論 文 内 容 の 要 旨

Background

Nowadays the demand of recombinant protein production has increased because of many applications in therapeutic and industrial fields. Various expression system has been used for over-expression of protein. Bacteria such as *Escherichia coli, Bacillus subtilis, B. megaterium* are the most widely used for the production of recombinant proteins because they are well characterized, easy to manipulate and their cultivation system is simple. Bacteria have been used as a host for production of industrial enzyme such as amylase, cellulase, lipase, etc, and therapeutic protein (Sahdev et al. 2008). However, bacteria lack post-translational modifications which are required for production of eukaryotic protein, often produce endotoxin, and the products form inclusion bodies. Yeast production system has advantage that it has availability of post-translational modification and grows fast (Porro et al. 2005). However, hyperglycosylation in yeast can affect the activity of the products (Wildt and Gerngross 2005). The production cost for large scale production of recombinant protein in yeast is high because rich medium is required. Transgenic plant production system offers several advantages, glycosylation pattern in plant is very similar to animal cells (Cabanes-Macheteau et al. 1999), recombinant protein produced can be enlarged on an agricultural scale (Whitelam et al. 1994). In spite of that, production process takes several years from transformation step to protein isolation, and protein purification from plant is laborious.

Microalgae are photosynthetic microorganisms that efficiently use light as three times of higher plants (Shimizu 1996), it can concert CO_2 into valuable compounds such as lipid, carbohydrates, and protein. Furthermore, microalgae contain 50-70% of protein in the fresh weight, which is higher than some of higher plant (Passwater 1997). Microalgae combine the merit of transgenic plant system and microorganism to be as rapid growth and easy scale up.

One of unicellular green algae, *Chlamydomonas reinhardtii* can be an ideal plant cell factory for production of recombinant protein because of short doubling time i.e., every 7-8 hours. Simple salt based nutrients and flexible scalability on demand are appealing merit of this alga. *C. reinhardtii* is well characterized among

microalgal species, the complete genome sequence is available. Methods for genetic manipulation has been well developed for *C. reinhardtii* over the last 20 years. Transformation of *Chlamydomonas* can be performed using particle bombardment (nuclear and chloroplast transformation) (Debuchy et al. 1989; Kindle et al. 1989), glass beads (Kindle 1998), electroporation (Shimogawara et al. 1998; Yamano et al. 2013) and silicon whisker (Dunahay et al. 1997). Required conditions to induce mating in *C. reinhardtii* has been well characterized. Thus, through mating between several transgenic lines generation of algal strains expressing multi-subunit recombinant protein is possible (Franklin and Mayfield 2005). Because of these reasons *C. reinhardtii* ranked top candidate for algal cell factory.

Recombinant protein can be expressed from nuclear and chloroplast genome of *C. reinhardtii.* Chloroplast-expressed recombinant protein accumulated within the chloroplast, no post-translational modification, and potential for high level accumulation of recombinant protein up to 10% of total soluble protein (TSP). Nuclear-expressed recombinant protein can secret to medium through endoplasmic reticulum (ER), also can be deposit in cytoplasm or targeted to other locations such as periplasmic space, flagella, chloroplast or mitochondrion (Franklin and Mayfield 2005; Lauersen et al. 2015), and the protein can be post-translationally modified (Griesbeck et al. 2006).

However, the major drawback of *C. reinhardtii* is the poor expression of transgenes located in the nuclear genome. The possible molecular mechanism are epigenetic transcriptional silencing of transgenes, ineffective transcription from promoters, mRNA instability (Cerutti et al. 1997). To improve transgenes expression, several strategies have been developed by *Chlamydomonas* researcher, such as improved expression system through codon usage optimization (Fuhrmann et al. 1999; Date et al. 2015); utilization of enhancer and promoters to enhance transcription level (Schroda et al. 2000; Ruecker et al. 2008; Scranton et al. 2016); development of expression system by fused ble gene to heterologous gene of interest via the foot-and-mouth-disease-virus (FMDV) 2A linker peptide. Using this expression cassette, two discrete protein (Ble protein and heterologous protein) are produced and the amount of interest protein is increased. This system can be result in 100-fold increase of production of fungal xylanase (0.25% of TSP) (Rasala et al. 2012); Another strategy is through strain improvement, Neupert at al. 1999 has successfully generated mutants, UVM4 and UVM11, by UV mutagenesis. These mutants were able to express transgenes from nuclear genome up to 0.2% of TSP (Neupert et al. 2009). However, these two mutants are still subjected to transgene

silencing, Recent studies showed, the expression of codon optimized synthetic cDNA of *Botryococcus SSL* genes have not been easily expressed even in UVM strains (Kong et al. 2014; Kong et al. 2015). Previously, we identified a tag-DNA insertional mutant which contain a tag at the *Chlamydomonas* unique maintenance type DNA methyltransferase 1 (*MET1*). This mutant (*met1-strain)* shows significant improved ability to express nuclear transgenes, the performance was almost same as UVM4 and UVM11 strains (Kong et al. 2015). However, these strain also not completely free from transcriptional repression irrespective of full disruption of *MET1* gene, which is the key enzyme for epigenetic gene silencing (Babinger et al. 2001).

Thus, until now no practical solution has been found to obtain transformants that is accumulating aimed protein expressed from nuclear genome of *Chlamydomonas* in high levels to other expression systems.

Research objective

Considering that DNA methylation is the well-described trigger for epigenetic transcriptional gene silencing, to develop *Chlamydomonas* strain that able to express transgenes much strongly and accumulate high amount of recombinant protein. We generated *Chlamydomonas* strain by further mutation of *met1*-strain through UV mutagenesis. 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 for transgene expression.

Result and discussion

UV-mediated mutagenesis was performed on selected transformants of *met-1* strain containing repressed expression of *ble-GFP*. The protein product of *ble-GFP* binds stoichiometrically with Zeocin to inhibit the DNA cleavage activity. Consequently, positive correlation is expected between *ble-GFP* expression level and Zeocin resistant level. Because of these reasons, fused *ble-GFP* was chosen as a marker gene to assess the degree of transcriptional gene silencing. In addition,

approximate protein level of Ble-GFP can be estimated by western-blot, and the Ble-GFP protein fluorescence can be observed through microscopic observation.

To select appropriate mother strains for UV-mutagenesis, transformants of *met1*-strain with weak Zeocin resistance caused by strong gene silencing on *ble-GFP* were selected. Seven transformants: no 26, 40, 45, 47, 55, 57, and 63, showed relatively weak Zeocin resistance. These strains grew slowly on 10 μg/mL Zeocin and faint growth on 60 μg/mL. Strains 40, 45, 47, and 57 were utilized as mother cells for further introduction of mutation by UV treatments based on their normal cell growth rate and motility. In this study these strains were represented with prefix of Ble(Low), (i.e., Ble(Low)-40, -45, -47, and -57). Higher Zeocin resistance and increased Ble-GFP protein, and enhanced transformation efficiency were assumed to be indicators of alleviated gene silencing.

After UV-mediated mutagenesis, the level of Zeocin resistance level of UV-mutants pool were checked using various concentration of Zeocin and subsequently Ble-GFP protein level were analyzed in mutants which the Zeocin resistant level were increased. Forty-one mutants that grew on 150 μg/mL of Zeocin plates were identified after spotting test assay, and the level of Ble-GFP protein were analyzed by western blotting. A significant increase of Ble-GFP protein level was detected on four mutants compared with their mother strains. These mutants: *met1*(UVM)-40 [a UV-mutant generated from the Ble(Low)-40 transformant of met1-strain], met1(UVM)-47A [Ble(Low)-47 transformant of met1-strain], met1(UVM)-47B [also a Ble(Low)-47 transformant of met1-strain], and met1(UVM)-57 [Ble(Low)-57 transformant of met1-strain]. These mutants were further characterized to evaluate their possibility as a molecular plant factory for recombinant protein production.

Transformation efficiency of these five mutants and their mother mother strains were performed to analyse whether these mutants have higher transformation rates or not comparing to the mother strains. Higher transformation rates are considered as one of the characteristic of mutants with alleviated gene silencing mechanism. Transformation efficiency was measured using *aad*A expression cassette which give spectinomycin resistance. Four of the five UV-mutants showed a higher transformation rates than their mother strains, *met1*(UVM)-40, *met1*(UVM)-45, *met1*(UVM)-47A, and *met1*(UVM)-47B (about two to ten-fold higher).

Most important required characteristic for desired mutant is the ability to express transgenes robustly irrespective of the integrated position in nuclear

genome with comparable level to yeast or bacteria protein expression system. To evaluate this ability, we analyzed expression level of newly introduced genes, such as codon-adjusted *VENUS*, *Chlamydomonas* domestic *SQS* gene, and a domestic radial spoken protein 3 gene (*RSP3*).

Ten various strains were analysed for VENUS expression level and strong western signal of VENUS was only detected in UVM4, Ble(Low)-47, *met1*(UVM)-47A, -47B, and *met1*(UVM)-57. Furthermore, in *met1*(UVM)-47A, -47B, and -57, the western positive ratios were above 70%. This results were in accordance with the enhanced accumulation of Ble-GFP in *met1*(UVM)-47A, -47B, and -57. While western-positive ratio was below 20% in *met1*-strain, CC-4533 (mother strain of of *met1* tag mutant), Ble(Low)-45, *met1*(UVM)-45, Ble(Low)-47, and Ble(Low)-57. These data suggest the repression of transgene expression in these three *met1* UV-mutants was lower than the other strains.

The average accumulated VENUS protein level were quantitatively analyzed by examination of five randomly selected transformants from three *met1*-UV mutants: *met1*(UVM)-47A, -47B, and -57 and their mother strain, also UV4 for comparison. VENUS expression level reach 0.22% of TSP (total soluble protein) in one of *met1*(UVM)-47A transformants. Until now, the highest protein accumulation level from *Chlamydomonas* nuclear is 0.25% ((Rasala et al. 2012; Barahimipour et al. 2016), so 0.22% is comparable with this level. The average accumulation level of VENUS in *met1*(UVM)-47A was exceeded that in UVM4, while in *met1*(UVM)-57 was almost same with UVM4. Relative expression level of SQS and RSP3 were high in three *met1* UV-mutants, *met1*(UVM)-47A, -47B, and -57. These results were consistent with VENUS expression level.

To confirm whether enhanced expression of transgenes in *met1* UV-mutant strains is caused by alleviation of transcriptional repression, the mRNA level of *ble-GFP* in *met1* UV-mutant and their mother strain were compared. Reverse-transcriptase (RT) PCR results showed that *ble-GFP* mRNA level in *met1*(UVM)-47A and -47B were 10 and 5-fold higher than the mother strain Ble(Low)-47 respectively. These results showed that low accumulation of Ble-GFP protein in Ble(low)-47 was due to limited amount mRNA by transcriptional repression.

It has been shown that some of *met1* UV-mutants able to overexpress various transgenes. The enzymes that involved in histone modification or protein that contribute in chromatin condensation through formation of hetechromatin which allow enhanced expression of transgenes integrated at any genomic region, might

have some mutation due to UV- mediated treatment. Because of this reason, histone H3 acetylation (H3Ac) and histone H3K4 di-methylation (H3K4 me2) level were analyzed. These two histone modifications were known to activate transcription in *Chlamydomonas.*

Significant increase in H3Ac was observed in *met1*(UVM)-47A being compare to the mother strain Ble(Low)-47. While no significant difference was found in the other strain. Therefore, *met1*(UVM)-47A may have mutation in histone modification related enzymes such as histone deacetylase. The level of H3K4 me2 in *met1*(UVM)-47A, -47B, and -57 were not different with the mother strains. The growth of three *met1* UV-mutants, *met1*(UVM)-47A, -47B, and -57 in TAP medium was marginally slower than their mother strains with cells concentration reach to 1.10^7 cells /mL at the end of stationary phase.

Conclusion

My experimental results suggest that *Chlamydomonas* has transgene repression system in which maintenance-type DNA methyltransferase is not involved. We succeed to improve nuclear transgene expression of *Chlamydomonas* through further mutagenesis of *met1*-strain, which is a maintenance-type DNA methyltransferase deficient mutant. Three *met1* UV-mutants, *met1*(UVM)-47A, -47B, and -57, showed high ability for overexpression of various transgenes along with enhanced transformation efficiency. Such strains may be useful for the production of recombinant proteins for medical applications and also for industrial applications.