at levels where the products were clearly detectable by western blotting using a monoclonal antibody. In contrast, western blot-positive SQS cassette transformants were frequently detected in the UVM4 and UVM11 strains, i.e., at an approximately 4.5 times higher frequency than that in the CC-124 wild-type strain. Moreover, transformants that accumulated large amounts of the SQS protein were obtained frequently in the UVM4 and UVM11 strains, i.e., the frequency was approximately 2.2 times higher than that in the CC-124 strain. However, a position effect of the integrated expression cassette was obviously detected not only in the wild-type but also in UVM strains. This suggests that the epigenetic repression mechanism of transgenic genes was not completely knocked out, even in the UVM strains. Further improved *Chlamydomonas*

strains are essential to facilitate high-throughput screening of transformants that express nuclear transgenes at a high level.

1.2 Introduction

The unicellular green alga, *Chlamydomonas reinhardtii*, is expected to be a potentially low-cost host for generating various products, e.g., biofuel, pharmaceutical terpenoid, and vaccine, because of its rapid phototrophic growth potency and the availability of several advanced molecular techniques (1). However, the nuclear expression levels of nondomestic cDNA genes are disappointingly poor (2, 3). Unsuitable codon usage in the open reading frame (ORF) is one of the proven causes of this problem (4). Random integration of transgenes in *C. reinhardtii* has been analyzed and proved using the RESDA-PCR method (5). The integration position of the construct dramatically affects the expression level (1, 6), which is referred to as the position effect. Recently, Neupert et al. (7) developed *C. reinhardtii* UVmutated (UVM) strains to overcome the inhibited expression of foreign genes. These cell wall-deficient strains, UVM4 and UVM11, harbor unknown and unmapped mutation(s), and it has been suggested that the epigenetic transgene suppression mechanisms have been successfully knocked out in these mutants. To the best of our knowledge, high expression in these UVM strains has only been demonstrated for green fluorescent protein (GFP) and yellow fluorescent protein (YFP) (7). Moreover, all of the reported genes are nondomestic genes, and their codon usage was not optimized for *C. reinhardtii*. This suggests that we might readily obtain transformants that accumulate high levels of target proteins if endogenous

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genes could be introduced into the UVM strains, because their codon usage would not interfere with their translation. 1-Deoxy-D-xylulose-5-phosphatesynthase (DXS1) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1) proteins are expected to be key enzymes involved with isoprene synthesis in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (8), while squalene synthase (SQS) protein catalyzes the first enzymatic step in the central isoprenoid pathway during sterol and triterpenoid biosynthesis (9). These three genes are unique and essential and contain a limited number of rare codons, which probably have no harmful effects on their translation levels. In this study, we examined the expression levels of the cDNA cassettes of *DXS1* and *SQS* in the wild-type strain CC-124, and that of *DXR1* in the wild-type strain CC-125. We also introduced the *SQS* expression cassette into the UVM4 and UVM11 strains to comparethe expression levels of the wild-type strain and the UVM mutants.

1.3 Materials and Methods

Construction of transformation vectors

The cassettes of *Hsp70A/RbcS2* promoter (containing the first intron of *RbcS2*) and *RbcS2* terminator were cloned into pUC18 (without the BamHI restriction enzyme site) as a SacI/KpnI fragment to generate the pHsp70A/RbcS2-plasmid. This artificial promoter also contains the first intron of *RbcS2* because of its enhancer activity (10). A gp64-tag sequence (11) was integrated into the respective constructs for analysis with western blotting. The fragments containing the gp64-tag sequences were as follows: gp64 tag 1: 5'-

GATGCTCGAGTCCTGGAAGGACGCGTCCGGCTGGTCCCATATGTAATAAAGT

ACTACTAGTGGATCCCCGCT-3′ and gp64 tag 2: 5′-
GATGCTCGAGAGATCTACACCGACTAGTTCCTGGAAGGACGCGTCCGGCTGG

TCCTAATAAGGATCCCCGCT-3′. These sequences were synthesized and cloned into

the *XhoI/BamH*I site of pHsp70A/RbcS2 plasmid to construct the pHsp70A/RbcS2-gp64

tag 1 and pHsp70A/RbcS2-gp64 tag 2 plasmids, respectively.

      DXS1, *DXR1*, and *SQS* cDNA were amplified using polymerase chain reaction

(PCR) with high-fidelity PrimeSTAR HS DNA polymerase (Takara, Japan), according to

the manufacturer's instructions. The specific sets of primers used for each amplification

reaction were as follows: DXS1fw (5′-AAG<u>AGATCT</u>ATGCTGCGTGGTGCTG-3′) and

DXS1rev (5′-AAG<u>ACTAGT</u>CGCTTGCAGCGCTGA-3′) for *DXS1*, which were

generated by introducing *Bgl*II and *Spe*I sites at the 5′ ends, respectively (the restriction

endonuclease sites are underlined); DXR1fw (5′-
AAG<u>CTCGAG</u>ATGCAGTCCGCGCAGC-3′) and DXR1rev (5′-
AAG<u>ACTAGT</u>GGCCATGGCCAGCACC-3′) for *DXR1*, which were generated by

introducing *Xho*I and *Spe*I sites at the 5′ ends, respectively; and SQSfw (5′-
<u>CATATG</u>GGAAAGTTAGGGGAGCTACTCTC-3′) and SQSrev (5′-
<u>GGATCC</u>TCACGCACGCCGCA-3′) for *SQS*, which were generated by introducing

*Nde*I and *BamH*I sites at the 5′ ends, respectively. The amplification conditions were as

follows: 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s,

annealing at 54°C for 15 s, and extension at 72°C for 2 min using a Thermal Cycler 2720

(Applied Biosystems, USA).

The *DXS1* cDNA fragment (~2.2 kb) was inserted into the pHsp70A/RbcS2-gp64 tag 1 plasmid as a *Bgl*II/*Spe*I fragment, generating the recombinant plasmid pHsp70A/RbcS2-gp64-DXS1. The *DXR1* cDNA fragment (~1.4 kb) was inserted into the pHsp70A/RbcS2-gp64 tag 1 plasmid as a *Xho*I/*Spe*I fragment, generating the plasmid pHsp70A/RbcS2-gp64-DXR1. The *SQS* cDNA fragment (~1.4 Kb) was inserted into the pHsp70A/RbcS2-gp64 tag 2 plasmid as a *Nde*I/*BamH*I fragment, generating the plasmid Hsp70A/RbcS2-gp64-SQS. The pHyg3 plasmid, which contained *aphVII* and conferred hygromycin resistance (10), was double digested with *Hind*III and subcloned into the Hsp70A/RbcS2-gp64-DXS1 plasmid in the sense orientation to generate the *DXS1* overexpression construct. The pSI103 plasmid, which contained *aphVIII* and conferred paromomycin resistance (11), was double digested with *Xba*I and *Kpn*I and then subcloned into the Hsp70A/RbcS2-gp64-DXR1 plasmid in the sense orientation to generate the *DXR1* overexpression construct. The pSP124S plasmid, which contained *ble* and conferred zeocine resistance (12), was double digested with *Xba*I and *Kpn*I and then subcloned into the Hsp70A/RbcS2-gp64-SQS plasmid in the sense orientation to generate the *SQS* overexpression construct. The DNA sequences of all the constructs were confirmed by direct sequencing using the dideoxy chain termination method (13). The final *DXS1*, *DXR1*, and *SQS* overexpression constructs are shown in Fig. 1-1.

Strains and transformation conditions

C. reinhardtii CC-124 (wild-type, mt^-) and CC-125 (wild-type, mt^+) were provided by the *Chlamydomonas* Resource Center (Duke University, USA), while the UVM4 and UVM 11 strains (6) were kindly provided by Dr. R. Bock (MPI-MP,

Germany). Unless stated otherwise, cells were cultivated mixotrophically at 25°C in Tris-acetate phosphate (TAP) medium (14) under moderate and constant white fluorescent light (20 μmol m^{-2} s^{-1}) with gentle shaking. Before transformation, vectors were linearized with a single restriction enzyme and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA), according to the manufacturer's protocols. Approximately 300 ng linearized plasmid DNA was used during each transformation experiment by electroporation, as described previously (15). Transformants of the *DXS1*, *DXR1*, and *SQS* overexpression constructs were selected directly on 1.5% TAP agar plates containing hygromycin B (30 mg L^{-1}), paromomycin sulfate (20 mg L^{-1}), or zeocin (15 mg L^{-1}), respectively, and the plates were incubated under continuous fluorescent light (20 μmol m^{-2} s^{-1}) at 25°C.

PCR screening of cotransformants

Transformants with antibiotic resistance were screened to detect the presence of the expression cassettes using a promoter-specific forward primer and a gene-specific reverse primer, the locations of which are shown in Fig. 1-1. Genomic DNA was isolated from independent transformants using the CTAB method (16). The specific sets of primers used to examine construct integration were as follows: forward, 5′-AAAATGGCCAGGTGAGTCGACG-3′and reverse, 5′-GATTGACCGAAGCCTCCGCG-3′ for *DXS1*; forward, 5′-CGTTTCCATTTGCAGGCTCGAGA-3′ and reverse, 5′-AGCAGCTTCACGTTGGAGCC-3′ for *DXR1*; and forward, 5′-TTGCAGGATGCTCGAGTCCTG-3′ and reverse, 5′-CCACGTCCAGTGCCATGTCA-

3′ for *SQS*. The amplification conditions were as follows: 95°C for 5 min; followed by 30 cycles at 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 using the Thermal Cycler 2720 (Applied Biosystems).

Western blot screening of transformants

Total soluble protein extracts were prepared by resuspending pelleted 5.0×10^6 *Chlamydomonas* cells in 50 µL of $2 \times$ SDS sample buffer (100 mM Tris, pH 6.8; 4% SDS; 1 mM DTT; and 30% glycerol). The samples were denatured at 95°C for 7 min. Subsequently, 5-µL aliquots of the supernatants from the denatured cell lysates were separated using 10% SDS-PAGE, transferred using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA) to polyvinylidene difluoride (PVDF) membranes (Hybond P; GE Healthcare, UK), and incubated overnight with standard transfer buffer (25 mM Tris, 20% methanol, 0.01% SDS, and 192 mM glycine). Immunobiochemical protein detection was performed using a monoclonal anti-BV envelope gp64 primary antibody (1/5,000) (Santa Cruz, CA, USA) and ECL peroxidase-labeled anti-mouse secondary antibody (1/20,000) (Sigma-Aldrich, Canada) with an ECL detection system (GE Healthcare).

1.4 Results and Discussion

1.4.1 *Construction of the expression vectors*

All of the vectors used for transformation contained one of the three expression cassettes with a marker gene cassette in the same strand (Fig. 1-1). The gp64 epitope tag for analysis with western blotting was introduced at the N-terminal end in *SQS*, whereas it was introduced at the C-terminal end in *DXS1* and *DXR1* (Fig. 1-1), given that the N-

terminal transit peptide region of DXS1 and DXR1 proteins are removed post-transnationally to localize these proteins in plastids.
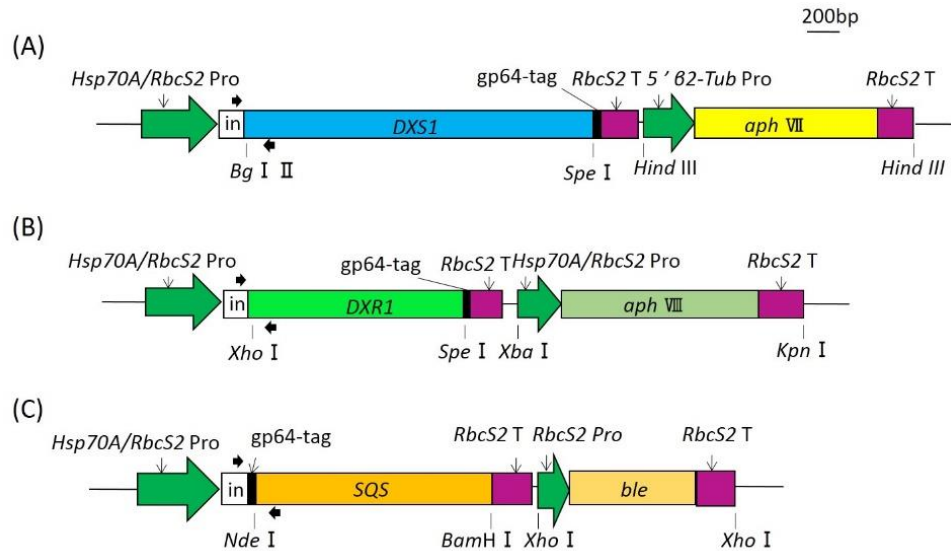
Figure 1-1. Schematic representation of the *DXS1* (A), *DXR1* (B), and *SQS* (C) transformation vectors. *Hsp70A/RbcS2* Pro, *Hsp70A/RbcS2* hybrid promoter; in, the first intron of *RbcS2*; *RbcS2*T, *RbcS2* terminator. The constitutive *Hsp70A/RbcS2* tandem promoter controlled the expression of *DXS1*, *DXR1*, and *SQS*, and three types of marker genes were constructed in the sense orientation, respectively. The restriction enzyme sites used to make the constructs are indicated. The locations of the primers used for PCR to determine the cotransformation ratio are indicated by black arrows.

1.4.2 *Expression levels of cDNA cassettes in C. reinhardtii wild-type strains*

The linearized plasmids for *DXS1* or *SQS* were introduced into the CC-124 strain (mt^-) to allow overexpression, whereas the linearized plasmid for *DXR1* was introduced into the CC-125 strain (mt^+). The antibiotic resistance characteristics used for selection guaranteed the integration of the marker gene in the transformants, but this does not guarantee the cointegration of the cDNA expression cassette in the transformants. To exclude transformants without the cointegrated expression cassette of the target gene, we

performed PCR to confirm the integration of the promoter region. PCR was performed using a set of primers, which were specific to 3′ end of the *Hsp70A/RbcS2* promoter and the N-terminal region of the cDNA expression cassette. For the PCR-positive transformants, we assumed that the intact cDNA cassette was integrated into the genome with the marker gene cassette; therefore, we tentatively counted these transformants as being successfully cotransformed (Table 1-1). The PCR results showed that the cotransformation ratios (number of PCR-positive transformants/number of transformants analyzed) for *DXS1* with *aphVII* (introduced into the CC-124 strain), *DXR1* with *aphVIII* (introduced into the CC-125 strain), and *SQS* with *ble* (introduced into the CC-124 strain) were 42.4% (112/264), 21.8% (55/252), and 51.9% (109/210), respectively (Table 1-1). The relatively lower cotransformation ratio of the *DXR1* cassette was probably because of the characteristics of the CC-125 strain. Recently, it was reported that the optimal transformation efficiency, using the electroporation method (17), for the CC-125 strain was approximately 66% of that for the CC-124 strain.

Western blotting was performed to detect the protein accumulation levels of the PCR-positive transformants. The results showed that the western blot-positive ratios (number of western blot-positive transformants/number of PCR-positive transformants) for the *DXS1* and *SQS* transformants in the CC-124 strain were 0.89% (1/112) and 4.6% (5/109), respectively (Table 1-1 and Fig. 1-2A). However, no *DXR1*-positive transformants were found among 55 PCR-positive transformants in the CC-125 strain (Table 1-1). The low expression levels of the target protein in the wild-type strains were in agreement with previous reports (1-3). The sensitivity of western blotting performed

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using a monoclonal antibody for gp64-tag was sufficiently high to detect the protein derived from gp64-tag-containing *aphVII* transformants (Fig. 1-2).

Gene name	Host strain	Cotransformation ratio[a]	Western blot-positive ratio[b]	Highly expressing transformant ratio[c]
DXS1	CC-124 (WT*)	112/264 (42.4%)	1/112 (0.89%)	1/1 (100%)
DXR1	CC-125 (WT)	55/252 (21.8%)	0/55 (0.00%)	0/55 (0.00%)
SQS	CC-124 (WT)	109/210 (51.9%)	5/109 (4.6%)	1/5 (20.0%)
	UVM 4	52/87 (59.8%)	11/52 (21.2%)	5/11 (45.5%)
	UVM 11	51/87 (58.6%)	9/5 (17.6%)	4/9 (44.4%)

Table 1-**1.** Expression of *DXS1*, *DXR1*, and *SQS* cDNA cassettes in various *Chlamydomonas reinhardtii* strains. Asterisk: wild type; [a]Number of PCR-positive transformants/number of transformants analyzed; [b]Number of western blot-positive transformants/number of PCR-positive transformants and [c]Number of high-protein accumulation transformants/ number of western blot-positive transformants.

1.4.3 *Expression levels of cDNA cassettes in UVM strains*

Encouraged by the successful overexpression of three nonendogenous fluorescence-related genes in the UVM4 and UVM11 strains (6, 18), we examined the overexpression of the *SQS* cassette in the UVM strains. The PCR results showed that the estimated cotransformation ratios for *SQS* in the UVM4 and UVM11 strains were approximately 59.8% (52/87) and 58.6% (51/87) (Table 1-1), respectively, which were only approximately 8% and 7% higher than those in the CC-124 strain. These results were not consistent with the results of Neupert *et al*. (2009), who showed that the cotransformation ratios of the full-length *GFP* cassette in the genomes of the UVM4 and

UVM11 strains were about five times higher than that of the control strain (CW-15, mt^+, *arg7*).
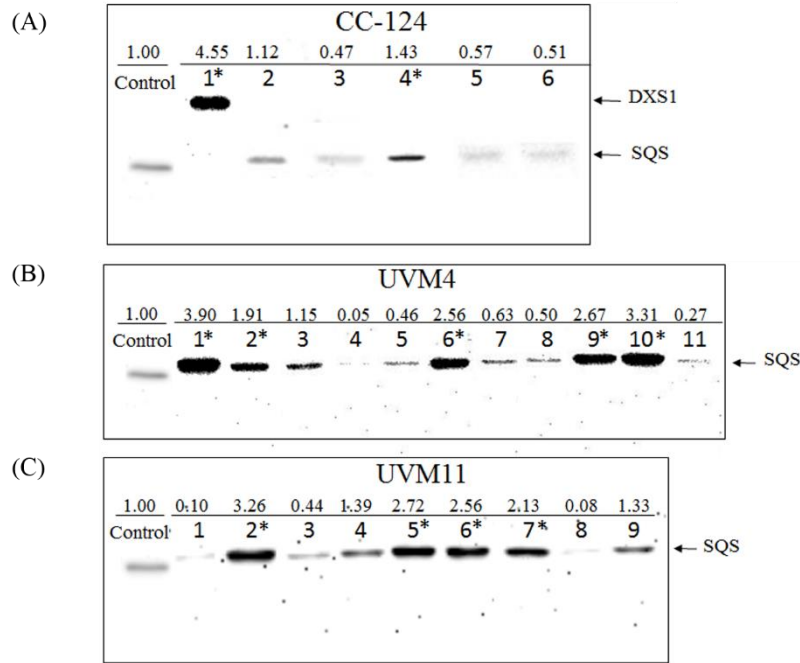
Figure 1-2. Identification of DXS1 and SQS protein-positive transformants by western blotting. The DXS1- and SQS-positive transformants in the CC-124 strain (A) and SQS-positive transformants in the UVM4 (B) and UVM11 (C) strains are shown. Control: gp64-tag-containing *aphVII* transformant. The bands were quantified using the Quantity One 1-D Analysis Program (Bio-Rad). The band intensities are shown as relative intensity units relative to the control in their respective gels. Band intensity values greater than 1.4 were defined as strongly expressing transformants in this study. Asterisk: transformants that expressed the target proteins at high levels.

1.4.4 *Endogenous cDNA cassettes are efficiently expressed in UVM strains*

However, the western blotting results detected dramatic differences between the UVM and wild-type strains. The western blot-positive ratios for *SQS* in the UVM 4 and UVM 11 strains were approximately 21.2% (11/52) and 17.6% (9/51) (Table 1-1 and Fig.

1-2B, 2C), respectively, which were about five and four times higher than the ratio in the CC-124 strain (4.6%, 5/109). Moreover, the high-protein accumulation ratios (number of high-protein accumulation transformants/number of transformants analyzed) of the western blot-positive clones were 45.5% (5/11) for the UVM4 strain and 44.4% (4/9) for the UVM11 strain (Table 1-1 and Fig. 1-2B, 2C). These ratios were approximately two times higher than those in the CC-124 strain (20%, 1/5) (Table 1-1 and Fig. 1-2A).

Unstable transgene expression has been observed frequently in *C. reinhardtii* (2). However, the *SQS* transformants generated from the UVM strains were cultured on antibiotic-containing plates for over 5 months, and they exhibited stable expression (data not shown). In addition, the growth rates of all the *SQS* protein-expressing transformants of the UVM4 and UVM11 strains did not differ significantly from the CC-124 strain (data not shown) in mixotrophic conditions (in TAP medium) with a 16/8-h light/dark cycle or continuous light. These results suggest that the UVM strains are highly suited to the endogenous cDNA transgene expression of moderate-molecular-weight proteins, and they are not restricted to the expression of low-molecular-weight proteins such as *GFP*.

The *SQS* cDNA cassette originated from domestic mRNA; therefore, its codon usage and substitution in the ORF must have been optimized by evolution. Thus, *SQS* may have fewer translational disadvantages than foreign genes. However, Fig. 1-2 shows that the SQS protein accumulation levels were not uniformly high even in the UVM strains, which was also the case in the CC-124 strain. It is reasonable to suspect that translational repression works uniformly against all *SQS* mRNAs, irrespective of the genomic locations of the *SQS* that generated the *SQS* mRNA. Therefore, the observed

differences in the levels of accumulated SQS protein must have been because of the variable transcriptional levels of the *SQS* located in different genomic regions.

Neupert *et al*. (2009) reported that all PCR-positive *GFP* transformants (9/9) had uniformly high protein accumulation levels. Based on this fact, they suggested the successful knock out of the transcriptional repression pathways of nuclear transgenes in the UVM strains. However, our results of *SQS* expression showed that epigenetic transcriptional repression was alleviated dramatically, although they were not completely free of it even in the UVM strains. This might have been due to the fact that the mutated gene(s) had not lost their activity completely, because these UVM strains were generated by UV treatments to introduce nucleotide mutations. The observed inconsistent characteristics of the UVM strains mentioned above might have been due to the different characteristics of the promoters used to drive the cDNAs, i.e., *GFP* was driven by either *PsaD* or *RbcS2*, whereas *SQS* was transcribed by the *Hsp70A/RbcS2*-hybrid promoter (19).

Further improvements are required to obtain truly epigenetic repression-free *Chlamydomonas* strains. The development of such strains would make *Chlamydomonas* an attractive host for producing biofuel and biopharmaceuticals.

1.5 References

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CHAPTER 2: Expression of heterologous genes by fusion to a selection marker in improved *Chlamydomonas* strains

2.1 Abstract

Chlamydomonas is a very attractive candidate plant cell factory. However, its main drawback is the difficulty to find the transformants that robustly express heterologous genes randomly inserted in the nuclear genome. We previously showed that domestic squalene synthase (*SQS*) gene of *Chlamydomonas* was much more efficiently overexpressed in a mutant strain [UV-mediated mutant (UVM) 4] than in wild type. In this study, we evaluated the possibility of a new mutant strain, *met1*, which contains a tag in the maintenance type methyltransferase gene that is expected to play a key role in the maintenance of transcriptional gene silencing. The versatile usefulness of the UVM4 strain to express heterologous genes was also analyzed.

We failed to overexpress *CrSSL3* cDNA, which is the codon-adjusted squalene synthase-like gene originated from *Botryococcus braunii*, using the common expression cassette in the wild-type CC-1690 and UVM4 strains. However, we succeeded to isolate the western-positive transformants through the combinational use of the UVM4 strain and ble2A expression system. It is noteworthy that even with this system, huge deviations in the accumulated protein levels were still observed among the UVM4 transformants.

2.2 Introduction

Recently, microalgae have attracted much attention as plant cell factories for the production of various commercial products, including biofuels, pharmaceutical terpenoid, nutraceuticals, and therapeutics (1-3). However, until now, only limited transgenic products have been commercialized mainly because of the difficulty in improving strains for abundant accumulation of a product of interest above the required levels.

The unicellular green alga, *Chlamydomonas reinhardtii*, has been a prominent model organism for genetic studies primarily because of the efficient genetic transformation techniques for mitochondria, chloroplasts, and nucleus (4-7). The genome (nuclear, plastid, and mitochondria) of *C. reinhardtii* has been fully sequenced (8), and large chemical and insertional mutant libraries have been established. On the other hand, the major disadvantage of *C. reinhardtii* is the poor expression of transgenes from the nuclear genome (9, 10). The molecular mechanism(s) of this is still uncovered, and a possible reason for this is closely related to the strong transcriptional silencing against transgenes (2, 11), which is mediated by both DNA methylation and DNA methylation-independent pathways (12, 13).

Over the past two decades, several advanced strategies have been developed to improve the expression of transgenes in *Chlamydomonas*, i.e., codon optimization (9, 10), utilization of endogenous intron(s) (14), and development of artificial strong promoter (15). Recently, UV-mediated mutant (UVM) strains of *Chlamydomonas* have been isolated for improved transgene expression. The UVM strains harbor unknown and unmapped mutation(s), and it has been suggested that their epigenetic transgene suppression mechanisms have been successfully knocked out (16).

Moreover, a novel nuclear expression system was reported to robustly express heterologous genes (2). The system utilizes the foot-and-mouth disease virus (FMDV) 2A "self-cleaving" peptide to transcriptionally fuse a transgene open reading frame (ORF) to the antibiotic resistance marker gene *ble* (referred to as ble2A system in this paper). The FMDV 2A peptide, which is a short peptide with approximately 20 amino acid sequences, mediates ribosome-skipping reaction during translation (17). Because of this reaction, when 2A is fused between two ORFs to generate a single transcriptional ORF, the resulting products are the two discrete proteins with the short 2A peptide sequence fused to the C-terminus of the first protein product, whereas the following protein has only one amino acid of the peptide covalently attached to the N-terminus. The efficient ability of the ble2A system to improve the heterologous expression of transgenes compared with the traditional nuclear expression vector in which the expression of the interest and selection marker genes controlled under independent promoters has been reported (2, 18).

Previously, we demonstrated that the *Chlamydomonas* squalene synthase (*SQS*) cDNA was much more efficiently expressed in the UVM strains than in wild-type strains (19). Recently, using the *Chlamydomonas* MmeI-based insertion site Sequencing (ChlaMmeSeq) method (20), an insertional mutant of *Cre10.g461750* was isolated. This gene encodes DNA methyltransferase 1 (*Dnmt1*) (20), which is expected to be involved in the maintenance of DNA methylation patterns (21, 22). Robust transcriptional gene silencing through DNA methylation is one of the major pathways for stable repression of transgenes. In this study, to expand the platform for efficient expression of various transgenes, we evaluated the potency of this tag-inserted strain. Moreover, using the ble2A system, codon-adjusted *SQS*-like 3 gene (*CrSSL3*) and *CrSSL1*, which originated

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in *Botryococcus braunii*, were heterogeneously expressed in wild-type strains and in the UVM4 strain. These *SSL* genes are key enzymes for the biosynthesis of botryococcene in *B. braunii* B-race (23). The expression levels of these enzymes were closely analyzed by western blotting using a monoclonal antibody against gp-64 epitope.

2.3 Materials and Methods

Construction of the transformation vectors

The construction of the transformation vector containing *SQS* expression cassette was shown in detail by Kong et al. (19). For the construction of the P$_{AR4}$::ble-2A-SSL::term expression vector, the *ble* sequence, which contained one copy of the *RbcS2* intron 1, was fused in frame to the codon-optimized FMDV 2A coding sequence (2), and synthesized as an *Xba*I-*Nde*I/*Kpn*I fragment. The ble-2A fragment was inserted into a pSTBlue-1 plasmid (EMD Biosciences, USA) as *Xba*I/*Kpn*I fragment, generating the recombinant plasmid pSTBlue-1-ble-2A. The *Hsp70A* promoter fragment was amplified by polymerase chain reaction (PCR) with high-fidelity PrimeSTAR HS DNA polymerase (Takara, Japan) from the pALM32 plasmid (24) using primers *Xba*I-Hsp70A-F (5′-AATCTAGAGACGGCGGGG-3′) and *Nde*I-*Hind*III-Hsp70A-R (5′-CATATGAACTGAAGCTTGAGTGGTTATGTA-3′). This fragment was inserted into the pSTBlue-1-ble-2A plasmid as a *Xba*I/*Nde*I fragment, generating the recombinant plasmid pHsp70A-ble-2A.The fragment containing the sequence of *RbcS2* 3′ untranslated region (UTR) terminator was excised from the pHsp70A/RbcS2-cgLuc plasmid (25) by *BamH*I-*Kpn*I digestion and cloned into pHsp70A-ble-2A, resulting in the recombinant plasmid pHsp70A-ble-2A- term. For the construction of four parallel copies of the first

intron (intron 1) of *RbcS2*, the sense and antisense single-stranded oligonucleotides intron 1-left (5′-CAGGTGAGTCGACGAGCAAGCCCGGCGGATCAGGCAGCGTGCTTGCAGATTT GACTTGCAACGCCCGCATTGTGTCGACGAAGGCTTTTGGCTCCTCTGT-3′) and intron 1-right (5′-TGCCTGCAGGAATTCGATTGGTCTTGGCCATCCTGCAAATGGAAACGGCGAC GCAGGGTTAGATGCTGCTTGAGACAGCGACAGAGGAGCCAAAAGCCTT-3′), respectively, were synthesized, annealed, and used as the templates to generate the fragment of intron 1 using the following primers: intron 1-left-F (5′-AAGCTTGATTGTCATGGCCAGGTGAGTCGACGAGCAAG-3′) and intron 1-right-R (5′-CCATGGGATATCGCATGCCTGCAGGAATTCGATTG-3′) by employing the overlap extension PCR (OE-PCR) (26) method. The fragment was then used as the template to amplify four parallel copies of intron 1 using the following primers: *Hind*III-c1-F (5′-AAGTAAAAGCTTGATTGTCATGGCCAG-3′) and *Sac*I-c1-R (5′-AAGTAAGAGCTCCCATGGGATATCGCATGC-3′) for intron 1-copy 1; *Sac*I-c2-F (5-AAGTAAGAGCTCGATTGTCATGGCCAGGTG-3′) and *Xba*I-c2-R (5′-AAGTAATCTAGACCATGGGATATCGCATGC-3′) for intron 1-copy 2; *Xba*I-c3-F (5′-AAGTAATCTAGAGATTGTCATGGCCAGGTG-3′) and *Sac*I-c1-R for intron 1-copy 3; *Sac*I-c2-F and *Kpn*I-c4-R (5′-AAGTAAGGTACCCCATGGGATATCGCATGC-3′) for intron 1-copy 4. These four fragments of copies of intron 1 were double digested using the introduced restriction enzymes and then ligated using the Mighty Mix DNA ligation kit (Takara) to generate four parallel copies of intron 1 of *RbcS2* as *Hind*III/*Kpn*I fragment. The *RbcS2* promoter fragment was generated by PCR from pHsp70A/RbcS2-

cgLuc plasmid using the primers *Kpn*I-RbcS2-Pro-F2 (5′-TAAGGTACCCCGGGCGCGCCA-3′) and *Nde*I-RbcS2-Pro-R2 (5′-CTTGGCCATATGTTTAGATGTTGAGTGACT-3′). The obtained fragment containing four copies of intron 1 and *RbcS2* promoter were digested by the *Hind*III-*Kpn*I and *Kpn*I-*Nde*I restriction enzyme couples, respectively, and then inserted into the *Hind*III/*Nde*I sites of pHsp70A-ble-2A-RbcS2 plasmid, generating the recombinant plasmid P_{AR4}::ble-2A::term expression vector.

The codon usage of *CrSSL-3* and *CrSSL-1* cDNAs was optimized for *Chlamydomonas* (http://www.kazusa.or.jp/codon), and the codon-adjusted 1 × gp64-tag (27) sequence was attached at the N-terminus. These sequences were synthesized as *Xho*I/*Kpn*I fragments. For the construction of the GS-linker-3 × gp64-tag fragment, the sense and antisense single-stranded oligonucleotides GS-linker-1 × gp64-tag (5′-ATGGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGCGGCTCCTGGAAGGAC GCGAGCGGCTGGAGCATCAGCGGCTCCTGGAAGGA-3′) and 2 × gp64-tag (5′-CGTGCCCTCAGTGGATCCTTATTAGCTCCAGCCGCTCGCGTCCTTCCAGGAGC CGCTCCAGCCGCTCGCGTCCTTCCAGGAGCCGCTGAT-3′) were synthesized, annealed, and used as the templates to generate the *Kpn*I-GS-linker-3 × gp64-tag-*BamH*I fragment by employing the OE-PCR method with the following primers: *Kpn*I-GS-linker-3 × gp64-tag-F (5′-AAGTAAGGTACCATGGGCGGCAGCGGC-3′) and *BamH*I-3 × gp64-tag-R (5′-CGTGCCCTCAGTGGATCCTTATTA-3′). The fragments of the *SSL-3* and *SSL-1* cDNA cassettes and GS-linker-3 × gp64-tag were double digested by the *Xho*I-*Kpn*I and *Kpn*I-*BamH*I restriction enzyme couples, respectively, and then cloned into the P_{AR4}::ble-2A::term vector as an *Xho*I/*BamH*I fragment to generate the *SSL-3* and

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SSL-1 transformation vector that is schematically shown in Fig. 2-1B and Fig. 2-9A. The DNA sequences of all the constructs were confirmed by direct sequencing using the dideoxy chain termination method (28).

C. reinhardtii strains, growth, and transformation conditions

The *C. reinhardtii* strains CC-124 (wild type, mt^-) and CC-1690 (wild type, mt^+) were provided by the *Chlamydomonas* Resource Center (Minnesota, USA), the C-9 strain (wild type, mt^-, NIES-2235) was provided from the National Institute for Environmental Studies (NIES, Japan), and the UVM4 strain (16) was kindly provided by Dr. R. Bock (MPI-MP, Germany). Unless otherwise stated, cells were cultivated mixotrophically at 25°C in Tris-acetate phosphate (TAP) medium (29) under moderate and constant white fluorescent light (84 µmol photons m^{-2} s^{-1}) with gentle shaking. Nuclear transformation was performed using the electroporation method (30). Briefly, the cells were grown to $1.0–2.0 \times 10^6$ cells/mL in TAP medium. Subsequently, 2.5×10^7 cells were harvested by centrifugation and suspended in 250 µL of TAP medium supplemented with 50 mM sucrose (TAP/sucrose). Electroporation was performed by applying an exponential electric pulse of 0.7 kV at a capacitance of 50 µF (BTX, USA), using 300 ng of linearized plasmids purified by agarose gel electrophoresis according to the manufacturer's instructions. The transgenic strains were selected directly on TAP/agar plates containing zeocin (15 mg/L), and the plates were incubated under continuous fluorescent light (20 µmol m^{-2} s^{-1}) at 25°C.

PCR screening and analyses of the transformants

Primary antibiotic-resistant transformants were screened for the presence of the expression cassettes by PCR as described previously (31, 32). In brief, cells (1.0–5.0 × 10^6) were resuspended in Tris–EDTA solution and incubated at 98°C for 10 min. Aliquots (1 µL) of the supernatants from denatured cell lysates were then used as template for 20 µL PCR, using promoter-specific forward and gene-specific reverse primers for *SQS* as previously described (19), and gene-specific forward primer (5′-AGATGGAGGCCAAGTGCGTC-3′) and terminator-specific reverse primer (5′-CCGCTTCAGCACTTGAGAGCA-3′) for *SSL-3*.

For semi-quantitative reverse transcription PCR (q-RT-PCR), total RNA was isolated as previously described (33, 34), using TRIzol reagent (Molecular Research Center, http://www.mrcgene.com/), according to the manufacturer's instructions. Purified total RNA was treated with DNase I (Takara, Japan) to remove the contaminated residual genomic DNA. First-strand cDNA was synthesized using an oligo(dT)18 primer or random heptamers and PrimeScript reverse transcriptase (Takara) according to the manufacturer's instructions. The cDNA fragment of *Cre10.g461750.t1.1* was amplified by Reverse transcription polymerase chain reaction (RT-PCR) using primers met1-exon-F: 5′-GCAGAACAGCATGGTCATGGCCT-3′ (part of the 31st and 32nd combined exons), and met1-exon-R: 5′AATGCACCTGCAGCGCTCCA-3′ (part of the 36th and 37th combined exons), and the cDNA fragment of *CrSSL1* was amplified by PCR using a set of primers CrSSL1-F (5′-ATGACTATCAAGCGCCTGCAGAG-3′), and CrSSL1-R (5′-CCGCTTCAGCACTTGAGAGCA-3′). The housekeeping gene *CBLP* (*Chlamydomonas* beta subunit-like polypeptide) was used as internal control, and the primers was as previously described (33).

Western blot screening of the transformants

Total cell extracts and soluble fractions were prepared as previously described (19, 33). For immunoblot analysis, proteins were separated on 12% SDS-PAGE and immunoblotted with the monoclonal anti-baculovirus envelope gp64 polypeptide antibody (1/5,000) (eBioscience, CA, USA) for detecting the gp64-tagged proteins, and anti-Histone H3 antibody (1/10,000) (Abcam, CA, USA) for detecting Histone H3 protein. The ECL detection system (Millipore) was used to detect immunoreactive proteins by utilizing anti-mouse or anti-rabbit secondary antibodies (1/20,000).

Spotting test to compare the relative zeocin resistance of the transformants

Spotting test was performed to estimate the level of zeocin resistance as previously described (34). Independent transgenetic strains were cultured to reach the logarithmic phase in TAP medium and subsequently subjected to serial dilutions (1:5) with TAP medium. Aliquots (1 µL) of the diluted samples were spotted on plates supplemented with various concentrations of zeocin (0, 30, 60, and 120 mg/L) and incubated for 7–10 days under white fluorescent light (20 µmol/m^2/s) at 25°C.

2.4 Results and Discussion

2.4.1 *Construction of the ble2A nuclear expression vector*

We previously constructed the expression vector for *SQS*, in which the target gene *SQS* and marker gene *ble* were expressed in independent ORFs (Fig. 2-1A) (19). Recently, a novel ble2A nuclear expression system was reported to express heterologous genes efficiently (2). We subcloned the *CrSSL3* or *CrSSL1* cDNA into the ble2A nuclear

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expression vector to generate ble2A-SSL fusion ORFs. The fused ORFs were placed under the control of the modified *Hsp70A/RbcS2* promoter (PAR4), which was revised to contain four copies of the first intron of *RbcS2* between the *Hsp70A* and *RbcS2* promoters (Fig. 2-1B, Fig. 2-9A).
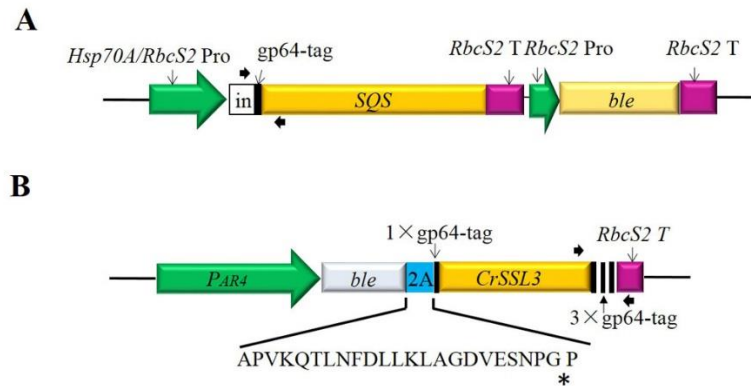

Figure 2-**1**. Schematic representation of the transformation vectors. (A) Expression cassette for *Chlamydomonas SQS* cDNA and *ble* marker gene. in, first intron of the *Chlamydomonas RbcS2* gene; *RbcS2* T, *Chlamydomonas RbcS2* terminator; gp-64-tag, gp-64 gene sequence for epitope tag peptide. The arrows show the location of the PCR primers used for the cotransformation assay. (B) Expression cassette for the codon-adjusted *Botryococcus braunii SSL3* cDNA. *PAR4, Hsp70A/RbcS2* promoter modified to contain four copies of the first intron of *RbcS2*; 2A, FMDV 2A peptide, *CrSSL3*, codon-optimized *SSL3* cDNA. The amino acid sequence of 2A is denoted by an asterisk to indicate the cleavage site.

2.4.2 *Enhanced nuclear transgene expression in a "met1" mutant of Chlamydomonas*

To date, two cytosine-specific DNA methyltransferases (i.e., *Cre10.g461750*, and *Cre12.g484600*) have been detected in the *Chlamydomonas* genome sequence (*Chlamydomonas* genomic information features v5.3.1). We isolated an insertion tagged mutant in *Cre10.g461750* tag mutant (20) (Fig. S1). In this tag mutant, the transcript was under detection level by semi-quantitative reverse transcriptionan PCR (q-RT-PCR)

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analyses, whereas it was evidently detected in the mother strain CMJ030 (Fig. S2). The enzyme coded in *Cre10.g461750* was estimated to function at the DNA replication foci. Therefore, this gene probably coded maintenance type DNA cytosine methyltransferase 1 (*Dnmt1* or *Met1*), while the other one may have coded a *de novo* cytosine methyltransferase. This strain contains an additional insertion whose position has not been identified because of the complex tag insertion and deletion detected by the sequence data of RESDA-PCR (35) products (data not shown). Therefore, the behavior of this tagged mutant is due to one of the two disrupted genes or mixed phenotypes of the two disrupted genes and untagged mutations caused by the transformation. The main characteristics of this tagged mutant may be different from those expected from the mutation of the maintenance type cytosine methylase; therefore, this tagged mutant has been here referred to as the "*met1*" mutant. Interestingly, the growth of this tag mutant in TAP was not different from that of the mother strain (data not shown).
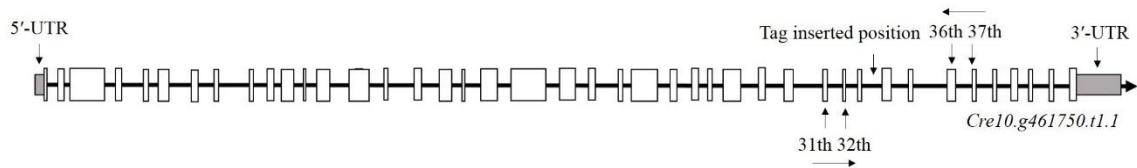

Figure 2-2. Schematically shown location of the *aphVIII* tag in *Cre10.g461750.t1.1* and the primer sites used for RT-PCR.

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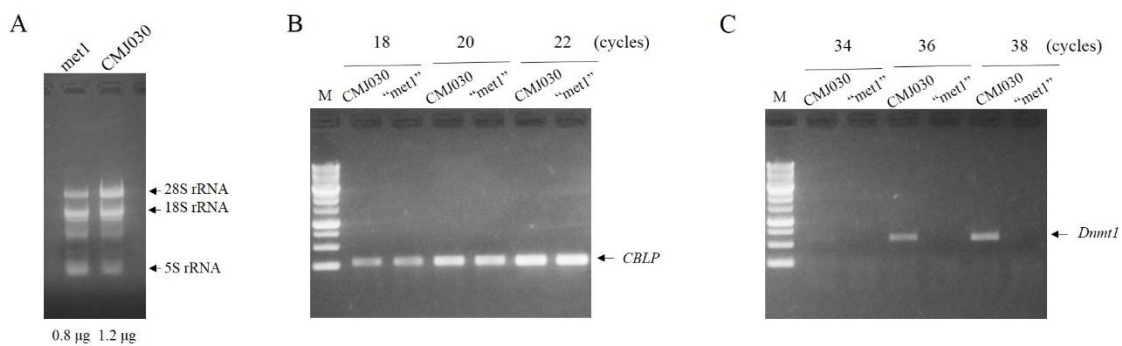
Figure 2-3. RT-PCR analysis of *Cre10.g461750* transcript. (A) Agarose gel electrophoresis of total RNA prepared from "met1" tag mutant (0.8 μg) and the mother cell CMJ030 (1.2 μg). Bands of 28S, 18S and 5S ribosomal RNAs were indicated by arrows. RT-PCR results for *CBLP* (B) and *Dnmt1* (C) using the same amount of cDNA prepared from *"met1"* tag mutant and the CMJ030 were shown. The housekeeping gene *CBLP* was used as internal control. M, 1-kb DNA ladder markers; *CBLP*, Chlamydomonas β subunit-like polypeptide; *Dnmt1*, DNA methyltransferase 1. The primers used for analysis of *Cre10.g461750* transcript were as follows: 5′-GCAGAACAGCATGGTCATGGCCT-3′ (part of the 31st and 32nd combined exons) and 5′-AATGCACCTGCAGCGCTCCA-3′ (part of the 36th and 37th combined exons). The primers for CBLP are shown in (33).

In this study, the endogenous *SQS* gene was overexpressed in the "*met1*" mutant and in wild-type CC-124 to compare the expression levels. The linearized cDNA expression cassette was transformed by electroporation. Transformants were selected on TAP/agar plates supplemented with 15 mg/L zeocin. Considering the occurrence of decoupling through introduction, zeocin-resistant transformants were further screened by
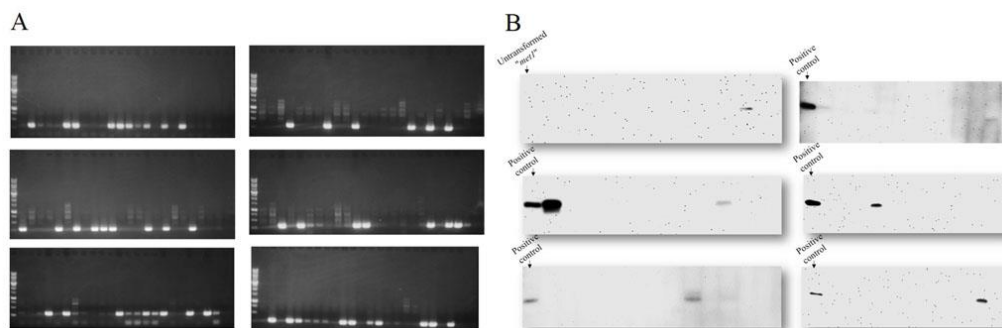
Figure 2-4. Cotransformation and western blot-positive ratios of *SQS* transformants in the "*met1*" strain. (A) PCR and (B) western blot screening of *SQS* transformants. Positive control, *SQS* transformant in the UVM4 strain (28). The untransformed "*met1*" strain was used as the negative control.

PCR for the cointegration of the *SQS* cDNA expression cassette with the marker gene *ble*. We considered these PCR-positive transformants as being successfully cotransformed (Table 2-1). The PCR results showed that the cotransformation ratio of *SQS* with *ble* in the "*met1*" strain was approximately 43.8% (63/144) (Table 2-1 and Fig. 2-4A), which is not significantly different from that of the wild-type CC-124 (109/210, 51.9%).

Host strain	Cotransformation ratio[a]	Western blot-positive ratio[b]	Ratio of highly expressing transformant[c] in Western blot-positive transformants
CC-124[e] (WT[d])	109/210 (51.9%)	5/109 (4.6%)	1/5 (20.0%)
UVM4[e]	52/87 (59.8%)	11/52 (21.2%)	5/11 (45.5%)
UVM11[e]	51/87 (58.6%)	9/51 (17.6%)	4/9 (44.4%)
"*met1*"	63/144 (43.8%)	10/53 (18.9%)	3/10 (30.0%)

Table 2-**1.** Expression of *SQS* cDNA cassette in various *C. reinhardtii* strains. [a]Number of PCR-positive transformants/number of transformants analyzed; [b]Number of western blot-positive transformants/number of PCR-positive transformants; [c]Number of high-protein accumulation transformants/number of western blot-positive transformants; [d]Wild-type and [e]Data were taken from our previous experiment in Kong et al. (19).

Western blotting was performed to analyze the protein accumulation levels of the PCR-positive transformants. The results showed that the western-positive ratio detectable after 10 min exposure for the *SQS* transformants in the "*met1*" strain was 18.9% (10/53) (Table 2-1 and Fig. 2-4B), which was 4 times higher than that in the CC-124 strain (4.6%,

5/109) (Table 2-1). Moreover, the high protein accumulation ratio of the western-positive transformants was 30.0% (3/10) for the "*met1*" strain (Table 2-1 and Fig. 2-5A), which was approximately 1.5 times higher than that in the CC-124 strain (20.0%, 1/5) (Table 2-1).
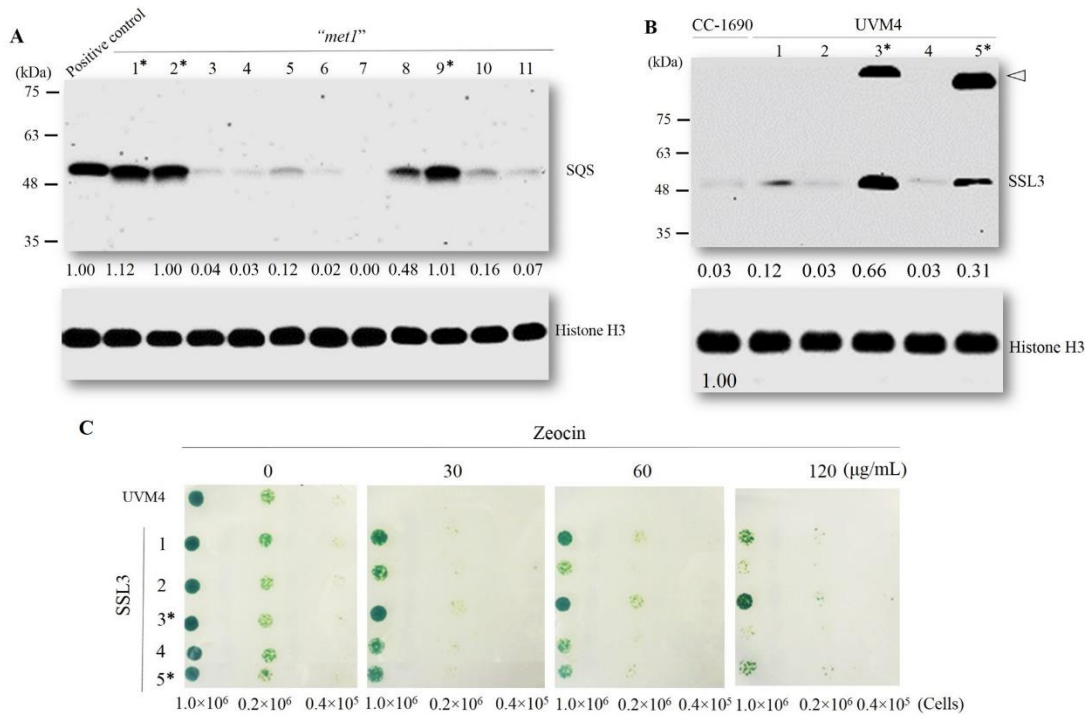
Figure 2-5. Expression levels of SQS and SSL3-positive transformants analyzed by western blotting. (A) The expression levels of SQS-positive transformants in the "*met1*" strain are shown in the upper gel, while the lower gel shows the result of histone H3 in which equal amounts of proteins were loaded. (B) The expression of SSL3-positive transformants in the CC-1690 and UVM4 strains (1 through 5) is shown. The amount of the proteins in the bands was quantified using the Image J software (http://imagej.nih.gov/ij/). The band intensities are shown as relative intensity units relative to the positive controls, which are one of the SQS transformants in the UVM4 strain (21) (A) and histone H3 (B). Signal intensity values above 1.0 for SQS and above 0.3 for SSL3 were attributed to strongly expressing transformants and marked with asterisks. The arrowhead indicates unprocessed fusion protein. (C) Zeocin resistance spotting test for SSL3 western-positive UVM4 transformants. Spotted cull numbers are shown below the spots.

This suggests the ability of "*met1*" to express the *SQS* cDNA is comparable to that of the UVM strains, which have been demonstrated to bear a high potential (16, 19).

We detected 21.2 % (11/52) and 17.6% (9/51) western-positive transformants and 45.5% (5/11) and 44.4% (4/9) of highly *SQS*-expressing strains in UVM4 and UVM11, respectively (19). The main advantage of using the "*met1*" strain over UVM strains is that it possesses mating ability, which facilitates the accumulation of useful traits by genetic crossing. Thus, the "*met1*" strain could be a novel promising host cell for robust transgene expression. The most probable reason for the high western-positive ratio is the successful disruption of the transcriptional silencing system caused by disabled *Dnmt1* gene, which is involved in the maintenance of DNA methylation patterns (21, 22). However, frequently detected western-negative transformants among PCR-positive transformants propose that the silencing ability has not been completely knocked out in the "*met1*" strain. This is also the case of the UVM strains (19). The retained silencing ability of the "*met1*" strain may be due to DNA methylation-independent silencing pathways (12, 13).

2.4.3 *Generation of ble2A-SSLs expressing transformants in wild-type strains*

First, we attempted to overexpress *CrSSL3* in the wild-type CC-1690 and UVM4 strains using an expression vector similar to that used for *SQS* expression, in which the *SQS* ORF was replaced with *CrSSL3* ORF and the *ble* marker gene was switched to *aadA*. However, no western-positive transgenic lines were found in the large number of PCR-positive transformants, which accounted for 261 transformants in CC-1690 and 294 in UVM4. This situation contrasts with that of *SQS* for which western-

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positive transformants were easily found (Table 2-1). Our previous success for *SQS* expression and current failure to find the western-positive *SSL3* transformants in the UVM4 strain clearly demonstrate that this strain is not a useful strain to heterologously express versatile genes of interest.

Then, we tested the *CrSSL* genes expression using the ble2A system to investigate whether it could be useful for heterologous expression of hydrocarbon production-related *Botryococcus* genes. The linearized ble2A expression vectors for *CrSSL3 and CrSSL1* were introduced into the wild-type CC-1690 and C-9 strains, respectively. Then, the transformants were screened by western blot, we found the SSL3 and SSL1 protein-positive transformants in both wild-type strains (Fig. 2-5B, 2-7B)

2.4.4 *Generation of ble2A-SSLs expressing transformants in UVM strains*

UVM strains were previously reported to be a good host to express heterologous genes than wild-type strains (16, 19). We tested that whether the powerful utilization of ble2A expression system could be enhanced in UVM strain. The ble2A expression vector for *CrSSL3* was also introduced into UVM4 strain. The cotransformation- and western-positive ratios for the *SSL3* in the UVM4 strain were approximately 51.3% (74/144) and 10.4% (5/48), respectively, which were 1.5 times and 5.0 times higher than those in the CC-1690 strain [34.0% (49/144) and 2.0% (1/49)] (Table 2-2, Fig. 2-6). Moreover, the protein accumulation ratio in the western-positive transformant was 40.0% (2/5) in the UVM4 strain, whereas no *SSL3* strongly expressing transformants were found in the CC-1690 strain (Table 2-2 and Fig. 2-5B). This intimates the high ability of the ble2A system for heterologous expression of cDNAs.

Host strain	Cotransformation ratio[a]	Western blot-positive ratio[b]	Ratio of highly expressing transformant[c] in Western blot-positive transformants
CC-1690 (WT[d])	49/144 (34.0%)	1/49 (2.0%)	0/1 (0.0%)
UVM4	74/144 (51.3%)	5/48 (10.4%)	2/5(40.0%)

Table 2-**2.** Expression of *SSL3* cDNA cassette in CC-1690 and UVM4 strains. See the footnotes of Table 2-1 for a, b, c, d and e.

In the *ble2A-CrSSL3* transformants, the fused products were specifically detected in highly SSL3-accumulating transformants, whereas in the *ble2A-CrSSL1* transformants, the fused products were detected even in the weakly *CrSSL1*-expressing transformants (Fig. 2-5B, Fig. 2-9B). These results suggest that the ribosome-skipping efficiency at the ble2A coding region is strongly affected by the following ORF. Moreover, we found that the ribosome-skipping efficiency of the *ble2A-SSL3* was not uniform even among the transformants (Fig. 2-5B). Transformant 3 is one of the highly SSL3-expressing transformants; in this transformant, the fused protein level was almost identical to that of the processed SSL3, whereas in transformant 5, which also highly expresses *CrSSL3*, the level of the fused product prominently exceeded that of the processed protein. Interestingly, in weakly expressing transformants, no fused protein was detected. The sequence of FMDV 2A induces ribosome-skipping during translation to generate discrete products from single ORFs (17, 36, 37), in which the translational release factors, eRF1 and eRF3, play an important role (38). However, the relation between the expression level of the protein and the ribosome-skipping ratios remains unknown.
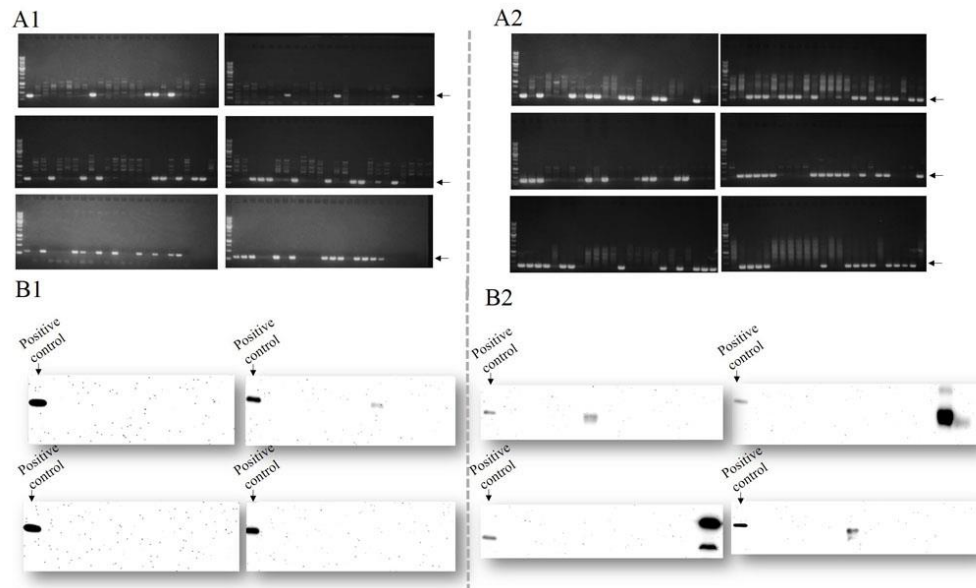
Figure 2-6. Cotransformation and western-positive ratios of SSL3 transformants in the CC-1690 wild-type and UVM4 strains. (A) PCR and (B) western blot screening of SSL3 transformants. Positive control, *aphVII* transformant (19). Arrow indicates the expected PCR product position. A1 and B1 are results of CC-1690, while A2 and B2 are those of UVM4.

2.4.5 *Improved expression level of SSLs by ble2A expression system in the UVM strain*

Through western analyses of the transformants, we showed that SSL3-positive transformants were much more frequently detected in the UVM4 strain (5/48) than in the wild-type strain CC-1690 (1/49). This result is in agreement with our previous experiments showing the efficient expression of the *SQS* cDNA from *Chlamydomonas* in the UVM4 and UVM11 strains (19). Therefore, UVM strains are much better hosts than wild-type strains, albeit not ideal, for the expression of various types of heterologous cDNAs.

In our previous experiment in which the *Hsp70A/RbcS2* promoter was used to stimulate the abovementioned *SQS* cDNA, the expression levels varied prominently

among transformants even in the UVM strains. Therefore, in this study, we used a modified *Hsp70A/RbcS2* promoter (PAR4), which contained four introns to enhance the transcriptional ability. However, strong position effect was still observed as in the case of the non-modified *Hsp70A/RbcS2* promoter (Fig. 2-5B, Fig. 2-9B), and the effects between the two promoters were not significantly different. This suggests that the PAR4 promoter is still not sufficiently strong to overcome the residual position effect in the UVM4 strain, regardless of its apparently alleviated silencing ability.

Therefore, the expression of a gene of interest using the ble2A system in the UVM or "*met1*" strains surely enhances the possibility to find transformants that highly accumulate the target protein. This combinational method may be useful for heterologous expression of almost all transgenes.

2.4.6 *Comparative analysis of the ble2A-CrSSL1 mRNA levels in the western-positive and -negative C-9 transformants*

To compare the *ble2A-CrSSL1* mRNA levels, total mRNA was isolated from C-9 (wild-type) and transformants of *ble2A-CrSSL1* that strongly (SSL1-5 and -34) and weakly (SSL-7 and -39) expressed CrSSL1 (Fig. 2-9). The results of RT-PCR clearly showed the relative abundance of *ble2A-CrSSL1* mRNA was higher in SSL1-5 and -34 than SSL-7 and -39 (Fig. 2-7). Thus, the data shows that the differences in the SSL1 protein levels are attributable mainly to variation in the mRNA levels. Therefore, the main mechanism responsible for the observed position effect must be closely related with epigenetic transcriptional repression systems.
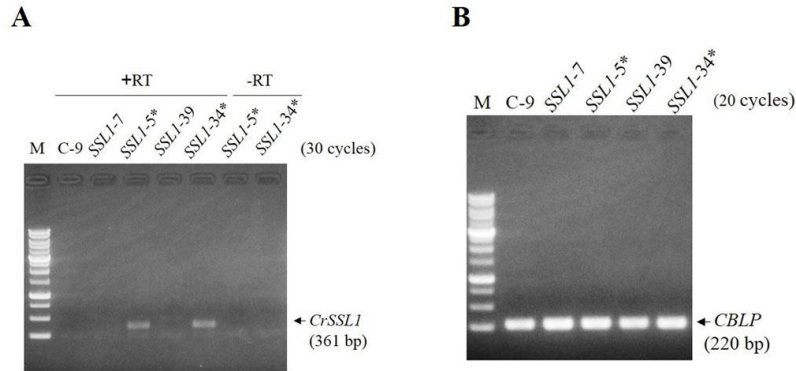
Fig. 2-**7**. Comparative analysis of the ble2A-CrSSL1 mRNA levels by semi-quantitative reverse transcription PCR. (A) RT-PCR results at the end of 30 cycles using cDNA prepared from SSL1-7, -5. -34, -39, and C-9 (untransformed wild-type) for partial amplification. Asterisk indicate the strains with high expression levels of CrSSL1 protein (see Fig. 2-9). +RT and -RT denote the reactions with or without reverse transcriptase, respectively. (B) RT-PCR results at the end of 20 cycles, which that equal amounts of mRNA were used. An endogenous *CBLP* cDNA was amplified. *CBLP, Chlamydomonas β* subunit-like polypeptide. See Fig. 2-8 for the details.
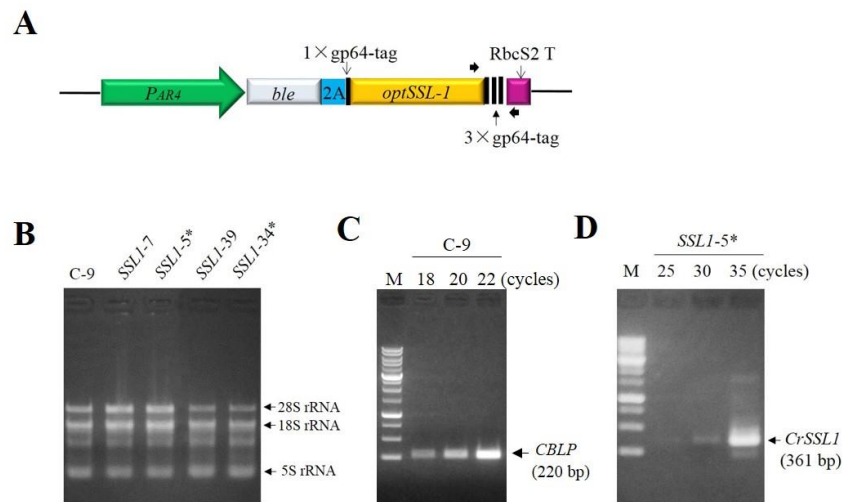
Fig. 2-**8**. (A) Arrows indicate the position of primers for the ble2A-CrSSL1 semi-quantitative reverse transcription PCR. (B) Agarose gel electrophoresis of total RNA prepared from SSL1-7, -5, -39, -34, and untransformed C-9 wild type. Asterisk indicate the strains with high expression levels of CrSSL1 protein. The bands of 28S, 18S and 5S ribosomal RNAs are indicated by arrows. (C) PCR cycle number-dependent

amplification of a part of *CBLP* cDNA prepared from C-9 wild-type mRNA. The number of the PCR cycle is shown at the top of the lane. (D) PCR cycle number-dependent amplification of a part of *CrSSL1* cDNA prepared from CrSSL1-5 mRNA. The number of PCR cycles is shown at the top of the lane. *CBLP, Chlamydomonas β* subunit-like polypeptide; M, M, 1-kb DNA ladder markers.

**2.4.7** *Determination of the relationship between target protein expression levels and zeocin resistance*

The mechanism of robust expression of recombinant proteins by transcriptionally fusing their genes to the *ble* marker gene is not yet well characterized. One of the probable reasons is that *ble* functions by sequestration of the antibiotic through one-to-one stoichiometric binding, but not through enzymatic inactivation of zeocin (18). Therefore, presumably high levels of *ble* expression are required for survival.

Ble and SSL proteins must be produced in 1:1 ratio under when mRNA is fully translated without breaks and ribosome-skipping occurs, as expected, perfectly at the 2A region. In turn, this leads to the simple speculation that zeocin resistance and amount of SSL should show a positive relationship. If so, screening of zeocin-resistant transformants could be a practical strategy to find highly expressed target gene. Based on this idea, the relationship between zeocin-resistance and SSL levels was analyzed. We measured the levels of zeocin resistance in SSL western-positive transformants by spotting transformant cells on agar plates containing various zeocin concentrations.

First, we confirmed that all the transformants chosen for analysis showed no significant growth differences on non-drug-containing TAP plates (Fig. 2-5C). UVM4 transformants 3 and 5 (UVM4-No. 3 and UVM4-No. 5, respectively) were expected to show relatively rapid growth on plates containing zeocin at high concentration, because

they accumulated relatively high levels of SSL3 protein compared to other transformants. As expected, UVM4-3 showed the best growth on the plate containing 120 mg/L zeocin, whereas the growth of UVM4-5 was slightly but significantly slower than that of UVM4-1 on the plates containing 30 mg/L or 60 mg/L zeocin, of which the SSL3 expression level was prominently lower than that of UVM4-5 (Fig. 2-5). Moreover, no transformants survived on the plate containing 200 mg/L zeocin. These results show that there is no strictly positive correlation between the target protein expression levels and the zeocin-resistance levels in the transformants obtained using the ble2A expression system. A similar result was observed in *CrSSL1* expressing C-9 wild-type transformants (Fig. 2-9). The transformants accumulating SSL1 protein at low levels (e.g., SSL1-39) showed growth rate not apparently different from that of the highly expressing transformants (e.g., SSL1-34) at the various zeocin concentrations (Fig. 2-9). Consistent with the results of these analyses, we failed to sort highly target protein-expressing transformants of *CrSSL1* among the transformants survived on the plate containing 120 mg/L zeocin, irrespective of the drastically decreased number of transformants on the plates (data not shown). Therefore, zeocin-resistance screening does not appear to be a practical method to identify the transformants highly expressing target proteins.

The above is also the case for the two independent expression cassette system, i.e., target gene expression cassette and marker gene expression cassette. Using the expression plasmid containing the independent cassettes of *CrSSL1* and *ble* in vicinity, we could not obtain the *SSL1*-highly expressing lines among the transformants appeared on the plate containing 120 mg/L of zeocin (data not shown).
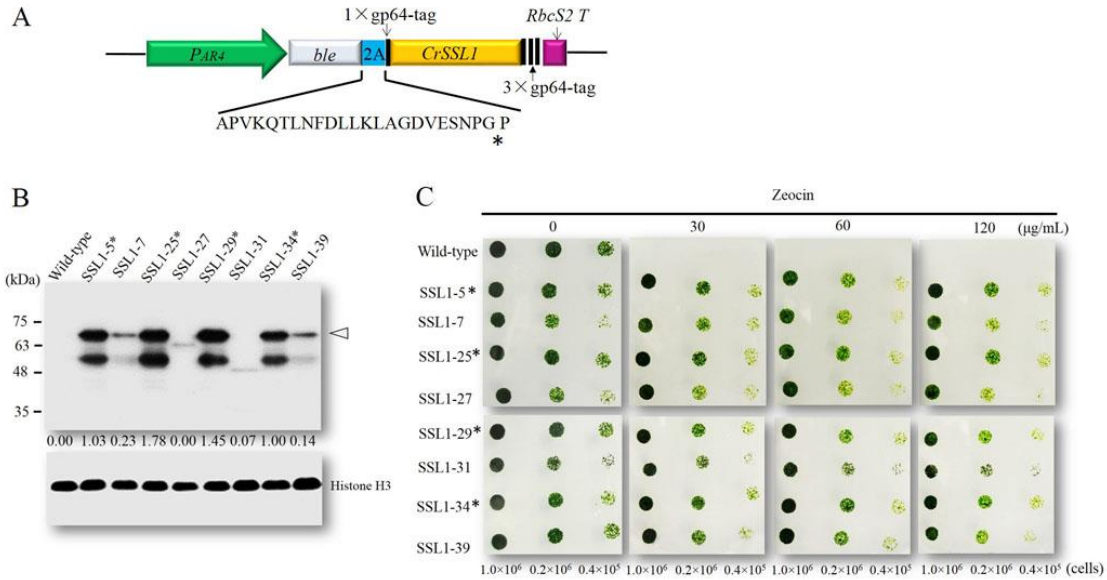
Figure 2-**9**. Protein expression levels of SSL1-positive transformants and their zeocin-resistance levels. (A) Schematic representation of the *SSL1* transformation vector. *PAR4,* modified *Hsp70A/RbcS2* promoter revised to contain four copies of the first intron of *RbcS2*; 2A, FMDV 2A peptide, *CrSSL1*, codon-optimized *SSL1* cDNA ORF. The asterisk indicates the cleavage site. Expression level analysis of SSL1-positive transformants by western blotting (B). Zeocin-resistance level analysis for western-positive transformants by spotting test (C). Wild-type, untransformed C-9 wild-type strain. The asterisk indicates transformants that expressed the target protein at high levels, and the arrowhead indicates the unprocessed fusion protein. Spotted cull numbers are shown below the spots

We suspect that the *ble* sequence may have an unknown effect leading to enhancement of translation or prevention of translational stall, and it appears to work efficiently only when the sequence is directly ligated to the target ORF. Very efficient expression of *ble-CrGFP,* in which *ble* is directly connected with *CrGFP,* is another example of a system showing similar ble-fusion effect (9).

Further studies are essential to unveil the mechanism of action of the ble2A system and to explain why it works efficiently in overcoming the silencing of

heterologous transgenes. The combinational use of the ble2A nuclear expression vector and UVM4 or "*met1*" strains for foreign gene expression could enhance the utility of *Chlamydomonas* as plant cell factory for producing biofuel, biopharmaceuticals, and other valuable compounds.

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CHAPTER 3: Generation and characterization of hybrid cells that expressing hydrocarbon-producing related enzymes

3.1 Abstract

Microalgae have presented themselves as strong platform for biofuels production to replace diminishing oil reserves. Squalene synthase-like (SSL) enzymes have been demonstrated to play an important role in the biosynthesis of botryococcenes in an oleaginous green algae *Botryococcus braunii* (B-race) of which colonies contain large amount of hydrocarbons. However, the slow growth rate and unavailable transformation technique hamper *B. braunii* as the platform for biofuel production. The model organism, *Chlamydomonas reinhardtii* is increasing attractiveness for the high potential to produce renewable biofuels. In this study, the third botryococcene biosynthesis related gene, (*SSL2*), was expressed in *C. reinhardtii*. The hybrids that expressing *SSL1/SSL2, SSL1/SSL3*, and *SSL1/SSL2/SSL3* were generated through genetic crossing. The protein expression levels of these SSL enzymes in the hybrids were not significantly different compared with the parental strains. Moreover, SSL enzyme activity of *SSL1/SSL3* hybrids, and botryococcene level of SSL3 expressing transformant were analyzed. Our results showed that expression of SSL3 enzyme is not enough to boost the intracellular botryococcene accumulation in *C. reinhardtii*. This suggested that if the activity of SSL enzymes activity are limited, irrespective of the existence of precursors in the cytosol, detectable levels of botryococcene is not produced in *C. reinhardtii*. Further biotechnological efforts are essential to produce botryococcene in *C. reinhardtii*.

Key words: biofuel, botryococcene, squalene synthase-like gene, *Botryococcus braunii*, *Chlamydomonas reinhardtii*

3.2 Introduction

Algae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms. Some of them are useful for the production of nutrients, cosmetics, pharmaceuticals, and biofuels (1, 2). Recently, biofuels production from algae attracts much more attention, due to the expected depletion of fossil fuels, and the increasing atmospheric greenhouse gases emission. Biofuels can be solids, liquids or gasses so long as they are derived directly from biological sources (3). Algae have potentiality to produce liquid fuels, such as triacylglycerol (TAG), which can be readily converted into biodiesel. Moreover, some algae can also produce complicated hydrocarbon, e.g., terpenoids, which are useful both as biofuel and various valuable coproducts (4). However, there is no innate ideal algae for the desirable biofuel-production, because many combined features are required for massive industrial production, some of which are not essential for survival in nature. (5). The B race of *Botryococcus braunii*, a colonial green microalga, is a potential source of renewable biofuel, because of the ability to produce large amounts of triterpenoid hydrocarbons which are known as botryococcenes (6). *B. braunii* B-race typically accumulates hydrocarbons to 30%-40% of their dry weight, with the optimized hydrocarbons contents up to 86% dry weight (7). C30 botryococcene is the precursor of all botryococcenes and is rapidly converted into homologues of up to C34 by methylation with sadenosylmethionine (8). Botryococcenes are biosynthesized through the isoprenoid pathway, and are similar in structure to another common triterpene, squalene (9). Recently, the unique mechanisms of triterpene botryococcne biosynthesis were identified in *B. braunii*. Niehaus et al. (10) reported that none of the three squalene synthase-like (*SSL*) genes in *B. braunii* is enough to produce

botryococcene by the single enzyme. *SSL1* catalyses presqualene diphosphate (PSPP) biosynthesis by utilizing farnesyl diphosphate (FPP) as substrate, and *SSL3* converts the PSPP into botryococcene; while *SSL2* is contributed to squalene production by conversion of the PSPP. Through experiments of the two SSL genes co-expression, robust botryococcene (SSL1+SSL3) or squalene biosynthesis (SSL1+SSL2) was observed in FPP highly expressing yeast. The reported maximum specific growth rate of *B. braunii* is $0.5 \, \mathrm{day}^{-1}$ (doubling time of 1.4 days) under most favorable growth conditions (11), however, such conditions are not practical for long term massive culture. Moreover, the whole genomic sequences and transformation methods for *B. braunii* are still not available, which hampers the development of *B. braunii* as the host for biofuel production (12).

The unicellular green alga, *Chlamydomonas reinhardtii*, has been a superb model organism for the basic studies, because of their rapid growth rate, cost-effective culturing, available genome sequences, and genetic manipulability (13-16). However, compared with the high-oil producing microalga *B. braunii*, *C. reinhardtii* yields limited hydrocarbons. In the past years, various genetic manipulations and metabolic engineering methods were applied to improve biofuel production in *C. reinhardtii*. Starchless *Chlamydomonas* mutants showed that TAG content could be increased from 0.5% to 20.5% (% of dry weight) (17, 18). Recently, overexpression of a DNA-binding-with-one-finger (Dof)-type transcription factor was also applied to increase the amount of TAG (19). However, so far, no successful studies have been reported that the production levels of isoprenoids were enhanced in *Chlamydomonas*.

It was reported that all isoprenoids are assembled through two common five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that are biosynthesized via Methyl-D-erythritol-4-phosphate (MEP) pathway in microalgae (20). IPP and DMAPP are the direct building blocks for the biosynthesis of botryococcene. However, if such precursors are large enough or not is unknown, for producing botryococcene. In this study, I attempted to produce botryococcenes in *C. reinhardtii*, through heterologously expressing the necessary genes in *Chlamydomonas*. However, major obstacle is the disappointingly poor expression of transgenes, irrespective of the high transformation frequency (21, 22). Recently, a novel ble2A nuclear expression system was reported to robustly express heterologous genes (23). Using this system, I succeeded to heterologously express *SSL1*and *SSL3* in *Chlamydomonas* wild-type strains through the ble2A nuclear expression system, respectively (unpublished data), as described in the previous chapters. In this chapter, I will report the success of *SSL2* expression and generation of *Chlamydomonas* strains that express two or three SSL genes. I generated the hybrids that expressing SSL1/SSL3, SSL1/SSL2, and *SSL1/SSL2/SSL3* with genetic crossing method. Moreover, the polyethylene glycol (PEG)-mediated cell fusion was used to generate *DXR1/SQS* expressing hybrids using cell wall-less UV-mutated (UVM) strain. The enzyme activity of hybrid cell (SSL1/SSL3) was analyzed and botryococcene production level of *SSL3* expressing transformant were determined by gas chromatography–mass spectrometry (GC/MS).

3.3 Materials and Methods

PCR screening of cotransformants

Primary transformants with antibiotic resistance were screened by *Chlamydomonas* colony PCR as described previously (24, 25) to detect the presence of the gene of interest. In brief, cells were resuspended in Tris–EDTA solution and heated to 98°C for 10 min. The cell lysate (1 µL) was then used as template for 20 µL PCR using Taq polymerase (Takara, Japan). The primers are as following: gene-specific forward primer (5′-ATGACTATCAAGCGCCTGCAGAG-3′) and terminator-specific reverse primer (5′-CCGCTTCAGCACTTGAGAGCA-3′) for *SSL1*, gene-specific forward primer (5′-CGGCATCCAGAAGAGCTGCA-3′) and terminator-specific reverse primer (5′-CCGCTTCAGCACTTGAGAGCA-3′) for *SSL2*, and gene-specific forward primer (5′-AGATGGAGGCCAAGTGCGTC-3′) and terminator-specific reverse primer (5′-CCGCTTCAGCACTTGAGAGCA-3′) for *SSL3*. PCR-based assay for mating type was performed as previously described (26), using the following primers: MTP2F (5′-GCTGGCATTCCTGTATCCTTGACGC-3′) and MTP2R (5′-GCGGCGTAACATAAAGGAGGGTCG-3′) for detecting the mating type plus (*mt*$^+$) allele, and MTM3F (5′-GCTGCACAATTCGTCTTGGCTGGTG-3′) and MTM3R (5′-CTGGCCTCGCCTTCACAAGAGAGC-3′) for detecting the mating type minus (*mt*$^-$) allele. The amplification conditions were as follows: 98°C for 5 min; followed by 30 cycles at 95°C for 15 s, 61°C for 30 s, and 72°C for 30 s; and a final step at 72°C for 7 min using a Thermal Cycler 2720 (Applied Biosystems).

Western blot screening of the transformants

Western blot was performed as described previously (27). For immunoblot analysis, total cell extracts and soluble fractions were prepared and separated on 12% SDS-PAGE and immunoblotted with the monoclonal anti-baculovirus envelope gp64 polypeptide antibody (1/5,000) (eBioscience, CA, USA) for detecting the gp64-tagged proteins, and anti-Histone H3 antibody (1/10,000) (Abcam, CA, USA) for detecting Histone H3 protein. The ECL detection system (Millipore) was used to detect immunoreactive proteins by utilizing anti-mouse or anti-rabbit secondary antibodies (1/20,000).

Chlamydomonas genetic crossing and PEG-mediated cell fusion

Genetic crossing was performed following (28) with some modification. The protocol is as following: gametes were induced by incubating mating type plus (mt^+) and mating type minus (mt^-) cells for 24 hours in 5 mL nitrogen-free liquid TAP medium. Mt^+ gametes were mixed with mt^- gametes in one to one ratio for 4 hours, and the mating reactions were plated on minimal medium containing 3% agar plates and maturated in the dark for 6 days. Unmated cells were scraped off to the side using a sterile razor blade, and the mated zygospores were remained buried in the agar plates. The zygospores containing agar were excised and slid onto TAP agar plates supplemented with 15 mg/L of zeocin and incubated in the light until colonies appeared. The independent colonies were diluted in TAP medium, and then spread on 15 mg/L of zeocin plates to isolate the single colony.

Polyethylene glycol (PEG)-mediated somatic fusion between cell wall mutants were followed (29). Briefly, cells were grown to $1.0–2.0 \times 10^6$ cells/mL in TAP medium and mixed in one to one ratio. The cells were centrifuged ($300 \times$ g, 2min), and then the

pellets were resuspended by 1/2 volume of 0.5 M mannitol and 1/2 volume of PEG solution (50 mM PEG 6000; 7.5 mM CaCl$_2$; 5 mM glycine; NaOH to make pH 8.0), and incubated at room temperature for 20–30 min. The cells were washed with equal volume of 0.5 M mannitol, pelleted by centrifuge, resuspended with TAP medium, and gently spread on TAP plates containing paromomycin sulfate (20 mg/L) and zeocin (15 mg/L). The plates were incubated under continuous fluorescent light (20 µmol m^{-2} s^{-1}) at 25°C.

SSL enzyme activities

Characterization of SSL enzymes assays was carried out followed (8), as cooperative work with Prof. Shigeru Okada (The University of Tokyo, Graduate School of Agricultural & Life Sciences).

3.4 Results and Discussion

3.4.1 *Generation of SSL expressing hybrid cells by genetic crossing*

CrSSL2-ble2A expressing vector was introduced into CC-1690 wild-type strain. The primary zeocin-resistance transformants were screened by genomic PCR, and the PCR-positive transformans identified were subjected to analyze by western blot (Fig. 3-1). The results suggested that *SSL2* cDNA cassette was successfully expressed in the CC-1690 by ble2A nuclear expression system, which is in accordance with previous
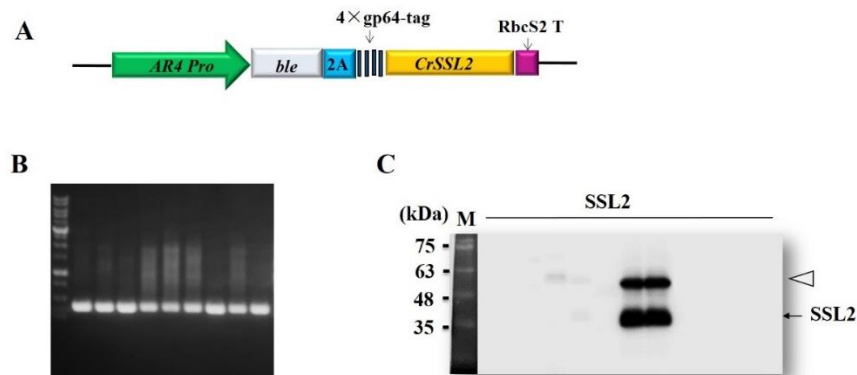
Figure 3-**1**. Generation and identification of *SSL2* expressing transformant. (A) Schematic representation of *SSL2* transformation vector. (B) and (C) The *SSL2* gene-and protein-positive transformants identified by PCR and western blot, respectively.

reports that heterologous genes can be efficiently expressed by fusion to a selective marker gene *ble* (unpublished data). However, molecular weight of the detected signals for SSL2 protein was apparently lower than expected (Fig. 3-1C), that is the case for the SSL2 expressed in yeast (personal communication). This might be due to SSL2 protein containing a transmembrane domain (10). In order to obtain *SSL1* and *SSL2* (*SSL1/SSL2*) expressing strain, we mated two independent transformants that stably express SSL1 and SSL2 proteins using the modified genetic crossing method (Fig. 3-2). The robust mating activity of the two different mating type cells
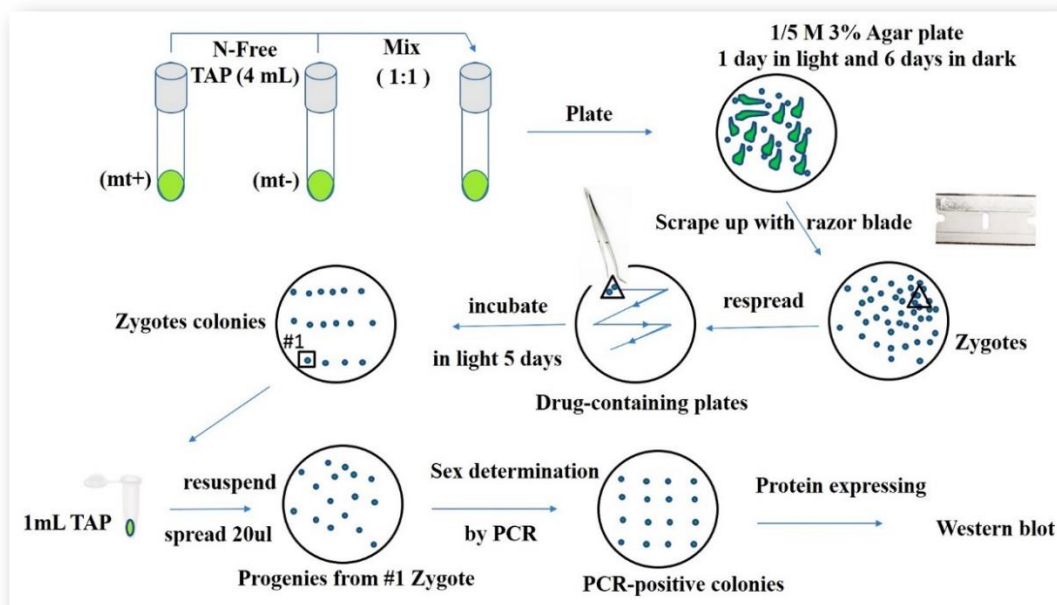
Figure 3-**2**. Schematic representation of *Chlamydomonas* genetic crossing and screening.

was confirmed by microscope observation (Fig. 3-3). The hatched progenies were selected on TAP agar plates containing 15 mg/L zeocin. The single colony was
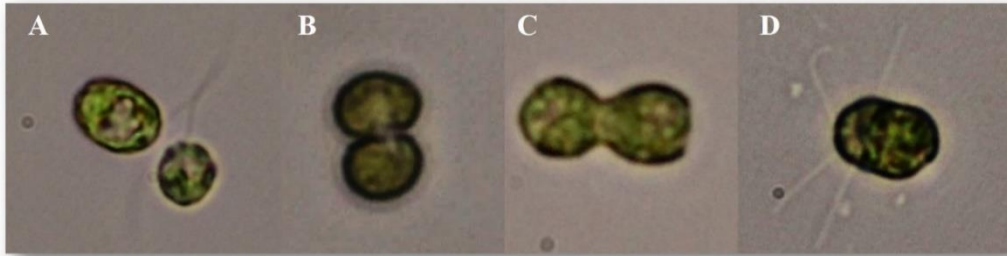
Figure 3-**3**. The mating process observed by microscope. (A) Gametic flagellar agglutination between cells of opposite mating type. (B) Activated mating structures fused to form a cytoplasmic bridge. (C) Early fused cell. (D) Four flagella containing fused cell.

obtained by separation of individual progeny on the 15 mg/L zeocin plates, and screened for the presence of *SSL1* and *SSL2* by PCR as described above. Approximately 35.7% (5/14) progenies from the independent zygospores are both *SSL1* and *SSL2* PCR-positive colonies (Fig. 3-4A, B). In addition to that, PCR-based mating-type-determination assay was performed to identify the hybrid cell is haploid or diploid. The result showed that all progenies had only one of the two mating-type specific regions (Fig. 3-4C), which indicate the hybrid cell is haploid. Western blot was performed to detect the protein expression levels of these PCR-positive colonies. The western blot results suggested that the PCR-positive colonies are expressing the target proteins (Fig. 3-4D) of which protein expression levels are not altered through mating and meiosis.
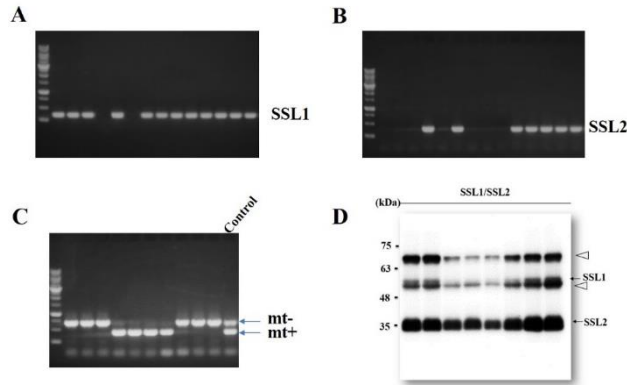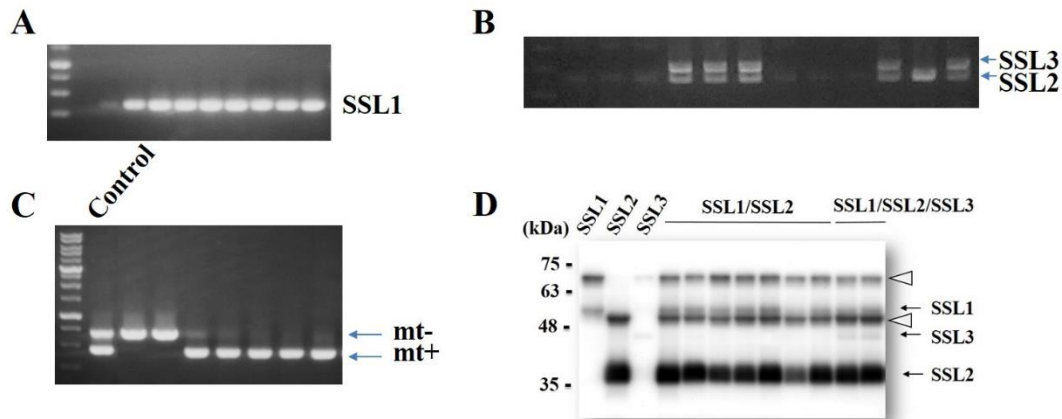
Figure 3-**4**. Identification of *SSL1/SSL2* expression in hybrid cells. (A) and (B) are the PCR results using *SSL1* and *SSL2* gene specific primers (C) PCR result using mating type minus (*mt⁻*) and plus (*mt⁺*) specific primers. (D) Identification of *SSL1/SSL2* expression in hybrid cells by western blot. The SSL1 and SSL2 proteins were shown by arrows, and the unprocessed SSL1 and SSL2 proteins were shown by the arrow heads.

Encouraged by the successful generation of *SSL1* and *SSL2* expressing cells, I attempted to generate SSL1/SSL2/SSL3 expressing cells by crossing. As shown in Fig. 3-5 and Fig. 3-6, we successfully generated the PCR- and western blot-positive hybrid cells that are expressing *SSL1/SSL3* and *SSL1/SSL2/SSL3*. No apparent change for protein expression levels was observed for them through the cross and meiosis (Fig. 3-5D, Fig. 3-6D).
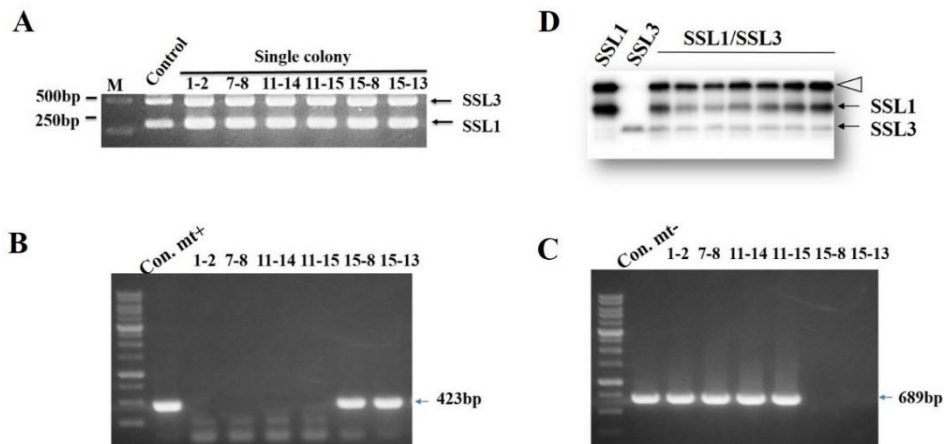
Figure 3-**5**. Identification of *SSL1/SSL3* expressing hybrid cells. The PCR results using *SSL1* and *SSL3* gene specific primers (A), and *mt*[+] (B) and *mt*[−] (C), were shown. (D) Identification of *SSL1/SSL3* expressing hybrid cells by western blot. The SSL1 and SSL3 proteins were show by arrows, and the unprocessed SSL1 protein was shown by the arrow head.


Figure 3-**6**. Identification of *SSL1/SSL2/SSL3* expressing hybrid cells. The PCR results using *SSL1*, *SSL2* and *SSL3* gene specific primers (A) and (B), and *mt*[−] and *mt*[+] specific primers (C) were shown. (D) Identification of *SSL1/SSL2/SSL3* expressing hybrid cells by western blot. The SSL1, SSL2 and SSL3 proteins were shown by arrows, and the unprocessed SSL1 and SSL2 proteins were shown by the arrow heads.

3.4.2 *Generation of DXR1/SQS genes overexpressing fusants by PEG-mediated cell fusion*

The cell wall-less UVM strains are reported to be useful hosts to express the gene of interest efficiently (27, 30). However, these strains have been lost the mating ability. I previously generated the *DXR1* and *SQS* expressing UVM4 transformants (27). In this study, I expected to obtain the *DXR1/SQS* expressing UVM fusants with PEG-mediated cell fusion method (29, 31). Only the hybrid fusion progeny can grow on the TAP plates containing paromomycin and zeocin. Fifteen both antibiotics resistant-fusants

were randomly selected and reinoculated to fresh selective plates. After 26 days, 26.6% (4/15) fusants showed to stable growth on the selection plates, while the others gradually changed into yellowish to die (Fig. 3-7A). This results suggested that some PEG-mediated cell fusants are genetically unstable, which is the case in red microalgae *Porphyridium sp.* as previously reported (32). Possible reason for this phenomenon is the multiploidy of fusants generated through PEG-mediated cell fusion, because of the large aggregates induced by PEG. Multiploidy might be prone to induce genetic rearrangements such as chromosome fusion, chromosome segregation, large chromosome deletion, which cause low survival ratio. The antibiotic resistance-fusion progeny was tested to determine whether they are expressing target proteins. The western blot results suggested that all the antibiotic resistant stable fusants were expressing the target proteins (Fig. 3-7B).
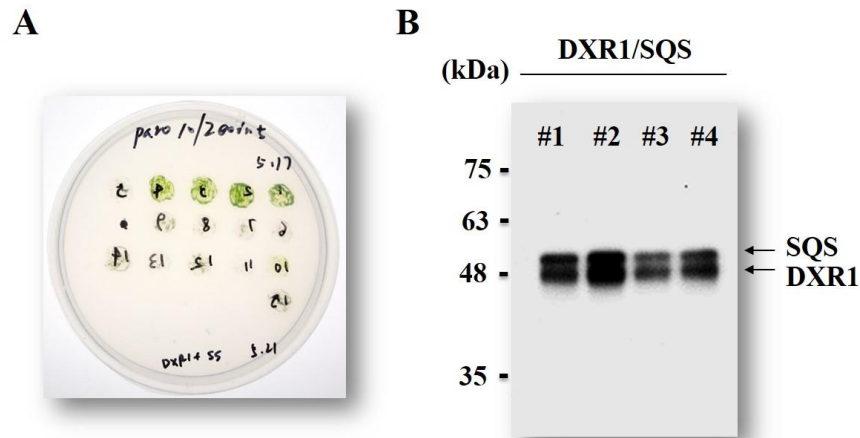
Figure 3-7. Generation of *DXR1/SQS* fusants by PEG-mediated cell fusion. (A) The fusants appeared on both paromomycin (20 mg/L) and zeocin (15 mg/L) containing TAP plates. (B) The target protein expression levels of the stable fusants.

These results demonstrate that somatic cell fusion method is available for cell wall-less UVM strains and useful to generate multiple transgenes expressing strains. This method must be useful to other *Chlamydomonas* strains and most probably other algal species as far as protoplasts are available.

3.4.3 *SSL enzyme activities and botryococcene product assays in Chlamydomonas transformants*

Using the *SSL1/SSL3* expressing transformants, preliminary experiments to detect the enzyme activities and the accumulation of botryococcene were carried out as co-operative work with Okada laboratory (The University of Tokyo). Unfortunately, so far there are no apparent data to show the enzymatic activities and existence of botryococcene in the *Chlamydomonas* transformants. These results suggested that further biotechnological efforts are necessary to enhance the enzyme activity and overproduce precursors for botryococcene production in *Chlamydomonas*.

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Achievements

1. List of publications

1) **Kong, F.**, Yamasaki, T., and Ohama, T*. Expression levels of domestic cDNA cassettes integrated in the nuclear genomes of various *Chlamydomonas reinhardtii* strains. *Journal of bioscience and bioengineering* 117, 613-616 (2014).

2) Hou, L., Wang, P., **Kong, F.**, Park, H., Kobiro, K., and Ohama, T*. Mesoporous TiO_2 nanoparticles: a new material for biolistic bombardment. *Phycol. Res.61*, 58-60 (2013).

3) **Kong, F.,** Yamasaki T., Sari D., Li X., Ivanova N., Okada, S. and Ohama, T* Robust expression of heterologous genes by selection marker fusion system in improved *Chlamydomonas* strains. Submitted (2014).

2. International and domestic conferences

1) The 4th Internatinal Symposium of Frontier Technology (ISFT) (2013, Shengyang, China, **Oral**).

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

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

3. Academic award and patent

1) KUT Academic Research Award 高知工科大学学術研究奨励賞 (2014)

2) Ohama, T., Yamasaki, T., Park, H., **Kong, F.**, Hou, L. (2013) Single cells isolation and culture method of the colonial oil-producing alga *Botryococcus braunii* 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 (WO

2013121509 A1).

APPENDIX-PUBLICATION