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Establishment of a Micro-Particle Bombardment Transformation System for *Dunaliella salina*

Congping Tan[1,2], Song Qin[1,*], Qun Zhang[1,2], Peng Jiang[1] and Fangqing Zhao[1,2]

[1]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, P.R.China*
[2]*Graduate School of Chinese Academy of Sciences, Beijing 100039, P.R.China*

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In this study, we chronicle the establishment of a novel transformation system for the unicellular marine green alga, *Dunaliella salina*. We introduced the CaMV35S promoter-*GUS* construct into *D. salina* with a PDS1000/He micro-particle bombardment system. Forty eight h after transformation, *via* histochemical staining, we observed the transient expression of *GUS* in *D. salina* cells which had been bombarded under rupture-disc pressures of 450 psi and 900 psi. We observed no GUS activity in either the negative or the blank controls. Our findings indicated that the micro-particle bombardment method constituted a feasible approach to the genetic transformation of *D. salina*. We also conducted tests of the cells' sensitivity to seven antibiotics and one herbicide, and our results suggested that 20 μg/ml of Basta could inhibit cell growth completely. The *bar* gene, which encodes for phosphinothricin acetyltransferase and confers herbicide tolerance, was introduced into the cells *via* the above established method. The results of PCR and PCR-Southern blot analyses indicated that the gene was successfully integrated into the genome of the transformants.

Key words: *Dunaliella salina,* micro-particle bombardment, *GUS, bar*

Dunaliella salina, a motile unicellular green alga with no cell wall, is one of the most halotolerant known eukaryotic organisms, and can be found in environments possessing salinity in a range from <0.1 M to near saturation (Avron, 1986). When exposed to high intensities of light, nutrient deprivation, and other stress conditions, the cells of *D. salina* accumulate abundant quantities of β-carotene, a highly valuable compound. The concentration of this compound can comprise up to 10% of the dry weight of the cells (Ben-Amotz, 1993; Leach *et al*., 1998). β-Carotene, also commonly referred to as pro-vitamin A, has multiple applications in the food, cosmetic, and pharmaceutical industries, and is used as a colorant, an antioxidant, and an anti-cancer agent (Burton and Ingold, 1984; Ben-Amotz and Avron, 1990; Bendich, 1991).

D. salina has a very simple structure and remarkable halotolerant ability, and can be cultured easily, rapidly, and inexpensively. This makes the organism a good bioreactor of recombinant proteins, particularly with regard to the production of pharmaceutical and industrial compounds. Thus far, though, the use of *Dunaliella* as a bioreactor has been limited by the absence of an efficient and stable transformation technique. Thus far, only an electroporation system has been established for the genetic transformation of *D. salina* (Geng *et al*., 2002; Geng *et al*., 2003). However, the transformation efficiency of this technique is somewhat low. Therefore, the development of a more efficient transformation system has clearly been required for quite some time. Our research focused on the establishment of a stable new genetic transformation system for *D. salina,* utilizing a more convenient and efficient method. We elected to use a micro-particle bombardment method, along with an appropriate selective marker.

* To whom correspondence should be addressed.
(Tel) 86-532-82898500; (Fax) 86-532-82880645
(E-mail) sqin@ms.qdio.ac.cn

Materials and Methods

Strains and medium

D. salina was allowed to grow in a modified Johnson medium (Johnson *et al*., 1968) at 23-25°C under illumination at a light intensity of 40-50 μmol photons/m^2/sec, with a photocycle of 14 : 10 (light : darkness). For selection and maintenance, the cells were grown in liquid medium supplemented with 20 μg/ml of Basta.

Vectors

The pBI221 vector (5.6 kb, Promega, USA) harbors a CaMV35S promoter-*GUS* construct. The p35S-bar vector, which harbors the CaMV35S promoter-*bar,* was kept in our laboratory. All vectors used in this study were prepared according to the method described by Sambrook (1992).

Sensitivity test

Streptomycin, kanamycin, hygromycin, geneticin (G418), Zeocin, spectinomycin, chloramphenicol, or the herbicide Basta were added to liquid media (5 ml) respectively, in cases in which *D. salina* was allowed to grow until logarithmic phase (approximately 1.0×10^6 cells/ml). The final concentrations of each of the antibiotics used, namely, streptomycin, kanamycin, hygromycin, spectinomycin, and G418, were 200, 400, 800, and 1,600 μg/ml, respectively. The final chloramphenicol concentrations were 100, 200, 400 and 800 μg/ml. The final Zeocin concentrations were 50, 100, 200 and 400 μg/ml. The final Basta concentrations were 5, 10, 15 and 20 μg/ml. The cells were counted under light microscopy.

Micro-particle bombardment

The Biolistic particle delivery system PDS1000/He (Bio-Rad, USA) was employed in this experiment. Cells were collected in logarithmic phase (1.0×10^6 cells/ml) *via* 30 sec of centrifugation at 6,000 g at 20°C, and were then transferred to sterilized plates for bombardment. 1.0×10^8 cells were bombarded in each of the treatments. The plasmid DNA was coated onto the gold particles used in the micro-particle bombardment according to the methods described by Jiang *et al.* (2002). Each of the bombardment treatments employed 1 μg of vector DNA, coated onto 60 μg of gold particles. The negative controls were bombarded with uncoated gold particles in an identical fashion. Three different rupture-disc pressures were applied at different bombardment distances (Table 1). All of the experiments were conducted in triplicate. After bombardment, the cells were cultured under dim light conditions for 24 h.

GUS assays

Histochemical staining for GUS activity was conducted 48 h after bombardment, as was described by Jefferson (1987). Before observing the samples *via* microscopy, the samples were immersed in 70% ethanol in order to remove the greenish background.

DNA isolation and PCR

D. salina cell cultures were allowed to grow to an approximate cell density of 1.0×10^7 cells/ml. The cells were then collected *via* centrifugation at 6,000 g for 5 min, then resuspended in 500 μl of SDS buffer (1% SDS, 20 mM Tris-HCl, 15 m M NaCl, 200 mM EDTA, pH 8.0). This mixture was then incubated for 2 h at 50°C, extracted with an equal volume of phenol/chloroform, and centrifuged. The aqueous layer was then transferred into a new 1.5 ml tube, and the DNA was precipitated with 2 vol of 100% ethanol. After precipitation, the DNA was pelleted for 10 min at 6,000 g. The pellet was then washed in 70% ethanol, and was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The PCR analysis for the detection of the integration of the bar gene into the *D. salina* genome was conducted using primer 1 (5' GCACCATCGTCAACCACTA 3') and primer 2 (5' CAGAAACCCACGTCATGC 3'), both of which were designed predicated on the portion of the coding region of the *bar* gene. Amplification was conducted with a Biometra T personal Thermocycler under the following cycle conditions: 94°C for 5 min, 30 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min), and then an extension step for 10 min at 72°C. The PCR products were evaluated via gel electrophoresis in 1.0% agarose gel. The primers used to amplify the plasmid p35S-bar were then used as a positive control in the PCR reactions.

Table 1. Transformation efficiency with different parameters

Rupture-disc pressure	Bombardment distance	Efficiency of transformation ($\times 10^{-4}$)
450 psi	6cm	1.12 ± 0.89
	9cm	0.53 ± 1.65
900 psi	9cm	0.14 ± 1.33
	12cm	0.57 ± 1.74
1,100 psi	12cm	0

The frequency of transformation was measured according to the number of blue cells among total checked cell. Mean ± SD (n=3)

PCR- Southern blot analysis

The PCR products were electrophoresed on 1.0% agarose gel, then transferred onto positively-charged nylon membranes, using the Bio-Rad Mini-PROTEAN II Cell blotter systems. Southern blots were conducted using the *bar* gene cleaved from the vector as a probe, in accordance with the instructions provided in the DIG DNA Labeling and Detection Kit (Roche, USA).

Results

Sensitivities to seven antibiotics and the Basta herbicide

Neither streptomycin, kanamycin, hygromycin, spectinomycin, nor G418 were determined to inhibit the growth of *D. salina,* even at a concentration of 1,200 μg/ml. The cells were determined to be sensitive to both chloramphenicol and Zeocin. 400 μg of chloramphenicol/ml or 100 μg of Zeocin/ml were determined to be sufficient to inhibit the growth of *D. salina.* The cells were also determined to be extremely sensitive to Basta, and a concentration of 20 μg/ml was sufficient to completely inhibit the growth of *D. salina* (Fig. 1). Basta can also be employed as a selective agent, and the bar gene is the most efficient selective marker.

Transient expression of GUS gene

The activity of GUS, i.e. the product of expressed *GUS,* was observed in the bombarded cells 48 h after bombardment under rupture-disc pressures of 450 psi and 900 psi (Fig. 2). None of the blue cells were ever detected in

either the blank or the negative controls. This result was confirmed to be reproducible in our laboratory. The optimal parameters for this procedure were found to be a bombardment distance of 6 cm, and a rupture-disc pressure of 450 psi. The maximal transformation efficiency was determined to be approximately 10^{-4} (blue cells/total checked cells).

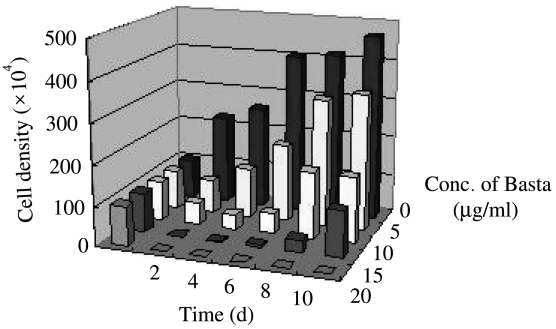

Fig. 1. Effects of the Basta herbicide on the growth of *D. salina*. The initial cell density was 1.0×10^6 cells/ml. Cell density was determined every 2 days after exposure to different concentrations (0, 5, 10, 15 and 20 μg/ml) of Basta.

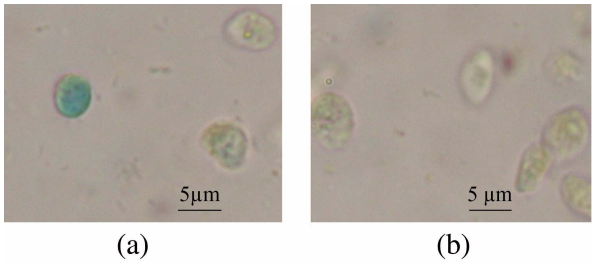

Fig. 2. Histochemical staining for GUS activity after 48 h of bombardment (magnification: 15 × 40).
(a) Transient *GUS* expression in transformed *D. sanila* cells, blue cells represent the transformants.
(b) Negative controls, cells were bombarded with uncoated gold particles.

Transformation of D. salina with the p35S-bar plasmid

After being transformed, the cells were incubated under dim light conditions (3 μmol photons/m^2/ sec) for 24 h. During Basta selection, the cells were carefully transferred to liquid medium supplemented with 20 μg/ml of Basta, then cultured under moderate light conditions (45 μmol photons/m^2/ sec) at a temperature of 24°C. The negative controls were then selected in the same fashion. After 2 weeks of culturing, the transformed *D. salina* cells were allowed to grow to a cell density of approximately 1.0×10^6 cells/ml, whereas none of the negative controls survived. Our findings indicated that the tolerant cells were the positive transformants.

PCR and PCR southern analysis of transformants

DNA samples obtained from the Basta-tolerant cells and the negative *D. salina* controls were analyzed *via* PCR, using a pair of specific primers, in order to confirm the presence of the *bar* gene. The expected PCR fragments (approximately 430 bp) were detected in the tolerant cultures, whereas no bands were visible in the negative controls. The PCR products were analyzed further *via* Southern blot analysis. After hybridization, we detected a band of approximately 430 bp in the transformants (Fig. 3). Our results indicated that the *bar* gene could be integrated stably into the *D. salina* genome, with no hybridizing bands seen in the negative control.

Discussion

The selection of transformants is the key procedure in the establishment of a transformation system. Geng *et al.* (2003) elected to use chloromycetin as a selective agent, and *CAT* as a selective marker. In this study, we determined that *D. salina* cells were extremely sensitive to Basta, indicating that the *bar* gene might be an efficient selective marker for the genetic transformation of *D.*

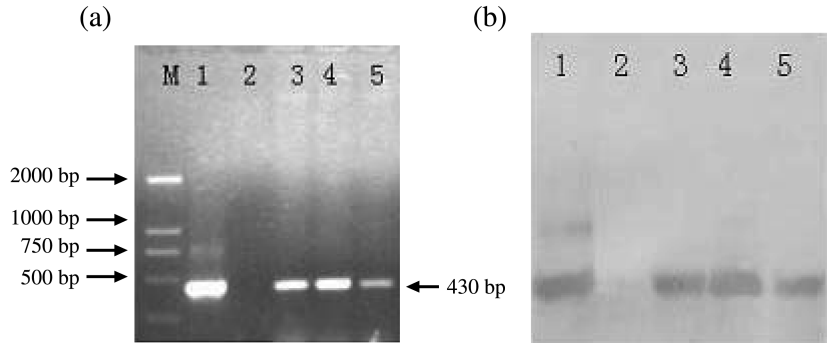

Fig. 3. PCR and PCR-Southern blot analysis of the transformed *D. salina*.
(a) PCR analysis of transformants. M, Marker DL2000 (Takara, Japan); Lane 1, positive control, PCR products (430 bp) from the p35S-bar plasmid; Lane 2, negative controls, PCR products from the wild-type *D. salina* cells; Lanes 3-5, PCR products (430 bp) from transformed *D. salina* cells.
(b) PCR-Southern blot analysis of transformants. Lane1, positive control, the hybridization of PCR productions from plasmid p35S-bar; lane2, negative control, the hybridization of PCR products from wild-type of *D. salina;* lanes 3-5, the hybridization of PCR products from the transformed *D. salina*.

salina. The *bar* gene encodes for phosphinothricin acetyltransferase (PAT), which confers tolerance to the herbicide phosphinothricin (PPT) - the active ingredient in the Basta herbicide, which is used extensively as a selective marker gene in the genetic transformation of many higher plants. The results of our experiment also verified that the cells were extremely sensitive to chloromycetin and Zeocin, but were not sensitive to streptomycin, kanamycin, hygromycin, spectinomycin, and G418. Moreover, the findings of our study indicated that the determination of selective pressure depends on cell density, and higher cell density requires an increase in selective pressure (data not shown).

Several methods have been used in the transformation of algal cells, including micro-particle bombardment (Boynton *et al*., 1988), electroporation (Chen *et al*., 2001), and agitation with glass beads (Kindle, 1990) or silicon fibers (Dunahay, 1993), mediated by *Agrobacterium tumefaciens* (Sasidharanpillai *et al*., 2004). Among these methods, micro-particle bombardment has proven to be the most powerful method for the transient and stable expression of foreign genes, in both macro- and micro-algae, including *Laminaria japonica* (Jiang *et al*., 2002), *Undaria pinnatifida* (Qin *et al*., 2003), and *Haematococcus pluvialis* (Teng *et al*., 2002), in our laboratory. The results of the present study demonstrate that micro-particle bombardment constitutes an effective method for the transformation of the economic halotolerant micro-alga, *D. salina,* after the optimization of transformation conditions. The results of this experiment indicate that lower bombardment pressure (≤ 900 psi) is superior to high pressure (1,100 psi). This is most likely attributable to *D. salina's* lack of a cell wall.

It is worth mentioning that the *GUS* reporter gene has been successfully transiently expressed in transformed *D. salina* cells. Another reporter gene, called *lac Z*, encodes for β-galactosidase. We had previously attempted to use *lacZ,* but met with no positive results. A third reporter gene, i.e. *EGFP,* which encodes for a fluorescent protein, was then employed, but we detected a green fluorescence background after the microscopic observation of a large amount of blank cells. After a long experimental period, we concluded that *EGFP* was not an appropriate reporter gene for the transformation of *D. salina* (Data not shown).

In our present study, the *bar* gene was introduced into *D. salina*, and Basta-resistant strains were assessed *via* molecular analysis. PCR and PCR-Southern blot analyses indicated that *bar* could be integrated into the *D. salina* genome. Our results indicate that we successfully transformed the unicellular green marine alga (*D. salina*) using *bar,* via a method which proved both efficient and reproducible-namely, the micro-particle bombardment method. Therefore, we are reporting the establishment of a novel genetic transformation system for *D. salina*. Further research, however, will be required before foreign genes can be introduced into *D. salina* cells in order to generate recombinant vaccines, mammalian antibodies, and added-value compounds, including carotenoids, polyunsaturated fatty acids, hydrogen, or bio-fuels.

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