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Stable transformation of the green algae *Acutodesmus obliquus* and *Neochloris oleoabundans* based on *E. coli* conjugation

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ABSTRACT

Microalgae are an ideal platform for the production of high-value chemicals, nutritional products and biofuels. Genetic engineering could speed up the development of microalgae derived products and reduce the overall production costs. Genetic methods such as particle bombardment, electroporation, *Agrobacterium tumefaciens* mediated transformation (ATMT), and agitation with glass beads and silicon carbide whiskers have been developed for the genetic transformation of microalgae. However, the transformation efficiency is species dependent, so a variety of transformation methods are required to engineer a wide range of microalgae species. The oleaginous microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* have a great potential as production platforms due to their ability to produce large amounts of triacylglycerol (TAG). Genetic modification techniques however are required to increase TAG levels further or to modify the fatty acid composition. Recently, a conjugation-based method for the delivery of episomes from bacteria to diatom microalgae has been reported. In this study, we have achieved the successful transformation of green oleaginous microalgal strains by transferring an expression vector via conjugation from *E. coli*. Since delivery of exogenous DNA into the microalgae cells is only the first step in obtaining transgenic microalgae, we further analyzed transformation efficiencies by PCR and expression of the Clover fluorescent protein in the targeted species.

1. Introduction

Microalgae are considered as promising feedstock for the production of numerous valuable commercial products which can be used for the production of biofuels, cosmetics, food and feed [1–7]. In order to allow a commercial-scale production of such compounds the integral process has to be economically feasible and thus biomass and product formation need to be enhanced. Genetic modification has become a powerful approach to achieve this goal [8,9]. Since microalgal cells are not able to take up exogenous DNA by nature, several genetic techniques have been developed for this purpose. Classical mutagenesis has been used for the creation of random mutations and a more directed approach to generate specific insertions, deletions or substitutions into the host strain in order to produce the desired phenotype [8].

The main bottleneck for the genetic modification of microalgae is the limited genetic toolbox that is currently available. Among transformation methods for the delivery of exogenous DNA the most common techniques are electroporation, *Agrobacterium tumefaciens*-mediated transformation (ATMT), ballistic systems and agitation with glass beads [10–13]. Although, most of these techniques have been proven to work with great success in model strains such as *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, and *Chlorella sp.*, there is a lack of efficient and stable transformation techniques that can be applied to a broader range of microalgae strains.

Genetic engineering of microalgal strains is considered to be an obstacle due to the great diversity of species with variety of cell sizes, cell wall structures and composition [14]. DNA delivery can be challenging since DNA has to be transferred through the cell wall, plasma membrane and nuclear membrane. Moreover, the cells have to be able to survive the chemical or mechanical treatment. Therefore, particular methods are needed for specific strains and thus a broader range of genetic tools have to be developed.

Electroporation became a successful genetic tool for the transformation of several microalgal species [10,15], however the protocol optimization is often challenging and time-consuming. We previously demonstrated that microalgal species have a different resistance to transformation and thus are more or less prone to take up exogenous molecules. Moreover, viability can decrease rapidly when high voltages are applied and variations in DNA fragment lengths or macromolecule sizes can also decrease the transformation efficiencies [15].

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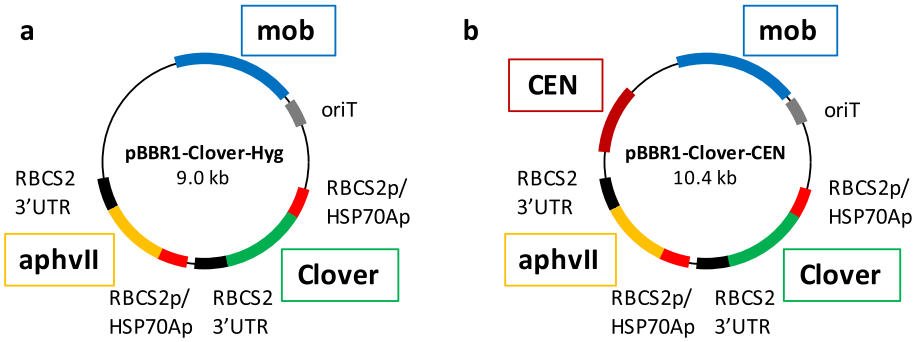

Fig. 1. Schematic map of the conjugative plasmids pBBR1-Clover-Hyg (**a**) and pBBR1-Clover-CEN (**b**).

Previous reports have demonstrated that the use of the bacterial pathogen *Agrobacterium tumefaciens* can be used as an efficient DNA delivery method in microalgae species such as *Chlorella*, *Scenedesmus* and *Ankistrodesmus* [16,17]. Moreover, Karas et al. 2015 [18] and Diner et al. 2016 [19] showed that episomal plasmids containing a yeast-derived centromeric sequence CEN6-ARSH4-HIS3 can be transferred by conjugation from *Escherichia coli* strains to the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. When the centromeric sequence was not present in the plasmids transformation occurred by random chromosomal integration, but at a lower efficiency. The transfer of genetic material via bacterial conjugation requires mainly an origin of transfer and *mob* genes which are essential for the DNA mobilization. *Mob* genes are needed for the DNA-transfer process, they are involved in the recognition and catalysis of the cleavage at the origin of transfer site (*oriT*), creating a single stranded DNA that will be transferred into recipient cells. This mechanism occurs naturally between bacteria, but it has been shown to be possible between prokaryotic and eukaryotic cells as well [20].

We report for the first time an efficient and stable transformation of the green microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* by transferring exogenous DNA from *Escherichia coli* via conjugation. We have designed and tested two plasmids for the integration and episomal transformation in both strains. *Acutodesmus obliquus* and *Neochloris oleoabundans* have been identified as potential candidates for the production of commercially relevant compounds. Both strains can accumulate a large amount of carbohydrates and lipids when grown under nutrient deficient conditions which makes them ideal for the production of biofuels such as bioethanol and biodiesel [21–24]. The transformation of microalgal species via bacterial conjugation offers an alternative for the delivery of exogenous DNA and reduces the cellular damage which occurs in other transformation methods. In this study, we provide a new genetic toolbox for the delivery of DNA into green microalgae which will enable the improvement of existing traits to enhance the economic potential and productivity of the studied strains.

2. Materials and methods

2.1. Strains and growth conditions

Acutodesmus obliquus SAG 276-6 obtained from the culture collection of algae at Göttingen University (SAG) and *Neochloris oleoabundans* UTEX 1185, (University of Texas) were grown in Freshwater (FW) medium as described by Breuer et al. 2012 [25]. Cultures were maintained photoautotrophically at 25 °C on light:dark cycles of 16:8 h under a light intensity of 40 μmol $m^{-2} s^{-1}$ on a rotary shaker (125 rpm). *Escherichia coli* S17 was grown at 37 °C in Luria-Bertani (LB) broth or agar (containing 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract and 10 g L^{-1} of sodium chloride) supplemented with 50 mg L^{-1} of kanamycin or 100 mg L^{-1} of ampicillin as needed.

2.2. Antibiotic sensitivity test

The sensitivity of *A. obliquus* and *N. oleoabundans* to hygromycin B, paromomycin and zeocin was tested. The effect of antibiotics on the viability of both strains was assessed either by plating on FW agar plates or by inoculating in FW liquid medium supplemented with the respective antibiotics. Antibiotics were added to the medium in a concentration range from 0 to 100 μg mL^{-1} when using hygromycin B and paromomycin and from 0 to 50 μg mL^{-1} when using zeocin. An initial cell concentration of 1×10^8 was plated or inoculated per assay. Each test was performed in triplicate for 15–20 days and incubated at 25 °C on light:dark cycles (16:8 h) and light intensity of 40 μmol $m^{-2} s^{-1}$. Flasks were continuously agitated at 125 rpm.

The effect of antibiotics in the strains studied was classified as non-inhibiting, inhibiting and completely inhibiting growth, which was determined by performing colony counting on plates and absorbance measurements at OD_{750} for liquid samples. We defined as non-inhibited, cultures that were able to grow, inhibited to those where no growth was observed without affecting viability and complete inhibition when viability decreased significantly compared to cultures in which no antibiotics were added to the medium.

2.3. Plasmid construction

In order to allow transfer of DNA to *A. obliquus* and *N. oleoabundans* via conjugation, two expression plasmids were constructed: pBBR1-Clover-Hyg and pBBR1-Clover-CEN (Fig. 1).

The conjugative plasmid pBBR1MCS-2 was obtained from Addgene and used as a backbone. pBBR1MCS-2 contains all the genes required for the plasmid transfer such as origin of transfer (*oriT*) and *mob* mobility genes. In order to allow reporter gene expression and antibiotic selection once the plasmid is transferred into the microalgae, two separate cassettes were introduced into the conjugative backbone. The gene encoding the green fluorescent protein variant Clover and the hygromycin B resistance marker *aphVII* were introduced into the backbone pBBR1MCS-2. The cassettes containing Clover and *aphVII* genes were obtained by digesting the plasmid pOpt-Clover-Hyg [26] with restriction enzymes *Kpn*I and *Pvu*I and then ligated into pBBR1MCS-2 to generate pBBR1-Clover-Hyg. *Clover* and *aphVII* gene expressions are both regulated under the *C. reinhardtii* heat shock 70A promoter/Rubisco small subunit 2 promoter (HSP70Ap/RBCS2p) and the 3′ untranslated region RBCS2. The ligation reaction mixture was transformed into *E. coli* S17 competent cells and colonies were selected on Luria-Bertani agar plates supplemented with 50 μg/L of kanamycin. The plasmid pBBR1-Clover-CEN has been constructed by introducing an additional yeast derived sequence CEN6-ARSH4-HIS3 fragment into pBBR1-Clover-Hyg plasmid. The CEN6-ARSH4-HIS3 fragment was obtained by PCR amplification from the episomal plasmid pPtPuc3 (Addgene) and introduced into pBBR1-Clover-Hyg by Gibson Assembly (New England Biolabs). Primers used for the amplification of the CEN6-ARSH4-HIS3 region were F-CEN: 5′-GGCGTATTTGAAGCGGACCCGGT

ACGCGAGCATCACGTGCTAT-3′ and R-CEN: 5′-ACTAAAGGGAACAAA AGCTGGGTACGTCAAGTCCAGACTCCTGTG-3′, both containing overlapping ends with pBBR1-Clover-Hyg previously linearized with restriction enzyme *Kpn*I. The PCR conditions were as follows: Initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 60.5 °C for 30 s and extension at 72 °C for 1 min, for 35 cycles, followed by a final extension at 72 °C for 10 min. The PCR was carried out using the proof-reading Phusion DNA Polymerase (New England Biolabs).

2.4. Bacterial transformation

Escherichia coli S17 competent cells were made by using the Mix & Go *E. coli* Transformation Kit and transformed with pBBR1-Clover-Hyg and pBBR1-Clover-CEN plasmids according to manufacturer's instructions (Zymo Research).

2.5. Transformation of Acutodesmus obliquus and Neochloris oleoabundans by conjugation

A. obliquus and *N. oleoabundans* were exponentially grown in FW medium and harvested by centrifugation at 5000 x *g* for 5 min at room temperature. The cell concentrations were adjusted to 1×10^{8} cells mL^{-1} and 250 μL was plated onto FW medium agar plates and incubated for 7 days at 25 °C under a 16:8 light:dark cycle with a light intensity of 60 μmol $m^{-2} s^{-1}$. FW medium was added to the plates, the cells were scraped and the concentration was adjusted to 1×10^{8} cells mL^{-1} by measuring the cell number on a Multisizer III (Beckman Coulter) using a 50 μm aperture tube.

Escherichia coli S17 strains containing pBBR1-Clover-Hyg and pBBR1-Clover-CEN were grown in 50 mL of Luria-Bertani broth at 37 °C with constant agitation at 250 rpm to a final OD_{600} of 1. Cells were spun down for 5 min at 5000 ×*g* and resuspended in 500 μL of SOC medium.

An equal volume of 100 μL of *A. obliquus/N. oleoabundans* and *E. coli* S17 culture were mixed, plated onto FW 5% LB agar plates and incubated in the dark at 30 °C for 2 h. After incubation, the plates were placed into a 25 °C incubation chamber with light:dark cycles of 16:8 h under a light intensity of 60 μmol $m^{-2} s^{-1}$ for 2 days.

Subsequently, the cells were scraped from plates and replated onto FW agar medium supplemented with 50 mg L^{-1} of hygromycin B and incubated for 15 days under conditions mentioned above.

2.6. Optimization of transformation conditions

In order to determine optimal conjugation conditions, we tested 3 independent incubation times. *A. obliquus* and *N. oleoabundans* were mixed and then incubated with *E. coli* at 30 °C for 60, 90 and 120 min. In addition, we investigated differences in transformation efficiencies when mixing 1:1 and 1:2 microalgal to bacterial ratios (v/v). Microalgal strains were adjusted to 1×10^{8} cells mL^{-1} and the bacterial inoculum was obtained from a culture with OD_{600} of 1.

2.7. Selection of transformant lines

Colonies were obtained from FW agar plates containing the respective antibiotics. Total genomic DNA was isolated from each colony as described by Muñoz et al. 2018 [15]. The *aphVII* gene was identified in the transformant lines by performing a polymerase chain reaction using primers 5′- AGTTCCTCATCGAGAAGTTCGACAG - 3′ and 5′-CCAGAAGAAGATGTTTGCCACCTC - 3′. The PCR reaction was carried out in a total volume of 25 μL with DreamTaq DNA Polymerase (Thermo Fisher). PCR conditions consisted of an initial denaturation at 95 °C for 1 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and with a final extension at 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel. As described by Karas et al. 2015 [18], in order to analyze episomal plasmids we transformed the extracted DNA into *Escherichia coli* S17 and colonies were selected on Luria-Bertani agar plates supplemented with 50 μg/L of kanamycin.

2.8. Fluorescent protein analysis

The cellular fluorescence of the green fluorescent protein Clover expressed in both microalgal strains was analyzed by fluorescence microscopy. Positive transformants were inoculated and grown in a 96-microwell plate containing FW medium with the respective antibiotic concentration. The transformants were incubated for 2 days at 25 °C on light:dark cycles of 16:8 h under a light intensity of 40 μmol $m^{-2} s^{-1}$ on a rotary shaker (125 rpm). Detection of Clover fluorescent protein was performed with the fluorescence microscope EVOS FL Auto Cell Imaging System incorporating a GFP excitation/emission cube (EVOS FL, ThermoFisher Scientific).

2.9. Statistical analysis

All the experiments in this study were performed in biological triplicates. The data were represented as mean ± standard deviation (SD).

3. Results

3.1. Genetic approach for microalgae transformation via conjugation

In order to achieve microalgal transformation via conjugation we followed the experimental design described in Fig. 2. We performed an antibiotic sensitivity test to select appropriate selection markers to be used for the identification of transformant lines. We evaluated the effect of hygromycin B, paromomycin and zeocin on the cell growth of *A. obliquus* and *N. oleoabundans*. To perform the sensitivity test, we either plated or inoculated the strains in FW agar plates or FW liquid medium supplemented with the respective antibiotics. To investigate whether conjugation can be used to transfer genes between *E. coli* and microalgae we constructed the plasmids pBBR1-Clover-Hyg and pBBR1-Clover-CEN. Both conjugative plasmids contain an origin of transfer (*oriT*), *mob* genes which are required for the DNA mobilization, the Clover gene as a green fluorescent reporter and an antibiotic resistance gene which confers resistance to the selected antibiotic. In order to obtain episomal replication in *A. obliquus* and *N. oleoabundans* we constructed and delivered the plasmid pBBR1-Clover-CEN. Finally, both plasmids were transferred to the conjugative strain *E. coli* S17. Bacterial and microalgal cells were co-cultivated first on liquid medium and subsequently plated on agar plates containing 95% (V/V) FW medium and 5% (v/v) LB medium. After 2 days of incubation the plates were scraped and re-plated on FW agar plates containing the selected antibiotic. All antibiotic resistant colonies were analyzed by PCR amplification of the antibiotic resistant gene and analysis of the green fluorescent Clover by microscopy.

3.2. Effect of antibiotics on Acutodesmus obliquus and Neochloris oleoabundans

Our tests showed that all 3 antibiotics resulted in complete growth inhibition of both strains when cells were exposed to concentrations over 50 μg mL^{-1}. As shown in Table 1, liquid media containing the antibiotics were more effective at inhibiting growth of both microalgae species compared to the effect observed on plates supplemented with equal antibiotic concentrations. *N. oleoabundans* showed to be highly inhibited by all 3 antibiotics when the test was performed in liquid medium even at very low concentrations. Cell growth of both species was strongly inhibited by zeocin. When zeocin was used either inhibition or no survival was observed at the lowest concentrations tested. The results indicate that the antibiotics tested are suitable and can be employed as selection markers. Hygromycin B at a concentration of 50 μg mL^{-1} showed a similar effect in both strains, which is sufficient to

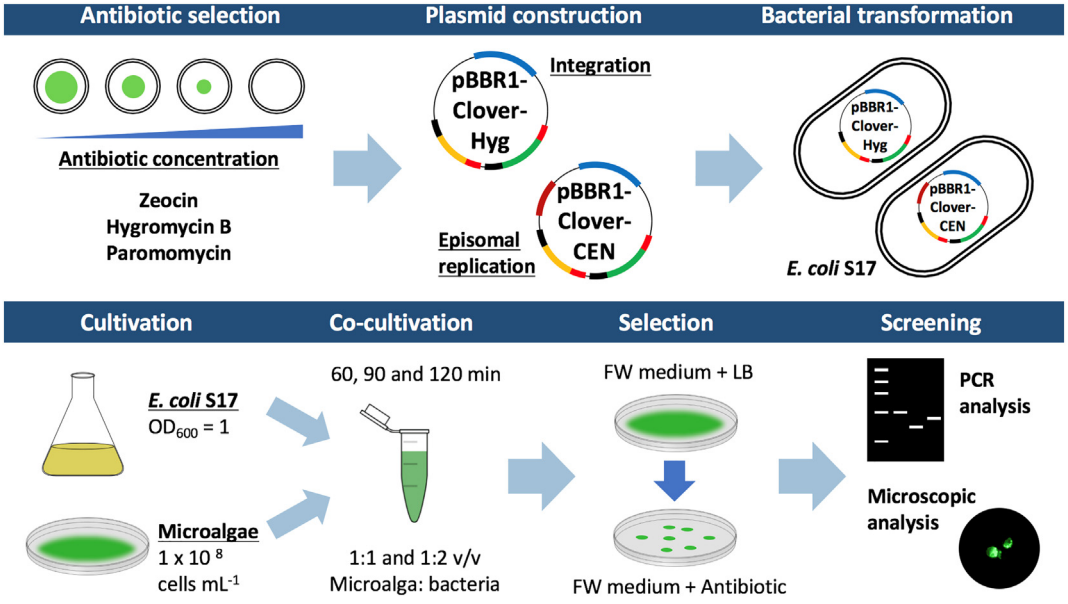

Fig. 2. Experimental design for the genetic transformation of *A. obliquus* and *N. oleoabundans* via bacterial conjugation.

completely inhibit growth. The *aphVII* gene conferring resistance to hygromycin B was chosen and included in the expression plasmids for the selection of positive transformants.

3.3. Transformation of Acutodesmus obliquus and Neochloris oleoabundans via conjugation

In our first attempt we performed conjugation by co-cultivating during 60 min, using a 1:1 ratio microalga:bacteria (v/v). We obtained colonies after 15 days of incubation on FW agar plates containing hygromycin B as a selective pressure. Around 56 and 52 hygromycin resistant colonies were obtained on plates, however only 10–14% were successfully identified as positive transformants for *N. oleoabundans* and *A. obliquus* colonies, respectively. To determine the optimal conjugation times and thus increase the transformation efficiencies, we investigated 3 different incubation times of microalgal strains with the donor strain. The experiment was performed by mixing 1×10^8 microalgae cells with the bacterial strain at OD_{600} of 1. The mixture was incubated at 30 °C for 60, 90 and 120 min. As shown in Fig. 3, longer incubation times resulted in more colonies on plates. Around 200 and 150 hygromycin B resistant colonies were obtained when *A. obliquus* and *N. oleoabundans,* respectively, were exposed to the longest conjugation time. The presence of hygromycin B resistance gene *aphVII* in the transformant lines was confirmed by PCR analysis. *A. obliquus* exposed to 120 min of co-cultivation with *E. coli*, resulted in 20% of positive transformants from the total colonies on plates. Co-cultivation with *N. oleoabundans* resulted in 17% positive transformants. Although more positive transformants were obtained while increasing incubation times, the overall transformation efficiency was not significantly improved considering that an increasing number of colonies not containing the *aphVII* gene were obtained on the selective medium. To further investigate transformation efficiencies, we performed the transformation for 120 min by using 1:1 and 1:2 (v/v) microalgae to bacteria ratios. When using 1:2 ratios we observed a higher variability in number of colonies and positive transformants among the triplicate plates but not a significant increase in the transformation efficiencies.

We also performed the transformation of *A. obliquus* and *N. oleoabundans* by transferring the plasmid pBBR1-Clover-CEN via conjugation (Fig. 3e–h). No significant differences were observed compared to the results obtained with the plasmid pBBR1-Clover-Hyg (Fig. 3a–d). Furthermore, in order to analyze episomal replication we performed the transformation of *E. coli* competent cells with the extracted genomic DNA from transformant lines, however no bacterial colonies were obtained on the selective plates. Since we were not able to recover the episomal plasmid from *A. obliquus* and *N. oleoabundans* strains containing the hygromycin resistance gene, indicating that random

Table 1
Antibiotic sensitivity test performed on *A. obliquus* and *N. oleoabundans*. The effect of hygromycin B, paromomycin and zeocin were tested on FW agar plates (**P**) and FW liquid medium (**L**). The response of both strains to different antibiotic concentrations were classified as non-inhibited (+), inhibited (−) and complete growth inhibition (−-). ND., not determined. The data represents the average of $n = 3$ replicate experiments.

Conc. (μg mL^{-1})	*Acutodesmus obliquus*						*Neochloris oleoabundans*					
	Hygromycin		Paromomycin		Zeocin		Hygromycin		Paromomycin		Zeocin	
	P	L	P	L	P	L	P	L	P	L	P	L
0	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	−	−	+	−	−	−	−	−
10	+	−	+	−	−	−	+	−	−	−	−	−
20	−	−	+	−	−	−	−	−	−	−	−	−
30	−	−	−	−	−	−	−	−	−	−	−	−
40	−	−	−	−	−	−	−	−	−	−	−	−
50	−	−	−	−	−	−	−	−	−	−	−	−
75	−	−	−	−	ND	ND	−	−	−	−	ND	ND
100	−	−	−	−	ND	ND	−	−	−	−	ND	ND

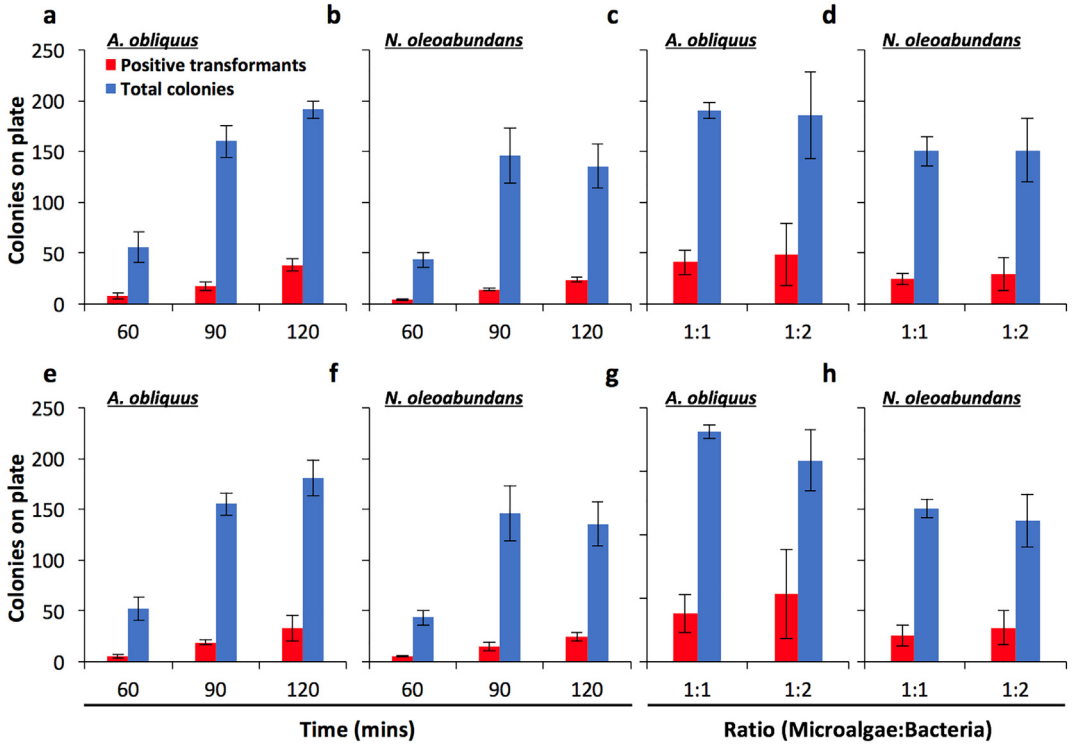

Fig. 3. Number of colonies obtained per transformation at different conjugation times and ratios microalgae:bacteria. *A. obliquus* and *N. oleoabundans* colonies obtained on plates when transformed with pBBR1-Clover-Hyg (**a**–**d**) and pBBR1-Clover-CEN (**e**–**h**) under different conjugation times (**a**, **b**, **e** and **f**). Ratios of 1:1 and 1:2 microalgae:bacteria were tested when exposed to 120 min of conjugation (**c**, **d g** and **h**). Positive transformants correspond to colonies containing the *aphVII* gene. The data represents the mean ± standard deviation (SD). The data represent the average of n = 3 replicate experiments. Standard deviation bars are shown.

integration of the plasmid in the genome might have occurred.

3.4. Expression of fluorescent reporter gene

The green fluorescent protein Clover was used as a reporter to evaluate gene expression. We selected and analyzed 10 positive transformants from each strain that were confirmed to contain the *aphVII* resistance gene after transformation with either pBBR1-Clover-Hyg or pBBR1-Clover-CEN. As shown in Fig. 4, the fluorescence emission of Clover protein is visualized as green and the auto-fluorescence from the chloroplast as red. The red auto-fluorescence of the chloroplast in the transformants was similar to the fluorescence observed in the wild type strains. The green fluorescence of Clover was clearly observed in the transformants and only a vague green fluorescence background is visualized in the wild types. Although the *Clover* reporter gene was stably and successfully expressed in all positive transformants, we visualized different fluorescence intensities in all the strains tested. Very high fluorescence signal was observed in transformants *N.o.*ST-1, *N.o.*ST-3, *N.o.*ST-5 and *A.o.*ST-4 compared to the wild type strains.

3.5. Stability of transformants

The strains *N.o.*ST-1, *N.o.*ST-3, *N.o.*ST-5 and *A.o.*ST-4 which showed high green fluorescence emissions were selected, inoculated in antibiotic-free medium and grown for 8 weeks. All the strains were re-inoculated each week to new fresh medium and transferred to FW agar plates in the last week. To analyze the stability of the transformed cell lines we performed the extraction of genomic DNA from colonies obtained on plates and we analyzed them by PCR amplification of the selective marker. The *aphVII* gene was detected at the expected size in all the tested transformants but the wild type strain. Moreover, no differences in green fluorescence were observed when analyzed with a fluorescent microscope.

4. Discussion

The genetic transformation of *A. obliquus* and *N. oleoabundans* was previously achieved by Guo et al. 2013 [27] and Chungjatupornchai et al. 2016 [28] respectively, by using an electroporation method to deliver the exogenous DNA. The major drawbacks of microalgal transformation methods such as electroporation and ballistics are the need of specialized equipment and the poor transformation efficiency when relatively long DNA fragments are delivered into the microalgae cells [15,29]. Moreover, they can result in high cell death since the cells are not able to recover after the treatment is applied [15]. Therefore, having a broader range of genetic tools available could offer a wider spectrum of capabilities. Bacterial conjugation is a natural mechanism for the gene transfer between bacterial strains. This mechanism has been extensively used as a transformation method for the delivery of cloning and expression vectors to other prokaryotic and eukaryotic cells [20]. In the current study, we adapted and optimized the conjugation conditions for the transformation of the green algae *A. obliquus* and *N. oleoabundans*. Firstly, we determined an appropriate selection marker for both microalgae strains. To identify the correct markers which would allow us to select positive transformants, we performed an antibiotic sensitivity test on plates and liquid medium supplemented with hygromycin B, paromomycin and zeocin. Our results revealed that all the antibiotics tested were effective as selective agents. Hygromycin B was chosen as a suitable selectable marker for both strains. We demonstrated that 50 $\mu g\,mL^{-1}$ is the lowest concentration needed to cause cell death in both strains which is a crucial factor for the selection of positive transformants. Hygromycin B has been reported to be an effective antibiotic for the selection of *A. obliquus* and *N. oleoabundans* strains carrying the *aphVII* gene [27,28].

In order to perform genetic transformation via conjugation the plasmids pBBR1-Clover-Hyg and pBBR1-Clover-CEN were constructed. The conjugative plasmids carry an origin of replication and a

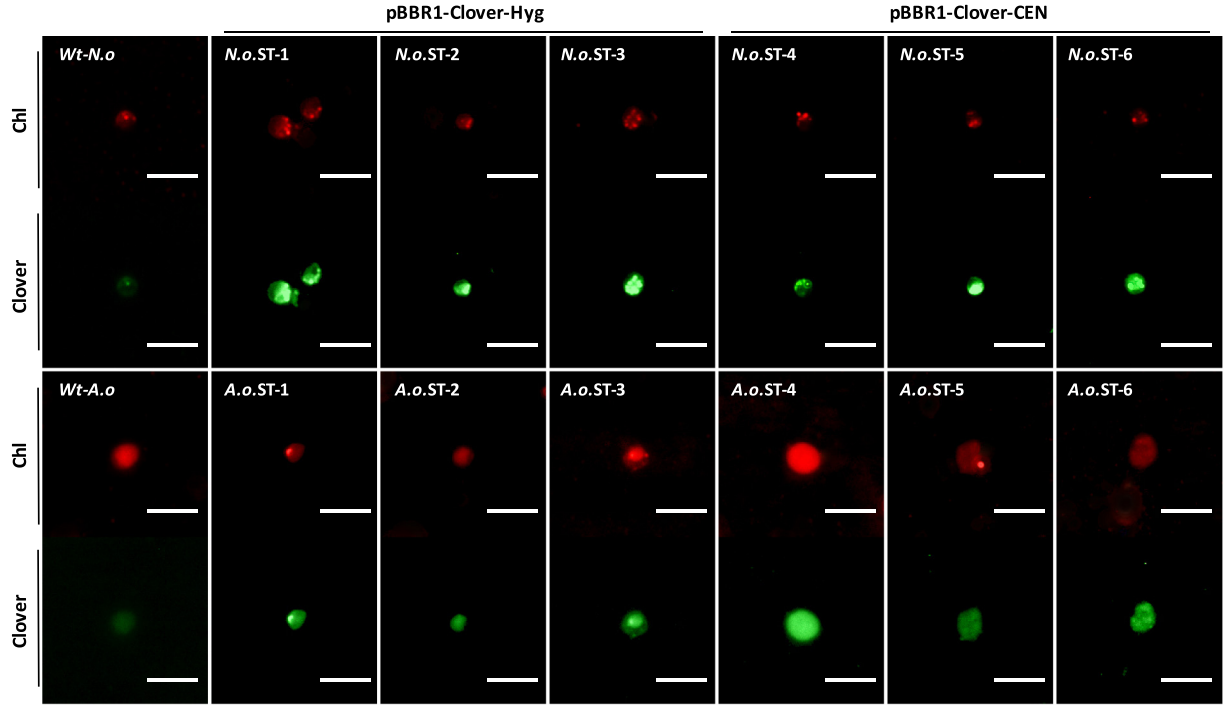

Fig. 4. Microscopic visualization of red chloroplast auto-fluorescence (**Chl**) and detection of green fluorescent protein Clover (**Clover**) in *A. obliquus* and *N. oleoabundans* strains (**ST**) transformed with pBBR1-Clover-Hyg and pBBR1-Clover-CEN plasmids. *A. obliquus* and *N. oleoabundans* wild-type strains (***Wt-A.o*** and ***Wt-N.o***) were used as negative controls. All scale bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

kanamycin resistance gene which allows replication and maintenance of the plasmid in the *E. coli* strain. In addition, both plasmids contain an origin of transfer and *mob* genes which are required for the DNA mobilization from the bacterial donor to microalgal cells [18]. Furthermore, we introduced the *aphVII* gene which confers resistance to hygromycin B and the green fluorescent protein Clover gene under the regulation of the *C. reinhardtii* heat shock 70A promoter/Rubisco small subunit 2 promoter (HSP70Ap/RBCS2p) and the 3′ untranslated region of RBCS2.

The plasmids were successfully delivered via bacterial conjugation to both *A obliquus* and *N. oleoabundans* strains. Positive transformants were identified by the amplification of the *aphVII* gene from genomic DNA, which indicates the integration of the plasmids in the microalgal genomes. We have achieved a higher percentage of positive transformants with *A. obliquus* than *N. oleoabundans* regarding the total colonies obtained on the selective plates. We hypothesized that the interaction microalgae:bacteria might be different when transferring DNA through different cellular barriers considering that both microalgae vary in cell size, cell wall structure and composition [30,31]. In our attempts to increase transformation efficiencies we were able to obtain more positive transformants per plate by increasing the conjugation times. These results show that a longer period of time of exposure of microalgae to bacterial cells provides more time for the DNA transfer to take place. However, the efficiencies were not increased significantly considering that an increasing number of positive and false-positive strains were obtained simultaneously on plates. On the other hand, when microalgae were exposed to twice the volume of bacterial culture we found high variations in positive and false-positives ratios among the triplicate plates. We suspect that higher biomass plated on the selective medium can result in different exposures to light and the antibiotic, which could lead to higher variabilities as observed in our results.

In order to obtain episomal replication in *A. obliquus* and *N. oleoabundans* we constructed and delivered the plasmid pBBR1-Clover-CEN into both strains. Although successful transformation was achieved, we were not able to recover the plasmid from the positive transformants. All transformant lines were confirmed to contain the antibiotic resistance marker even after 8 weeks of incubation in antibiotic-free medium. In previous work using episomal plasmid transformation conducted in *Nannochloropsis oceanica* and *P. tricornutum*, episomal plasmids were rescued; moreover, subculturing the cells in antibiotic-free medium and growing them for 10 and 30 days, respectively, was an efficient strategy to lose the episomal plasmids [19,32,33]. Thus, since we could not rescue any episomal plasmids from genomic extraction and we have seen stable transgene expression subculturing cells in antibiotic-free medium for 8 weeks, our results suggest that chromosomal integration events may have occurred, as observed with the non-episomal plasmid pBBR1-Clover-Hyg. The centromere sequence used in this study has been specifically identified and adapted for diatom microalgae strains. Diner et al. 2016 [19] demonstrated that several combinations of the elements present in the centromere sequence and variations in GC content can lead to higher transformation efficiencies in *Phaeodactylum tricornutum*. Therefore, a more appropriate centromeric sequence has to be identified to allow episomal maintenance and replication in *A. obliquus* and *N. oleoabundans*.

To evaluate the expression of the positive transformants we used the GFP-variant Clover as reporter gene for its high fluorescence emissions and photostability [34]. All positive transformants tested showed the expression of the reporter gene. We visualized different fluorescence intensities due to random integration of our expression vectors. It is likely that multiple integration or integration positional effects may be causing different expression levels [35].

Our work demonstrated for the first time that it is possible to transform green microalgae via *E. coli* conjugation. We reported stable transgene expression in two different species, moreover we performed optimization of the transformation protocol. The development of this novel protocol offers advantages over traditional methods for its simplicity, no need of expensive equipment such as electroporator or gene gun, minimal disruption of algae cells and transfer of relatively large plasmids which could allow the expression of large set of genes. This method therefore can facilitate metabolic engineering approaches for

the creation of green microalgae with improved production traits.

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Authors contribution

CFM and MHJS designed the experiments. CFM performed the experiments, analyzed, interpreted the data and wrote the manuscript. MHJS, RAW, SD and RHW supervised the project and edited the manuscript. All authors contributed to the work, discussed the results, read and approved the final version of this manuscript.

Conflict of interest statement

We declare that there are no conflicts of interest related to this work.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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