J. Phycol. **44,** 768–776 (2008)

DOI: 10.1111/j.1529-8817.2008.00508.x

CONDITIONAL PRODUCTION OF A FUNCTIONAL FISH GROWTH HORMONE IN THE TRANSGENIC LINE OF *NANNOCHLOROPSIS OCULATA* (EUSTIGMATOPHYCEAE)[1]

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Plasmid phr-YPGHc, containing the fish growth hormone (GH) cDNA driven by a heat shock protein 70A promoter and a RUBISCO SSU 2 promoter, was transferred into the protoplast of marine microalga *Nannochloropsis oculata* (Droop) D. J. Hibberd by electroporation. Four transgenic clones were obtained in which the transferred phr-YPGHc was integrated into the genome and existed stably at least until the 50th generation. When we treated these transgenic microalgae by heat shock, the heterologous fish GH was produced in the amount of 0.42 to 0.27 μg · mL^{-1} from the 50 mL of medium. We incubated artemia with the wildtype and transgenic *N. oculata* for 6 h and then fed these microalgae-treated artemia to red-tilapia larvae. After feeding, the growth of larvae that were fed artemia incubated with transgenic microalgae was greater (i.e., statistically significant: $P < 0.05$) than that of larvae that were fed artemia incubated with nontransgenic microalgae: 316% versus 104% in weight gain, and 217% versus 146% in body length increase, respectively. Therefore, the *N. oculata* enables production of functional GH, and we propose that it might be an excellent bioreactor material.

Key index words: **bioreactor; gene transfer; inducible promoter; microalga; *Nannochloropsis;* protoplast**

Abbreviations: **ARS, arylsulfatase; bp, base pair; EPA, eicosapentaenoic acid; GH, growth hormone; kb, kilobase pair; kDa, kilodalton; kV, kilovolt; NaCl, sodium hydrochloride; YP, yellowfin porgy; YPGH, yellowfin porgy growth hormone**

The global market for aquaculture products like fish and shellfish ranges over US$40–50 billion per year (New 1999) with a tremendously increasing trend (8% p.a.), especially in Asia–Pacific regions. Some microalgae are considered excellent sources of proteins, carbohydrates, lipids, and vitamins and have been used indispensably as food and/or feed additives for aquacultural larvae for >40 years (Rocha et al. 2003). In addition, microalgae are a ''green-water'' maker for larvae tanks (Fulks and Main 1991, Lubzens et al. 1995). Therefore, microalgae have important functions in aquaculture hatchery in terms of the nutritional value from the natural food chain and the maintenance of water quality in the hatchery environment (Rodolfi et al. 2003).

More than 40 species of microalgae are used in aquaculture worldwide, depending on the special requirements of local seafood production. *Nannochloropsis* (Hibberd 1981), which was previously known as *Chlorella*, is a marine unicellular alga belonging to the class Eustigmatophyceae (Hibberd 1990, Karlson et al. 1996). Several species of *Nannochloropsis* were identified (Lubzens et al. 1995) since the genus was first identified (Chini Zittelli et al. 1999). *Nannochloropsis* is commonly cultivated in fish hatcheries as a food source for zooplankton production, such as rotifers and copepods, which in turn is used as feed for rearing larvae of many species of mollusks, crustaceans, and fish (Lavens and Sorgeloos 1996), because *Nannochloropsis* is well known as a source of pigments, such as chl *a*, zeaxanthin, canthaxanthin, and astaxanthin (Sukenik 1999), and a source of polyunsaturated fatty acid, such as EPA (20:5n3) (Chini Zittelli et al. 1999, Sukenik 1999). Astaxanthin is beneficial for human consumption because of its usefulness in preventing several diseases (Chini Zittelli et al. 1999). Therefore, *Nannochloropsis* is a potentially

[1]Received 4 February 2007. Accepted 27 September 2007.
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good material for a bioreactor to carry a valuable heterologous protein because of easy culture, low cost, high expression, and safety in the environment and in food (Hawkins and Nakamura 1999). However, to date, there have been no studies of the transgenesis of *Nannochloropsis*.

In this study, we develop an effective approach for transferring DNA fragments to *N. oculata* and demonstrate that transgenic *N. oculata* expresses the introduced fish GH cDNA under the control of an inducible promoter. The heterologous fish GH cDNA is proven to be transmitted stably for at least 50 generations in the transgenic *N. oculata*. Furthermore, the growth of tilapia fry is enhanced significantly after the fry are fed this transgenic *N. oculata* strain, suggesting that transgenic microalgae expressed a functional fish GH. This is the first report that expresses the foreign protein under the inducible system in *Nannochloropsis*. We propose that it might have high potential for being a new bioreactor material for application, especially in aquaculture.

MATERIALS AND METHODS

Culture conditions and preparation of algal protoplast. The f/2 medium (Guillard 1975) was used for culturing *N. oculata* as described by Rodolfi et al. (2003). The 100 $\mu g \cdot mL^{-1}$ of ampicillin and kanamycin were added in the f/2 medium to prevent bacterial contamination. Growth condition was 28°C for 5–7 d under a 14:10 light:dark (L:D) photoperiod at 35–40 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$. To prepare protoplasts, 5 mL *N. oculata* cells (grown to $\sim 1 \times 10^7$ cells $\cdot$ mL^{-1}) were collected by centrifugation at 8,000*g* for 10 min at room temperature. The pellet was washed twice with 500 μL of fresh f/2 medium and resuspended in 500 μL of fresh f/2 medium. Several enzyme mixtures were added to examine the efficacy of cell wall digestion. They were (i) 4% cellulose (Sigma Inc., St. Louis, MO, USA) and 4% macerase (Sigma Inc.); (ii) 4% hemicellulase (Sigma Inc.) and 2% driselase (Sigma Inc.); (iii) 4% cellulose, 4% macerase, and 4% hemicellulase; and (iv) 4% cellulose, 4% macerase, 4% hemicellulase, and 2% driselase. Cells and enzyme mixture were incubated at 37°C for 8 h in the dark with gentle shaking. After the treatment with these enzymes, the protoplast production was confirmed by Calcofluor white (Sigma Inc.) staining. Calcofluor is a fluorescent material that binds to cellulose components of the cell wall and exhibits a blue color when visualized by fluorescence microscopy. This fluorescence indicated removal of the cellulose component from the cell wall.

Construction of phr-YPGHc plasmid for gene transfer. We constructed a 12 kb plasmid, phr-YPGHc, in which GH cDNA (YPGHc) of yellowfin porgy (YP, *Acanthopagrus latus*; Tsai et al. 1993) was driven by a fusion promoter consisting of a promoter of heat shock protein 70A gene (*HSP70A*) and a promoter of RUBISCO SSU 2 (RBCS2) derived from the green alga *Chlamydomonas reinhardtii* (Schroda et al. 1999). This plasmid was prepared in large scale, as described by Sambrook et al. (1989), extracted by using the VIOGENE V500 kit (VIOGENE, Sunnyvale, CA, USA), digested by *Xba*I, and isolated by a gel-recovery kit (Geneaid, Taipei, Taiwan). The purified linear DNA plasmid was resuspended in filtered sterilized seawater at a concentration of 20 $\mu g \cdot \mu L^{-1}$.

Gene transfer by electroporation. *Nannochloropsis oculata* protoplasts were centrifuged at 400*g* for 5 min and were washed twice with fresh f/2 medium at room temperature. The pellet was gently resuspended with 5 mL of f/2 medium containing 0.6 M sorbitol (Sigma Inc.) and 0.6 M mannitol (Sigma Inc.), and then 20 μg linear DNA fragments were added. After incubation on ice for 10 min, the foreign DNA was introduced by electroporator (BTX, Holliston, MA, USA) under a combination of different parameters, such as voltage (ranged from 0.5 to 2 kV) and pulse time (ranged from 10 to 20 μs), to generate the transgenic *N. oculata*. Before electroporation, the protoplast cells were stained with Calcofluor white, and we calculated the cell numbers that appeared blue under fluorescence microscopy. After electroporation, we also calculated the cell numbers that were regenerated to a normal circular-shape and appeared green under LM. The survival rate was calculated by the proportion of number of cells after elecroporation among the total number of cells before electroporation.

Regeneration and growth. After electroporation, the cells were transferred to a glass tube containing 5 mL fresh f/2 medium and cultured at 28°C for 3–5 d with shaking at 200 rpm. The cell pellet of *N. oculata* was collected by centrifugation at 8,000*g* for 5 min. After the pellet was resuspended in 20 μL of fresh f/2 medium, the cells were spread on the f/2 medium plate at 28°C for 5–7 d. Each colony was picked up and cultured in liquid media as described above for subsequent analyses.

Genomic extraction. The genomic DNA extraction of *N. oculata* was modified according to Dawson et al. (1997). Cultured cells from 50 mL ($\sim 1 \times 10^7$ cells $\cdot$ mL^{-1}) were harvested and resuspended in a 500 μL buffer solution (54 mM hexadecyltrimethylammonium bromide, 0.25 mM Tris [pH 8.0], 1.4 M NaCl, 10 mM EDTA, and 2% β-mercaptoethanol). The mixture was incubated at 65°C for 2 h and shaken every 15 min. After incubation, an equal volume of phenol–chloroform was added, and the aqueous phase was recovered after 5 min of centrifugation at 8,000*g* for 10 min. The mixture was extracted several times until the aqueous layer was no longer cloudy. The genomic DNA was precipitated with 2 volumes of 100% ethanol, centrifuged at 8,000*g* for 15 min, washed with 70% ethanol, dried, and resuspended in 30 μL TE (10 mM Tris [pH 8.0] and 1 mM EDTA [pH 8.0]) buffer.

PCR analysis. Two oligonucleotide primers were synthesized for detection of the existence of the transferred DNA fragment, YPGH cDNA, by PCR analysis: a forward primer (5′-GGTCCC-TTTGTGCGCCGTTA-3′) and a reverse primer (5′-CTGTCG-TTGCTCCTCAGTCAG-3′). The primers for detection of the endogenous 18S rRNA gene, which served as an internal control, were the forward primer 5′-GCGGAGGAAAAGAAC-TAACCAGGATT-3′ and the reverse primer 5′-AACGCCATGG-CACAACCGC-3′. Each PCR sample consisted of 20 μL of solution containing 10–20 ng of template, 10 pmol of each primer, 25 μM of each dNTP, and 5 U of Viotaq enzyme (VIOGENE), in a 10× PCR buffer. Amplification was performed with a Perkin-Elmer Cetus DNA Thermal Cycler (Norton, OH, USA). PCR consisted of 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by 10 min extension at 72°C. PCR products (10 μL) were subjected to electrophoresis on a 3% NuSieve GTG agarose gel (Cambrex, Rockland, ME, USA).

Genomic Southern analysis. *Xba*I-digested genomic DNA isolated from *N. oculata* was separated on an agarose gel and transferred onto a nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA, USA). The procedures of probe preparation and Southern blot analysis involved the use of the digoxigenin (DIG; Roche, Florence, SC, USA) system as previously described (Chen et al. 2006). Equipment for the gel electrophoresis and Southern blot was purchased from Protech Technology Enterprise Co. (Taipei, Taiwan).

Induction, extraction, and Western blot analysis of the produced YPGH. Culture of 50 mL ($\sim 1 \times 10^7$ cells $\cdot$ mL^{-1}) of the transformed *N. oculata* was heat shocked by shifting to 42°C for

protein expression with a different induction time. The protein extracted from the transformed *N. oculata* was modified from Hawkins and Nakamura (1999). Briefly, after microalgae were collected by centrifuging for 10 min at 8,000*g* in 4°C, the pellet was resuspended in 500 μL extraction buffer (Franklin et al. 2002). The cell suspension was transferred to a microtube (Axygen, Union City, CA, USA) containing 0.5 g glass beads (Sigma), and cells were broken by a mini-beadbeater (Biospec Products, Bartlesville, OK, USA). Cells were broken at room temperature for 30 s and then put on ice for 20 s. We repeated this procedure at least 15 times. The supernatant was collected by centrifuging for 15 min at 10,000*g* at 4°C, and 50 μL of sample loading buffer (1 mM EDTA, 250 mM Tris-HCl [pH 6.8], 4% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue, 50% glycerol) was added. Prior to running SDS-PAGE analysis, samples were boiled for 10 min and then centrifuged for 10 min at 10,000*g*. The supernatants were electrophoresed on a 12% SDS–polyacrylamide gel. The detection of Western analysis was performed by NBT/BCIP system (Roche, Florida, SC, USA). The final dilution of polyclonal antiserum against YPGH was 1:2000, and alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz, Santa Cruz, CA, USA) was used as the secondary antibody. Equipment for the gel electrophoresis and Western blot was purchased from BioRad Laboratories (Hercules, CA, USA).

ELISA. The yield of heterologous YPGH protein expressed by the transformed *N. oculata* was determined by ELISA. The protein extracted from the transformed *N. oculata* was described as above. The purified histidine-tagged YPGH recombinant protein was used as a standard protein, and the rabbit polyclonal antibody against YPGH protein was used as the primary antibody at 1:1000 final dilution. The optical density (OD_{405}) was measured with a CERES UV900C ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) to determine the concentration of heterologus YPGH produced by *N. oculata*.

The stable inheritance of transferred DNA fragment. The transformed *N. oculata* was inoculated at the initial concentration of 1×10^3 cells $\cdot$ mL^{-1} in fresh f/2 medium and cultured until it reached $\sim 1 \times 10^7$ cells $\cdot$ mL^{-1}. We then continuously transferred the cells to fresh f/2 medium at the initial concentration. Serial inoculation of the transformed *N. oculata* was carried out. We used PCR to detect the existence of the inserted YPGH in the genome of the transformed *N. oculata* after each renewal inoculation.

Biological activity of YPGH produced by N. oculata. Two thousand larvae aged 1 week and derived from red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) were collected and cultured for another week (in laboratory) prior to performing the feeding experiment. In total, 240 larvae similar in size were selected (~0.20–0.26 g in weight; 1.32–1.38 cm in length) and divided into four groups. They were fed the brine shrimp, which were incubated with (i) wildtype *N. oculata* for 4 h, (ii) transformed *N. oculata* for 4 h, (iii) wildtype *N. oculata* for 6 h, and (iv) transformed *N. oculata* for 6 h. Each group consisted of 20 larvae and was cultured in a 120 L tank filled with 90 L of water in triplicate. Because fish larvae cannot consume *N. oculata* directly, brine shrimp (*Artemia nauplius*) are commonly used as a grazer of the microalgae since brine shrimp remove the cell wall of *N. oculata*. Thus, we took advantage of using brine shrimp to release the fish GH from *N. oculata*. Brine shrimp (O.S.I. Marine Lab. Inc., Snowville, UT, USA) were cultured in 2 L cup-shaped containers filled with 30‰ seawater at room temperature and starved for 5 d after hatching. About 2×10^5 brine shrimp were incubated with 1×10^8 cells of either transformed or wildtype *N. oculata* for 4 or 6 h. After incubation, the brine shrimp were collected by a screen net (60 μm in mesh) and evenly dispersed on the surface of a 90 L tank in which 20 red tilapia larvae were raised. The final numbers of the treated brine shrimp were ~2 individuals $\cdot$ mL^{-1}. We fed the fry once a day for 4 weeks with the brine shrimp containing microalgae. Growth enhancement was evaluated in terms of the increase in length (L) and weight (W) of the fish larvae every week. Condition factor was calculated as $W \times L^{-3} \times 1{,}000$ where W is wet weight (g) and L is total length (cm) (Takeuchi 1988) after the termination of the 4-week treatment.

Statistical analysis. The body lengths and body weights from each tank were analyzed statistically with the general linear models procedure (SAS Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) was used to compare the mean values between the mock-treated control group and the experimental group. A Duncan's multiple range test was used to separate sample means. A significance level of $P < 0.05$ was used in all statistical analyses.

RESULTS AND DISCUSSION

Preparation of N. oculata *protoplasts.* After *N. oculata* was cultured at log-phase in a density of 1×10^7 cells, it was treated with four different enzyme mixtures. Results showed that the mixture of 4% hemicellulase and 2% driselase enabled efficient digestion of the cell wall: >85% of the cells of the enzyme-treated microalgae were negative by Calcofluor staining (data not shown), indicating that most of them became protoplasts. The other three enzyme mixtures digested <50% of *N. oculata*, even with prolonged treatment of >2 h. Nevertheless, when we treated 1×10^5 of *N. oculata* cells with the mixture of 4% hemicellulase and 2% driselase for 1 h, almost all cells became protoplasts. The efficiency of protoplast formation demonstrated here is as good as that reported by Kim et al. (2002) for *Chlorella ellipsoidea*. Thus, we prepared protoplasts for electroporation under similar conditions.

The effect of electroporation in N. oculata. In the last decade, many methodologies of algal transformation were attempted to develop a bioreactor: microprojectile bombardment, electroporation, glass-bead, silicon carbide whiskers, polyethylene glycol, and *Agrobacterium tumifaciens*. Achievements have been reported in many algal species, including *C. reinhardtii*, *Volvox carteri*, *Chlorella* sp., and *Haeodactylum tricornutum* (reviewed by Walker et al. 2005). However, extremely little attention has been paid to microalgae, such as *Nannochloropsis,* which are well known material for fish larval aquaculture (Rocha et al. 2003, Rodolfi et al. 2003). In this study, we developed an electroporation system that allows a foreign gene to be transferred into *N. oculata* effectively and stably. To optimize the electroporation condition for transferring the foreign gene into the protoplasts of *N. oculata*, we examined the effect of voltage and pulse number on the survival rate of microalgae. As shown in Figure 1, the higher the voltage and pulse number used, the lower the survival rate obtained. Voltage played a more important role than that of pulse number. A higher voltage is required to introduce the foreign DNA through the cell wall, but it also may be harmful to the

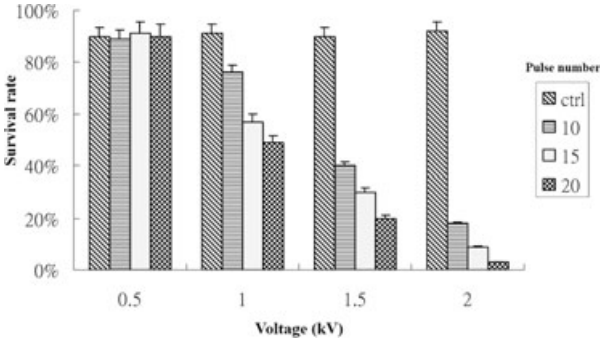

FIG. 1. Effect of voltage and pulse time of electroporation on the survival rate of *Nannochloropsis oculata*. Plasmid DNA was prepared at the concentration of 20 $\mu g \cdot \mu L^{-1}$ in the filtered seawater. After electroporation, each group of microalgae were transferred into a glass tube filled with f/2 medium and cultured at 28°C for 3–5 d. The survival rate (mean ± SD) was calculated as the proportion of cells that were of normal circular-shape with green color among the total number of cells prior to electroporation treatment.

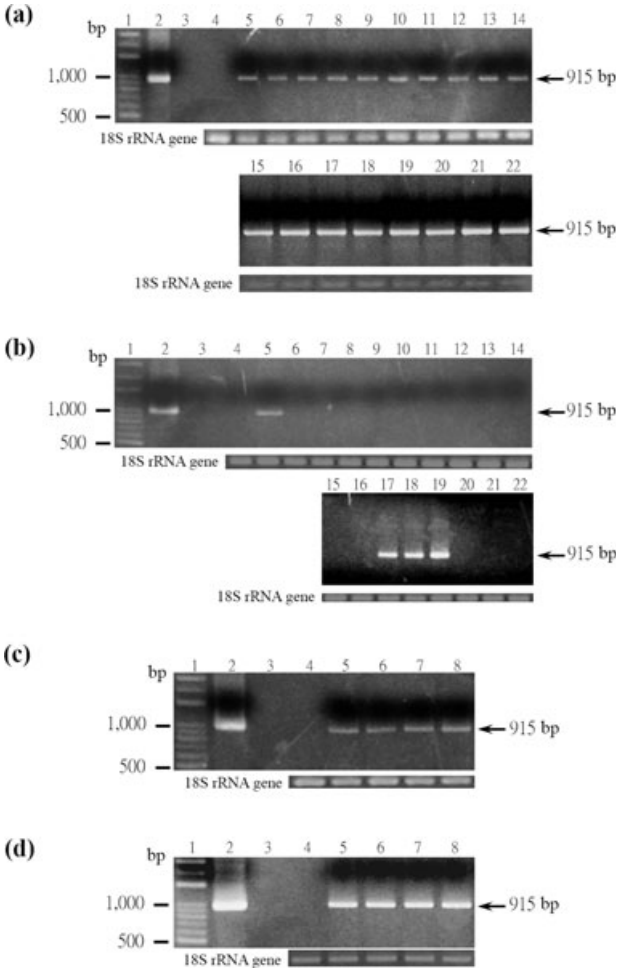

FIG. 2. PCR detection of exogenous DNA fragment in the genomic DNA isolated from transgenic *Nannochloropsis oculata* clones. Lane 1, 100 bp DNA molecular marker. Lane 2, phr-YPGHc served as a template (positive control). Lane 3, no template was added (negative control). Lane 4, untreated wildtype. Lanes 5–22: (a) the microalgal clones after electroporation of linear phr-YPGHc, (b) transgenic *N. oculata* clones were cultured after the 15th generation. Lanes 5–8: (c) transgenic *N. oculata* clones were cultured after the 30th generation, (d) transgenic *N. oculata* clones were cultured after the 50th generation. The 915 bp PCR product was amplified from the transgene YPGH cDNA as indicated, whereas the 450 bp shown on the bottom was amplified from the endogenous 18S rRNA gene (internal control).

transformed cells. Thus, the lower the voltage we used to treat the host cells, the better the quality of transformants we obtained. In our preliminary test, the voltage of electroporation used for intact cells of *N. oculata* that were not treated with enzymatic digestion was higher (2.0 kV) than that used to electroporate the protoplasts (1.0 kV) and was close to what was used in *Chlorella vulgaris* (1.8 kV; Chow and Tong 1999). However, it was lower than the voltage used for *Dunaliella salina* (6.0 kV; Geng et al. 2003), which has no cell wall structure. Finally, we chose a voltage of 1.0 kV and a pulse number of 1.5 for gene transfer of *N. oculata*.

Screening the transgenic clones of N. oculata *by PCR analysis.* After electroporation, the transformed *N. oculata* was plated on the f/2 medium plate for culture. An antibiotic-resistant gene has been widely used for serving as a selection marker of the transformed plant cells, but it is rarely used in microalgae because of the natural resistance (Polne-Fuller and Gibor 1990, Apt et al. 1996). We treated *N. oculata* with ampicillin, chloramphenicol, kanamycin, and streptomycin and determined that there was no growth inhibition, even when antibiotics were added in concentrations as high as 200 $\mu g \cdot mL^{-1}$. In this study, the exogenous DNA fragment phr-YPGHc we used did not consist of an antibiotic-resistant gene. We did not use antibiotics to serve as a selection marker or pressure. The purpose of using ampicillin and kanamycin in the culture plates in our experiment was to prevent bacterial contamination. Not using an antibiotic-resistant gene as a selection marker for transgenic microalgae was a large benefit considering biological safety concerns when culturing microalgae outdoors. Thus, transgenic *N. oculata* is a safe organism for feeding aquaculture species; it even serves as a bioreactor for pharmaceutical purposes or human food. We screened the transformants by PCR analysis of genomic DNA extracted from *N. oculata*. When we examined part of the 500-cell colonies grown on agar plate for the first generation, a single PCR product with a molecular weight of 915 bp was found (Fig. 2a, lanes 5–22). This positive band corresponded to that amplified from the DNA fragment used for gene transfer (Fig. 2a, lane 2). Among colonies from the first generation of microalgae we examined, we observed that almost all the colonies had a 915 bp PCR product, suggesting that the gene transfer rate was extremely high under the conditions we set for electroporation for *N. oculata*. However, after we continued to culture those transgenic microalgae until the 15th generation, we determined that the PCR-positive signal was

shown in only four transgenic clones among 500 clones examined (Fig. 2b, lanes 5, 17–19), suggesting that the exogenous DNA fragment in the host genome was unstable. From our results, the heterologous DNA fragment was detected in the transformed *N. oculata* prior to the 10th generation. However, after these transformed *N. oculata* were cultured for 1.5 months (11th to 15th generations), only 0.8% of the transformants (4 out of 500 clones) contained the transferred fragment (Fig. 2, a and b). The more generations that passed, the lower the transfer became, suggesting that the transferred DNA fragment was unstable in the transformed microalgae. This phenomenon has been described also in reports of transgenic *Chlorella* with antibiotic pressure (Hawkins and Nakamura 1999, Kim et al. 2002). Because of its lack of a rigid cell wall, *D. salina* is more easily used for introducing foreign genes (Sadka et al. 1991). However, it is significant that, under the conditions we used in this study, we can transfer material into *N. oculata* as successfully as when cell-wall-free *D. salina* is used. We believe this technology can also be applied in other microalgae, even though they contain a cell wall.

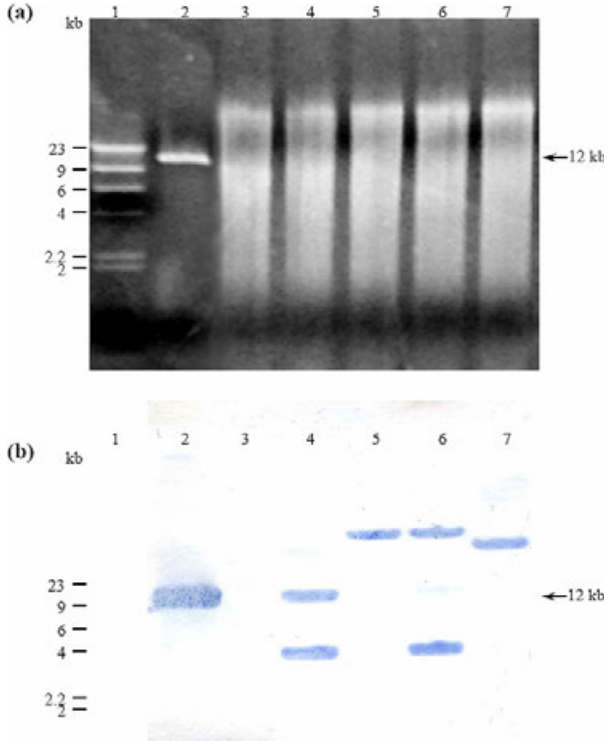

FIG. 3. (a) Southern blot analysis of the genomic DNA extracted from four transgenic *Nannochloropsis oculata* clones after they were cultured, 15th generation. (b) Southern blot analysis of the corresponding DNAs performed using a digoxigenin-labeled YPGHc as a probe for hybridization. Lane 1, λ/*Hin*dIII DNA molecular marker; lane 2, *Xba*I-cut phr-YPGHc, which was used for gene transfer (positive control); lane 3, the *Xba*I-cut genomic DNA from the untreated wildtype strain (negative control); lanes 4–6, the *Xba*I-cut genomic DNAs from four transgenic clones. Arrow indicates the molecular size of the linear plasmid (12 kb), phr-YPGHc.

Genomic Southern blot analysis of transgenic N. oculata. After genomic DNA isolated from these four transgenic clones was digested with *Xba*I and Southern blot analysis was performed, we observed positive band(s) with molecular masses higher than the 12 kb DNA used for transgenesis (Fig. 3, lanes 4–7), indicating that the transgene was integrated in the host genome. But we also noticed that some positive bands appeared smaller than 12 kb (Fig. 3, lanes 1 and 3), suggesting these integrative DNA fragments may be deleted or rearranged during integration. On the basis of Southern blot analysis of the genomic DNA isolated from these four clones, we determined that the foreign gene was integrated into the genome of *N. oculata* because the molecular weights of the positive hybridization bands were higher than that of phr-YPGHc (Fig. 3), suggesting that the nonhomologous recombination end-joining of the transgene occurred, such as rearrangements, partial deletions, and terminal modifications as reported in cell lines (Nakai et al. 1999), transgenic animals (Chou et al. 2001, Hsiao et al. 2001), and microalgae (Kim et al. 2002, Geng et al. 2003). Because of the random integration into the genome of *N. oculata*, the inserted sites and copy numbers vary among these four clones (Fig. 3, lanes 4–7).

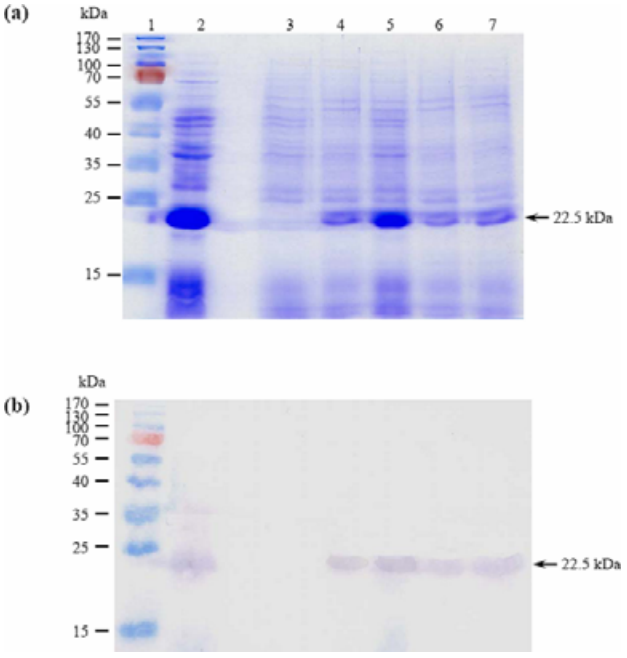

FIG. 4. (a) Western blot analysis of the proteins extracted from four transgenic *Nannochloropsis oculata* and analyzed by SDS-PAGE. (b) Western blot analysis of the corresponding proteins performed using polyclonal antiserum against yellowfin porgy growth hormone (YPGH). Lane 1, protein molecular marker; lane 2, recombinant YPGH expressed by *Escherichia coli* (positive control); lane 3, proteins from the untreated wildtype *N. oculata*; lanes 4–7, proteins from four transgenic clones of transgenic *N. oculata* after induction. Arrow indicates the molecular mass of the fish YPGH (22.5 kDa).

Fish GH was produced by the transgenic N. oculata. In our preliminary data, the optimal duration of heat-shock induction for transgenic *N. oculata* to express exogenous GH was 2 h at 42°C. The total proteins extracted from the wildtype and the transgenic *N. oculata* were analyzed by SDS-PAGE. As shown in Figure 4, a band with molecular weight of 22.5 kDa was observed in these four transgenic clones (Fig. 4a, lanes 4–7). This band was also positive for Western blot analysis when the polyclonal antiserum against fish GH was used (Fig. 4b, lanes 4–7). However, this 22.5 kDa band was absent in the total proteins extracted from the wildtype strain (Fig. 4a, lane 3), which was also negative by Western blot analysis (Fig. 4b, lane 3). To determine the expression level of YPGH in these four transgenic *N. oculata* clones, we used the ELISA analysis to determine the amount of fish GH produced by the transgenic *N. oculata*. Compared to the known standard curve, the yields of the YPGH protein produced by these four transgenic clones ranged from 0.41 (clone 2) to 0.27 μg (clone 3) per mL (Fig. 5). Like transgenic higher plants and animals, promoter strength is one important factor that determines the expression level of transgenes in *N. oculata*. In this study, we combine RBCS2 promoter with an inducible HSP70A promoter from *C. reinhardtii* (Schroda et al. 2000). After using SDS-PAGE to analyze the proteins extracted from transgenic *N. oculata*, we determined that a protein band with a molecular mass of ~22.5 kDa was present, which corresponds with the recombinant GH produced by *Escherichia coli*.

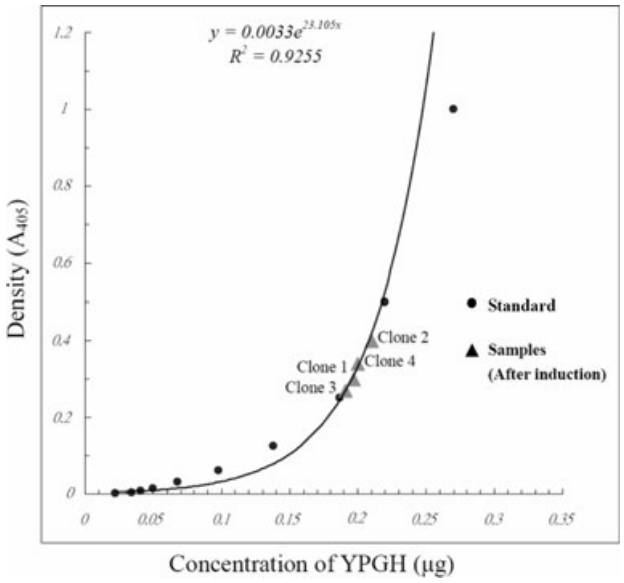

FIG. 5. The yield of yellowfin porgy growth hormone (YPGH) produced by each transgenic clone of *Nannochloropsis oculata* was determined by ELISA. The filled circles indicate the OD_{405} values of known YPGH protein concentration with different dilutions. The filled triangles indicate the OD_{405} values of four transgenic *N. oculata* clones (clones 1, 2, 3, and 4).

Furthermore, this band is also positive for the Western blot analysis, indicating that fish GH is successfully expressed in the transformed *N. oculata* (Fig. 4, a and b). In this study, transgenic microalgae clone 2 has the highest yield of fish GH production after heat induction: the expression level of GH increases 13-fold after induction (from 0.031 to 0.41 $\mu g \cdot mL^{-1}$). This rate is lower than Schroda et al. (2000) reported, that is, the arylsulfate (ARS) enzyme increased 26-fold in *C. reinhardtii* after induction. The lower rate reported in this study may be because the promoter they used originates from *C. reinhardtii*, but the promoter we used does not form *N. oculata* per se. The efficacy of the heterologous promoter may be less than that of homologous origins.

The various integrations of transgene in these four clones results in different levels of YPGH being expressed (Fig. 5). The highest yield of YPGH produced by one of the transgenic *N. oculata* is ~0.41 $\mu g \cdot mL^{-1}$ (1×10^7 cells $\cdot mL^{-1}$), which is 10-fold greater than the GH that was expressed in *C. ellipsoidea* (0.41 $\mu g \cdot mL^{-1}$ in 1×10^8 cells $\cdot mL^{-1}$; Kim et al. 2002). However, compared with other algae, the cost of culture medium is lower and the large-scale culture is easy for *N. oculata*, suggesting that this microalga system is very promising for the production of eukaryotic proteins, including pharmaceutically important proteins.

Stable inheritance of the transferred gene. Although the existence of exogenous DNA fragment was detected in almost all the colonies from the first generation, we found that the PCR-positive signal was shown in only four transgenic clones among 500 clones after we continued to culture those transgenic microalgae until the 15th generation (Fig. 2b, lanes 5, 17–19). We continued to culture these four transgenic clones until the 30th generation. At that time, PCR analysis of the transferred gene revealed that a single 915 bp PCR band was still detected (Fig. 2c); these four clones also expressed this band at the 50th generation (Fig. 2d). These results indicate that the stable lines that inherit the exogenous gene in the transgenic microalgae do exist, although they may be difficult to find. The four clones we screened kept the transferred gene until at least 50 generations when they were cultured in the f/2 medium without antibiotics (Fig. 2d). This finding is comparable to the results reported by Geng et al. (2003) for transgenic *D. salina*. They reported that the introduced gene was kept and expressed stably at least for 60 generations without adding chloramphenicol. Introducing foreign genes to *D. salina* is made easier by its lack of a rigid cell wall (Sadka et al. 1991). However, it is significant that, under the conditions we used in this study, we can transfer material into *N. oculata* as good as that of cell-wall-free *D. salina*. We believe this technology can also be applied in other microalga, even though they contain a cell wall.

TABLE 1. The incubation time and saturation rate required for artemia fed the microalga *Nannochloropsis oculata*.

Groups	Incubation time of feeding (min)	Saturation rate (%)[a]
1	15	7 ± 1
2	30	10 ± 1
3	45	33 ± 2
4	60	50 ± 2
5	75	60 ± 3
6	90	70 ± 4
7	120	75 ± 5
8	150	80 ± 7
9	240	85 ± 8
10	360	90 ± 10

[a]Data are shown as mean with standard errors of the mean. Each group was examined three times in a 250 mL beaker with about 1,000 artemia and 100 mL *N. oculata* (1×10^5 cells). The calculation of saturation rate was the proportion occupied by microalga in the artemia intestine under the fluorescent microscope.

Growth performance of fish larvae fed transgenic microalgae. Because *N. oculata* contains cell walls that cannot be digested completely by fish larvae, we designed the experiment using artemia, which ingest the microalgae and digest cell walls, releasing the fish GH. Before we carried out the biological function of GH produced by microalgae, we needed to know the rate of consumption of *N. oculata* by artemia. We evaluated the magnitude of presence of microalgae in the artemia intestine with a fluorescent microscope after feeding. As shown in Table 1, approximately half of the intestinal space of artemia was occupied by microalgae at 1 h. In cases in which we incubated *N. oculata* with artemia for 6 h or longer, we observed that >90% of intestinal space of artemia was occupied by microalgae.

Next, we determined whether fish GH was released from the transgenic *N. oculata* after being digested by artemia. We applied the extraction buffer of artemia as described by Tanguay et al. (2004) to examine whether the YPGH is released after artemia digestion. But we did not extract the proteins of *N. oculata* by using this extraction buffer, indicating the proteins we extracted are from artemia, not from microalgae. After microalgae were induced to produce the fish GH at 42°C for 2 h and were fed artemia, total proteins were extracted and analyzed by SDS-PAGE and Western blot. As shown in Figure 6, a band with a molecular mass of 22.5 kDa was observed in the artemia groups that were incubated with transgenic *N. oculata* after 7, 5, 3, 1, and 0 h (Fig. 6a, lanes 4–8). This band was also positive in Western blot analysis (Fig. 6b). However, this 22.5 kDa band was not observed in the artemia group that was not fed with microalgae (Fig. 6, a and b, lane 3). These findings indicate that the exogenous fish GH is released from the transgenic microalgae after being digested by artemia for 4 and 6 h.

The highest GH-expression clone (clone 2) of transgenic *N. oculata* was chosen to carry out the feeding experiment. The transgenic and wildtype *N. oculata* that were incubated at 42°C for 2 h were incubated with artemia for 4 or 6 h. These artemia were then used to feed red tilapia larvae as the only source of food. As shown in Table 2, body weight and length were not different in each group at the beginning of the experiment. After 4 weeks, the fish fed artemia that had been incubated with transgenic microalgae for 4 and 6 h increased 232 ± 7.1% and 316 ± 9.7% in weight, respectively. In the parallel control group (fed artemia incubated with wildtype microalgae for 4 and 6 h), fish increased in weight 87 ± 3.2% and 104 ± 4.1%, respectively. The weight gain of experimental groups was enhanced greatly. The body length of experimental groups that were fed with artemia that were incubated with transgenic microalgae increased significantly: 197 ± 7.5% and 217 ± 8.9% of experimental groups for 4 and 6 h, respectively, versus 139 ± 4.2% and 146 ± 5.5% of control groups. Comparing the weight gain and length increment among the control and experimental groups for 4 and 6 h showed a significant difference ($P < 0.05$). The growth enhancement between the control groups of 4 and 6 h was

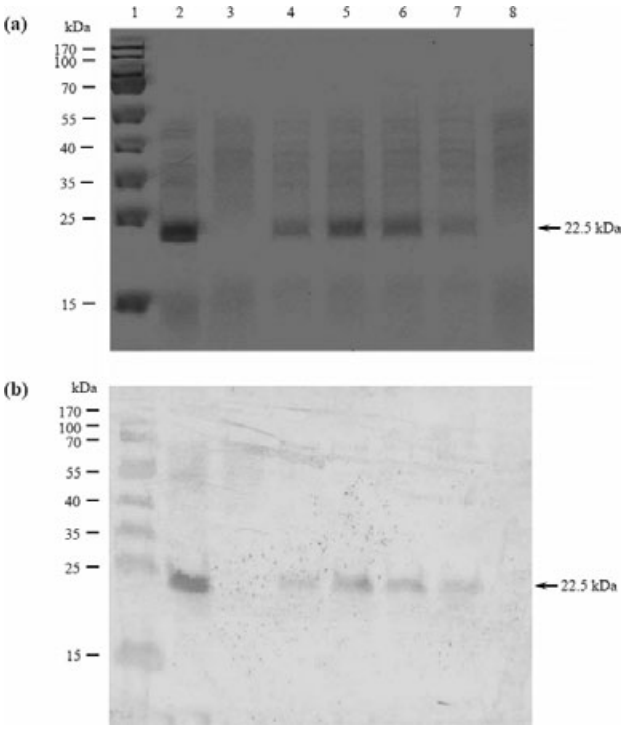

FIG. 6. Protein analysis of microalgae after they were fed on by artemia. (a) SDS-PAGE was used to analyze the proteins extracted from *Nannochloropsis oculata* after they were fed on by artemia. (b) Western blot analysis of the corresponding proteins performed using polyclonal antiserum against yellowfin porgy growth hormone (YPGH). Lane 1, protein molecular marker; lane 2, proteins from the transgenic *N. oculata* (positive control); lane 3, proteins from artemia without feeding *N. oculata* (negative control); lanes 4–8, proteins from artemia after they were fed transgenic *N. oculata* for 7, 5, 3, 1, and 0 h, respectively. Arrow indicates the molecular mass of the expressed YPGH (22.5 kDa).

TABLE 2. Comparison of the growth performance in red tilapia larvae by feeding artemia, which take up the wildtype and transgenic *Nannochloropsis oculata*.

Incubation time (h)	Groups of feeding artemia	Average body weight (g)		Weight gain (%)	Average body length (cm)		Length increment (%)	Condition factor
		Initial	Final		Initial	Final		
4	Wildtype	0.23 ± 0.01^{a}	0.43 ± 0.07^{a}	87 ± 3.2^{a}	1.36 ± 0.01^{a}	3.27 ± 0.10^{a}	139 ± 4.2^{a}	12.0 ± 0.01^{a}
	Transgenic	0.23 ± 0.01^{a}	0.77 ± 0.09^{b}	232 ± 7.1^{b}	1.36 ± 0.01^{a}	3.98 ± 0.17^{b}	197 ± 7.5^{b}	12.1 ± 0.02^{a}
6	Wildtype	0.23 ± 0.01^{a}	0.47 ± 0.06^{c}	104 ± 4.1^{c}	1.36 ± 0.01^{a}	3.35 ± 0.13^{c}	146 ± 5.5^{c}	12.2 ± 0.02^{a}
	Transgenic	0.23 ± 0.01^{a}	0.98 ± 0.14^{d}	316 ± 9.7^{d}	1.36 ± 0.01^{a}	4.38 ± 0.23^{d}	217 ± 8.9^{d}	12.0 ± 0.01^{a}

Data are shown as mean ± SD ($n = 20$ individuals of red tilapia larvae for each group). Means in the same column sharing a common superscript letter (a, b, c, d) are not significantly different ($P < 0.05$); 100 mL *N. oculata* (1×10^5 cells).

significantly different in terms of body weight and body length (Table 2). We reason that microalgae per se are a natural nutrient for fish larvae (Lavens and Sorgeloos 1996). The longer incubation time of larvae with microalgae, the more nutrition from the microalgae should be taken by fish larvae, resulting in the greater growth rate of larvae. Therefore, in the control groups, the 6 h incubation larvae produced faster growth than the 4 h incubation larvae. In the case of the experimental groups, the exogenous GH should be another factor that enhanced the growth rate of fish larvae after they consumed the GH-containing microalgae. Although the feeding of transgenic microalgae containing fish GH greatly promoted the growth rate of red tilapia larvae, the condition factor was close (Table 2), indicating that fish GH enhances both weight and length. We also noticed that the increases in body weight of experimental groups were already observed after only 1 week (Fig. 7). The function of GH administration orally involves the food-uptake mechanism. It is known that fish can take up protein molecules by pinocytosis (McLean and Donaldson 1990), and Jeh et al. (1998) reported that oral administration of recombinant fish GH resulted in a 24% increase in average weight and a 12% increase in average length of juvenile flounder after 7 weeks of feeding. Similarly, Kim et al. (2002) also reported that oral administration of recombinant flounder GH resulted in a 25% increase in total body length and width of juvenile flounder after 30 d of feeding. However, compared to using recombinant protein, there are several advantages to using transgenic microalga as a novel bioreactor system in aquaculture, such as the ease and low cost of culture for maintenance and sustenance, a short life cycle, and their use as a natural feed for fish and shellfish larvae. Unlike the genetically modified fish, which contains the exogenous GH gene in their genome, fish fed with genetically modified microalgae do not contain the transgenic GH gene. When humans consume the adult fish that have been fed GH-containing microalgae several months or years ago, it is extremely unlikely that these fish still harbor the exogenous GH gene after digestion. We believe it is safer for humans to consume nontransgenic fish, which are fed GH-containing microalgae for a temporary

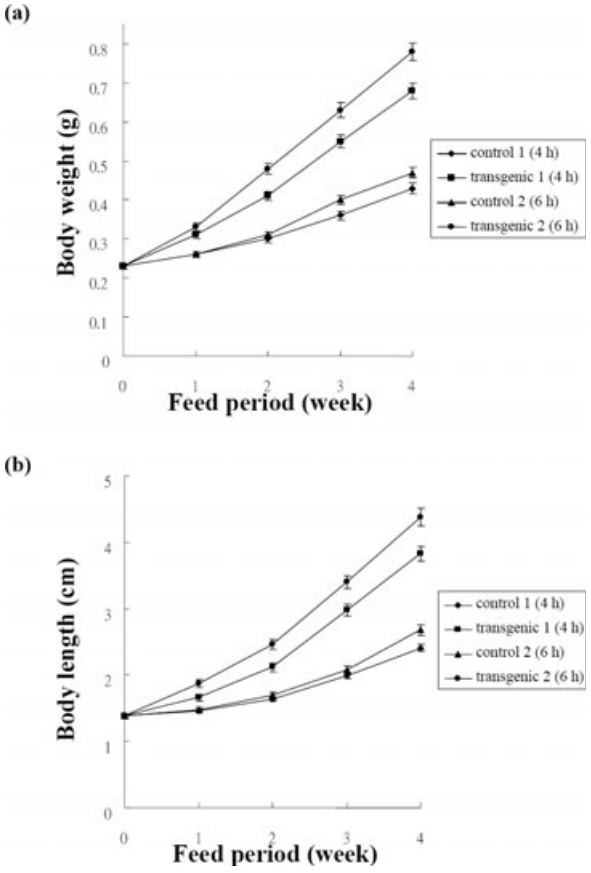

FIG. 7. The growth performance of fish larvae. The body weight (a) and body length (b) of red tilapia larvae after they were fed artemia (since artemia enables larvae to digest microalgae and enables release of the fish growth hormone produced by transgenic *Nannochloropsis oculata*). After artemia were incubated with nontransgenic (control; diamond and triangle) or transgenic *N. oculata* for 4 h (square) and 6 h (circle), they were used to feed red tilapia larvae. The growth performance was measured weekly.

period, rather than directly consuming transgenic GH-containing fish. In addition, if the transgenic GH-containing microalgae are applied outdoors, the process may still be controlled. First, exogenous fish GH is not expressed except by heat shock, and the life span of microalgae is no longer than 3 h after heat-shock induction. Second, transgenic GH-containing microalgae are only fed on by fish larvae in a transient treatment manner. Third, microalgae are

asexually reproductive organisms. Their lack of gametes may greatly reduce the risk of horizontal transmission of transgenes in the environment. Fourth, the recycled closed water system is popular for the microalgae culture industry. This system is also a good strategy for preventing the release of the transgenic *N. ouculta* into the environment.

In conclusion, we have demonstrated an effective way to electroporate a foreign DNA fragment into the protoplast of *N. oculata*. We screened out some transformants of *N. oculata* that transmit the transferred gene stably. The active form of fish GH can be expressed in the transgenic microalgae by using a heterologous double promoter. This report highlights the potential usage of *N. oculata* as a bioreactor for producing foreign proteins, which may be of benefit for both pharmacological and aquaculture industries.

We thank Dr. H. M. Su, Biotechnology Division of the Fisheries Research Institute, Tungkang, Taiwan, for providing the *N. oculata* strain. We also thank Dr. M. Schroda, Institut Für Biolie III, Universität Freiburg, Germany, for kindly providing the pCB740 plasmid. Further, we thank Prof. Y. L. Song, Institute of Zoology, National Taiwan University, for helping with the ELISA analysis. This work was partially supported by the National Science Council (H. J. T.).

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