Heterologous overexpression of sfCherry fluorescent protein in *Nannochloropsis salina*

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A B S T R A C T

Oleaginous microalgae of the *Nannochloropsis* genus are considered excellent candidates for biofuels and value-added products owing to their high biomass productivity and lipid content. Here, we report the first overexpression and detection of a heterologous sfCherry fluorescent protein in *Nannochloropsis salina* in order to develop a transformation toolbox for future genetic improvements. Particle bombardment was employed for transformation, and expression of Sh*ble* under the control of TUB and UEP promoters, cloned from *N. salina*, was used to confer resistance to Zeocin antibiotics, resulting in 5.9 and 4.7 transformants per 10^8 cells, respectively. Stable integration of the markers into the genome was confirmed using a restriction enzyme site-directed amplification (RESDA) PCR. The expression of sfCherry fluorescent protein was confirmed by Western blot analysis and confocal microscopy. These results suggest new possibilities of efficient genetic engineering of *Nannochloropsis* for the production of biofuels and other biochemicals.
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1. Introduction

Growing concerns about potential energy crises and environmental problems caused by a dependence on traditional fossil fuels has increased our interest in alternative energy sources. Microalgae are among the most promising feedstocks for biofuels and natural products owing to their high lipid content and fast growth rate compared with other biofuels feedstocks, such as crops and other land plants. Moreover, they do not require arable land or fresh water for cultivation, and can grow using CO_2 in flue gas, thereby reducing levels of this important greenhouse gas [1,2].

Nannochloropsis sp. are attractive industrial production strains for biofuels and value-added products such as eicosapentaenoic acid (EPA) by virtue of their fast growth and high lipid content [3]. Various cultivation methods have been developed as part of an effort to efficiently produce biofuels from *Nannochloropsis* [4]. In addition, genomic and transcriptomic analyses of several *Nannochloropsis* strains have been reported [3]. These data can be used for genetic improvements of *Nannochloropsis* designed to achieve commercial production of biofuels. Recent reports have demonstrated the transformation of *Nannochloropsis*, indicating that the creation of 'smart' microalgae with high lipid productivity is now conceivable [5,6]. Subsequent studies have reported improved transformation efficiency through delivery of a PCR product instead of an intact plasmid [7] and by using top agar for recovery [6] or conditioned medium [8]. It also has been reported that metabolic pathway related genes are knocked out using the homologous recombination method [9].

Three main types of transformation methods have generally been used for microalgae: agitation with glass beads, electroporation, and particle bombardment. Agitation with glass beads and electroporation are commonly used to transform microalgae that lack or have weakened cell walls. For microalgae with hard cell walls, such as diatoms, particle bombardment has been employed as a transformation method [10]. It is also the method of choice for organellar transformation. Notably, chloroplast transformation can be used in a microalgal cell-factory setting for producing heterologous protein and value-added products [11,12].

An established method for localization of the transgene integration site within the host genome is thermal asymmetric interlaced polymerase chain reaction (TAIL PCR). In TAIL PCR, the insertion site can be found efficiently using nested specific primers

and relatively short, arbitrary, degenerate primers [13]. RESDA (restriction enzyme site-directed) PCR is an improved version of TAIL PCR that uses degenerate primers containing restriction enzyme sites to increase the efficiency of TAIL PCR [14].

In addition, the applications of fluorescent proteins involve using them for selection marker, checking the expression level of fusion protein, and exploiting for imaging of the localization and dynamics of specific organelles [15]. However, it should be noted that proper choice of fluorescent protein is important not only for preventing the interference of autofluorescent signal from cells, but also for greater brightness and photostability [16]. Fluorescent protein has been genetically modified in order to allow them to do efficient folding and to maintain strong fluorescence [17].

In this study, we report the first overexpression and detection of a heterologous sfCherry fluorescent protein, a genetically modified mCherry, as part of an effort to develop a genetic-manipulation toolbox in Nannochloropsis salina [17]. To introduce a heterologous protein, we used particle bombardment to transform N. salina with plasmids containing a marker and/or a reporter. Transformants were further analyzed by RESDA PCR to determine if the introduced constructs were integrated into the genome [14]. Finally, in order to validate that our transformation technique resulted in the successful production of a heterologous protein, we expressed a construct of the sfCherry fluorescent protein in N. salina, and confirmed its expression by Western blot analysis and confocal fluorescence microscopy. It is expected that this work will facilitate the genetic engineering of Nannochloropsis for the production of biofuels and bioproducts.

2. Materials and methods

2.1. Microalgae strain and culture conditions

N. salina CCMP1776 (National Center for Marine Algae and Microbiota) was maintained in sterile modified F2N media [9] composed of the following: 15 g/L sea salt (Sigma–Aldrich, USA), 10 mM Tris–HCl (pH 7.6), 427.5 mg/L $NaNO_3$, 30 mg/L $NaH_2PO_4\cdot2H_2O$, 5 mL/L trace metal mixture (4.36 g/L Na_2 $EDTA\cdot2H_2O$, 3.15 g/L $FeCl_3\cdot6H_2O$, 10 mg/L $CoCl_2\cdot6H_2O$, 22 mg/L $ZnSO_4\cdot7H_2O$, 180 mg/L $MnCl_2\cdot4H_2O$, 9.8 mg/L $CuSO_4\cdot5H_2O$, 6.3 mg/L $Na_2MoO_4\cdot2H_2O$), and 2.5 mL/L vitamin stock (1 mg/L vitamin B_{12}, 1 mg/L Biotin, 200 mg/L thiamine·HCl) [18]. Cells were cultivated in a 200 mL working volume in 250 mL Erlenmeyer baffled flasks at 25 °C with agitation (120 rpm) under fluorescent light (120 μmol photons/m^2/s). Air mixed with 2% CO_2 was directly supplied to the culture at a rate of 0.5 vvm (volume gas per volume medium per minute).

2.2. Vector construction

The plasmid, pNsTShble (Fig. 1a), harboring the endogenous TUB promoter, the Shble gene, which confers resistance to Zeocin (Invitrogen, USA), and the TUB terminator was constructed and used for transformation. Other plasmids used in this study include pNsUShble (UEP promoter, Shble, and UEP terminator) and pNssfCherry (TUB promoter, the gene encoding sfCherry fluorescent protein, and TUB terminator). The pNssfCherry vector also harbors the Shble gene as a selection marker flanked by the UEP promoter and the UEP terminator (Fig. 2a). All plasmids were constructed using the Gibson assembly technique [19].

2.3. Particle bombardment

N. salina cultivated in modified F2N media was harvested after 7 days in the mid-exponential phase ($OD_{680\,nm}=6$), and cell number was determined using a hemocytometer. A 47-mm-diameter cellulose acetate membrane filter (Sartorius Stedim Biotech, Germany) was placed on F2N agar media, and 10^8 cells were placed on the membrane filter for a single bombardment. Plasmids, linearized by treatment with XbaI, were coated onto
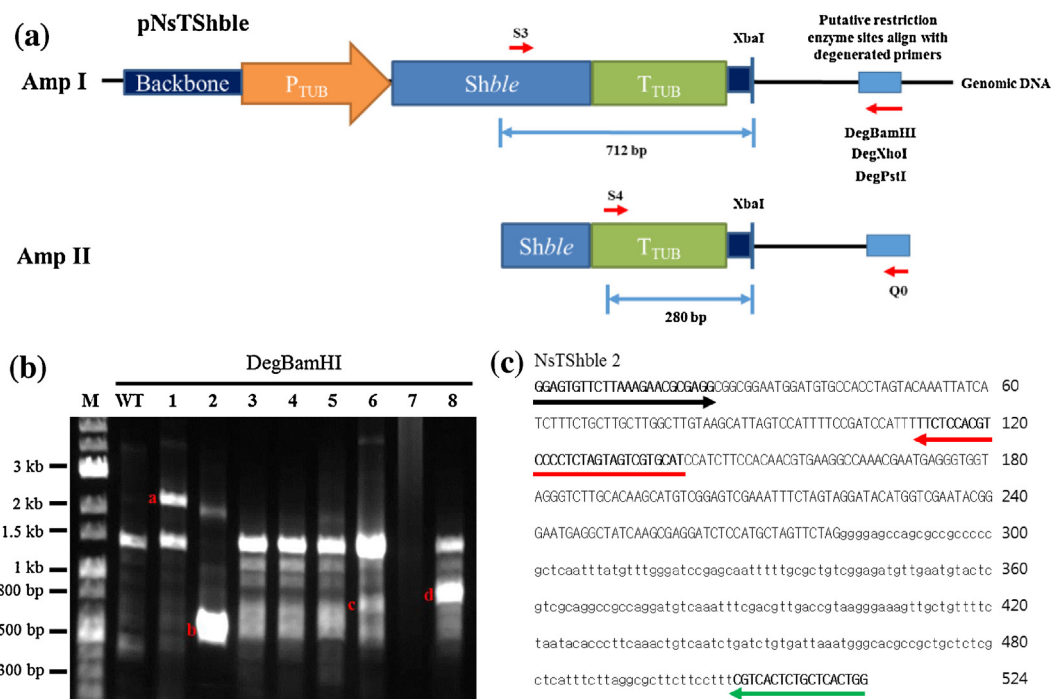
Fig. 1. Results of RESDA PCR for N. salina wild type and NsTShble transformants 1 through 8. (a) A schematic depiction of RESDA PCR for the NsTShble transformants of N. salina. (b) The results of Amp II using DegBamHI. The bands 'a' to 'd' in the gel from DegBamHI samples were recovered and sequenced. (c) Examples of sequences obtained from NsTShble 2. Capital letters represent the plasmid region, and lowercase letters show the flanking genomic sequence. Black, red, and green arrows represent the S4, S5, and Q0 primers, respectively. Abbreviations: M, marker; WT, wild type.
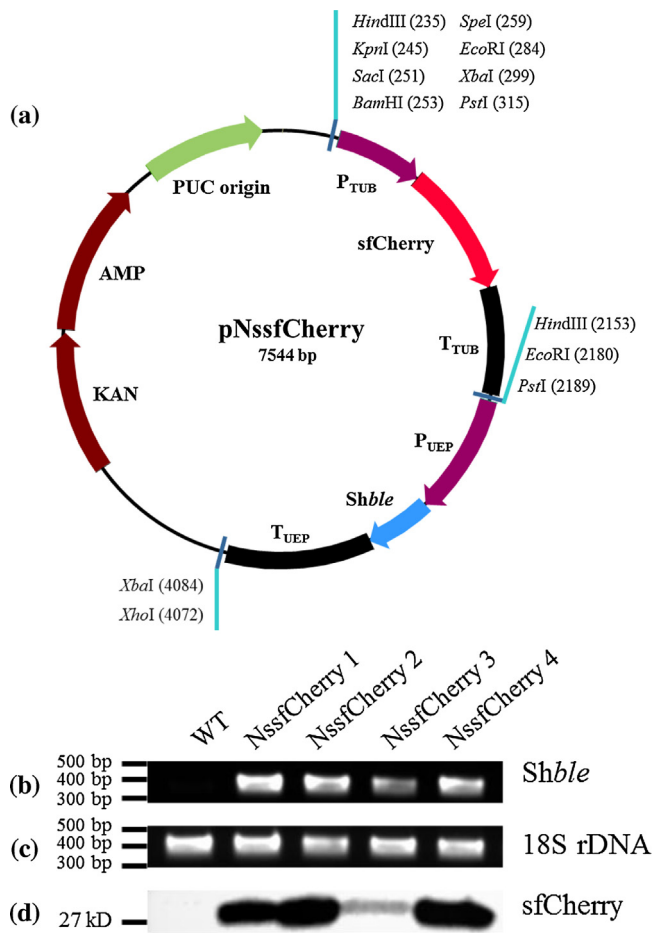
Fig. 2. Analyses of NssfCherry transformants. (a) The vector map of pNssfCherry. Agarose gel electrophoresis for the verification of (b) the Sh*ble* PCR product and (c) 18S rDNA. (d) sfCherry fluorescent protein accumulation determined by western blotting.

microcarrier gold particles (Bio-Rad, USA) for transformation. A mixture of plasmid, 2.5 M CaCl$_2$, and 0.1 M spermidine was prepared in 25% glycerol containing gold particles and vigorously mixed for 3 min, after which the coated particles were allowed to settle by gravity at room temperature for 10 min. After discarding the supernatant, the coated particles were washed with 70% ethanol and then resuspended in 100% ethanol. Particle bombardment was performed using a low-pressure gene delivery system (GDS-80; Wealtec, USA) under the following conditions: 625 μg gold particles coated with 1 μg of linearized plasmid per shot by 700 psi of helium and 3 cm target distance [20]. After transformation, the cells were recovered in modified F2N media and incubated at 25 °C under fluorescent light (5 μmol photons/m^2/s) without agitation for 1 day. All cells were plated onto selective F2N agar media containing 2.5 μg/mL Zeocin. After 3–4 weeks, colonies that appeared on the selective media were selected for further analyses.

2.4. Analytical procedures

2.4.1. PCR analysis of N. salina transformants

Zeocin-resistant *N. salina* colonies were harvested and washed with distilled water, and crude DNA was isolated using Instagene Matrix (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, 200 μL of Instagene Matrix was added to the cells and mixed. The mixtures were first incubated at 56 °C for 20 min and then at 100 °C for 8 min. After centrifugation, the supernatant was

Table 1
Primers used in this study.

Primer	Sequence 5′–3′	Purpose
SR6	GTCAGAGGTGAAATTCTTGG	18S rDNA
SR9	AACTAAGAACGGCATGCAC	
S1	AAGTTGACCAGTGCCGTTCCGGTG	Sh*ble*
S2	CTCGGCCACGAAGTGCACGCAGTT	
S3	ATGACCGAGATCGGCGAGCA	RESDA PCR
S4	GGAGTGTTCTTAAAGAACGCGAGG	
S5	ATGCACGACTACTAGAGGGGACGTGGAGAA	
DegPstI	CCAGTGAGCAGAGTGACG IIIIINNS CTGCAG W	
DegXhoI	CCAGTGAGCAGAGTGACG IIIIINNS CTCGAG W	
DegBamHI	CCAGTGAGCAGAGTGACG IIIIINNS CCTAGG W	
Q0	CCAGTGAGCAGAGTGACG	

used for PCR. S1 (forward) and S2 (reverse) primers were used to detect the Sh*ble* gene in *N. salina* transformants (Table 1). 18S rDNA, used as a positive control, was detected using SR6 (forward) and SR9 (reverse) primers (Table 1) [21]. PCR amplification was carried out using Ex-taq polymerase (Takara, Japan) with 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The expected sizes of Sh*ble* and 18S rDNA PCR fragments were 357 and 380 bp, respectively.

2.4.2. RESDA PCR

RESDA PCR was used to identify the insertion sites of the plasmid in the genomic DNA as described previously [14] and depicted schematically in Fig. 1a. Each PCR reaction was carried out in a final volume of 50 μL using Ex-taq polymerase (Takara, Japan). REDSA PCR consists of three stages: amplification I (Amp I), amplification II (Amp II), and re-amplification (Re-Amp). For Amp I, PCR was performed with the S3 primer and three degenerate primers (Table 1) using genomic DNA as the template. The PCR conditions for Amp I were 5 min at 96 °C followed by 20 cycles of 1 min at 95 °C, 1 min at 60 °C and 3 min at 72 °C, then 10 cycles of 1 min at 95 °C, 1 min at 40 °C, 3 min at 72 °C, and a final step of 10 min at 72 °C. For the Amp II step, PCR was conducted with S4 and Q10 primers using 1 μL of the PCR product from the Amp I step as the template. The PCR conditions for Amp II were 5 min at 96 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C, 3 min at 72 °C, and a final step of 10 min at 72 °C. Insertion sites in genomic DNA were identified by sequencing the specific PCR bands between approximately 300 bp and 2 kb after purification with a gel extraction kit (Qiagen, USA). A Re-Amp step was performed to confirm that the PCR bands selected from the Amp II step were correct. The template for Re-Amp PCR was obtained from the PCR product in the Amp II analytical agarose gel by directly inserting a 200-μL pipette tip into the corresponding band and shaking the tip in 50 μL of distilled water. Re-Amp PCR was performed with S4 and S5 primers using the water containing the extracted Amp II PCR product as a template. The PCR conditions for the Re-Amp step were 5 min at 96 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 3 min at 72 °C, and a final step of 10 min at 72 °C.

2.4.3. Western blot analysis

The expression of sfCherry in transformed cells was examined by extracting proteins with 1.5× Laemmli sample buffer, which is composed of the following components: 62.5 mM Tris–HCl (pH 7.6), 7% sodium dodecyl sulfate (SDS), 25% glycerol 5% β-mercaptoethanol, and 0.02% bromophenol blue. Cells obtained at mid-exponential phase were washed with distilled water, suspended in 1.5× Laemmli sample buffer, and then heated at 100 °C for 5 min. After centrifugation at 13,000 rpm for 5 min, the supernatants were separated by SDS-PAGE (polyacrylamide gel electrophoresis) on 4–15% gradient gels and transferred to a PVDF (polyvinylidene difluoride) membrane using a Trans-Blot Turbo system (Bio-Rad).

After blocking with 5% skim milk in phosphate buffered saline (PBS), the membranes were probed with a rabbit anti-mCherry antibody (Abcam, UK). Membranes were then washed three times in 5% skim milk and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, USA). After washing membranes three times with PBS, immunoreactive proteins were detected using enhanced chemiluminescence (ECL) reagents and the ChemiDoc system (Bio-Rad).

2.4.4. Visualization of sfCherry fluorescence

Fluorescence was visualized using an A1 Plus confocal microscope (Nikon, USA). sfCherry fluorescence of the NssfCherry transformant was detected using an mCherry filter (excitation, 561 nm; emission, 595/50 nm), and N. salina auto-fluorescence was detected using a FITC (fluorescein isothiocyanate) filter (excitation, 488 nm; emission, 525/50 nm).

3. Results and discussion

3.1. Transformation by particle bombardment and confirmation of stable integration

One major problem associated with microalgal transformation is that transgene expression is lost over time due to transgene silencing [22]. This phenomenon has hampered the development of microalgal genetic engineering techniques. The long-term stability of transformed microalgal cells without selection is one of the main issues in microalgal transformation. Compared to glass bead agitation and electroporation, bombardment appears to provide more stable transformation in the absence of selection [10]. Unfortunately, the exact mechanism how the gene delivery method affects the stability of the heterologous expression is unknown. However, there are some reports describing the long-term stability of transformants according to transformation methods. Based on statistical analysis, the long term stability of phenotype has been reported in 90.6% of papers whose authors used the bombardment method, and in 66.6% and 33.3% of those that used glass beads and electroporation, respectively [10]. This statistical analysis implies that after bombardment, the presence of exogenous DNA in the genome of the transformants correlates positively with long term survival compared to the glass bead and electroporation methods.

Therefore, in order to express heterologous protein in N. salina, we first tested transformation efficiency when using particle bombardment. N. salina was transformed with plasmid expression constructs for the Sh*ble* marker gene under the control of the TUB or UEP promoter. The transformants were selected on agar plates containing Zeocin, and their transformation efficiencies were determined, as summarized in Table 2. These analyses showed that 5.9 and 4.7 transformants per 10^8 cells were obtained using TUB and UEP promoter constructs, respectively. These results are consistent with a previous report that used electroporation method for transformation of Nannochloropsis gaditana [5]. Here, it is worth to note that transformation efficiency can be improved

by modification of transformation conditions, and that size and concentration of DNA fragment, which will be transferred into cells, affect the transformation efficiency. For instance, Li et al. suggested that transformation efficiency significantly increased, when using short DNA fragment for Nannochloropsis sp. transformation [7]. This is likely due to the fact that short DNA fragments improve their chances of integration. Therefore, an integrated development of bombardment based transformation is necessary to increase transformation efficiency.

To validate stable integration of the transgene in the genomic DNA and to identify the integration site, we employed RESDA PCR. The overall strategy and a vector map are shown in Fig. 1a. Fig. 1b shows the results of the second round of PCR (Amp II) using DegBamHI during RESDA PCR. The results obtained are consistent with a previous report that, in general, the average band size after Amp II ranges from 300 bp to 2 kb [14]. There were unique bands in many NsTShble transformant lines, denoted as 'a' through 'd'. DNA from each band was then purified and sequenced to identify integration patterns because different band patterns represent different insertion sites of transgenes. Sequencing of one example transformant, NsTShble 2, revealed sequences of the transformed vector flanked by the genomic sequence of N. salina CCMP1776, in addition to the landmark primer sequences of S4, S5, and Q0 (Fig. 1c). Other identified sequences are shown in Fig. S1. Importantly, the size of each band and the length of the corresponding sequence coincided, indicating that the amplification and sequencing of the integration sites were correct. It has been recently reported that the organellar genomes of N. salina are more than 97% identical to genomes of N. gaditana [23]. Therefore, the flanking regions of each transformant were identified based on N. gaditana genome information [24], then we could indirectly estimate where transgenes were integrated as shown in Table S1.

The expression of heterologous protein is primarily influenced by the genomic environment in which the transgene is integrated. This is known as the position effect of transgenes [25]. Transgene silencing is the most devastating problem in genetic engineering; it can be caused by the position effect and/or by transcriptional and post-transcriptional silencing complexes identified in algae and plants [26,27]. Such silencing effects can be counteracted using boundary elements called matrix attachment regions (MARs) to enhance transgene expression [28], as shown in Dunaliella salina [29]. Identification of the transgene integration sites in N. salina will help to clarify the integration patterns and could assist in the design of better strategies for achieving the stable expression of heterologous protein. For instance, we are able to find specific flanking sites resulting in better and stable expression of target protein through statistical analysis with lots of integration patterns of transformants.

Taken together, strategies combined with identification of stable integration sites and bombardment transformation is likely very useful for the production of stable transformants.

### 3.2. Heterologous overexpression of sfCherry fluorescent protein

In order to assess the expression of a heterologous protein, we transformed N. salina with an expression construct for the gene encoding sfCherry fluorescent protein (Fig. 2a) and analyzed its expression by Western blot analysis and confocal microscopy. Transformant candidates were initially analyzed by PCR using S1 and S2 primers (Table 1) to amplify part of the Sh*ble* gene as well as the positive control, 18S rDNA (Fig. 2b and c). PCR-positive candidates were subsequently analyzed for expression of the sfCherry fluorescent protein by western blotting. These analyses showed the expression of a protein with a molecular weight of about 27 kD, consistent with the expected size of 27.39 kD based on the mCherry amino acid sequence (Fig. 2d). To further validate the

Table 2
Transformation efficiency of N. salina achieved by particle bombardment.

Promoter	Colonies/10^8 cells	Colonies/μg DNA
TUB	5.9 ± 1.6	5.9 ± 1.6
UEP	4.7 ± 2.0	4.7 ± 2.0
No plasmid[a]	0	0

Data are means ± SD (n = 3).
[a] No plasmid indicates a control bombardment transformation carried out without DNA.
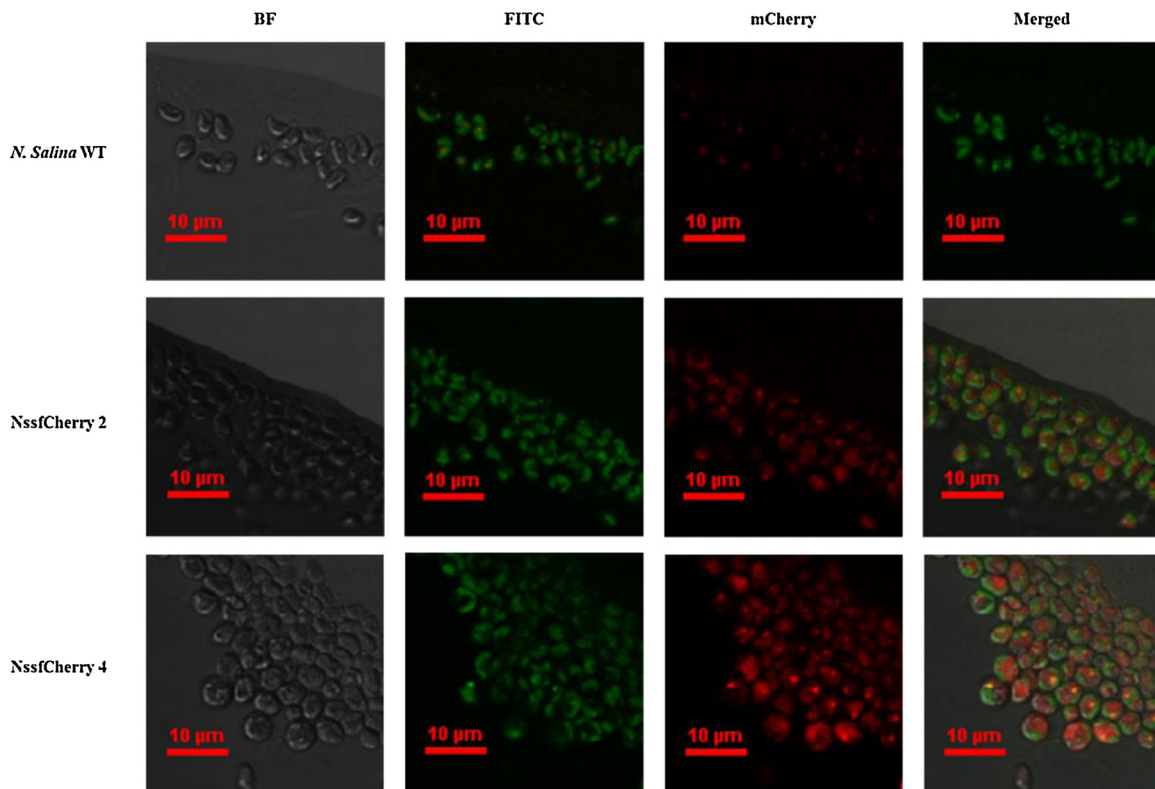
Fig. 3. Confocal microscopic images of NssfCherry 2 and 4 compared with wild-type control. *First column:* bright field (BF) images; *second column:* FITC filter images showing autofluorescence; *third column:* images obtained with the sfCherry filter. Merged images of the three channels are shown in the last column.

expression of functional sfCherry fluorescent protein, we analyzed transformants NssfCherry 2 and 4, which Western blot analyses showed were strongly positive for sfCherry, using confocal fluorescence microscopy (Fig. 3). FITC fluorescence (auto-fluorescence) was detected in both wild-type controls and transformants, but only transformants expressed sfCherry-specific fluorescence (excitation: 561 nm, emission: 595/50 nm). The intensity of sfCherry fluorescence in NssfCherry 4 was stronger than that of NssfCherry 2. This is likely due to the position effect, which can affect the expression level of fluorescent protein [25,28]. These results indicate that particle bombardment can stably deliver DNA into N. salina cells, and produce correctly folded functional proteins.

Fluorescence proteins have been used for structural and functional studies in various cell lines [30]. In addition, there is an increasing trend toward the application of flow cytometry and fluorescent markers, which have been available for use with other organisms as well as microalgae [31]. We previously transformed N. salina with expression constructs for cyan fluorescence protein (CFP), but were unable to detect CFP fluorescence signal owing to the high background (Fig. S2), most likely reflecting the cellular pigments present in Nannochloropsis. In contrast, N. salina cells were successfully transformed and confirmed to express functional sfCherry fluorescent protein without interference from endogenous pigments. sfCherry was developed by directed mutagenesis for efficient folding and strong fluorescence [17,32]; thus, this protein has great potential for the structural and functional analyses necessary for the genetic engineering of Nannochloropsis. In particular, sfCherry fluorescent protein can be applied for development of selection marker, monitoring the real time expression level of fusion protein, and imaging of the localization and dynamics of specific organelles [15].

Furthermore, because microalgal chloroplasts can correctly fold complex proteins, microalgae, especially Chlamydomonas reinhardtii, represent an emerging source for the production of various bioproducts, such as nutraceuticals and therapeutics [11,33]. Another attractive feature of C. reinhardtii as a heterologous protein factory is that a relatively short time is required to yield bioproducts [12]. Although Nannochloropsis will still require genetic improvements to be used as a strain for the production of biofuels and bioproducts such as EPA, our demonstration of heterologous protein expression in Nannochloropsis suggests the possibility of this strain as a cell factory for biofuels and bioproducts.

4. Conclusions

Heterologous protein, sfCherry fluorescent protein, was overexpressed and visualized in N. salina for the first time. To do transformation, particle bombardment was employed, and TUB and UEP promoters were used to express the Shble gene, yielding 5.9 and 4.7 transformants per 10^8 cells, respectively. Genomic integration, confirmed by RESDA PCR, ensured the stable expression of the transgenes. Furthermore, transgenic expression and correct function of the sfCherry fluorescent protein were confirmed by western blotting and confocal microscopy. These results provide techniques for the genetic manipulation of Nannochloropsis that may be useful for the stable transformation and production of bioproducts.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.08.004.

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