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1 Robust expression of heterologous genes by selection marker fusion system in
2 improved *Chlamydomonas* strains

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23 Short title: Heterologous expression of squalene synthase-like gene

24

25

1 **Abstract**

2 *Chlamydomonas* is a very attractive candidate plant cell factory. However, its
3 main drawback is the difficulty to find the transformants that robustly express
4 heterologous genes randomly inserted in the nuclear genome. We previously showed
5 that domestic squalene synthase (*SQS*) gene of *Chlamydomonas* was much more
6 efficiently overexpressed in a mutant strain [UV-mediated mutant (UVM) 4] than in
7 wild type. In this study, we evaluated the possibility of a new mutant strain, *met1*,
8 which contains a tag in the maintenance type methyltransferase gene that is expected
9 to play a key role in the maintenance of transcriptional gene silencing. The versatile
10 usefulness of the UVM4 strain to express heterologous genes was also analyzed. We
11 failed to overexpress *CrSSL3* cDNA, which is the codon-adjusted squalene synthase-
12 like gene originated from *Botryococcus braunii*, using the common expression
13 cassette in the wild-type CC-1690 and UVM4 strains. However, we succeeded in
14 isolating western blot-positive transformants through the combinational use of the UVM4 strain
15 and ble2A expression system of which expression cassette bears a fused ORF of the target
16 gene and the antibiotic resistance gene *ble* via the foot-and-mouth disease virus
17 (FMDV) self-cleaving 2A sequence. It is noteworthy that even with this system, huge
18 deviations in the accumulated protein levels were still observed among the UVM4
19 transformants.

1 **Introduction**

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

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

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

25 Moreover, a novel nuclear expression system was reported to robustly express

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

14 Previously, we demonstrated that the *Chlamydomonas* squalene synthase
15 (*SQS*) cDNA was much more efficiently expressed in the UVM4 and UVM11 than in
16 wild-type strains (19). Recently, using the *Chlamydomonas* MmeI-based insertion site
17 Sequencing (ChlaMmeSeq) method (20), an insertional mutant of *Cre10.g461750* was
18 isolated. This gene encodes DNA methyltransferase 1 (*Dnmt1*) (20), which is
19 expected to be involved in the maintenance of DNA methylation patterns (21, 22).
20 Robust transcriptional gene silencing through DNA methylation is one of the major
21 pathways for stable repression of transgenes. In this study, to expand the platform for
22 efficient expression of various transgenes, we evaluated the potency of this tag-
23 inserted strain by comparing domestic *SQS* gene expression levels in four strains CC-
24 124 (wild-type), UVM4, UVM11, and the insertional mutant of *Cre10.g461750*
25 (“*met1*” see below). Moreover, to evaluate the ability of the ble2A system for

1 overexpression of codon-optimized transgenes, a codon-adjusted *SQS*-like 3 gene
2 (*CrSSL3*) and *CrSSL1*, which originated in *Botryococcus braunii*, were
3 heterogeneously expressed in wild-type strains and in the UVM4 strain. These *SSL*
4 genes are key enzymes for the biosynthesis of botryococcene in *B. braunii* B-race
5 (23). The expression levels of these enzymes were closely analyzed by western
6 blotting using a monoclonal antibody against gp-64 epitope.

7

8 **Materials and Methods**

9 ***Construction of the transformation vectors***

10 The construction of the transformation vector containing *SQS* expression
11 cassette was shown in detail by Kong et al. (19). For the construction of the P_{AR4}::ble-
12 2A-SSL::term expression vector, the *ble* sequence, which contained one copy of the
13 *RbcS2* intron 1, was fused in frame to the codon-optimized FMDV 2A coding
14 sequence (2), and synthesized as an *XbaI-NdeI/KpnI* fragment. The ble-2A fragment
15 was inserted into a pSTBlue-1 plasmid (EMD Biosciences, USA) as *XbaI/KpnI*
16 fragment, generating the recombinant plasmid pSTBlue-1-ble-2A. The *Hsp70A*
17 promoter fragment was amplified by polymerase chain reaction (PCR) with high-
18 fidelity PrimeSTAR HS DNA polymerase (Takara, Japan) from the pALM32 plasmid
19 (24) using primers *XbaI-Hsp70A-F* (5'-AATCTAGAGACGGCGGGG-3') and *NdeI-*
20 *HindIII-Hsp70A-R* (5'-CATATGAACTGAAGCTTGAGTGGTTATGTA-3'). This
21 fragment was inserted into the pSTBlue-1-ble-2A plasmid as a *XbaI/NdeI* fragment,
22 generating the recombinant plasmid pHsp70A-ble-2A. The fragment containing the
23 sequence of *RbcS2* 3' untranslated region (UTR) terminator was excised from the
24 pHsp70A/*RbcS2*-cgLuc plasmid (25) by *BamHI-KpnI* digestion and cloned into
25 pHsp70A-ble-2A, resulting in the recombinant plasmid pHsp70A-ble-2A- term. For

1 the construction of four parallel copies of the first intron (intron 1) of *RbcS2*, the
2 sense and antisense single-stranded oligonucleotides intron 1-left (5'-
3 CAGGTGAGTCGACGAGCAAGCCCGGCGGATCAGGCAGCGTGCTTGCAGAT
4 TTGACTTGCAACGCCCGCATTGTGTGTCGACGAAGGCTTTTGGCTCCTCTGT-
5 3') and intron 1-right (5'-
6 TGCCTGCAGGAATTCGATTGGTCTTGGCCATCCTGCAAATGGAAACGGCGA
7 CGCAGGGTTAGATGCTGCTTGAGACAGCGACAGAGGAGCCAAAAGCCTT-
8 3'), respectively, were synthesized, annealed, and used as the templates to generate
9 the fragment of intron 1 using the following primers: intron 1-left-F (5'-
10 AAGCTTGATTGTCATGGCCAGGTGAGTCGACGAGCAAG-3') and intron 1-
11 right-R (5'-CCATGGGATATCGCATGCCTGCAGGAATTCGATTG-3') by
12 employing the overlap extension PCR (OE-PCR) (26) method. The fragment was then
13 used as the template to amplify four parallel copies of intron 1 using the following
14 primers: *Hind*III-c1-F (5'-AAGTAAAAGCTTGATTGTCATGGCCAG-3') and *Sac*I-
15 c1-R (5'-AAGTAAGAGCTCCCATGGGATATCGCATGC-3') for intron 1-copy 1;
16 *Sac*I-c2-F (5-AAGTAAGAGCTCGATTGTCATGGCCAGGTG-3') and *Xba*I-c2-R
17 (5'-AAGTAATCTAGACCATGGGATATCGCATGC-3') for intron 1-copy 2; *Xba*I-c3-
18 F (5'-AAGTAATCTAGAGATTGTCATGGCCAGGTG-3') and *Sac*I-c1-R for intron
19 1-copy 3; *Sac*I-c2-F and *Kpn*I-c4-R (5'-
20 AAGTAAGGTACCCCATGGGATATCGCATGC-3') for intron 1-copy 4. These four
21 fragments of copies of intron 1 were double digested using the introduced restriction
22 enzymes and then ligated using the Mighty Mix DNA ligation kit (Takara) to generate
23 four parallel copies of intron 1 of *RbcS2* as *Hind*III/*Kpn*I fragment. The *RbcS2*
24 promoter fragment was generated by PCR from pHsp70A/*RbcS2*-cgLuc plasmid
25 using the primers *Kpn*I-*RbcS2*-Pro-F2 (5'-TAAGGTACCCCGGGCGCGCCA-3') and

1 *NdeI*-RbcS2-Pro-R2 (5'-CTTGGCCATATGTTTAGATGTTGAGTGACT-3'). The
2 obtained fragment containing four copies of intron 1 and *RbcS2* promoter were
3 digested by the *HindIII*-*KpnI* and *KpnI*-*NdeI* restriction enzyme couples, respectively,
4 and then inserted into the *HindIII*/*NdeI* sites of pHsp70A-ble-2A-RbcS2 plasmid,
5 generating the recombinant plasmid P_{AR4}::ble-2A::term expression vector.

6 The codon usage of *CrSSL3* and *CrSSL1* cDNAs was optimized for
7 *Chlamydomonas* (<http://www.kazusa.org.jp/codon>), and the codon-adjusted 1 × gp64-
8 tag (27) sequence was attached at the N-terminus. These sequences were synthesized
9 as *XhoI*/*KpnI* fragments. For the construction of the GS-linker-3 × gp64-tag fragment,
10 the sense and antisense single-stranded oligonucleotides GS-linker-1 × gp64-tag (5'-
11 ATGGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGCGGCTCCTGGAAGG
12 ACGCGAGCGGCTGGAGCATCAGCGGCTCCTGGAAGGA-3') and 2 × gp64-tag
13 (5'-
14 CGTGCCCTCAGTGGATCCTTATTAGCTCCAGCCGCTCGCGTCCTTCCAGGA
15 GCCGCTCCAGCCGCTCGCGTCCTTCCAGGAGCCGCTGAT-3') were
16 synthesized, annealed, and used as the templates to generate the *KpnI*-GS-linker-3 ×
17 gp64-tag-*BamHI* fragment by employing the OE-PCR method with the following
18 primers: *KpnI*-GS-linker-3 × gp64-tag-F (5'-
19 AAGTAAGGTACCATGGGCGGCAGCGGC-3') and *BamHI*-3 × gp64-tag-R (5'-
20 CGTGCCCTCAGTGGATCCTTATTA-3'). The fragments of the *SSL3* and *SSL1*
21 cDNA cassettes and GS-linker-3 × gp64-tag were double digested by the *XhoI*-*KpnI*
22 and *KpnI*-*BamHI* restriction enzyme couples, respectively, and then cloned into the
23 P_{AR4}::ble-2A::term vector as an *XhoI*/*BamHI* fragment to generate the *SSL3*
24 transformation vector that is schematically shown in Fig. 1. The DNA sequences of all
25 the constructs were confirmed by direct sequencing using the dideoxy chain

1 termination method (28).

2

3 ***C. reinhardtii* strains, growth, and transformation conditions**

4

5 The *C. reinhardtii* strains CC-124 (wild type, mt^-) and CC-1690 (wild type,
6 mt^+) were provided by the *Chlamydomonas* Resource Center (Minnesota, USA), the
7 C-9 strain (wild type, mt^- , NIES-2235) was provided from the National Institute for
8 Environmental Studies (NIES, Japan), and the UVM4 and UVM11 strains (16) were
9 kindly provided by Dr. R. Bock (MPI-MP, Germany). Unless otherwise stated, cells
10 were cultivated mixotrophically at 25°C in Tris-acetate phosphate (TAP) medium (29)
11 under moderate and constant white fluorescent light ($84 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) with
12 gentle shaking. Nuclear transformation was performed using the electroporation
13 method (30). Briefly, the cells were grown to $1.0\text{--}2.0 \times 10^6$ cells/mL in TAP medium.
14 Subsequently, 2.5×10^7 cells were harvested by centrifugation and suspended in 250
15 μL of TAP medium supplemented with 50 mM sucrose (TAP/sucrose).
16 Electroporation was performed by applying an exponential electric pulse of 0.7 kV at
17 a capacitance of 50 μF (BTX, USA), using 300 ng of linearized plasmids purified by
18 agarose gel electrophoresis according to the manufacturer's instructions. The
19 transgenic strains were selected directly on TAP/agar plates containing zeocin (15
20 mg/L), and the plates were incubated under continuous fluorescent light ($20 \mu\text{mol m}^{-2}$
21 s^{-1}) at 25°C.

22

23 ***PCR screening and analyses of the transformants***

24 Primary antibiotic-resistant transformants were screened for the presence of
25 the expression cassettes by PCR as described previously (31, 32). In brief, cells (1.0--
26 5.0×10^6) were resuspended in Tris-EDTA solution and incubated at 98°C for 10 min.

1 Aliquots (1 μ L) of the supernatants from denatured cell lysates were then used as
2 template for 20 μ L PCR, using promoter-specific forward and gene-specific reverse
3 primers for *SQS* as previously described (19), and gene-specific forward primer (5'-
4 AGATGGAGGCCAAGTGCGTC-3') and terminator-specific reverse primer (5'-
5 CCGCTTCAGCACTTGAGAGCA-3') for *SSL3*. The amplification conditions were
6 as follows: 98°C for 5 min; followed by 30 cycles at 95°C for 15 s, 58°C for 30 s, and
7 72°C for 30 s; and a final step at 72°C for 7 min using a Thermal Cycler 2720
8 (Applied Biosystems).

9 For semi-quantitative reverse transcription PCR, total RNA was isolated as
10 previously described (34) using TRIzol reagent (Molecular Research Center,
11 <http://www.mrcgene.com/>), according to the manufacturer's instructions. The purified
12 total RNA was treated with DNase I (Takara, Japan) to remove residual genomic
13 DNA contamination. First-strand cDNA was synthesized using an oligo(dT)18 primer
14 or random heptamers and PrimeScript reverse transcriptase (Takara) according to the
15 manufacturer's instructions. The cDNA fragment of *CrSSL1* was amplified by PCR
16 using a set of primers: *CrSSL1*-F (5'-ATGACTATCAAGCGCCTGCAGAG-3'), and
17 *CrSSL1*-R (5'-CCGCTTCAGCACTTGAGAGCA-3').

18

19 ***Western blot screening of the transformants***

20 Total cell extracts and soluble fractions were prepared as previously described
21 (19, 33). For immunoblot analysis, proteins were separated on 12% SDS-PAGE and
22 immunoblotted with the monoclonal anti-baculovirus envelope gp64 polypeptide
23 antibody (1/5,000) (eBioscience, CA, USA) for detecting the gp64-tagged proteins,
24 and anti-Histone H3 antibody (1/10,000) (Abcam, CA, USA) for detecting Histone

1 H3 protein. The ECL detection system (Millipore) was used to detect immunoreactive
2 proteins by utilizing anti-mouse or anti-rabbit secondary antibodies (1/20,000).

3

4 ***Spotting test to compare the relative zeocin resistance of the transformants***

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

11

12 **Results and Discussion**

13 ***Enhanced nuclear transgene expression in a “met1” mutant of *Chlamydomonas****

14 To date, two cytosine-specific DNA methyltransferases (i.e., *Cre10.g461750*,
15 and *Cre12.g484600*) have been detected in the *Chlamydomonas* genome sequence
16 (*Chlamydomonas* genomic information features v5.3.1). We isolated an insertion
17 tagged mutant in *Cre10.g461750* tag mutant (20) (Fig. S1). The enzyme encoded by
18 *Cre10.g461750* was expected to function at the DNA replication foci because of the
19 presence of a protein domain (IPR022702, <http://www.ebi.ac.uk/interpro/IPR022702>)
20 that functions to target this enzyme toward the replication foci (35). Thus, this gene
21 probably encodes a maintenance-type DNA cytosine methyltransferase 1 (*Dnmt1* or
22 *Met1*), while the other one may encode a *de novo* cytosine methyltransferase. In this
23 tagged mutant, the transcript was under detection level by semi-quantitative reverse
24 transcription PCR analyses, whereas it was evidently detected in the mother strain
25 CMJ030 (Fig. S2). This strain contains an additional insertion whose position has not

1 been identified because of the complex tag insertion and deletion detected by the
2 sequence data of RESDA-PCR (36) products (data not shown). Therefore, the
3 behavior of this tagged mutant is due to the disrupted *MetI* gene or unidentified
4 gene(s). Considering this fact, we refer to this tagged mutant as the “*metI*” mutant.

5 The main characteristics of this tagged mutant may be different from those expected
6 from the mutation of the maintenance type cytosine methylase; therefore, this tagged
7 mutant has been here referred to as the “*metI*” mutant. Interestingly, the growth of
8 this tag mutant in TAP was not different from that of the mother strain (data not
9 shown). This suggests that the loss of DNA methylation has no significant effect on
10 transcriptional repression required for homeostasis and effective silencing of
11 transposons in *Chlamydomonas*.

12 In this study, the endogenous *SQS* gene was overexpressed in the “*metI*”
13 mutant and in wild-type CC-124 to compare the expression levels. The linearized
14 cDNA expression cassette was transformed by electroporation. Transformants were
15 selected on TAP/agar plates supplemented with 15 mg/L zeocin. Considering the
16 occurrence of decoupling through introduction, zeocin-resistant transformants were
17 further screened by PCR for the cointegration of the *SQS* cDNA expression cassette
18 with the marker gene *ble*. We considered these PCR-positive transformants as being
19 successfully cotransformed (Table 1). The PCR results showed that the
20 cotransformation ratio of *SQS* with *ble* in the “*metI*” strain was approximately 43.8%
21 (63/144) (Table 1 and Fig. S3A), which is not significantly different from that of the
22 wild-type CC-124 (109/210, 51.9%).

23 Western blotting was performed to analyze the protein accumulation levels of
24 the PCR-positive transformants. The results showed that the western-positive ratio

1 detectable after 10 min exposure for the *SQS* transformants in the “*metI*” strain was
2 18.9% (10/53) (Table 1 and Fig. S3B), which was 4 times higher than that in the CC-
3 124 strain (4.6%, 5/109) (Table 1). Moreover, the high protein accumulation ratio of
4 the western-positive transformants was 30.0% (3/10) for the “*metI*” strain (Table 1
5 and Fig. 2A), which was approximately 1.5 times higher than that in the CC-124
6 strain (20.0%, 1/5) (Table 1). This suggests the ability of “*metI*” to express the *SQS*
7 cDNA is comparable to that of the UVM strains, which have been demonstrated to
8 bear a high potential (16, 19).

9 We detected 21.2 % (11/52) and 17.6% (9/51) western-positive transformants
10 and 45.5% (5/11) and 44.4% (4/9) of highly *SQS*-expressing strains in UVM4 and
11 UVM11, respectively (19). The main advantage of using the “*metI*” strain over UVM
12 strains is that it possesses mating ability, which facilitates the accumulation of useful
13 traits by genetic crossing. Thus, the “*metI*” strain could be a novel promising host cell
14 for robust transgene expression (Fig. S4). The most probable reason for the high
15 western-positive ratio is the successful disruption of the transcriptional silencing
16 system caused by disabled *Dnmt1* gene, which is involved in the maintenance of DNA
17 methylation patterns (21, 22). However, frequently detected western-negative
18 transformants among PCR-positive transformants propose that the silencing ability
19 has not been completely knocked out in the “*metI*” strain. This is also the case of the
20 UVM strains (19). The retained silencing ability of the “*metI*” strain may be due to
21 DNA methylation-independent silencing pathways (12, 13).

22 The above results show that this tag-inserted mutant is one of the promising
23 hosts for plant cell factories. However, this strain bears another tag besides that at the
24 *MetI* gene. It is essential to generate a backcrossed strain that bears only one tag at
25 the *MetI* gene for a robust genetic background. Considering this observation, in this

1 study, expression analysis in “*met1*” was limited to the *Chlamydomonas SQS* gene.

2

3 ***Improved expression of heterologous SSL genes by the ble2A expression system in***
4 ***the UVM4 strain***

5

6 First, we attempted to overexpress *CrSSL3* in the wild-type CC-1690 and
7 UVM4 strains using an expression vector similar to that used for *SQS* expression, in
8 which the *SQS* ORF was replaced with *CrSSL3* ORF and the *ble* marker gene was
9 switched to *aadA*. However, no western-positive transgenic lines were found in the
10 large number of PCR-positive transformants, which accounted for 261 transformants
11 in CC-1690 and 294 in UVM4. This situation contrasts with that of *SQS* for which
12 western-positive transformants were easily found (Table 1). Our previous success for
13 *SQS* expression and current failure to find the western-positive *SSL3* transformants in
14 the UVM4 strain clearly demonstrate that this strain is not a useful strain to
15 heterologously express versatile genes of interest.

16 Then, we tested the *CrSSL* genes expression using the ble2A system to
17 investigate whether it could be useful for heterologous expression of hydrocarbon
18 production-related *Botryococcus* genes. We subcloned the *CrSSL3* or *CrSSL1* cDNA
19 into the ble2A nuclear expression vector to generate ble2A-SSL fusion ORFs. The
20 fused ORFs were placed under the control of the modified *Hsp70A/RbcS2* promoter
21 (PAR4), which was revised to contain four copies of the first intron of *RbcS2* between
22 the *Hsp70A* and *RbcS2* promoters (Fig. 1B). Then, the linearized plasmids were
23 introduced into the wild-type CC-1690 and UVM4 strains. The cotransformation- and
24 western-positive ratios for the *SSL3* in the UVM4 strain were approximately 51.3%
25 (74/144) and 10.4% (5/48), respectively, which were 1.5 times and 5.0 times higher

1 than those in the CC-1690 strain [34.0% (49/144) and 2.0% (1/49)] (Table 2, Fig. S5).
2 Moreover, the protein accumulation ratio in the western-positive transformant was
3 40.0% (2/5) in the UVM4 strain, whereas no *SSL3* strongly expressing transformants
4 (0/1) were found in the CC-1690 strain (Table 2 and Fig. 2B). This intimates the high
5 ability of the *ble2A* system for heterologous expression of cDNAs.

6 In *ble2A-CrSSL3* transformants of UVM4, the fused products were
7 specifically detected in highly *SSL3*-accumulating transformants, whereas in *ble2A-*
8 *CrSSL1* transformants of C-9 (the wild-type), the fused products were detected even
9 in weakly *CrSSL1*-expressing transformants (Fig. 2B, Fig. S6B). These results
10 suggest that ribosome skipping efficiency at the *ble2A* coding region is strongly
11 affected by the following ORF or by the different characteristics of the strains used.
12 Moreover, we found that the ribosome-skipping efficiency of the *ble2A-SSL3* was not
13 uniform even among the transformants (Fig. 2B). Transformant 3 is one of the highly
14 *SSL3*-expressing transformants; in this transformant, the fused protein level was
15 almost identical to that of the processed *SSL3*, whereas in transformant 5, which also
16 highly expresses *CrSSL3*, the level of the fused product prominently exceeded that of
17 the processed protein. Interestingly, in weakly expressing transformants, no fused
18 protein was detected. The sequence of FMDV 2A induces ribosome-skipping during
19 translation to generate discrete products from single ORFs (17, 37, 38), in which the
20 translational release factors, eRF1 and eRF3, play an important role (39). However,
21 the relation between the expression level of the protein and the ribosome-skipping
22 ratios remains unknown.

23 Through western analyses of the transformants, we showed that *SSL3*-positive
24 transformants were much more frequently detected in the UVM4 strain (5/48) than in
25 the wild-type strain CC-1690 (1/49). This result is in agreement with our previous

1 experiments showing the efficient expression of the *SQS* cDNA from
2 *Chlamydomonas* in the UVM4 and UVM11 strains (19). Therefore, UVM strains are
3 much better hosts than wild-type strains, albeit not ideal, for the expression of various
4 types of heterologous cDNAs.

5 In our previous experiment in which the *Hsp70A/RbcS2* promoter was used to
6 stimulate the above-mentioned *SQS* cDNA, the expression levels varied prominently
7 among transformants even in the UVM strains. Therefore, in this study, we used a
8 modified *Hsp70A/RbcS2* promoter (PAR4), which contained four introns to enhance
9 the transcriptional ability. However, prominent variation of transgene expression
10 levels was still observed as in the case of the non-modified *Hsp70A/RbcS2* promoter
11 (Fig. 2B, Fig. S6B), and the effects between the two promoters were not significantly
12 different. This suggests that the PAR4 promoter is still not sufficiently strong to
13 overcome the remaining variation of transgene expression levels in the UVM4 strain,
14 regardless of its apparently alleviated silencing ability.

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

19

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

22

23 To compare the ble2A-CrSSL1 mRNA levels, total mRNA was isolated from C-9
24 (wild-type) and transformants of ble2A-CrSSL1 that strongly (SSL1-5 and -34) and
25 weakly (SSL-7 and -39) expressed CrSSL1 (Fig. S7). The results of RT-PCR clearly

1 showed that the relative abundance of ble2A-CrSSL1 mRNA was higher in SSL1-5
2 and -34 than in SSL-7 and -39 (Fig. 3). Thus, the data shows that the differences in
3 the SSL1 protein levels are attributable mainly to variation in the mRNA levels.
4 *Chlamydomonas* has no RNA-dependent RNA polymerase gene (40) that is essential
5 to exert strong RNA interference (41). Therefore, the observed variation in transgene
6 expression might be mainly due to position-dependent levels of transcriptional gene
7 silencing (TGS) but not due to post-transcriptional gene silencing (PTGS), which has
8 been suggested to be the main factor in position-dependent differences in single
9 transgene expression in *Arabidopsis* (42, 43).

10

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

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

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

1 transformants by spotting transformant cells on agar plates containing various zeocin
2 concentrations.

3 First, we confirmed that all the transformants chosen for analysis showed no
4 significant growth differences on non-drug-containing TAP plates (Fig. 2C). UVM4
5 transformants 3 and 5 (UVM4-3 and UVM4-5, respectively) were expected to show
6 relatively rapid growth on plates containing zeocin at high concentration, because
7 they accumulated relatively high levels of SSL3 protein compared to other
8 transformants. As expected, UVM4-3 showed the best growth on the plate containing
9 120 mg/L zeocin, whereas the growth of UVM4-5 was slightly but significantly
10 slower than that of UVM4-1 on the plates containing 30 mg/L or 60 mg/L zeocin, of
11 which the SSL3 expression level was prominently lower than that of UVM4-5 (Fig.
12 2). Moreover, no transformants survived on the plate containing 200 mg/L zeocin.
13 These results show that the correlation between the target protein expression levels
14 and the zeocin-resistance levels is not so strict in the transformants obtained using the
15 *ble2A* expression system. A similar result was observed in *CrSSL1* expressing C-9
16 wild-type transformants (Fig. S6). The transformants accumulating SSL1 protein at
17 low levels (e.g., SSL1-39) showed growth rate not apparently different from that of
18 the highly expressing transformants (e.g., SSL1-34) at the various zeocin
19 concentrations (Fig. S6). Consistent with the results of these analyses, we failed to
20 sort highly target protein-expressing transformants of *CrSSL1* among the
21 transformants survived on the plate containing 120 mg/L zeocin, irrespective of the
22 drastically decreased number of transformants on the plates (data not shown).
23 Therefore, zeocin-resistance screening does not appear to be a practical method to
24 identify the transformants highly expressing target proteins.

25 The above is also the case for the two independent expression cassette system,

1 i.e., target gene expression cassette and marker gene expression cassette. Using the
2 expression plasmid containing the independent cassettes of *CrSSL1* and *ble* in
3 vicinity, we could not obtain the *SSL1*-highly expressing lines among the
4 transformants appeared on the plate containing 120 mg/L of zeocin (data not shown).

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

10 Further studies are essential to unveil the mechanism of action of the ble2A
11 system and to explain why it works efficiently in overcoming the silencing of
12 heterologous transgenes. The combinational use of the ble2A nuclear expression
13 vector and UVM4 or “*met1*” strains for foreign gene expression could enhance the
14 utility of *Chlamydomonas* as plant cell factory for producing biofuel,
15 biopharmaceuticals, and other valuable compounds.

16

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25

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7

8 **Figure legends**

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

18

19 **Figure 2.** Expression levels of SQS and SSL3-positive transformants analyzed by
20 western blotting. (A) The expression levels of SQS-positive transformants in the
21 “*met1*” strain are shown in the upper gel, while the lower gel shows the result of
22 histone H3 in which equal amounts of proteins were loaded. (B) The expression of
23 SSL3-positive transformants in the CC-1690 and UVM4 strains (1 through 5) is
24 shown. The amount of the proteins in the bands was quantified using the Image J
25 software (<http://imagej.nih.gov/ij/>). The band intensities are shown as relative

1 intensity units to the positive controls, which are one of the SQS transformants in the
2 UVM4 strain (19) for (A) and histone H3 for (B). Signal intensity values above 1.0
3 for SQS and above 0.3 for SSL3 were attributed to strongly expressing transformants
4 and marked with asterisks. The arrowhead indicates unprocessed fusion protein. (C)
5 Zeocin resistance spotting test for SSL3 western-positive UVM4 transformants.
6 Spotted cell numbers are shown below the spots.

7

8 **Fig. 3.** Comparative analysis of the ble2A-CrSSL1 mRNA levels by semi-quantitative
9 reverse transcription PCR. (A) RT-PCR results at the end of 30 cycles using cDNA
10 prepared from SSL1-7, -5, -34, -39, and C-9 (untransformed wild-type) for partial
11 amplification. Asterisks indicate the strains with high expression levels of CrSSL1
12 protein (see Fig. S5). +RT and –RT denote the reactions with and without reverse
13 transcriptase, respectively. (B) RT-PCR results at the end of 20 cycles, which show
14 that equal amounts of mRNA were used. An endogenous *CBLP* cDNA was amplified.
15 *CBLP*, *Chlamydomonas* β subunit-like polypeptide. See Fig. S6 for details.

Table 1. Expression of *SQS* cDNA cassette in various *C. reinhardtii* strains.

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%)
“ <i>met1</i> ”	63/144 (43.8%)	10/53 (18.9%)	3/10 (30.0%)

^aNumber of PCR-positive transformants/number of transformants analyzed.

^bNumber of Western blot-positive transformants/number of PCR-positive transformants.

^cNumber of high-protein accumulation transformants/number of Western blot-positive transformants.

^dWild-type.

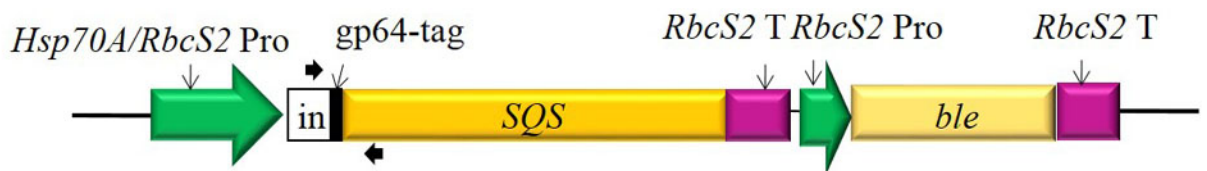
^eData were taken from our previous experiment in Kong et al. (19).

Table 2. Expression of SSL3 cDNA using the ble2A system in CC-1690 and UVM4 strains.

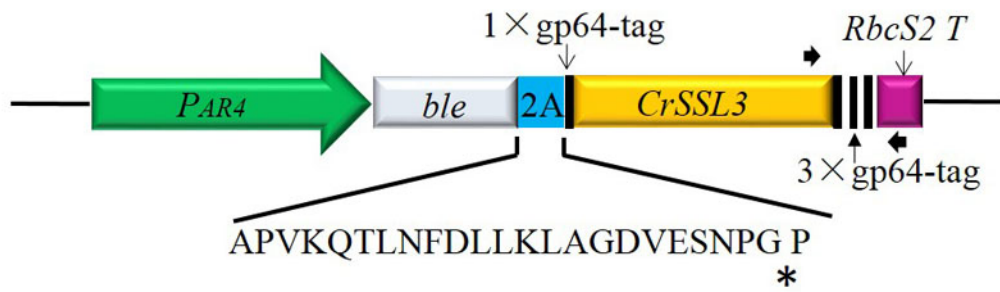
Host strain	Cotransformation ratio ^a	Western blot-positive ratio ^b	Ratio of highly expressing transformant ^c in Western blot-positive transformants
CC-1690 (WT ^d)	49/144 (34.0%)	1/49 (2.0%)	0/1 (0.0%)
UVM4	74/144 (51.3%)	5/48 (10.4%)	2/5(40.0%)

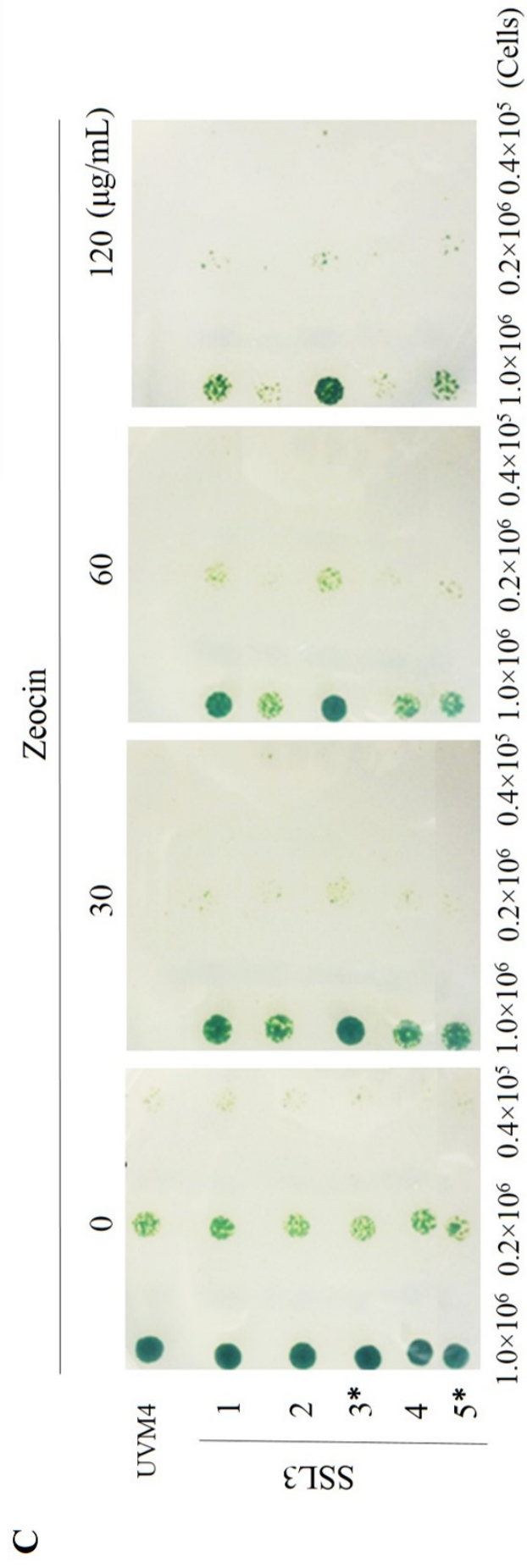
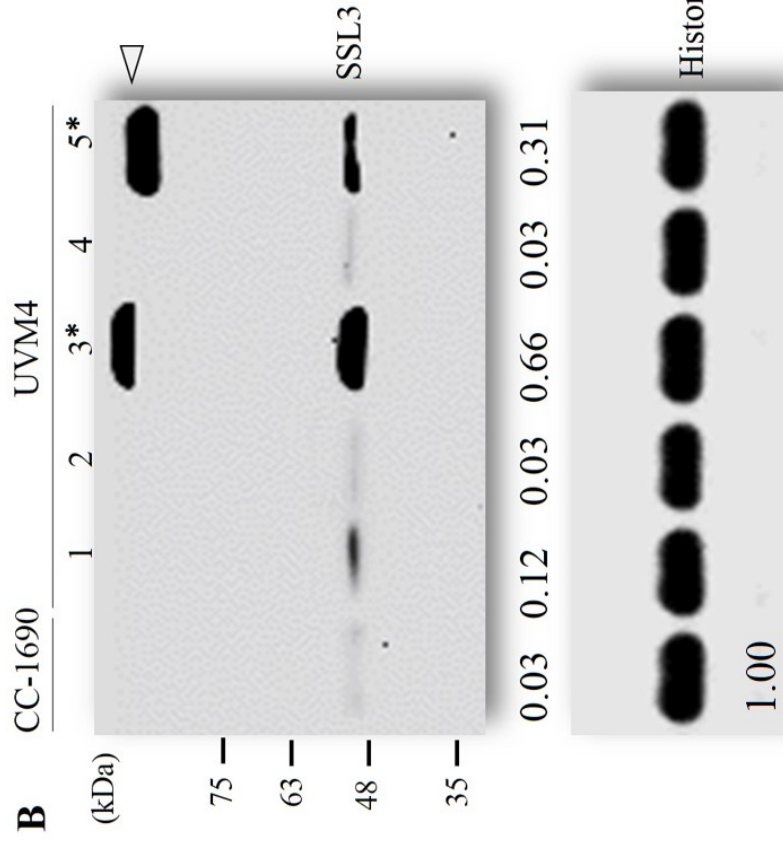
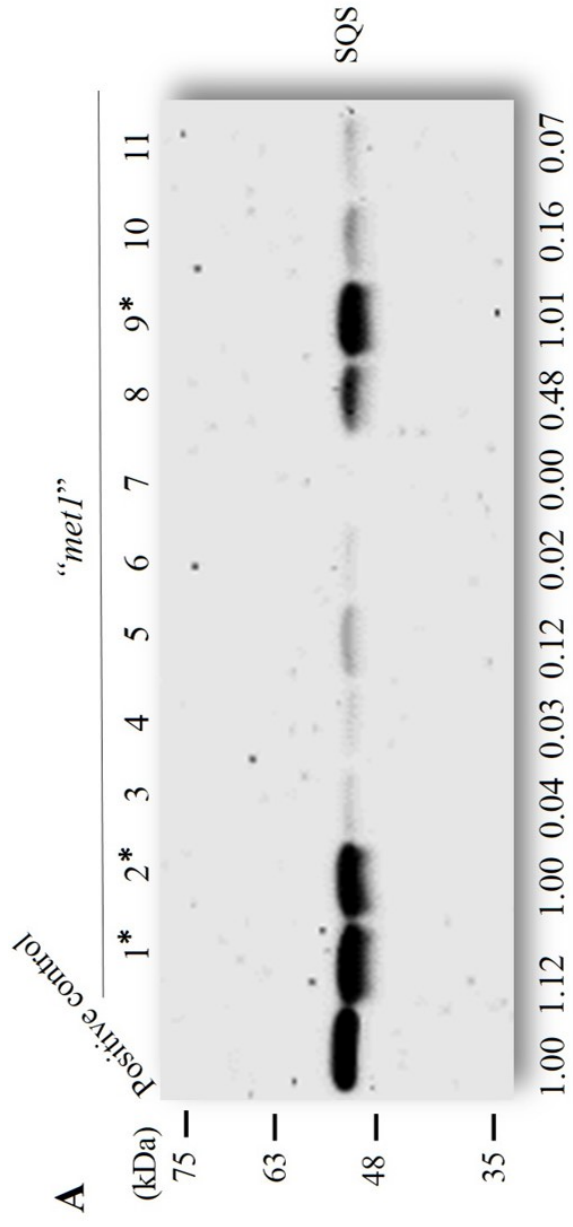
See the footnotes of Table1 for a, b, c, and d.

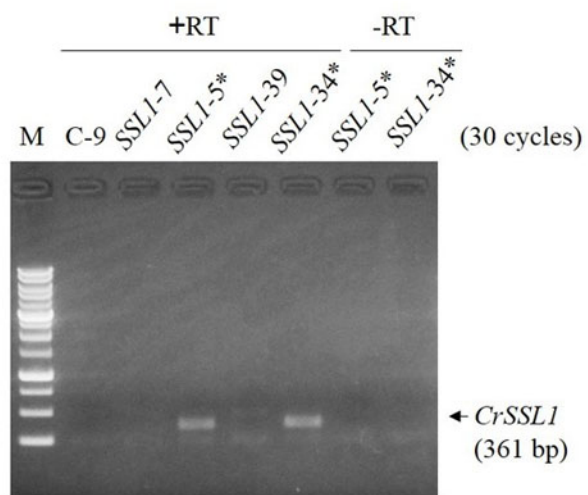
A



B





A**B**