PLASTID ENGINEERING OF A MARINE ALGA, *NANNOCHLOROPSIS GADITANA*, FOR CO-EXPRESSION OF TWO RECOMBINANT PEPTIDES

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The purpose of this study was to establish a plastid transformation system for expressing recombinant proteins in *Nanochloropsis gaditana*. On the basis of the sequenced plastid genome, the homologous flanking region, 16S-*trnI/trnA*-23S, and the endogenous regulatory fragments containing the *psb*A promoter, *rbc*L promoter, *rbc*L terminator, and *psb*A terminator were amplified from *N. gaditana* as elements of a plastid transformation vector. Then, the herbicide-resistant gene (*bar*) was used as a selectable marker, regulated by the *psb*A promoter and *rbc*L terminator. Finally, two codon-optimized antimicrobial peptide-coding genes linked by endogenous ribosome binding site (RBS) in a polycistron were inserted into the constructed vector under the regulation of the *rbc*L promoter and *psb*A terminator. After microparticle bombardment, the positive clones were detected using polymerase chain reaction (PCR), and Southern and Western blotting were used to assess the co-expression of the two antimicrobial peptides from the plastid. *Nanochloropsis gaditana* showed the potential to express recombinant proteins for biotechnological applications, for example, for the development of oral vaccines in aquaculture.

**Key index words:** 16S-*trnI/trnA*-23S region; antimicrobial peptide; microparticle bombardment; *Nanochloropsis gaditana*; plastid transformation; ribosome binding site

**Abbreviations:** FACHB, Freshwater Algae Culture Collection of the Institute of Hydrobiology; RBS, ribosome binding site

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Nannochloropsis gaditana is a single-celled marine alga classified as Heterokontophyta, Eustigmatophyceae (Andersen et al. 1998), which has been used as aquaculture feed due to the intracellular abundance of nutrients, especially, polyunsaturated fatty acids (Li et al. 2014). In addition, N. gaditana is one of the few algae that have been successfully cultivated outdoors on a commercial scale (Radakovits et al. 2012), which has received considerable attention in biofuel applications (Jinkerson et al. 2013). Hence, genetic engineering of N. gaditana is required to develop it as a model microalga with important application prospects.

Until now, the nuclear transformation system with targeted integration has been established for Nannochloropsis sp. (Li et al. 2014, Kang et al. 2015). The initial attempt was on Nannochloropsis oculata, in which foreign promoters from Chlamydomonas reinhardtii or viral promoters were used; however, the transformants lost the foreign genes after 1.5 months of unselective cultivation (Chen et al. 2008). This problem was also observed in other algae, mainly because the expression of foreign genes from heterologous promoters was affected by the unique nuclear characteristics of host microalgae. Endogenous promoters are more efficient in generating stable transformants and driving foreign gene expression than heterologous promoters (Walker et al. 2005, Radakovits et al. 2012, Ajawi et al. 2017, Jackson et al. 2019). However, nuclear genetic engineering still remains challenging owing to the occurrence of gene silencing and position effects, resulting in poor expression of foreign genes (Li et al. 2016).

Compared to nuclear transformation, plastid genetic engineering shows many advantages, such as co-expression of multigenes, high expression and accumulation of foreign proteins, and homologous recombination without position effect (Quesada-Vargas et al. 2005, Wang et al. 2009, Wani et al. 2010). Currently, plastid transformation systems have been established in only a few microalgal species, such as Chlamydomonas reinhardtii, Phaeodactylum tricornutum, Haematococcus pluvialis, and Nannochloropsis oceanica (Boynton et al. 1988, Xie et al. 2014, Galarza et al. 2018, Gan et al. 2018). The key to successful plastid transformation is the selection of the intergenic insertion sites, transformation methods, marker genes, and efficient endogenous regulatory sequences (Narra et al. 2018). Generally, two adjacent genes are selected as homologous recombination fragments, such as rbcL/aceD, rps7/ndhB, and trnI/trnÄ (Wang et al. 2009, Adem et al. 2017, Narra et al. 2018), which can harbor foreign DNA fragments without affecting the function of any endogenous gene. Various transformation methods for microalgae have been developed, such as the biolistic method, Agrobacterium-mediated transformation, and electroporation (Nymark et al. 2016, Mini et al. 2018, Gan et al. 2018). To the best of our knowledge, only one species in Nannochloropsis, N. oceanica, harbors a mature plastid transformation system, which expresses the selectable marker, ble, under an endogenous promoter (Gan et al. 2018). However, plastid engineering of N. gaditana has not been possible owing to the lack of efficient plastid genetic tools, especially suitable homologous recombination fragments and polycistron constructs for multigene co-expression, which has hindered further studies and the application of this alga.

In this study, a plastid transformation system of Nannochloropsis gaditana was established for expressing recombinant proteins. For verifying the feasibility of this system, two short peptides were assembled into the newly constructed chloroplast transformation vector and successfully expressed in N. gaditana. This technique will improve genetic engineering of N. gaditana and enable the production of recombinant proteins of biotechnological importance, such as those used in aquaculture.

MATERIALS AND METHODS

Strain and cell growth. Nannochloropsis gaditana strain CCAP 849/5 was obtained from Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. The cells were grown in modified F/2 medium at pH 7.6 - 7.8 and 22°C and illuminated using a 12:12 h light:dark cycle under a light intensity of roughly 75 µmol photons·m⁻²·s⁻¹ (Li et al. 2014).

Cell density (OD₆₀₀) of wildtype Nannochloropsis gaditana and transformants was determined at 600 nm using a UV-visible spectrophotometer TU-1810 (Puxi, China) and measured after every 2 d during 12 d of culture (Chu and Takiguchi 2015). The initial cell density of the cultures (day 0) was set to OD₆₀₀ = 0.1. All OD₆₀₀ data indicated the average of three experiments with standard deviation (± SD).

Cloning the elements for Nannochloropsis gaditana plastid transformation plasmid. The Nannochloropsis gaditana cultures were harvested via centrifugation at 8,000g for 5 min, and genomic DNA was extracted using the plant genomic DNA extraction kit (Tiangen Biotech, China) per the manufacturer's instructions. Gene-specific primers with homologous linkers and proper restriction enzyme sites were designed according to the plastid genome sequence of N. gaditana (https://www.ncbi.nlm.nih.gov/). All primers were synthesized by Ruibo Company (China) and are summarized in Table 1. The N. gaditana endogenous regulatory fragments, psbA promoter, rbcL terminator, rbcL promoter, and psbA terminator, were amplified using primer pairs S3-for/S3-rev, S4-for/S4-rev, S5-for/S5-rev, and S6-for/S6-rev, respectively. Similarly, the homologous flanking fragments, 16S-trnA and trnA-23S, were amplified using primer pairs S1-for/S1-rev and S2-for/S2-rev, respectively.

The herbicide Basta-resistant bar gene was used as the selectable marker and amplified from the pSBV vector (Thermo Fisher, USA) using a primer pair bar-for/bar-rev. Codon bias is an important determinant for protein expression in microalgal plastids. In this study, two antimicrobial peptide-coding genes (n21114, No.6k50_A; piscidin-4, No. AKA60777.2) were codon-optimized to resemble the plastid codon of Nannochloropsis gaditana (66.4% AT content; Table S1 in the Supporting Information) and were then determined to be ant1 and ant2 after codon optimization and synthesis by Ruibo Company. The primer pairs F1-for/F1-rev and

| MATERIALS AND METHODS | PREFERENCES FOR DESIGN | OUTCOMES | CONCLUSIONS |
|-----------------------|------------------------|----------|-------------|
| Cell density (OD₆₀₀)  | 0.1                    |          |             |
| OD₆₀₀ data            | Average of three       | ± SD     |             |
| Cloning elements      | Nannochloropsis gaditana |          |             |
| psbA promoter         | rbcL terminator        | rbcL    | psbA        |
| bar gene              |                        |            |             |
| Codon bias            |                        |            |             |
| Optimized codon       |                        |            |             |
| Antimicrobial peptides |                       |           |             |
| Basta-resistant bar   |                        |           |             |

Table 1. The results of cloning elements for Nannochloropsis gaditana plastid transformation plasmid.
F2-for/F2-rev, containing proper restriction enzyme sites, homologous linkers, and 6 × His tags were used to amplify ant1 and ant2, respectively. All the PCR products were electrophoresed on 0.8% agarose gel and the target bands were recovered using Quick Clean II gel extraction kit (GenScript, China). The purified fragments were sequenced by Ruibo Company.

Plasmid construction. To construct the plastid transformation vector of Nannochloropsis gaditana, homologous flanking fragments, endogenous promoters/terminators, the selectable marker bar, and the two short peptide genes were amplified as described method (Cui et al. 2012), using a range of restriction enzyme sites. The restriction sites were: HindIII and SacI for T1; AatII and XbaI for T2; and SbfI and HindIII for T3.

Selection of transformants. The restored cells were transfected with N. gaditana/pMD-F1 plasmid containing proper restriction enzyme sites. The bar-rev primers were used to confirm the presence of the bar gene, and the con-for and con-rev primers were used to confirm the insertion of the entire expression cassette. PCR with genomic DNA isolated from wildtype Nannochloropsis gaditana cells was used as the blank control.

Samples that were positive in PCR were further analyzed using Southern blotting. Genomic DNA (~ 4 μg) from resistant clones and wild Nannochloropsis gaditana was digested with Hinc II and BamHI and electrophoresed on 0.8% agarose gel. Subsequently, the digested DNA samples were transferred to positively charged GeneScreen nylon membranes (PerkinElmer, USA) using the electro transfer system (Bio-Rad, USA) after denaturation and neutralization. Digoxin-labeled probes were prepared using the PCR products of bar, ant1, and ant2 as templates. Probe labeling and hybridization were conducted as per the protocol of the DIG DNA labeling and detection kit (Roche, Germany). Southern blotting was performed using the DIG High Prime DNA labeling and detection starter (Roche, USA) and monitored using the ChemiDoc XRS + system (Bio-Rad).

Western blotting was performed to verify the expression of the two short peptides. Total soluble protein was extracted from cultures (200 mL per transformant) at post-logarithmic phase (OD600 at 1.2) using plant total protein extraction kit (Sangon Biotech, China). The proteins (~10 μg per sample) were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gradient gels and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were blocked with 5% skim milk in TBST (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h. The membranes were then washed with TBST and incubated with 1:2000 dilution of anti-His antibody (Invitrogen, USA) at 4°C overnight. The membranes were washed again with TBST and incubated with 1:2000 dilution of HRP-conjugated anti-mouse secondary antibody (Santa Cruz, USA) for 1 h. The membranes were washed again with TBST and incubated with ECL substrate. The signals were visualized using X-ray film (Fujifilm).
Positive transformation efficiency was calculated using the following equation (Gutierrez et al. 2012):

\[ \text{TE} = \frac{x}{y} \times \text{surviving cells/\mu gDNA} \]

**RESULTS**

Plastid transformation vector of Nannochloropsis gaditana was successfully constructed. Four endogenous elements were amplified to regulate the expression of foreign genes: psbA promoter (486 bp), rbcL promoter (486 bp), psbA terminator (230 bp), and rbcL terminator (220 bp). In addition, 16S-trnI and trnA-23S homologous flanking fragments were amplified as 1,030 bp and 1,000 bp products.

Using the elements above, the plasmid pT1/ch/bar-anti was constructed as shown in Fig. 1. In pT1/ch/bar-anti, the psbA promoter was used to drive the expression of bar, which was followed by the rbcL terminator. Downstream of the bar expression cassette, ant1 and ant2 were linked by RBS under the regulation of the rbcL promoter and psbA terminator in a polycistron.

Two short peptides were co-expressed from the Nannochloropsis gaditana plastids. The pT1/ch/bar-anti vector was transformed in Nannochloropsis gaditana via microparticle bombardment. After multiple rounds of selection, Basta-resistant colonies were visible on agar plates spread with pT1/ch/bar-anti treated cultures, while the Basta-sensitive cells died out. Finally, 40 clones were separated from the agar plates and cultured in liquid BG-11 medium (Fig. S1 in the Supporting Information).

After five rounds of subculture in the presence of Basta, the genomic DNA from these colonies were extracted and used as templates for PCR. Using bar-for and bar-rev primers, bar was amplified from the positive transformants as a 550-bp product, while this fragment was not amplified from wildtype Nannochloropsis gaditana (Fig. 2a).

To further confirm the insertion of foreign genes, Southern blotting was performed using the PCR-positive M1 transformants. Single strong signals were detected using digoxin-labeled bar, ant1, and ant2 probes in the M1 strain, while the wild Nannochloropsis gaditana did not show any signal (Fig. 2b). Furthermore, 2.1 kb (16S-trnI/trnA-23S) and 4.1 kb bands (expression cassette) were amplified using the con-for and con-rev primers from the M1 strain (Fig. 2c), while the wildtype strain showed only a 2.1-kb band. Thus, the integration of foreign fragments via homologous recombination in the plastid genome of the N. gaditana transformant M1 was confirmed. Based on the proportion of the positive transformants in total tested colonies, the final transformation efficiency was calculated to be 217 ± 47 clones · μg DNA⁻¹.

The expression of two antimicrobial peptides was verified using Western blotting. After hybridization, two bands of approximately 4.55 kDa and 9.56 kDa, which are similar to the weights of ANT1 and ANT2, were detected in the M1 strain (Fig. 2d), while no bands were detected in wildtype Nannochloropsis gaditana. Therefore, the two peptides were successfully co-expressed from the N. gaditana plastid.

The Nannochloropsis gaditana transformants grew normally. To assess the effect of foreign gene expression on cellular viability, the growth rate of the transformant M1 strain was determined. After 12 d of culture, similar growth curves of the M1 strain and wildtype Nannochloropsis gaditana were obtained, as shown in Figure 3. Both M1 and wildtype N. gaditana entered the logarithmic growth phase after 2 days of inoculation at a fixed concentration (OD600 = 0.1) and continued to grow for 8 days. The maximum cell densities (OD600) of the M1 and wildtype strains were 1.41 and 1.37, respectively, which were obtained on the 10th day of culture.

**DISCUSSION**

In recent years, the use of microalgae as recombinant platforms for biosynthesis has attracted increasing attention. Microagal plastids are considered ideal bioreactors for expressing transgenes due to high level of expression of foreign proteins and multigenes from engineered plastids (Adem et al. 2017). Hence, in this study, we developed a plastid transformation system of the microalgae, Nannochloropsis gaditana.

Insertion of foreign genes should not affect the function of the original genes post-plastid transformation. Previous studies in higher plants and microalgae have used two adjacent genes as homologous recombination fragments, namely rbcL/accD, psbA/trnK, and trnA/trnL, etc. (Wang et al. 2009, Adem et al. 2017, Narra et al. 2018). Among them, the trnA/trnL region was mostly used as an insertion site, which may be related to the localization of the region. trnA/trnL is located in the inverted repeat region of the plastid genome, and the orfA located in trnL facilitates rapid replication of foreign genes to two copies in one plastid genome (Narra et al. 2018). However, previous studies on Nannochloropsis oceanica have used chdl, a gene encoding a light-independent protoclorophyllide reductase subunit, as the insertion site (Gan et al. 2018). The introduction of exogenous genes perturbs chdl expression, which is detrimental for cell growth in the dark. Therefore, the trnL/trnA region as is more suitable...
as an insertion site for *N. gaditana* and other species of *Nannochloropsis* than other regions.

Although plastid transformation systems have been established in several microalgal species (such as *Chlamydomonas reinhardtii*, *Haematococcus pluvialis*, and *Nannochloropsis oceanica*), the gene regulatory sequences are scarce (Boynton et al. 1988, Xie et al. 2014, Galarza et al. 2018, Gan et al. 2018). In fact, the commonly used regulatory sequences in plastid transformation are endogenous promoters and terminators, such as the *psbA2* promoter, *rbcL* promoter, *psbA2* terminator, and *rbcL* terminator (Galarza et al. 2018, Gan et al. 2018). Considering that *psbA2* and *rbcL* encode high levels of enzymes related to photosynthesis, the endogenous regulatory sequences of *psbA2* and *rbcL* are generally believed to possess high regulatory activity (Lilly et al. 2002, Gutierrez et al. 2012, Gimpel et al. 2015). Hence, these four endogenous regulatory sequences were selected for regulating the expression of *ant1* and *ant2* in this study. Our results also confirmed that these four endogenous elements can be used to regulate the expression of foreign proteins in the chloroplast of *N. gaditana*.

Despite limited reports on plastid engineering, multigene co-expression with a polycistronic construct is one of the advantages of plastid transformation (Lee et al. 1999). An efficient RBS is crucial for the co-expression of multigenes; hence, an endogenous RBS was introduced between *ant1* and *ant2* to form a polycistronic structure in this study. The results showed stable expression of two proteins,

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**Fig. 1.** Construction of pT1/ch/bar-anti vector. a, F1 fragment fused via fusion PCR using primer pair S1-for/S5-rev; b, F2 fragment fused via fusion PCR using primer pair S6-for/S2-rev; c, pT1/ch/bar vector and F3 fragment were digested with *Xba*I and *BamH*I restriction enzymes, resulting in pT1/ch/bar-anti vector. [Colour figure can be viewed at wileyonlinelibrary.com]
ANT1 and ANT2, indicating that it is feasible to co-express two or more genes in *Nannochloropsis gaditana* plastids.

Appropriate selection markers are also important for plastid transformation. The herbicide Basta (phosphinothricin or glufosinate), commonly used in agriculture, has an inhibitory effect on glutamine synthetase and can rapidly prevent photosynthesis (Day and Goldschmidt-Clermont 2011). Unlike other herbicides, it is characterized by low toxicity, high activity, and good environmental compatibility (Chèvre et al. 1997). The *bar* gene encoding phosphinothricin acetyltransferase has been cloned from *Streptomyces hygroscopicus*, which inactivates phosphinothricin by catalyzing its aminoacetylation, and has previously been used as a selection marker in tobacco (Day and Goldschmidt-Clermont 2011). In green microalgae, the phosphinothricin-resistant *bar* gene was successfully used in *Tetraselmis subcordiformis* plastid as a single selectable marker (Cui et al. 2014). This report shows that the selection strategy with phosphinothricin/*bar* gene is suitable for *Nannochloropsis* species.

Microparticle bombardment used in this study resulted in high transformation efficiency in *Nannochloropsis gaditana*. Considering the advantages of plastid engineering, *N. gaditana* can be engineered to produce oil or express high-value recombinant proteins. However, even after 3 months of screening, we were unable to obtain any homoplastic transformant (Fig. 2c; M1 strain showed both 2.1 kb and 4.1 kb bands). The homogenization of the plastid genome is a requirement for transgene stability of the transformants; therefore, continuous selection will be performed in future.

*Nannochloropsis gaditana* is a microalga with potential for application in biotechnology, such as for the development of oral feed for aquaculture. Antimicrobial peptides can be expressed in this microalga as a “functional feed” to reduce the risks in aquaculture (Dorrington and Gomez-Chiarri 2008, Li and Tsai 2009, Mu et al. 2012). *Nannochloropsis oculata* expressing codon-optimized bovine lactoferrin in cytoplasm showed a bactericidal effect on *Vibrio parahaemolyticus* and increased the survival rate of *Oryzias latipes* after oral-in-tube delivery (Li and Tsai 2009, Mu et al. 2012).
Fig. 3. Growth curves of M1 strain and wild Nannochloropsis gaditana. Cells were cultured in modified F/2 medium of pH 7.6 - 7.8 and illuminated using a 12 h/12 h light/dark cycle under a light intensity of 60 μmol photons - m⁻² - s⁻¹. P < 0.05 indicated significant difference. Each value represents mean ± SD (n = 3). WT, wildtype N. gaditana. [Colour figure can be viewed at wileyonlinelibrary.com]

2009). Considering the major advancements in plastid engineering, this study will further promote the improvement of this important microalgal species.

CONCLUSIONS

In the present study, the plastid transformation system of Nannochloropsis gaditana was established for expressing recombinant proteins. This is the first report of the co-expression of two recombinant peptides from a N. gaditana plastid. This study is expected to accelerate the application of N. gaditana in biotechnology.

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Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

Figure S1. Selection of transgenic Nannochloropsis gaditana with Basta.

Table S1. The sequences of ant1, ant2, and RBS used in this study.