Generation of *Chlamydomonas* strains for robust expression of nuclear transgenes

A Dissertation Submitted to Kochi University of Technology

in Partial Fulfillment of the Requirements

for the Degree of
Doctor of Engineering
by

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September, 2016

ABSTRACT

Chlamydomonas reinhardtii is a green freshwater microalga that can be a promising cell factory for the production of valued recombinant proteins because of its rapid growth in simple salt based media. However, a significant major drawback in the use of this species is the very poor expression of transgenes integrated into the nuclear genome, which is conceivably due to severe transcriptional silencing of transgenes irrespective of their genomic positions. In this study, we challenged to isolate *Chlamydomonas* mutants to overcome this disadvantage by conducting UV-mediated mutagenesis of a null tag-mutant of maintenance type DNA methyltransferase. We successfully obtained several mutants with a drastically enhanced ability to overexpress various transgenes, in which western signal positive transformants were detected very frequently and transgenes were expressed nearly uniformly high irrespective of the integrated location. As expected, most mutants had prominently elevated transformation efficiencies. *Chlamydomonas* must bear several pathways for transgene repression in addition to that involves the maintenance type DNA methyltransferase as a key role player, because we succeeded to isolate such strains by further mutagenesis of a maintenance-type DNA methyltransferase deficient mutant. This is in line with the fact that DNA methylation occurs infrequently in this species.

Keywords: *Chlamydomonas*, UV-mediated mutation, heterologous gene expression, transcriptional gene silencing, maintenance-type DNA methyltransferase.

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Chapter 1 Introduction

1.1 Background

Bacteria have been widely used as hosts for production of various recombinant proteins (e.g., Terpe, 2006). However, bacteria lack most of post-translational modification systems that are essential to confer full ability of eukaryotic proteins (e.g., Sahdev et al. 2008). As the alternative of bacteria, eukaryotic unicellular microalgae are recently considered as promising low-cost platform for production of eukaryotic proteins such as antibodies and antigens for vaccine (e.g., Spolaore et al. 2006; Rasala et al. 2012). Among unicellular green algae, *Chlamydomonas reinhardtii* (hereafter, *Chlamydomonas*) has attracted the interest of biologists for the accumulated genetic technics and its rapid growth in simple salts-based medium (Mussgnug 2015).

On the other hand, critical drawback of this alga is the disappointingly poor heterologous expression of nuclear transgenes. In *Chlamydomonas*, nuclear transformation occurs primarily by random insertion through non-homologous end joining (Zhang et al. 2014), while the expression level of the transgene is significantly affected by the inserted genomic region. This so called 'position effect' is known to be caused by the locally varied levels of transcriptional repression (Cerutti et al. 1997; Neupert et al. 2009b; Jinkerson and Jonikas 2015). In *Chlamydomonas*, severe gene silencing seems to exert over almost of the whole genome. Therefore, it is a laborious work to find transformants in which the expression cassette is happened to be inserted at a rare jackpot to accumulate the product in satisfactory high enough level. To overcome this disadvantage, several advances have been developed; i.e., codon usage optimization (Fuhrmann et al. 1999), utilization of enhancers and specialized promoters to boot up the transcription (Schroda et al. 2000; Ruecker et al. 2008), fusion ORF expression system with 2A peptide that induces ribosome skip (Rasala et al. 2012; Rasala et al. 2013; Plucinak et al. 2015). However, these maneuvers worked insufficiently to solve this obstacle.

Considering such difficulties, recently UV mutagenesis of *Chlamydomonas* was conducted to generate mutants (Neupert et al. 2009b), and the isolated UV-mutant strains such as UVM4 and UVM11 showed elevated expression ability for nuclear transgene expression (e.g., Lauersen et al. 2013; Lauersen et al. 2015). Currently, these two mutants are widely used as versatile tools. However, these strains are apparently not free of transgenetic gene silencing. In fact, codon optimized synthetic cDNA of *Botryococcus* SSL genes were not easily expressed even in these UVM-strains (Kong et al. 2014; Kong et al. 2015). In our previous study, through comparison of the expression ability in various *Chlamydomonas* mutants, we found a tag-DNA insertional mutant, which bears a tag at the *Chlamydomonas* unique maintenance type DNA methyltransferase 1 (here after, *met1*), shows significantly improved ability to express nuclear transgenes (Kong et al. 2015). The ability was not less than UVM4 and UVM11 (Kong et al. 2015).

However, as it is so in the UVM-mutants, this strain was also not completely free of transcriptional repression (Kong et al. 2015) regardless of the complete disruption of the unique maintenance type methyltransferase in *Chlamydomonas*, which is a key enzyme for epigenetic gene silencing (Babinger et al. 2001). Thus, up to now no general solution has been found to swiftly obtain the transformants that are accumulating the aimed protein in satisfactory high enough levels.

1.2 Research objective and outline

Considering that DNA methylation is the well-studied principle trigger for epigenetic transcriptional gene silencing while mutated genes in UVM4 and UVM11 are uncovered (Neupert et al. 2009b), we conducted further mutation of *met1* strain to develop dedicated *Chlamydomonas* strain in which nuclear transgenes are expressed robustly irrespective of the inserted genomic local position. To isolate such a strain, we selected transformants of *met1*-strain in which *ble-GFP* fused gene is expressed in low level to confer limited Zeocin resistance. Then, through UV-mediated mutagenesis of these strains, we selected UV-mutants that apparently showed higher Zeocin resistance as a sign for the alleviated epigenetic silencing.

In this work, we analyzed the basic characteristics of such UV-mutants of *met1*-strain to ascertain if these ones are useful as a platform for low-cost production of recombinant proteins.

First, low expressing *ble-GFP* transformant were selected then through mutagenesis of these strains, mutants that expressed *ble-GFP* in high level were selected as a sign for alleviated of epigenetic silencing. Analysis of transformation efficiency and expression level of several genes were performed to select the best mutant strain (**Figure 1-1**).

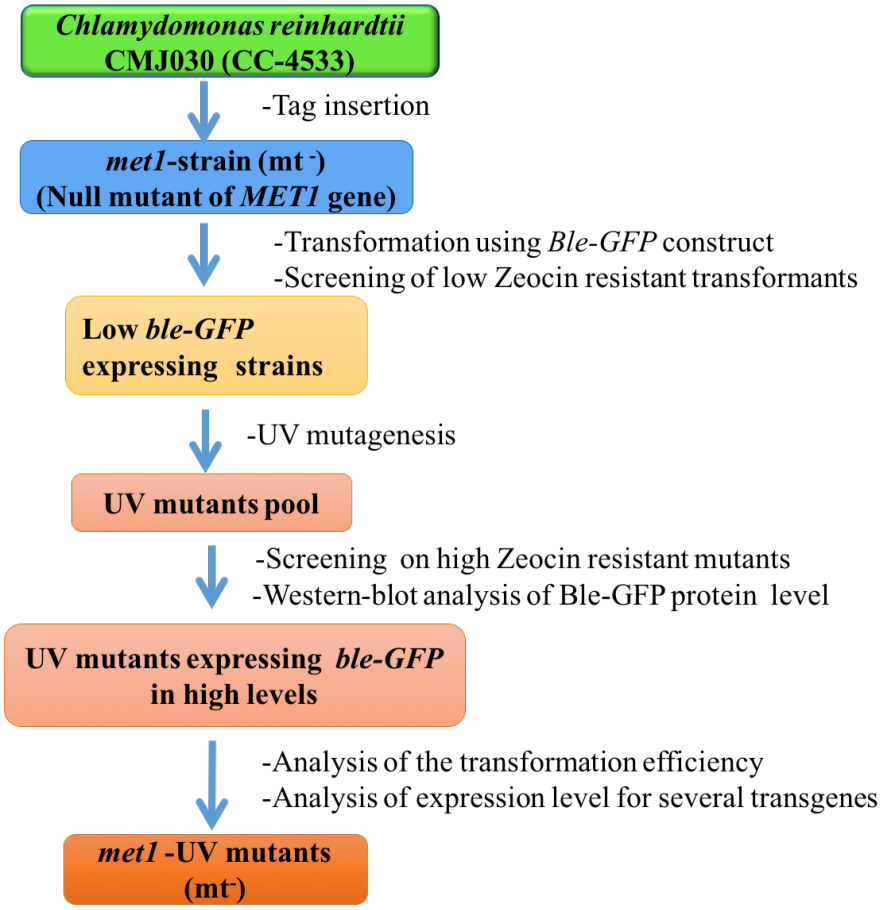

Figure 1-1. Outline of research strategy

Chapter 2 Materials and methods

2.1 *Chlamydomonas* strains and culture conditions

Cell-wall deficient strain CMJ030 (*cw15*, *mt*⁻) (deposited as CC-4533 at the *Chlamydomonas* resource center) and an insertional mutant of Cre10.g461750 (Zhang et al. 2014) that encodes DNA methyltransferase1 (Bird 2002; Fuks et al. 2003) were provided by Dr. Martin Jonikas (Carnegie Institution for Science, Stanford, CA, USA). UV-mutated strains, UVM4 and UVM11 (Neupert et al. 2009b) were by Dr. R. Bock (MPI-MP, Germany). These cells were cultured mixotrophically in Tris-acetate phosphate (TAP) medium at 25°C under constant white fluorescent light (84 mmol photons m^{-2} s^{-1}) with gentle shaking.

2.2 Construction of expression cassettes

Chlamydomonas reinhardtii squalene synthase (*SQS*) cDNA region was amplified using a primer set, SQS_*Bgl*II-F (5'-GATGAGATCTTCCTGGAAGGACGCGTCCG-3') and SQS_*EcoRI*-R (5'-CTCCAGAATTCTCACGCACGCCGCAGTACC-3') (restriction sites were underlined), and the *SQS* expression cassette containing plasmid as a template (Kong et al. 2015). Then, enzyme treated PCR product was used to replace the *VENU*S ORF region of p_Opt_Venus_Hyg expression cassette (Lauersen et al. 2015), and the generated construct was named p_ Opt_SQS_Hyg expression cassette. Therefore, these two expression cassettes share the promoter (*HSP70A/RBCS2* hybrid promoter) and the *RBCS2* terminator. Basic structure of these constructs are shown in **Figure 2-1**. To insert spectinomycin resistance cassette into pEK*RSP3* (radial spoke protein 3 gene expression cassette) (Kozminski et al. 1993), enzyme treated PCR product of *aadA* expression cassette containing *EcoR*V *and Xho*I restriction site was inserted at downstream of *RSP3* cassette, and generated construct was named pEK*RSP3*_aadA (Figure 2-1).

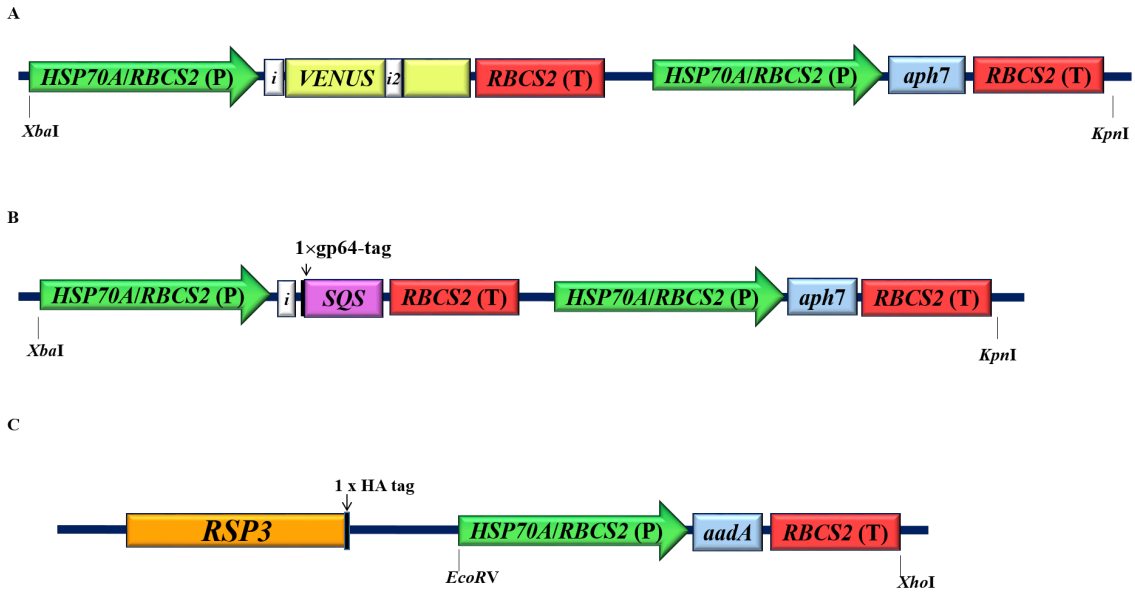

Figure 2-1. Constructs used in this study are shown schematically. A, *VENUS* expression cassette (p_Opt_Venus_Hyg) driven by *HSP70A/RBCS2* hybrid promoter; B, *Chlamydomonas* SQS expression cassette (p_SQS _Venus_Hyg plasmids) driven by *HSP70A/RBCS2* hybrid promoter; C, *Chlamydomonas* flagella *RSP3* (pEKRSP3_aadA) expression cassata driven by the own promoter. '*i*" and "*i2*" indicate first and second introns, respectively

2.3 Nuclear transformation

All transformations were performed by the electroporation method (Yamano et al. 2013) using Nepa 21 electroporator (Nepa gene, Chiba, Japan). About 200 ng of linearized plasmids were contained in a cuvette irrespective of the difference of the expression cassettes through this study. Transformants of *met1*-strain with linearized pMF59 (*Kpn*I/*Spe*I) that contains *ble-GFP* expression cassette (Fuhrmann et al. 1999), were selected on TAP plates containing 5 µg/mL Zeocin (Invitrogen). Among the obtained transformants, we selected the low Zeocin resistant ones as mother cells for subsequent UV-mediated mutagenesis (see below). To make linearized expression cassettes of p_Opt_Venus_Hyg and p_SQS _Venus_Hyg plasmids, *Xba*I and *Kpn*I were used, respectively, while pEK*RSP3*_aadA was treated by *Xho*I. Hygromycin B (100 µg/mL) or spectinomycin (100 µg/mL) was used to select the transformants.

To assess the transformation efficiency of *met1*-strain and the ones, pALM32 harboring the codon optimized *aadA* expression cassette (Meslet-Cladière and Vallon 2011) and pHyg3 containing *aph7* expression cassette (Berthold et al. 2002) were utilized.

2.4 Screening of appropriate *ble-GFP* transformants of *met1*-strain for UV-mediated mutagenesis

Through spotting test (Yamasaki et al. 2008) using various concentrations of Zeocin plates (0μg/mL, 5μg/mL, 10 μg/mL, 60 μg/mL, and 100 μg/mL), we isolated *ble-GFP* transformants of *met1*-strain which show comparatively low Zeocin resistance to select the severely silenced *ble-GFP* gene(s) harbouring transformants: We carried out 10-fold dilution of the stationary phase cells grown in TAP, then 5 μL of it was spotted on Zeocin plates to know the resistance level.

2.5 UV-mediated mutagenesis of low-level Zeocin resistant transformants

Low Zeocin resistant transformants of *met1*-strain were grown in liquid TAP medium until cell density reached to approximately 4×10^6 cell/mL, and cells were harvested by centrifugation (3,000 ×*g,* 5 min). The pellet was resuspended in 20 mL TAP to adjust the concentration to 5×10^6 cell/mL. Twenty milliliters of cells in a petri dish were exposed to UV-C light using a three UV-C tubes (15 Watt each) placed at 15 cm above the target cells. Three different durations (30, 60, and 120 sec) of UV-light exposure were conducted over gently shaking the plastic petri dish without the lid, and incubated for 24 hours in dark to minimize light-activated repair, then harvested by centrifugation. The pellets were resuspended in TAP, and 2.7×10^6 cells were spread on a TAP plate supplemented with 80 μg/mL Zeocin to select mutants that improved the expression level of the previously introduced *ble-GFP* expression cassette(s). For mutants that can grow on 80 μg/mL Zeocin plates, their antibiotic drug resistance levels were further analyzed by spotting the cells on higher concentrations of Zeocin plates.

2.6 Analysis of accumulated protein levels

To probe the accumulation levels of heterologously expressed gene products in the UV-mediated mutants of *met1*-strain, we used three expression cassettes, p_Opt_Venus_Hyg, p_ Opt_ SQS_Hyg, and pEKRSP3_aadA. Accumulated protein levels of VENUS, SQS, and a *Chlamydomonas* flagella radial spoke protein 3 (RSP3) protein were analysed by the western blotting method shown below. For quantification of VENUS protein level, the total soluble protein (TSP) extracts were prepared from 50 mL of late log phase culture. Cells were harvested by centrifugation (5,000×g, 5 min), and resuspended in 0.5 mL lysis buffer (10 mM PBS, 0.1% Tween-20, 1 mM phenylmethanesulfonylfluoride), then lysed by sonication using Sonics vivra cell sonicator (Sonics Materials Inc., CT. USA) (20% amplitude of the maximum level, for 2 min). Debris were sedimented by centrifuge at 16,000×*g* for 10 min at 4°C. Total soluble protein concentration was measured using Bradford reagent kit (Bio-Rad, USA) following the manufacturer's instructions. Samples containing 12 μg of TSP were denatured at 95°C for 5 min. To facilitate quantification of the accumulated Ble-GFP or VENUS protein in the TSP, we used a dilution series of standard recombinant GFP of the known concentration (see below).

On the other hand, to determine relative protein expression levels, total soluble proteins were prepared form about 3×10^6 cells in 20 μL of 2×SDS sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 1mM DTT; and 30% glycerol). The suspension was denatured at 95°C for 5 min, followed by centrifugation at 12,000×g for 2 min to precipitate the debris. Heat denatured protein (10 μL) were electrophoresed by denaturing 12.5 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond P PVDF 0.2; GE Healthcare, UK) using a wet-blotting apparatus (Trans-blot electrophoretic transfer cell, Bio-Rad, USA). Overnight transfer to the membrane was carried out using transfer buffer (25 mM Tris-base, 20% methanol, 0.01% SDS, and 192 mM glycine).

Immunobiochemical protein detection was performed using a monoclonal anti-GFP primary antibody (1/10,000 dilution) (ab186734; Abcam, CA, USA) to

detect GFP and also VENUS proteins, while anti-Histone H3 antibody (1/10,000) (ab1791; Abcam, CA, USA) was used for histone H3, which is used as an internal control to prove the equal loading of the total soluble protein. ECL detection system (Millipore) was used to detect immunoreactive proteins by applying anti-mouse or anti-rabbit or anti-rat secondary antibodies (1/20,000) (Sigma-Aldrich). To detect RSP3 protein conjugated with a HA-tag peptide, monoclonal antibody against HA rat (1/10,000) (3F10, Roche) was used. Anti-gp64-tag antibody (1/5,000) (eBioscience, USA) was used to detect gp64 conjugated SQS protein.

Moreover, accumulated levels of Ble-GFP fused protein in nucleus or VENUS in cytosol were estimated through microscopic observation of fluorescence using Olympus IX with mirror unit U-MNIBA3 (Olympus) for GFP, while Las X Widefield system fluorescence microscope (Leica microsystems, Germany) for VENUS.

2.7 Analysis of histone modifications

To analyze the acetylation and di-methylation levels of H3 histone, polyclonal antibody against di-methylated H3K4 (H3K4me2) (1/1,000) (ab7766; Abcam, CA, USA) and acetylated H3 (H3Ac) (0.1 μg/mL) (06-599; Millipore) were used for the western blot. Image Quant LAS-100 (Fuji film, Japan) was used to detect the ECL signal.

2.8 Comparative mRNA level analyses for UV-mediated mutants of *met1*-strain and the mother strains by RT-PCR.

To compare *ble-GFP* mRNA level using semiquantitative RT-PCR, total RNA was isolated as previously described (Yamasaki et al. 2008) using TRIzol reagent (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized by PrimeScript reverse transcriptase (Takara, Japan) using an oligo(dT)18 primer or random heptamers following the manufacturer's instructions. Limited part (about 200 bp) of the *Ble-GFP* cDNA was amplified by PCR using a set of primers: Ble-

F1036 (5’-GTCGTGTCCACGAACTTCCG-3’), and GFP-R1250 (5’-GAGAACTTGTGGCCGTTCACG-3’).

Chapter 3 Results and discussion

3.1 Screening of UV-mediated mutants of *met1*-strain

Generally maintenance type DNA methyltransferase (MET1) is one of the well-known key players to establish robust transcriptional gene silencing, which usually accompanies chromatin condensation (Babinger et al. 2001; Bird 2002; Fuks et al. 2003). *MET1* is the unique gene in *C. reinhardtii* for this role (Babinger et al. 2007). Kong et al. (2015) showed that *MET1* disrupted *Chlamydomonas* (*met1* strain) acquired the ability to express nuclear transgenes more strongly than wild types. In fact, heterologous expression of *Chlamydomonas SQS* cDNA was drastically improved in the *met1*-strain than the wild type strain CC-124 (Kong et al. 2015), and it was not less than UVM4 and UVM11 that were UV-mediated mutants of Elow47 transformant [derived from arginine autotrophic strain *cw15*-302 (cell-wall deficient, mt^+, *arg7*)] (Neupert et al. 2009b). However, *met1*-strain was not completely free of transcriptional gene silencing as exemplified by the widely varied levels of accumulated SQS protein among the transformants of *met1*-strain (Kong et al. 2015). These suggest *Chlamydomonas* harbours DNA methylation independent transgene silencing system(s).

Considering it, we executed UV mediated mutagenesis for *met1*-strain to generate strains that obtained characteristics to express nuclear transgenes in much higher levels through disruption or debilitation of the remained epigenetic gene silencing system(s). UV-mediated mutagenesis was carried out for selected transformants of *met1*-strain that harbouring strongly repressed *ble-GFP* gene, then Zeocin resistance lifted UV-mutants were selected as candidates of gene silencing relieved mutants. Product protein of Ble-GFP binds stoichiometrically to Zeocin to inhibit the DNA cleavage activity. Therefore, over wide range of Zeocin concentration, much strict positive correlation is expected between the *ble-GFP* gene expression level and the Zeocin resistant level. Considering the above, in this study *ble-GFP* fusion gene was chosen as a marker gene to assess the gene silencing state. Additional advantage of *ble-GFP* gene is that it allows to estimate the

expression level promptly through microscopic observation of the fluorescence intensity.

As appropriate mother strains for UV-mutagenesis, we decided to select transformants of *met1*-strain which express *ble-GFP* very weakly to show limited Zeocin resistance because of the strong gene silencing exerting on the transgene. We totally selected 76 *ble-GFP* transformants that can grow on 5 µg/mL Zeocin plates but cannot grow on 100 µg/mL Zeocin plates. Then, for 13 transformants, second screening was performed to access the closer antibiotic drug resistance level (Figure 3-1). Seven strains, No. 26, 40, 45, 47, 55, 57, and 63, showed relatively weaker Zeocin resistance among them (grow very slowly on 10 µg/mL, while the growth is faint on 60 µg/mL) (Figure 3-1), while in No. 26 and 55 and the Ble-GFP protein signals detected by preliminary western blot analysis were much larger than expected molecular weight (41 KDa), suggesting the insertion of unexpectedly modified expression cassette(s) (data not shown) in these transformants. For No. 63 transformant, we could not find swimming cells. Therefore, No.40, 45, 47 and 57 were utilized as mother cells to introduce further mutation(s) by UV-treatment.

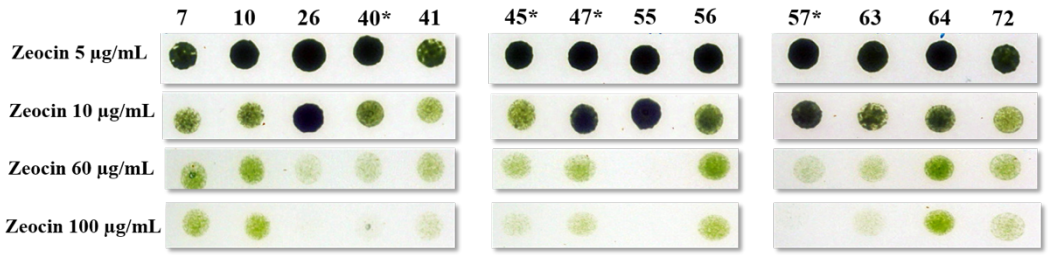

Figure 3-1. Spotting test to select limited expression of *Ble-GFP* transformant. Selected transformants as mother strain for UV mediated-mutagenesis are shown with asterisk (*).

In this study, these strains were represented with prefix of Ble(Low), [i.e., Ble(Low)-40, -45, -47, and -57]. Higher Zeocin resistance, brighter GFP fluorescence, increased Ble-GFP protein, and enhanced nuclear transformation rate than the mother strain was adopted as the expected characteristics of the mutants of alleviated gene silencing. As first selection of such UV-mutants, we carried out the renovated resistant level assay using various concentration of Zeocin plates, and

subsequently the Ble-GFP protein level was analyzed for mutants apparently showing increased Zeocin resistant level.

Through the spotting test, 41 mutants that grew on 150 μg/mL Zeocin plate were selected, and for them the relative accumulation levels of Ble-GFP were analyzed by western analysis. Significant increase of Ble-GFP level was detected for 4 mutants when their protein levels were compared with each of their mothers, i.e., *met1*(UVM)-40 [one of UV-mutants generated from Ble(Low)-40 transformant of *met1*-strain], *met1*(UVM)-47A [Ble(Low)-47 transformant of *met1*-strain], *met1*(UVM)-47B [also Ble(Low)-47 transformant of *met1*-strain] and *met1*(UVM)-57 [Ble(Low)-57 transformant of *met1*-strain] (Table 1, Figure 3-2). The GFP fluorescence of these 4 mutants were increased compare to their mother strains (

Figure **3-3**), this results were in line with western blot data. It is possible that these UV-mutants harbor mutation(s) on genes related to epigenetic gene silencing, and such mutation(s) enfeebled the strong repression exerting on the previously integrated *ble-GFP* gene to increase the product. Further basic characteristics of these mutants were assayed as shown below to confirm if these mutants are appropriate as platform for algal cell factory.

Table 1. Relative expression level of Ble-GFP in UV mutants

Strain	**Expression Level** (Relative level to mother strain)
Ble(Low)-40(mother strain)	1.0
***met1*(UVM)-40**	39.8
Ble(Low)-45 (mother strain)	1.0
***met1*(UVM)-45**	1.6
Ble(Low)-47(mother strain)	1.0
***met1*(UVM)-47A**	256.9
***met1*(UVM)-47B**	198.5
Ble(Low)-57(mother strain)	1.0
***met1*(UVM)-57**	69.7

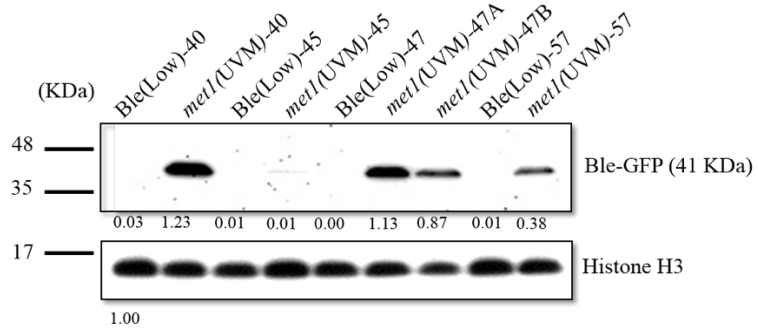

Figure 3-2. Accumulation level BLE-GFP in the five mutants of *met1*-strain and their mother strain for comparison. BLE-GFP levels (upper gel) were analysed by western blotting, while histone H3 level in the lower gel are internal control to prove the equal amount of loaded protein. Signal level were quantified using ImageJ software, and normalized using the H3 signal level of Ble(low) as 1.00.

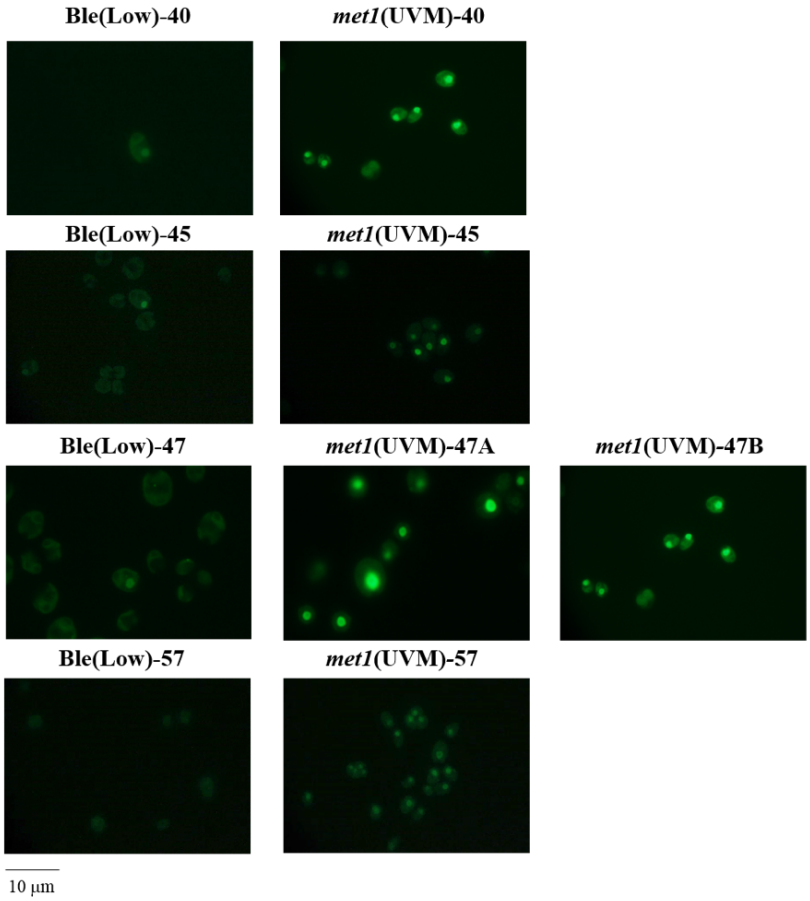

Figure 3-3. Microscopic observation of GFP fluorescence in four UV-mutants of *met1*-strain and their mother strain.

3.2 Transformation efficiency

Enhanced transformation ratio is one of the expected characteristics of the mutant harboring alleviated gene silencing capability. In such mutants, despite the transgene inserted genomic region, most of the intact transgenes are anticipated to confer the sufficient drug resistance to grow on the plate containing minimum concentration of antibiotic drug to kill the non-transformed cells. Based on such expectation, we assayed if obtained five mutants reveal higher transformation rates than each of their mother strains.

First transformation efficiency was assayed using codon adjusted *aadA* expression cassette driven by *Chlamydomonas HSP70A* promoter (Meslet-Cladière and Vallon 2011), which confers spectinomycin resistance. Moreover, to investigate the spectrum of drug resistant levels, number of transformants appeared on various concentration of spectinomycin plates were assayed. Four of the five UV-mediated mutants of *met1*-strain showed higher transformation ability than their mother strains, *met1*(UVM)-40, *met1*(UVM)-45, *met1*(UVM)-47A, and *met1*(UVM)-47B, despite the difference of the spectinomycin concentration (80 μg/mL, 100 μg/mL, 150 μg/mL): Apparently elevated transformation ratios were observed in *met1*(UVM)-45, *met1*(UVM)-47A, and *met1*(UVM)-47B (about 4-10 fold higher), while moderate increase was observed for *met1*(UVM)-40 (about twice). Unexpectedly, *met1*(UVM)-57 showed lesser transformation efficiency than the mother strain Ble(Low)-57 (about half) (**Figure 3-4**).

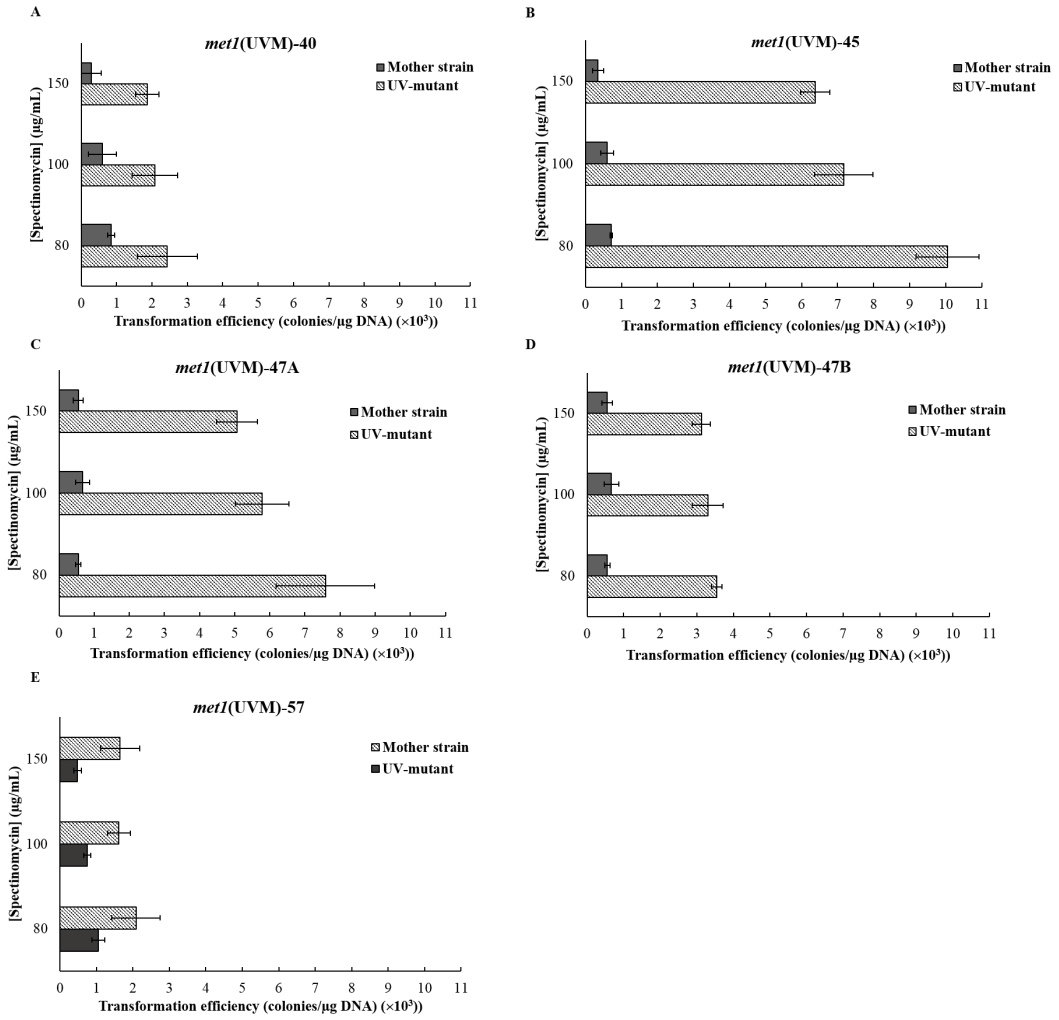

Figure 3-4. Transformation efficiencies of *aadA* casette in five mutants of *met1*-strain and the related strains. Transformed cells were spread on TAP agar of different spectinomycin concentration (80, 100, amd 150 μg/mL). Transformation ratios of *met1*-strain derived UV-mutant were compared with their mother strains.

Besides *aadA*, we also used *aph7,* which is driven by *Chlamydomonas* domestic *β*-tubulin promoter and confers hygromycin resistance (Berthold et al. 2002), to investigate the transformation ratio. The results were mostly in accordance with the *aadA* probed transformation ratio: Moderately increased *aph7* transformation ratios in *met1*(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-45 were consistent with the results of *aadA*. While apparently increased transformation ratio in *met1*(UVM)-57, and non-improved transformation ratio of *met1*(UVM)-40 were conflicting with the data obtained using the *aadA* (Figure 3-5). Such

disagreements are conceivably ascribing to the troublesome to prepare vital single cells for transformation, because *met1*-strain and all the derivative mutants have fairly short single cell period in their cell cycle (see below for detail).

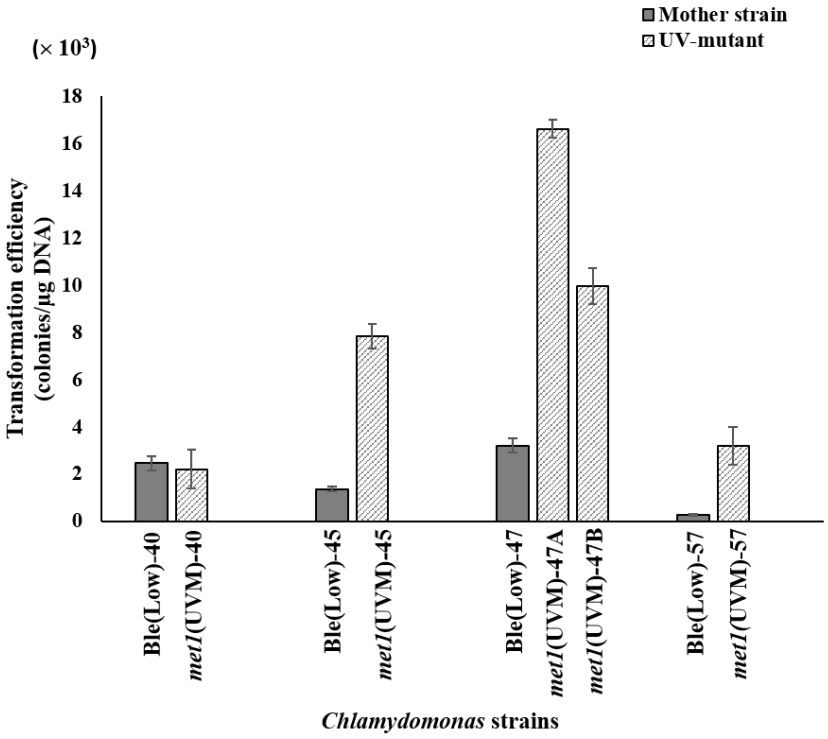

Figure 3-5. Transformation ratio of *aph7* expression casette in several UV-mutant of *met1*-strain and their mother strains.

Drastically increased transformation ratios in *met1*(UVM)-47A and -47B for *aadA* and *aph7* genes suggest these UV-mutants are very promising candidates for high expression of transgenes irrespective of their integrated locations. It is also noteworthy that transformation efficiency in ARGONOUTE 3 null tag-mutant (Yamasaki et al. 2015), which is one the key enzymes for RNAi, was not significantly different from that of a wild type (data not shown). This suggests RNAi in *Chlamydomonas* is strictly limited to post-transcriptional gene silencing but conceivably not linked to transcriptional gene silencing of the nuclear transgenes.

3.3 Expression levels of transgenes

The most critically required nature for our desired mutant is the ability to express transgene robustly irrespective of the integrated position in the nuclear genome. To directly assay this ability, we analyzed the expression levels of newly introduced genes, i.e., codon adjusted *VENUS* driven by *HSP70A/RBC2* hybrid promoter, *Chlamydomonas* domestic *SQS* gene also driven by *HSP70A/RBC2* hybrid promoter, and a domestic radial spoke protein 3 gene (*RSP3*) driven by the original promoter (Figure 1-1B) in *met1*(UVM)-45, *met1*(UVM)-47A, *met1*(UVM)-47B, *met1*(UVM)-57, and the related strains for comparison. As shown details in below, several *met1* UV-mutants that we isolated in this study showed high accumulation levels of these proteins. Therefore, these *met1*-UV mutants must have largely overcome the transgene expression problem, and very useful as an efficient production platform.

3.3.1 VENUS accumulation levels

We introduced a codon adapted *VENUS* gene controlled by a *HSP70*A/*RBC*S2 hybrid-promoter (Lauersen et al. 2015). Then, to roughly estimate the gene silencing alleviation level, we assayed the western-positive ratio in the randomly chosen PCR-positive transformants.

Among analysed 10 various strains, strong western signal was detected only in UVM4, Ble(Low)-47, *met1*(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-57 (Table 2). Furthermore, in *met1*(UVM)-47A, -47B, and -57, the western-positive transformation ratios were above 70%. This is in accordance with the enhanced accumulation of Ble-GFP in *met1*(UVM)-47A, -47B, and -57 (Table 1, Figure 3-2). While the ratio was below 20% in *met1*-strain, CC-4533 (mother cell of *met1* tag-mutant), Ble(Low)-45, *met1*(UVM)-45, Ble(Low)-47, and Ble(Low)-57 (Table 2). These data suggest that in these three *met1* UV-mutants, *met1*(UVM)-47A, -47B, and -57, transgene repression is working much weaker than the other strains throughout the genome.

Table 2. Expression of VENUS in various *Chlamydomonas reinhardtii* strains

Host strain	Co-integration ratio[a]	Western blot-positive transformant ratio in PCR positive[b]	Ratio of highly expressing transformants in western positive[c]
CC-4533 (Wild-type)	35/96 (36.5%)	5/33 (15.2%)	0/5 (0%)
UVM4	98/125 (78.4%)	16/98 (16.3%)	6/16 (37.5%)
met1-strain	53/96 (55.2%)	7/53 (13.2%)	0/7 (0%)
Ble(Low)-45 (mother strain)	32/96 (33.3%)	1/32 (3.1%)	0/1 (0%)
***met1*(UVM)-45**	43/144 (29.9%)	1/43 (2.3%)	0 /1 (0%)
Ble(Low)-47(mother strain)	30/96 (31.3%)	10/30 (33.3%)	2/10 (20%)
***met1*(UVM)-47A**	54/96 (56.3%)	26/54 (48.1%)	22/26 (84.6%)
***met1*(UVM)-47B**	25/48 (52.1%)	7/25 (28%)	5/7 (71.4%)
Ble(Low)-57(mother strain)	26/80 (32.5%)	0/26 (0%)	NA
***met1*(UVM)-57**	43/96 (44.8%)	12/43 (27.9%)	9/12 (75%)

[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, *i.e.*, the signal intensity above 1.2-fold relative to Ble-GFP signal in *met1*(UVM)-47A.

NA : Not applicable

We quantitatively analysed the deviation and average levels of accumulated VENUS protein by analysing five randomly selected transformants in the above three *met1*-UV mutant strains with shown higher performance, and also in their mother strains, *i.e.*, Ble(Low)-47 and Ble(Low)-57, and UVM4 for comparison. In one of the *met1*(UVM)-47A transformants, *met1*(UVM)-47A-22, the VENUS concentration reached to 0.22 % of the total soluble proteins (Figure 3-6E). To our knowledge, 0.25 % accumulation of fungal Xylanase (Rasala et al. 2012) using *ble*-2A-fusion protein expressing system in a wild-type CC-1690 and 0.25% of codon optimized HIV antigen P24 (Barahimipour et al. 2016) in UVM11 is the highest level of nuclear transgene products in *Chlamydomonas*, so 0.22 % is comparable to this level. Average accumulation level of VENUS in *met1*(UVM)-47A exceeded

that in UVM4. Moreover, it is noteworthy that the VENUS expression levels in *met1*(UVM)-47A were nearly uniformly high throughout the analysed five transformants (Figure 3-6). The level in *met1*(UVM)-47B seemed to be some extent lesser than UVM4, and it was accompanied by significant deviation (**Figure 3-6**F). Unexpectedly, non-mutagenized Ble(Low)-47 revealed some elevated potency to express *VENUS*, which is apparently higher than that of Ble(Low)-57. This suggests that Ble(Low)-47, which is the mother cell of *met1*(UVM)-47A and -47B, had been acquired some desirable mutation(s) through *ble-GFP* gene transformation process preceding the UV-mutagenesis.

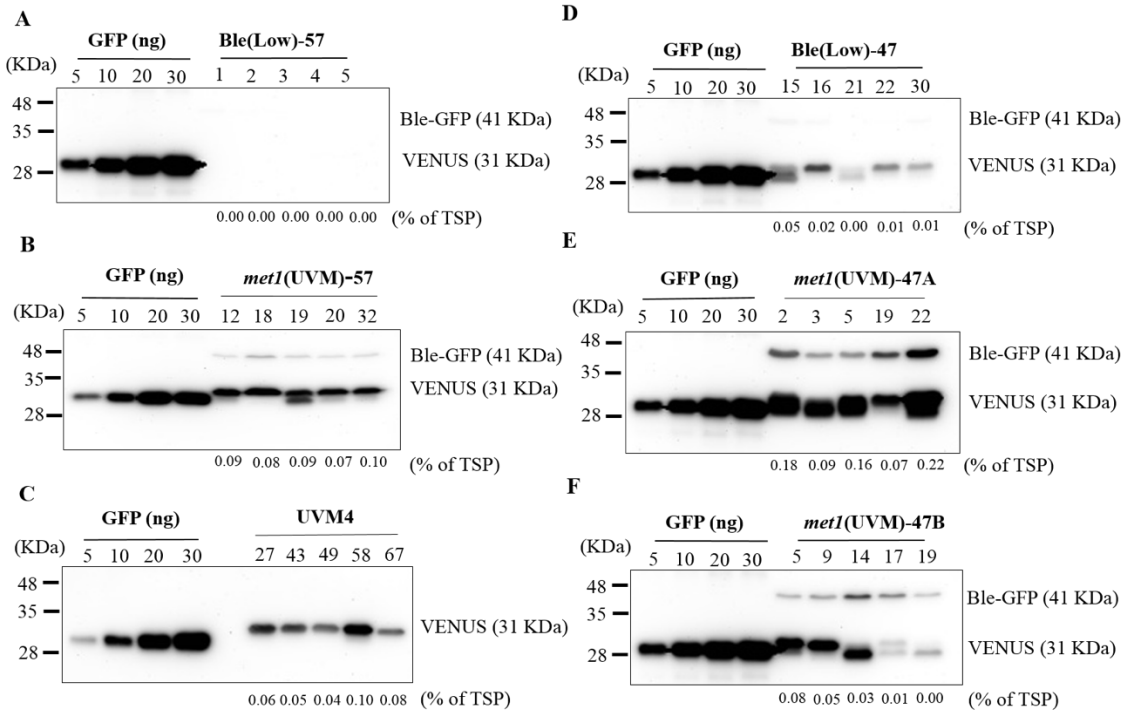

Figure 3-6. Accumulation levels of VENUS in randomly selected five transformants from each of the three UV-mutants of *met1*-strain and the related strains. 12 μg of total soluble protein (TSP) were loaded in each lane. Dilution series of standard GFP protein (5, 10, 20, amd 30 ng) was included for quantification of VENUS. Based on signal level comparison, weight percent of the VENUS in TSP were calculated, and shown below of each lane. To calculate it, if the signal if doublet, to signal strengths are joined into one.

To ensure the western data of VENUS accumulation, we microscopically observed the fluorescence of it. Observed fluorescence levels were agreeable with the western results (Figure 3-7).

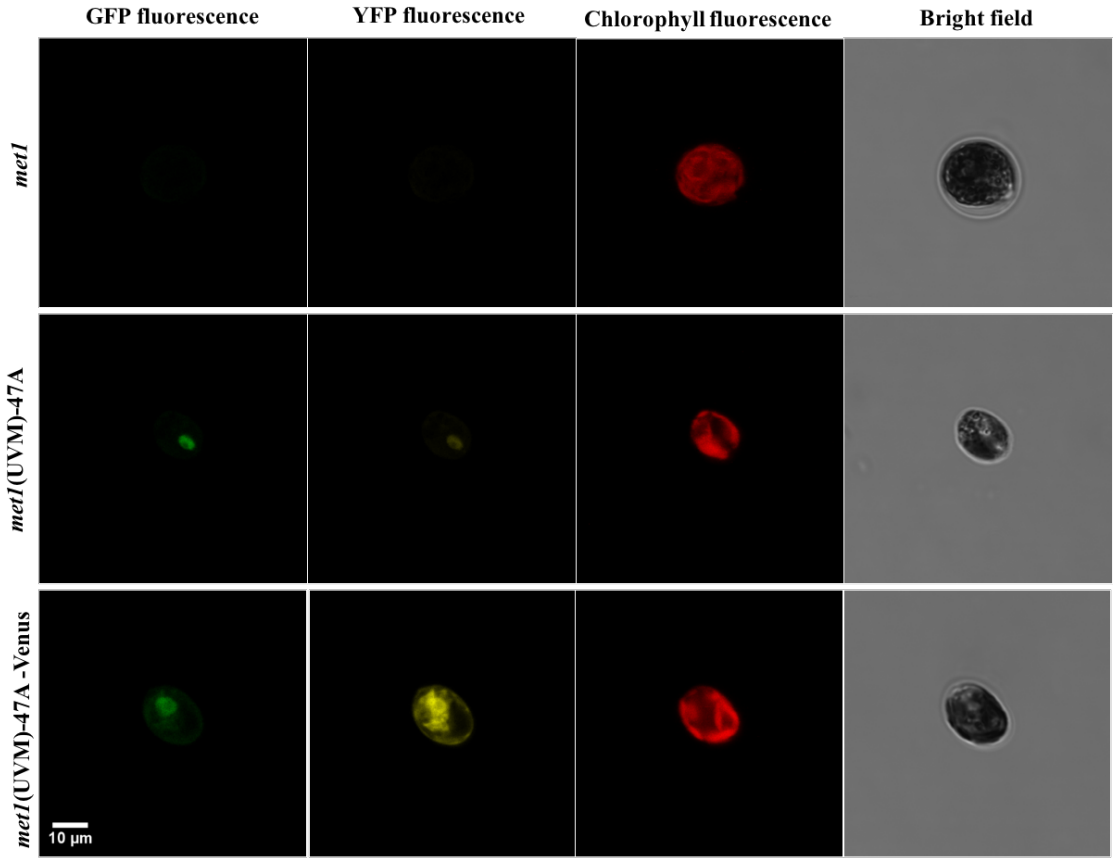

Figure 3-7. Microscopic observation of GFP and VENUS fluorescence of *met1*(UVM)-47A and *met1*-strain

3.3.2 Flagella radial spoke protein 3 accumulation levels

pEKRSP3 contains the cDNA of *Chlamydomonas* flagella radial spoke protein 3 (RSP3) with modification to contain single HA-epitope tag at the C-terminus, which expression is controlled by the original promoter (Kozminski et al. 1993). Linearized pEKRSP3 was introduced to three UV-mutants of *met1*-strain, *met1*(UVM)-47A, -47B, and -57, and also the related 5 strains, CC-4533, UVM4, *met*-strain, Ble(Low)-47, and Ble(Low)-57. Western-positive *RSP3* transformants were readily obtained in all of the above strains, while strongly *RSP3* expressing

ones were very frequently obtained in the three UV-mutants of *met1*-strain. The ratios were 73.9%, 59%, and 47.6% for *met1*(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-57, respectively. On the other hand, it was limited to about 20-40% in the non-*met1*(UVM) strains, CC-4533, UVM4, *met1*, Ble(Low)-47, Ble(Low)-57 (**Table 3**). Accordingly, high accumulation of RSP3 protein in *met1*(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-57 were proved by *RSP3* as well as Ble-GFP and VENUS.

3.3.3 SQS protein accumulation levels

For further investigation of transgene expression ability in the UV-mutants of *met1*-strain, we introduced *Chlamydomonas* SQS cDNA expression cassette under the control of *HSP70A*/*RBCS2* hybrid promoter (**Figure 2-1**B). SQS has enzymatic activity to convert FPP (farnesyl diphosphate) to squalene (Kajikawa et al. 2015). Strong western signals were detected only in the transformants of *met1*(UVM)-47A, -47B, -and -57 with the frequency of about 48%, 25%, and 50%, respectively, while it was not observed in CC-4533, UVM4, *met1*-strain, Ble(Low)-47, and Ble(Low)-57) (**Table 4**). Therefore, observed transgene expression ability for SQS expression cassette in these strains was consistent with the results of VENUS (Table 2) and RSP3 (**Table 3**).

Table 3. Expression of radial spoke protein3 (RSP3) in various *Chlamydomonas reinhardtii* strains.

Host strain	Co-integration ratio[a]	Western blot-positive transformant ratio in PCR positive[b]	Ratio of highly expressing transformants in western positive[c]
CC-4533 (Wild-type)	34/96 (35.4%)	12/34 (35.3%)	3/12 (25%)
UVM4	50/96 (52.1%)	18/44 (40.9%)	7/18 (38.9%)
met1-strain	37/96 (38.5%)	14/37 (37.8%)	4/14 (28.6%)
Ble(Low)-47 (mother strain)	49/96 (51.0%)	16/44 (36.4%)	4/13 (30.8%)
***met1*(UVM)-47A**	44/96 (45.8%)	23/44 (52.3%)	17/23 (73.9%)
***met1*(UVM)-47B**	45/96 (46.9%)	22/44 (50%)	13/22 (59%)
Ble(Low)-57 (mother strain)	36/96 (37.5%)	10/36 (27.8%)	2/10 (20%)
***met1*(UVM)-57**	40/96 (41.7%)	21/40 (52.5%)	10/21 (47.6%)

[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, *i.e.*, the signal intensity above 1.2-fold relative to CC-124 strain harbouring *aph7*-2xHA tag.

Table 4. Expression of squalene synthase (SQS) in various *Chlamydomonas reinhardtii* strains.

Host strain	Co-integration ratio[a]	Western blot-positive transformant ratio in PCR positive[b]	Ratio of highly expressing transformants in western positive[c]
CC-4533 (Wild-type)	15/35 (42.9%)	0/15 (0%)	NA
UVM4	22/58 (37.9%)	0/22 (0%)	NA
met1-strain	30/96 (31.3%)	3/24 (12.5%)	NA
Ble(Low)-47 (mother strain)	42/96 (43.8%)	9/40 (22.5%)	NA
***met1*(UVM)-47A**	58/124 (46.8%)	25/50 (50%)	12/25 (48%)
***met1*(UVM)-47B**	41/136 (30.14%)	9/30 (30%)	4/10 (25%)
Ble(Low)-57 (mother strain)	17/48 (35.42%)	0/17 (0%)	NA
***met1*(UVM)-57**	41/96 (42.7%)	8/41 (19.5%)	4/8(50%)

[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, *i.e.*, the signal intensity above 1.0-fold relative to CC-124 strain harbouring *aph7*-2xgp64 tag.

NA : Not applicable

3.4 Relationship between the levels of mRNA and accumulated Ble-GFP protein

One of the highly possible molecular mechanism for the limited nuclear transgene expression in *Chlamydomonas* is the transcriptional repression (Cerutti et al. 1997; Neupert et al. 2009b; Jinkerson and Jonikas 2015), while elevated expression of transgenes in the *met1*-UVM strains are conceivably owe to the alleviation of transcriptional repression. To confirm this scenario, mRNA levels of *ble-GFP* in

met1-UV mutants and those in their mother strains were compared. RT-PCR showed that the relative *ble-GFP* mRNA levels in *met1*(UVM)-47A and *met1*(UVM)-47B were around 10- and 5-fold higher than their mother strain Ble(Low)-47 respectively (Figure 3-8), while the protein levels were increased around 260 and 200 times for 47A and 47B, respectively (Table 1).

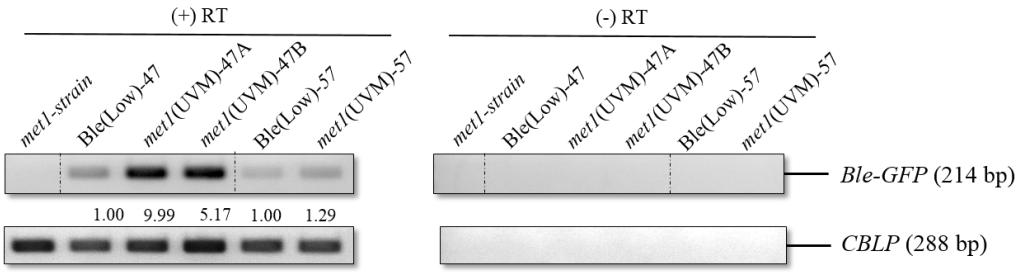

Figure 3-8. Comparative analysis of *Ble-GFP* mRNA level in UV-mutants of *met1*-strain by RT-PCR. RT-PCR products obtained at the end of 30 cycles using cDNA prepared from UV-mediated *met1*-strain and their parents. (+)RT and (-)RT denote the reaction with and without reverse transcriptase respectively.

This clearly shows that low accumulation of Ble-GFP protein in Ble(Low)-47 is apparently due to the limited amount of the mRNA, and through UV mutagenesis transcription of the previously integrated *ble-GFP* was enhanced. Moreover, it was shown that increase of mRNA has drastic effect to increase the product, since the product increase ratios overcome those of the mRNA in 20-26 times; e.g., 10-fold increase of mRNA resulted in 260-fold increase of the Ble-GFP product in *met1*(UVM)-47A. This is also the case for *met1*(UVM)-57: around 1.3-fold of the mRNA increase resulted in the around 70-fold of the product (Figure 3-8, Table 1).

3.5 Analysis of representative histone modification levels

We have shown that some UV-mutants of *met1*-strain can overexpress various transgenes, and in such strains transcription of previously introduced *ble-GFP* gene was prominently increased. UV-mediated malfunction at the histone modification

enzymes or chromatin condensation related proteins possibly able to confer such ability through limited heterochromatin formation to allow enhanced expression of transgenes integrated at any genomic position. Considering these, we analysed the histone H3 acetylation (H3Ac) and histone H3K4 di-methylation (H3K4me2) levels using polyclonal antibodies, both of which are representative histone modifications to activate transcription in *Chlamydomonas* (Bernstein et al. 2002; Casas-Mollano et al. 2008).

H3K4me2 levels were not significantly different in analysed five strains, Ble(Low)-47, *met1*(UVM)-47A, *met1*(UVM)-47B, Ble(Low)-57, and *met1*(UVM)-57. No significant increase of H3K4me2 levels were observed for *met1*(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-57 when their levels were compared with each of their mother strain Ble(Low)-47 or Ble(Low)-57 (Figure 3-9). While statistically significant increase of H3Ac level was detected in *met1*(UVM)-47A (**Figure 3-9**). So, it is possibly that *met1*(UVM)-47A bears mutation(s) in histone modification related enzyme(s) such as histone deacethylase. Currently, we have no evidential clue to explain the molecular mechanism for the enhanced transgene expression potential observed in *met1*(UVM)-47B and -57, because no substantial change of the H3Ac and H3K4me2 levels were detected in these strains.

The cooperative effect between DNA methylation and histone modifications is not closely analyzed in *Chlamydomonas*, however some transgene repressions seem to be DNA methylation independent (Cerutti et al. 1997). In fact DNA methylation levels in *Chlamydomonas* is limited to around 5.39% of CpG (Feng et al. 2010). Moreover, *met1*-strain that is a null mutant of unique maintenance type DNA methyltransferase apparently bear transgene repression ability (Kong et al. 2015). Therefore, histone modifications might be the main cue for heterochromatinization against transgenes. Casas-Mollano et al. (2009) have isolated a *Chlamydomonas* mutant defective in a Ser/Thr protein kinase, which phosphorylates histones H3 and H2A, to show the enzyme is necessary for heritable epigenetic silencing of transgenes and transposons.

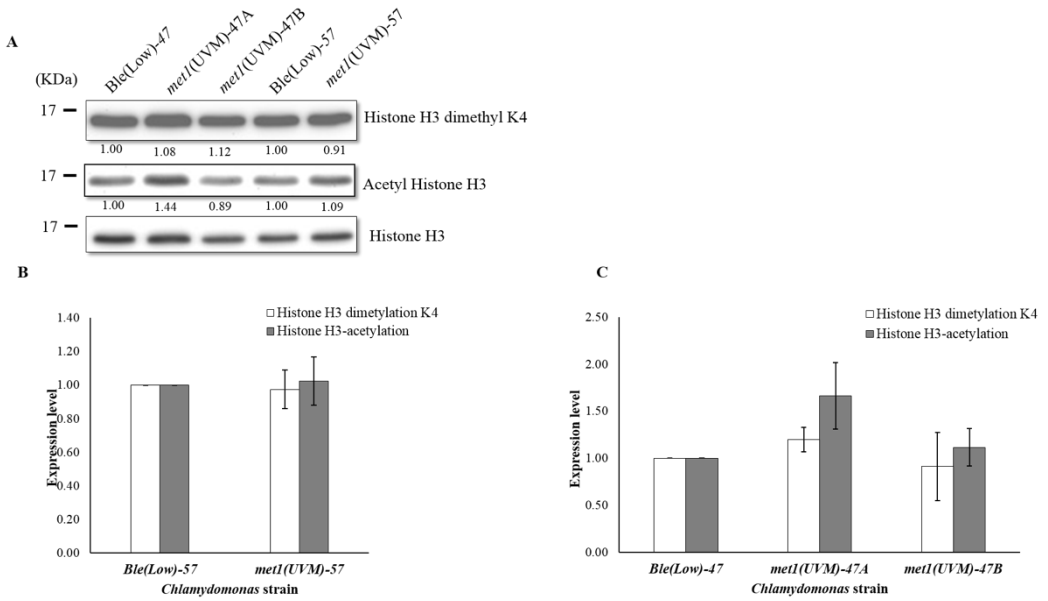

Figure 3-9. Relative H3 histone acetylation and H3 di-methylation at K4 in *met1*(UVM)-47A, -47B, -57, their mother strain Ble(Low)-47, and -57. A) Result of western analysis; B) Summarized results of western analyses that independently carried out five times. H3 histone signals were used as internal control to show the equal amount of protein loading.

Post translational histone modification and heterochromatinization systems must be indispensable for proper tuning of domestic genes. Generally, tag-insertion often generates null-mutants and only rarely yields mutations of decreased enzyme activity, while UV mutagenesis easily generates mutants of reduced enzyme activity. Most probably in related with it, Neupert et al (2009) failed to isolate mutants harbouring robust transgene expression capability by tag-induced mutagenesis. So, UV-mutagenesis might be a superior method to isolate mutants that can express transgenes robustly with keeping homeostatic stability in long term culture.

3.6 Relationship between the transformation efficiency and the accumulated transgene product levels

Relieved transcriptional repression exerting on whole nuclear genome should lead to the enhanced nuclear transformation ratio and the lifted product level at once. In this study, we showed it is robustly true for *met1*(UVM)-47A and -47B. These two strains revealed drastic increase of transformation efficiency for *aadA* (Figure 3-4) and *aph7* (Figure 3-5) with accompanying evidently increased protein levels for VENUS (Table 2), RSP3 (Table 3) and SQS (Table 4) were evidently increased. However, what makes the relationship more complicated one is that *met1*(UVM)-45 did not show the improved accumulation of VENUS and Ble-GFP (no data for SQS and RSP3) regardless of the significantly increased transformation efficiency. Moreover, *met1*(UVM)-57, which showed no increased *aadA* transformation efficiency and fairly modest increase for *aph7*, unexpectedly revealed the robustly increase of heterologously expressed VENUS (Table 2), RSP3 (Table 3), and SQS (Table 4). Such conflicting relationships might be due to the unexpected large fluctuation of transformation efficiency, and it is highly likely caused by the difficulty to normalize the cell conditions (see below).

3.7 Co-integrationbr efficiency

Neupert et al (2009) have shown that their UV mutant strains (UVM4 and UVM11) show absolutely high co-transformation efficiency of a drug resistant marker gene and the aimed gene being located at independent plasmids. To assess if there is any positive relationship between co-transformation ratio and transgene expression ability, we assayed the co-transformation ratio of *VENUS* gene and hygromycin resistance *aph7* gene using p_Opt_Venus_Hyg plasmid that harbors two genes on the same strand (**Figure 2-1**). It was assayed in *met1*(UVM)-45, -47A, -47B, and -57, and also in other related strains by exerting PCR to detect the two genes for at least 48 transformants per strain.

In our assay, UVM4 showed the very high co-transformation ratio (about 78 %) supporting the report (Neupert et al. 2009), while co-transformation ratios in

met1(UVM)-47A, *met1*(UVM)-47B, and *met1*(UVM)-57 were limited to about 56%, 52%, 45%, respectively, and these ratios were not significantly different from those in their mother strains (31-33%) and that in *met1*(55%), which have lower expression ability than the above *met1* (UVM)-mutants. Thus, we could not show the convincing positive relationship between the co-transformation efficiency ratio and the expressed protein levels.

3.8 Mixotrophic and photoautotrophic growth

Mixotrophic growth of the three *met1*-mutants, *met1*(UVM)-47A, -47B, and -57 in TAP medium was a little bit slower than each of their mother strains, and CC-4533 (mother strain of *met1*) (Figure 3-10). On the other hand under a photoautotrophic condition in TAP medium without acetate under constant light, *met1*-strain and the derivative ones displayed much slower growth than in normal TAP medium. Moreover, the cell concentration at stationary phase was limited to around 2×10^6 cell/mL in TAP without acetate (data not shown), while in the normal TAP it reached to around 1×10^7 cell/mL (Figure 3-10).

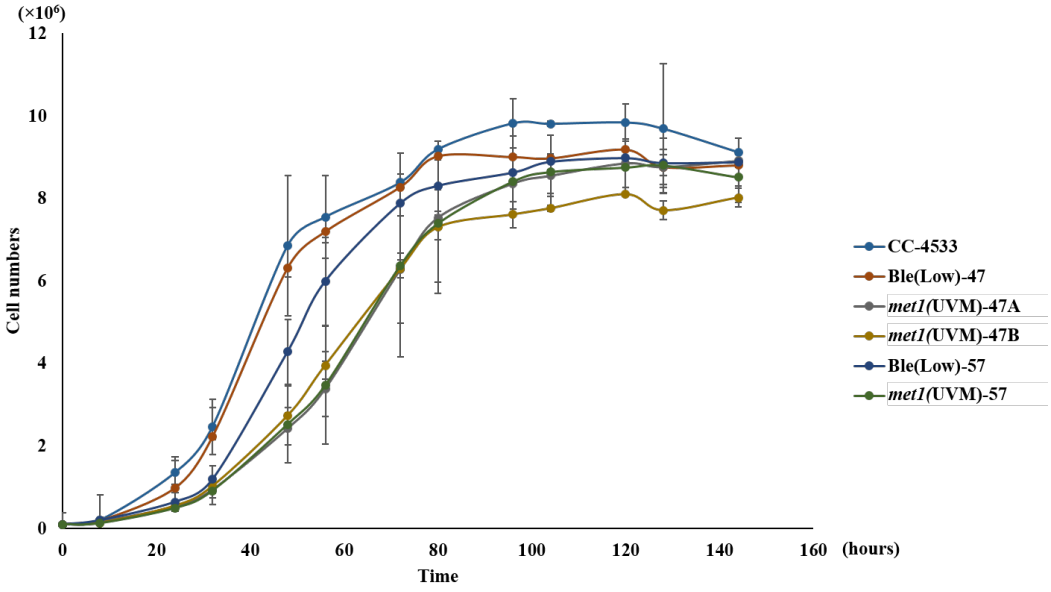

Figure 3-10. Mixotrophic growth in the UV-mutants of *met1*-strain and the related strains. Three times of independent experiments were carried out.

Microscopic observation showed *met1*(UVM)-47A, -47B, and -57 bear swimming ability, and we have confirmed that they have mating capability by trial mating with a wild type CC-125(mt^+) (data not shown). Potentially interesting characteristic is that *met1*-strain and all the *met1*-strain derivative strains seem to have abnormal cell cycle: Until these cells reach to the early stationary phase (OD_{750} ≒1.1), almost of all cells are at the pre-hatching stage, i.e., four or eight daughter cells are enclosed under a mother cell-wall (Figure 3-11). This suggests DNA methylation is potentially associated to regulate the cell cycle.

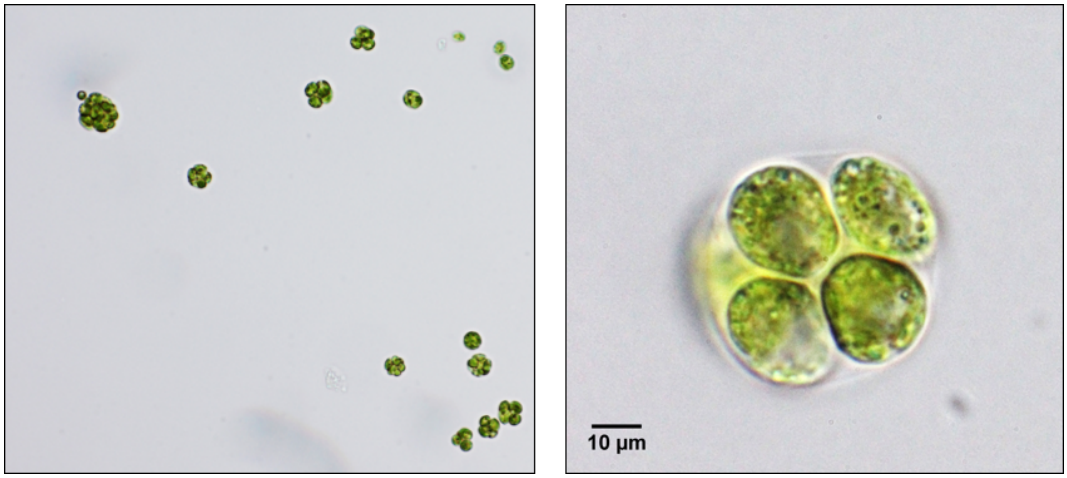

Figure 3-11. *met1*(UVM)-47B cells at mid-log phase. Almost of all cells are in pre-hatching stage encapsulated with mother cell-wall (sporangial cell wall).

Chapter 4 Conclusion and suggestion

4.1 Conclusion

Our data suggest *Chlamydomonas* bears several pathways for transgene repression in addition to that involves the maintenance type DNA methyltransferase as a key player, because we succeeded to isolate strains that show robustly improved expression of nuclear transgenes by further mutagenesis of *met1*-strain, which is a maintenance-type DNA methyltransferase deficient mutant. Several UV-mutants of *met1*-strain, *i.e*., *met1*(UVM)-47A, -47B, and -57, showed accomplished ability for heterologous expression of various transgenes with the highest expression level was 0.22% of TSP along with the prominent transformation efficiency. Such strains must be very useful as platform for production of human and animal therapeutic proteins and industrial enzymes.

4.2 Suggestion

To improve the ability of *met1*-UV mutants ability in expressing transgenes up to 5% of TSP, further mutation or genome shuffling strategy can be utilized. *Chlamydomonas* strain that able to express nuclear transgenes up to 5% of TSP must be useful for industrial enzyme and biomedical proteins.

Chapter 5 Reference

Babinger P, Kobl I, Mages W, Schmitt R (2001) A link between DNA methylation and epigenetic silencing in transgenic *Volvox carteri*. Nucleic Acids Res 29:1261–71. doi: 10.1093/nar/29.6.1261

Babinger P, Völkl R, Cakstina I, Maftei a., Schmitt R (2007) Maintenance DNA methyltransferase (Met1) and silencing of CpG-methylated foreign DNA in Volvox carteri. Plant Mol Biol 63:325–336. doi: 10.1007/s11103-006-9091-1

Barahimipour R, Neupert J, Bock R (2016) Efficient expression of nuclear transgenes in the green alga *Chlamydomonas*: synthesis of an HIV antigen and development of a new selectable marker. Plant Mol Biol. doi: 10.1007/s11103-015-0425-8

Bernstein BE, Humphrey EL, Erlich RL, Schneider R, Bouman P, Liu JS, Kouzarides T, Schreiber SL (2002) Methylation of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A 99:8695–8700. doi: 10.1073/pnas.082249499

Berthold P, Schmitt R, Mages W (2002) An Engineered Streptomyces hygroscopicus aph 7″ Gene Mediates Dominant Resistance against Hygromycin B in *Chlamydomonas reinhardtii*. Protist 153:401–412. doi: http://dx.doi.org/10.1078/14344610260450136

Bird A (2002) DNA methylation patterns and epigenetic memory DNA methylation patterns and epigenetic memory. Genes Dev 6–21. doi: 10.1101/gad.947102

Casas-Mollano JA, Jeong B-R, Xu J, Moriyama H, Cerutti H (2008) The MUT9p kinase phosphorylates histone H3 threonine 3 and is necessary for heritable epigenetic silencing in *Chlamydomonas*. Proc Natl Acad Sci U S A 105:6486–6491. doi: 10.1073/pnas.0711310105

Cerutti H, Johnson a M, Gillham NW, Boynton JE (1997) Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell 9:925–45. doi: 10.1105/tpc.9.6.925

Feng S, Cokus SJ, Zhang X, Chen P-Y, Bostick M, Goll MG, Hetzel J, Jain J, Strauss SH, Halpern ME, Ukomadu C, Sadler KC, Pradhan S, Pellegrini M, Jacobsen SE (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci U S A 107:8689–8694. doi: 10.1073/pnas.1002720107

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

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278:4035–4040. doi: 10.1074/jbc.M210256200

Jinkerson RE, Jonikas MC (2015) Molecular techniques to interrogate and edit the *Chlamydomonas* nuclear genome. Plant J. doi: 10.1111/tpj.12801

Kajikawa M, Kinohira S, Ando A, Shimoyama M, Kato M, Fukuzawa H (2015) Accumulation of Squalene in a Microalga *Chlamydomonas reinhardtii* by Genetic Modification of Squalene Synthase and Squalene Epoxidase Genes. PLoS One 10:e0120446. doi: 10.1371/journal.pone.0120446

Kong F, Yamasaki T, Kurniasih SD, Hou L, Li X, Ivanova N, Okada S, Ohama T (2015) Robust expression of heterologous genes by selection marker fusion system in improved *Chlamydomonas strains*. J Biosci Bioeng 120:239–245. doi: 10.1016/j.jbiosc.2015.01.005

Kong F, Yamasaki T, Ohama T (2014) Expression levels of domestic cDNA cassettes integrated in the nuclear genomes of various *Chlamydomonas reinhardtii* strains. J Biosci Bioeng 117:613–6. doi: 10.1016/j.jbiosc.2013.10.025

Kozminski KG, Diener DR, Rosenbaum JL (1993) High level expression of nonacetylatable α-tubulin in *Chlamydomonas reinhardtii*. Cell Motil Cytoskeleton 25:158–170. doi: 10.1002/cm.970250205

Lauersen KJ, Berger H, Mussgnug JH, Kruse O (2013) Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. J Biotechnol 167:101–10. doi: 10.1016/j.jbiotec.2012.10.010

Lauersen KJ, Kruse O, Mussgnug JH (2015) Targeted expression of nuclear transgenes in *Chlamydomonas reinhardtii* with a versatile, modular vector toolkit. Appl Microbiol Biotechnol. doi: 10.1007/s00253-014-6354-7

Meslet-Cladière L, Vallon O (2011) Novel shuttle markers for nuclear transformation of the green alga *Chlamydomonas reinhardtii*. Eukaryot Cell 10:1670–8. doi: 10.1128/EC.05043-11

Mussgnug JH (2015) Genetic tools and techniques for *Chlamydomonas reinhardtii*. Appl Microbiol Biotechnol 5407–5418. doi: 10.1007/s00253-015-6698-7

Neupert J, Karcher D, Bock R (2009) Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. Plant J 57:1140–50. doi: 10.1111/j.1365-313X.2008.03746.x

Plucinak TM, Horken KM, Jiang W, Fostvedt J, Nguyen ST, Weeks DP (2015) Improved and versatile viral 2A platforms for dependable and inducible high-level expression of dicistronic nuclear genes in *Chlamydomonas reinhardtii*. Plant J. doi: 10.1111/tpj.12844

Rasala B a, Barrera DJ, Ng J, Plucinak TM, Rosenberg JN, Weeks DP, Oyler G a, Peterson TC, Haerizadeh F, Mayfield SP (2013) Expanding the spectral palette of fluorescent proteins for the green microalga *Chlamydomonas reinhardtii*. Plant J 74:545–56. doi: 10.1111/tpj.12165

Rasala B a, Lee P a, Shen Z, Briggs SP, Mendez M, Mayfield SP (2012) Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS One 7:e43349. doi: 10.1371/journal.pone.0043349

Ruecker O, Zillner K, Groebner-Ferreira R, Heitzer M (2008) Gaussia-luciferase as a sensitive reporter gene for monitoring promoter activity in the nucleus of the green alga *Chlamydomonas reinhardtii.* Mol Genet Genomics 280:153–162. doi: 10.1007/s00438-008-0352-3

Sahdev S, Khattar SK, Saini KS (2008) Production of active eukaryotic proteins through bacterial expression systems: A review of the existing biotechnology strategies. Mol Cell Biochem 307:249–264. doi: 10.1007/s11010-007-9603-6

Schroda M, Blöcker D, Beck CF (2000) The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. Plant J 21:121–31.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96. doi: 10.1263/jbb.101.87

Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72:211–222. doi: 10.1007/s00253-006-0465-8

Yamano T, Iguchi H, Fukuzawa H (2013) Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. J Biosci Bioeng 115:691–4. doi: 10.1016/j.jbiosc.2012.12.020

Yamasaki T, Kim EJ, Cerutti H, Ohama T (2015) Argonaute3 is a key player in miRNA-mediated target cleavage and translational repression in *Chlamydomonas*. Plant J 258–268. doi: 10.1111/tpj.13107

Yamasaki T, Miyasaka H, Ohama T (2008) Unstable RNAi effects through epigenetic silencing of an inverted repeat transgene in *Chlamydomonas reinhardtii*. Genetics 180:1927–44. doi: 10.1534/genetics.108.092395

Zhang R, Patena W, Armbruster U, Gang SS, Blum SR, Jonikas MC (2014) High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. Plant Cell 1–13. doi: 10.1105/tpc.114.124099

ACKNOWLEDGEMENT

First of all, I would like to say sincerely gratitude to my supervisor, Prof. Takeshi Ohama for giving me opportunity to join his laboratory as one of doctoral student. His thoughtful kindness, guidance, encouragement and all kinds of help and support from him during my research or my daily life in Japan. I learned a lot from Sensei from his patient, positive attitude and his passion on research. He always encourages me to enjoy research even during though time during my doctoral study and also in my life planning.

Moreover, I also would like to express my thankfulness to my co-adviser, Associate Professor Sakae Horisawa, Associate Professor Osamu Ariga, and Prof. Yusuke Kamachi for their friendly discussions and suggestions during biological seminars.

Secondly, I would like to express my gratitude for Dr. Tomohito Yamasaki, for helping me in solving some experimental problem and assistance in developing biotechnological tools. His patience and willingness to help, improve my ability of troubleshooting as a researcher. Furthermore, I want to express my appreciation for my laboratory members sincerely, Mr. Fantao Kong, Ms. Hou Liyuan, Ms. Resnanti Utami Handayani and Ms. Dwiyantari Widyaningrum, their support and friendship gave me a lot happiness they are just like my brothers and sisters.

Thirdly, I would like to give my special thanks to all of International Relation Center members for their kindly taking care of me and make me feel like home, especially Ms. Mari Yamasaki. I also would like to say my thank you to my friend Reisa, Handi, Eliza for their friendship and support and to Keiichiro my niece who always smile and play with me.

Last, I would like to say thank you to my mother Mrs. Netty Kurniati for her support and love to me. To my father Mr. Denny Indrawan for his support and encouragement. Also to my brother and sister, Febi and Nilam. Their support encourage me to pursuit my dreams.

ACHIEVEMENTS

1. List of publications

Kurniasih SD, Yamasaki T, Kong F, Okada S, Widyaningrum D, Ohama T. (2016).UV-mediated *Chlamydomonas* mutants with enhanced nuclear transgene expression by disruption of DNA methylation-dependent and independent silencing systems. *Plant Molecular Biology (in-press)*

Kong F, Yamasaki T, **Kurniasih SD**, Hou L, Li X, Ivanova N, Okada S, Ohama T.(2015). Robust expression of heterologous genes by selection marker fusion system in improved *Chlamydomonas* strains. *Journal of Bioscience and Bioengineering,* 120. 239-245.

2. International conferences

1) 16th International *Chlamydomonas* meeting. June 26th – July 1st , Kyoto, Japan, **ORAL**
2) Academic Symposium "China-Japan Innovation Forum on New Energy Utilization and Sustainable Development. November 15th – 17th 2014, Hefei, China, **ORAL**
3) The 5th International Symposium of Frontier Technology (ISFT) July24th– 26th 2015, Kunming, China, **ORAL**