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Transformation of coccolithophorid *Emiliania huxleyi* harboring a marine virus (Coccolithoviruses) serine palmitoyltransferase (SPT) gene by electroporation*

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Abstract *Emiliania huxleyi* is the most prominent modern coccolithophore, a group of marine unicellular eukaryotes that play a critical role in ocean biogeochemistry. Coccolithoviruses are large double stranded DNA viruses, which is responsible for the demise of large oceanic blooms formed by *E. huxleyi*. *E. huxleyi* virus (EhVs) acquired a series of enzyme-coding genes predicted to be involved in the sphingolipid biosynthesis by horizontal gene transfer between virus-host. Currently, there is limited experimental validation identifying the functions of these genes in EhV. Genetic transformation of eukaryotic cells is a powerful tool to get an insight into gene functions of the studied organisms. Serine palmitoyltransferase (SPT) catalyzes the first committed step in de novo sphingolipid biosynthetic pathway. Here, a novel vector system for the transformation of *E. huxleyi* was designed. It contained fragments of promoter and terminator sequences of *E. huxleyi* endogenic fucoxanthin chlorophyll *a*/*c*-binding protein gene "*fcp*" and harbored EhV-99B1 *spt* gene. The resultant recombinant transformation vectors pEhux-I-*spt* and pEhux-II were co-transferred into *E. huxleyi* BOF92 by electroporation. Transformants were obtained upon glufosinate-ammonium selection, and confirmed by Southern hybridization, genome PCR, qRT-PCR and Western blot screening of *spt* gene, which indicated that *spt* gene was integrated into the nuclear genome and was expressed at the mRNA and protein levels. The expression of the viral *spt* gene led to differences in lipid compositions analyzed using thin-layer chromatography (TLC). The results present the genetic transformation system for *E. huxleyi*, providing additional genetic resource with potential for exploring basic biological questions such as the virus-host interactions.

Keyword: *Emiliania huxleyi*; coccolithovirus; genetic transformation; serine palmitoyltransferase (SPT); total lipid

1 INTRODUCTION

Coccolithophore (haptophytes) is a group of globally important unicellular marine microalgae. One prominent feature of this alga is the ability to produce "the coccoliths". It is considered the most productive calcifying organism on earth and becoming a major factor in the global carbonate cycle and climate changes (Westbroek et al., 1993; Read et al., 2013; Hernández et al., 2018). *Emiliania huxleyi* is one of the most abundant and widely distributed coccolithophore in modern oceans. It is considered the world's major producer of calcite and an important factor in determining the exchange of CO_2 between the oceans and the sediments (Dymond and Lyle, 1985), Since its ability to synthesis long-chain alkenones, as a suite of organic biomarkers providing a highly characteristic record in the sedimentary archive (Westbroek et al., 1993).

Viruses that infect phytoplankton play a key role in

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shaping the evolution and dynamics of the oceanic microscale ecosystem (Suttle, 2005). In natural marine ecosystem, some *E. huxleyi* species could be infected by the specific large dsDNA lytic viruses (EhVs, genus Coccolithovirus), and its bloom collapsing has been frequently linked to virus control in the marine environment (Bratbak et al., 1993; Brussaard, 2004). The EhV-86 virus genome sequence identified a series of enzyme-coding genes apparently involved in sphingolipid metabolism (Wilson et al., 2005). Phylogenetic evidence demonstrated the occurrence of horizontal gene transfert of these genes between *E. huxleyi*-EhV system (Monier et al., 2009). An unusual glucosylceramide was isolated from EhV-86 infected host cells (Rose et al., 2014), which attributed to coordinated interactons between host- and viral-encoded sphingolipid biosynthetic enzymes (Michaelson et al., 2010; Rosenwasser et al., 2014), providing a fascinating new paradigm for host-virus interactions. Sphingolipids are essential structural components of all eukaryotic membranes and are important signaling lipids in diverse cellular pathways, such as apoptosis, and they play a crucial role in the life cycle of intracellular pathogens (Schneider-Schaulies and Schneider-Schaulies, 2015). It was attractive that the EhV-encoded serine palmitoyl transferase (vSPT), the first-rate limiting enzyme in de novo sphingolipid synthesis exhibited a novel metabolic strategy leading to the production of a unique suite of viral-specific glycosphingolipids (vGSLs) (Ziv et al., 2016). In agreement, vGSLs were found to act as signaling lipids to induce host programmed cell death (PCD) (Vardi et al., 2009; Liu et al., 2018). More recently, we further revealed that the host lipidome (both lipid content and composition) significantly changed in response to EhV infection (Zeng et al., 2019). Currently, there is still limited direct experimental evidence for the EhV genes proposed to play a role in sphingolipid biosynthesis. Therefore, it will be important to experimentally identify the viral genes involved in host sphingolipid metabolism, particularly given the proposed role for this sphingolipid in apoptosis and virus mediated sphingolipid metabolic regulatory mechanism.

Genetic transformation of eukaryotic cells is a valuable tool for the elucidation of gene functions and certain metabolic pathways, and allows us to study biochemical processes as well as viral infection mechanisms of the studied organisms. The ability to manipulate microalgae via genetic engineering in order to introduce or optimize desired traits will facilitate more extensive exploitation of these organisms since interest in the use of microalgae for research as well as commercial applications has increased in recent years (Hlavova et al., 2015; Xue et al., 2015; Velmurugan and Deka, 2018). However, information on transgene expression and genetic engineering for understanding molecular mechanisms and strain developments is very limited in coccolithophores (Endo et al., 2016).

The aim of this work is to design a novel vector construct for the transformation of the coccolithophore *E. huxleyi* and identify the possible function of EhV-*spt*. Thus, we developed the methods of transient and stable transformation for *E. huxleyi*. The utility of glufosinate-ammonium selection marker (*bar*) and green fluorescent protein reporter genes (*gfp*) were examined for use in *E. huxleyi*. The genetic transformation was investigated using electroporation and the transformation conditions were determined with the *gfp* gene. The endogenous promoter and terminator of fucoxanthin chlorophyll *a*/*c*-binding protein gene "*fcp*" were tested for the expression of *bar* and *spt* genes. The recombinant vector containing EhV-99B1-*spt* was transformed for a preliminary functional identification of viral *spt* gene.

2 MATERIAL AND METHOD

2.1 Cultivation of *Emiliania huxleyi*

Emiliania huxleyi BOF 92 strain, obtained from the Biology Department of Bergen University (Bergen, Norway) was grown in liquid 70% f/2-Si medium (Guillard, 1975) for usual cultivation. The culture conditions were 16±1 °C and the light regime was a 14-h:10-h light:dark cycle with about 50 μmol·photons/(m^2·s) cool white fluorescent lights. The cultures were incubated with 2×10^4 cells/mL.

2.2 Construction of expression vectors

All the genes fragments were obtained by PCR and the sequence of the primers and the PCR conditions were summarized in Table 1. Glufosinate-ammonium selection marker gene-*bar* (552 bp) was amplified by PCR from the commercial plasmid pCAMBIA3300 (Biovector Science Lab, Inc.) as a *Spe* I/*Mlu* I fragment using the primers A1/A2. The gene encoding green fluorescent protein-*gfp* (717 bp) was amplified by PCR from pGFP vector (Clontech) as a *Spe* I/*Eco*R I fragment using the primers A3/A4. As an attempt to increase gene transfer efficiency and the transformant stability in *E. huxleyi* cells, constructs

Table 1 Primers and PCR programs used in this study

Gene	Primer	5′→3′ sequence	Restriction site	PCR condition
Bar	A1:Bar_F	**ACT AGT** ATG AGC CCA GAA CGA CGC	*Spe* I	94 °C 30 s, 64 °C 30 s, 72 °C 45 s
	A2:Bar_R	**ACG CGT** TCA GAT CTC GGT GAC GGG C	*Mlu* I	33 cycles
Gfp	A3:Gfp_F	**ACT AGT** ATG AGT AAA GGA GAA GAA CTT	*Spe* I	94 °C 30 s, 54 °C 30 s, 72 °C 45 s
	A4:Gfp_R	**GAA TTC** TTA TTT GTA TAG TTC ATC C	*Eco*R I	33 cycles
FAP	A5:Fap_F	**AGA TCT** TGT GTG GCT TGA GTA AC	*Bgl* II	94 °C 30 s, 60 °C 30 s, 72 °C 45 s
	A6:Fap_R	**ACT AGT** GGT GAG GAA GGA GAG GTG A	*Spe* I	33 cycles
FBP	A7:Fbp_F	**GCG GCC GC**T GTG TGG CTT GAG TAA	*Not* I	94 °C 30 s, 60 °C 30 s, 72 °C 45 s
	A8:Fbp_R	**GAA TTC** GAA CTG GGA GTT TAC CTT G	*Eco*R I	33 cycles
FAT1	A9:Fat1_F	**ACG CGT** GCT GCC CCC GGC AGC GT	*Mlu* I	94 °C 30 s, 60 °C 30 s, 72 °C 45 s
	A10:Fat1_R	**GCG GCC GC**G GCT GTG GGA GGG GTA GT T	*Not* I	33 cycles
FAT2	A11:Fat2_F	**AAG CTT** GTC CCC CCC CCC GTG T	*Hind* III	94 °C 30 s, 60 °C 30 s, 72 °C 45 s
	A12:Fat2_R	**CTC GAG** TGG AGG ACC GCG CCA	*Xho* I	33 cycles
Spt	A13:Spt_F_1	**CCC GGG** ATG TAC ACG GCC GTA TTC	*Sma* I	94 °C 15 s, 50 °C 30 s, 68 °C 2 min
	A14:Spt_R_1	**AAG CTT** CAT TGA CAG ATA CTC AAT CAT AAA C	*Hind* III	35 cycles

Bold means restriction endonuclease restriction sites.

containing larger fragments of promoter and terminator were considered. The endogenous *fcpA* promoter regions (FAP 484 bp and FBP 300 bp) of the fucoxanthin chlorophyll *a*/*c*-binding protein gene in *E*. *huxleyi* BOF92 were amplified by PCR as *Bgl* II/*Spe* I and *Not* I/*Eco*R I fragments respectively, with *E*. *huxleyi* genome as the template using the primer sets A5/A6 and A7/A8 respectively. Similarly, the *fcpA* terminator regions (FAT1 600 bp and FAT2 600 bp) were obtained as *Mlu* I/*Not* I and *Hind* III/*Xho* I fragments by PCR using primers sets A9/A10 and A11/A12 respectively. *Spt* gene (2 613 bp) was amplified by PCR as *Sma* I/*Hind* III fragment with *E*. *huxleyi* virus (EhV-99B1) genome as the template using the primer sets A13/A14. Premiers for both *fcp* promoters and terminators were designed based on the whole genomic sequences of *E*. *huxleyi* CCMP1516 (http://genome.jgi.doe.gov/Emihu1/Emihu1.home.html). Primers for *spt* were designed based on the genomic sequences of *E*. *huxleyi* virus 86 (EhV 86) (https://www.ncbi.nlm.nih.gov/nuccore/NC_007346). These products were cloned into the pMD19T (TaKaRa) vector respectively, for sequencing and analysis through tools from PLACE database (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioniformatics.psb.ugent.be/webtools/plantcare/html/).

The plasmid pSP73 (Promega Corporation, Madison, WI, USA) was used as the backbone transformation vector. Construction of the plasmids pEhux-I and pEhux-II used for transformation of *E*. *huxleyi* were shown as Fig.1a & b. Plasmid pEhux-I (Fig.1a) was constructed in several stages. The first step involved subcloning the 600 bp *fcp* terminator FAT2 into the *Hind* III/*Xho* I sites of pSP73 to form the plasmid pSP73-FAT2. The genes of FAP (*fcp A* promoter), *bar*, FAT1 (*fcp* terminator) and FBP (*fcp A* promoter) were ligated by *Spe* I, *Mlu* I, and *Not* I respectively as a *Bgl* II/*Eco*R I fragment, which were inserted into pMD19-T vector for sequencing. The FAP/Bar/FAT1/FBP gene fusion (as a *Bgl* II /*Eco*R I fragment) was then linked into the *Bgl* II /*Eco*R I sites of pSP73-FAT2 to form the final basic construct pEhux-I (pSP73-FAP-*bar*-FAT1-FBP-FAT2). The pSP73 multiple cloning site (MCS) including *Eco*R I, *Sma* I, *Xba* I, and *Hind* III were preserved intact into which gene of *spt* was inserted as *Sma* I/*Hind* III fragment. The recombinant vector harboring *spt* gene was designed as pEhux-I-*spt* (Fig.1c). The expression vector pEhux-II (Fig.1b), in which *gfp* is controlled by FAP promoter, was constructed by first inserting the 600 bp *fcp* terminator FAT2 gene (as a *Hind* III/*Xho* I fragment) into the corresponding sites of pSP73. The FAP/*gfp* gene fusion 1 201 bp (as a *Bgl* II /*Eco*R I fragment) was then inserted into the *Bgl* II/*Eco*R I sites. The construct was designated pEhux-II (pSP73-FAP-*gfp*-FAT2).

2.3 Transformation of *Emiliania huxleyi* by electroporation

pEhux-I-*spt* bearing a marker gene "*bar*" and pEhux-II carrying the reporter gene "*gfp"* were co-

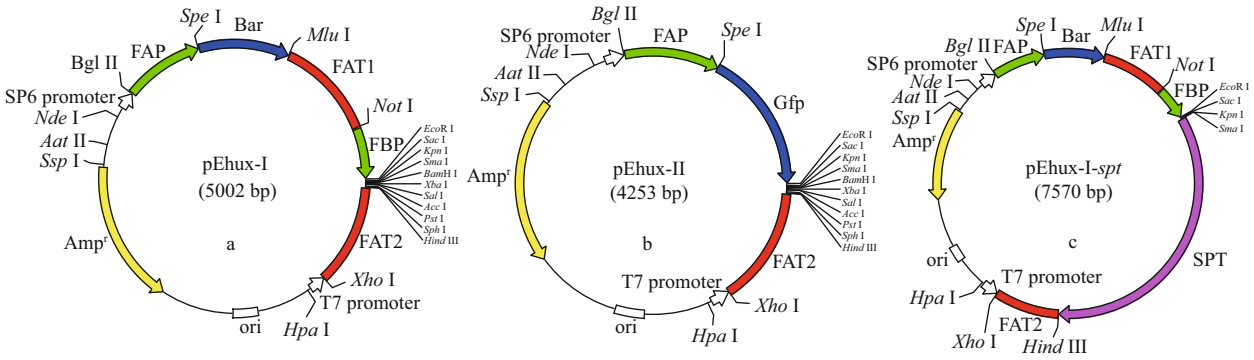

Fig.1 Construction of plasmids pEhux-I (a), pEhux-II (b), and pEhux-I-*spt* (c) used to co-transform *Emiliania huxleyi* BOF 92 strain

a. the 552-bp *bar* gene from pCAMBIA3300 was isolated as a *Spe* I/*Mlu* I fragment and cloned in pSP73 to generate pSP73-fcp-bar; b. the 717-bp *gfp* gene from pGFP was isolated as a *Spe* I/*Eco*R I fragment and cloned in pSP73 to generate pSP73-fcp-gfp; c. the 2 613 bp -*spt* gene from EhV99B1 was isolated as *Sma* I/*Hind* III fragment and cloned in pEhux-I to generate pEhux-I-*spt*. *Fcp* promoter (FAP/FBP) and terminator (FAT1/FAT2) sequences obtained by PCR from *E. huxleyi* BOF92 strain were subsequently inserted (see more information in Section 2).

transformed into *E. huxleyi* which should facilitate an identification of positively transformed clones. *E. huxleyi* cells were harvested at the mid-logarithmic phase (cell density of (1.5–2.0)×10^6 cells/mL) by centrifuging (1 500×*g*, 5 min, 4 °C) and decalcified by suspension with 0.5-mol/L MES-NaOH buffer (pH 5.5) (Sekino and Shiraiwa, 1996) at 15 °C for 1 h. After the decalcification, cells were collected by centrifuging (1 500×*g*, 2 min, 4 °C) and suspended in 1-mL electric shock buffer (0.08-mol/L KCl, 0.005-mol/L $CaCl_2$, 0.2-mol/L mannitol, 0.2-mol/L sorbitol and 0.01-mol/L Hepes, pH 7.2) containing approximately 5.6×10^7 cells. The created transformation vectors pEhux-I-*spt* and pEhux-II were re-suspended in distilled water and added to the above mentioned solution at the final concentration of 10 μg/mL. After incubation for 15 min at 4 °C without shaking, 300 μL of suspension was transferred into the electroporation chamber (Pulser/MicroPulser Cuvette, 0.4 cm gap; Bio-Rad Laboratories). The transformation of *E. huxleyi* was performed by electroporation using an electroporation system (Gemini SC, BTX, USA). The parameters, electrical field strength, used in electroporation was determined using the following equation: Critical electrical field strength *E* (V/cm)=10 000/(1.5×cell radius) (Čgovnik and Novaković, 2004). By observing electroporated cells under microscope to examine their mortality ratio, electroporation parameters were set with electrical field strengths of 1 500 V/cm and electrical shock time of 1.0–4.0 ms. To achieve high transformation efficiency, the pulsed cells were kept in fresh 70% f/2-Si non-selective medium under constant illumination for 24 h to allow recovery and then glufosinate-ammonium (PPT) was added to these cultures at the final concentration of 50 μg/mL. The cultures were allowed to grow under standard culture conditions for one week before spreading on selection plates. Transformants were examined for *gfp* fluorescence by fluorescence microscope (Axio Vert A1, ZEISS, Germany).

2.4 Screening of transformants on solid medium

Growth experiments on solid media were performed in f/50 media supplemented with 1.5% agar (Sigma Aldrich) (Laguna et al., 2001). The media were autoclaved before adding 50 μg/mL PPT and ampicillin. Transformants cultures were collected by centrifugation at 1 300×*g* for 2 min and resuspended in 1-mL selective medium (~8×10^7 cells). Transformed cells were screened on f/50 selective plates and were incubated photosynthetic conditions (14 h:10 h light-dark cycle) in upright position for the culture volume to integrate into the agar medium and then turned upside down for 2–3 weeks until the pigmented colonies appeared (wild type *E. huxleyi* as the control).

2.5 Southern hybridization

Transformants cultures re-growth experiments in liquid media were performed by scraping cells off the surface of the plates using disposable plastic inoculating rings and transferring them into liquid selective f/2 medium for 12 days under constant illumination. Genomic DNAs were extracted from transformed five strains (Nos. 1–5) following the method of hexadecyl-trimethyl-ammonium bromide (CTAB) (Ausubel et al., 1999) with some modifications. In briefly, algal cultures (250 mL) were

collected by centrifugation at 1 300×*g* for 5 min at 4 °C. Cell pellets were suspended in 1-mL pre-warmed lysis buffer (0.5% SDS, 20 μg/mL proteinase K) and incubated at 55 °C for 30 min. Added 160-μL 5-mol/L NaCl and 100-μL pre-warmed 10% CTAB solution at 65 °C. After incubation at 65 °C for 10 min, the lysate was extracted with chloroform: isoamyl alcohol (24∶1). The aqueous phase was collected and CsC1 was added to a final concentration of 1.2 g/mL, and ethidium bromide of 0.6 mg/mL. The solution was centrifuged at 200 000×*g* using a T-8100 rotor (Sorvall Discovery 100S centrifuge) for 6 h. The DNA band was removed by pipetting under UV irradiation and further purified using the ethanol precipitation method. Wild-type *E. huxleyi* genomic DNA was used as a negative control. 6.5 μg of purified DNA were digested with *Bgl* II and transferred to a nylon membrane. The Biotinylated (dUTPs) *spt* specific probe was prepared by a random-prime method (Synbio Technologies) and hybridized with the membrane. Signals were detected by streptavidin-horseradish peroxidase conjugate and visualized using Chemiluminescence Detection kit (Advansta, WesternBrightTM ECL, USA).

2.6 Genomic PCR and qRT-PCR

Genomic PCR was performed using the DNA acquired above and PCR products were separated by electrophoresis in 1.0% agarose gel. Total RNA were isolated from transgenic *E. huxleyi* cells (1.84×10^5 cells) using Plant RNA Isolation Reagent (Invitrogen Corporation, USA). The quality of the RNA sample was determined with a NanoDrop Spectrophotometer (ND-1000; Thermo Fisher Scientific Inc., USA). The first-strand cDNA was synthesised using the FastQuant RT kit (with gDNase) (TIANGEN, China). The cDNA were then subjected to qRT-PCR using standard methods to determine the expression level of *spt* gene in the transformed *E. huxleyi* cells. pEhux-I-*spt* vector (bearing *spt* gene) was used as the standard and decimal dilutions of plasmid pEhux-I-*spt* were tested to normalize the absolute expression level of *spt* gene in transgenic cells. *Spt* gene (140 bp) was amplified using the following primers: *spt*-f_2: 5′-AGTCCGGTAT-CGTCTTGTCG-3′ and *spt*-r_2: 5′-TACACCTTCAA-CCAAAACATAGA-3′.

A Thermo Scientific PikoReal PCR instrument (Thermo Fisher Scientific Inc., USA) and SYBR Premix Ex Taq™ II (TaKaRa) were used for qRT-PCR analysis. The assays were performed in a total volume of 10 μL containing 0.5 μL of the above-described cDNA, 0.1-μL SYBR Green I, 1-μL 10×PCR Buffer, 0.8-μL dNTP (10 mmol/L), 0.4-pmol/L each of the 3′ and 5′ primers, 0.5-U *Taq* HS, 3.4-mmol/L $MgCl_2$, and RNase-free water. The qRT-PCR reactions were subjected to an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 20 s. A melting curve of reaction was used to determine the specificity of amplified products, which was obtained by performing a thermal cycle of 95 °C for 20 s, decreased to 60 °C for 1 min, increased again to 95 °C, with the temperature increase stepwise by 0.5 °C every 10 s. Template-free, negative, pEhux-I-*spt* and single primers controls were established before the examination. All samples were analyzed separately, whereas triplicate C_t values of the same sample were averaged before drawing a standard curve, and the standard curve was used for statistical analysis.

2.7 Protein electrophoresis and Western blot

To examine *spt* protein expression, total protein was extracted from the transformants of *E. huxleyi* and wild type control using a Protein Extraction Kit (AR0102-10, Boster Biological Technology, USA) and protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Proteins (40 μg/well) were separated by SDS-PAGE (10% acrylamide) and electrotransferred onto nitrocellulose membranes (0.45 μm, ThermoFisher Scientific) for Western blot analysis.

Anti-LCB_2 polyclonal antibody (Abmart, Shanghai, China) was developed in rabbit using the recombinant EhV-99B1-LCB_2 (the catalytic subunit of EhV-99B1-*spt*) corresponding to the N-terminal region of *spt* (in 1∶500 dilution). Protein bands were visualized using horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (1∶200 dilution) and a chemiluminescence detection system (Advansta, WesternBrightTM ECL, USA). The purified recombinant EhV-99B1-LCB_2 protein was used as a positive control.

2.8 Lipid extraction and thin-layer chromatography (TLC) analysis

Cultures in liquid were harvested by centrifugation at 1 300×*g* for 5 min at 4 °C. The dry powder of cell pellets were resuspended in 300 μL of methanol, and homogenized using the bead-based homogenizer (Tissuelyser-24, Shanghai Jingxin Industrial Development Co., Ltd., China) at 65 Hz for 2.5 min. Next, 600 μL of chloroform and 250 μL of Milli-Q

water was added to the homogenate successively. The mixture was thoroughly vortexed after the solvent addition, followed by centrifugation at 12 000 r/min for 10 min at 8 °C to form a two-phase system. The down-layer was vacuum-dried in a Speedvac concentrator (Thermo Scientific, USA), and then was dissolved in 100 μL of methanol and separated by TLC on 50 mm×100 mm plates of silica gel GF 254 (Qingdao, China) developed in the same direction with two different solvent mixture of methyl acetate∶isopropanol∶chloroform∶methanol∶potassium chloride (0.9%) (25∶25∶25∶10∶9) and hexane∶diethyl ether∶glacial acetic acid (80∶20∶2), respectively. Spots in the TLC plates were visualized by exposing the plates to copper acetate (3%) in an oven for 10 min at 160 °C.

3 RESULT AND DISCUSSION

3.1 Vector elements construction

Transformation protocols require effective selection markers and reporter genes to discriminate successful transformants from untransformed cells. Genes that confer resistance to antibiotics or herbicide have been used successfully as selectable markers for marine algal transformants (Muto et al., 2013; Mussgnug, 2015). Previously, we chose ampicillin, kanamycin, streptomycin, G418, novobiocin, chloramphenicol, puromycin, and glufosinate-ammonium as selectable markers. In the resistant test, only PPT and G418 proved to be effective at killing *E. huxleyi* at low concentrations (25 μg/mL) (data not shown here). The *bar* gene encodes for glufosinate-ammonium acetyltransferase that confers resistance to PPT. Since PPT has broad spectrum activity against bacteria, fungi and plants, it is useful as a selective agent for the construction of vectors in many organisms (Radakovits et al., 2010). In comparison to G418, PPT has some advantages, such as high activity, low toxicity, environmental friendly, and small side effects; therefore the *bar* gene is the most efficient selective marker for *E. huxleyi*. The green fluorescent protein (*gfp*) has been used as a universal reporter of gene expression and in subcellular localization analyses in various marine algae **(**Miyagawa et al., 2009; Watanabe et al., 2011**)**. The diatom fucoxanthin-chlorophyll *a*/*c* binding protein gene (*FCP*) promoter is effective in marine diatoms and other marine algae (Li et al., 2009; Miyagawa-Yamaguchi et al., 2011; Qin et al., 2012; Muto et al., 2013). Attempting to increase the transformation efficiency and the transgene copy number, the larger fragments of 484-bp promoter/600-bp terminator (Fig.1a) and 300-bp promoter/600-bp terminator sequences of the fucoxanthin chlorophyll *a*/*c*-binding protein A gene (*fcpA*) in *E. huxleyi* BOF 92 were used to construct the cassettes (Fig.1b).

3.2 Development of transformation system for *Emiliania huxleyi*

Two general-purpose transformation vectors, pEhux-I and pEhux-II were constructed to facilitate efficient introduction of heterologous genes in the *E. huxleyi*. pEhux-I harbored *bar* cassettes and MCS cassette. The primary selection for *E. huxleyi* cells harboring the vector was PPT resistance, encoded by the *bar* gene, which was flanked by the *fcp A* promoter (FAP) and the *fcp A* terminator (FAT1) (Fig.1a, Supplementary Fig.S1). pEhux-II contained the promoter *fcp A* (FAP) and terminator (FAT2) regions flanked the *gfp* gene and MCS to promote *gfp* gene expression effectively (Fig.1b, Supplementary Fig. S2). The promoter *fcp A* (FBP) and terminator (FAT2) regions flanked the MCS to promote efficient expression of the inserted *spt* gene (Fig.1c, Supplementary Fig.S3). Several methods have been used in the transformation of algal cells, such as particle bombardment, electroporation, polyethylene glycol (PEG)-mediated transfer and agitation with glass beads or silicon fibers. Among these methods, electroporation has proven to be a powerful and economic method for the transient and stable expression of foreign genes in microalgae, including *Chlamydomonas reinhardtii*, *Chlorella* spp., *Dunaliella salina*, *Haematococcus pluvialis*, *Nannochloropsis* sp., and *Phaeodactylum tricornutum* (Brown et al., 1991; Coll, 2006; Kilian et al., 2011; Niu et al., 2012, 2013). In this study, the binary-vectors pEhux-I-*spt* and pEhux-II were introduced into the *E. huxleyi* BOF92 simultaneously by electroporation. All the clones analyzed retained the non-selectable reporter gene under conditions selective only for the antibiotic resistance gene. It might be important to maintain selection pressure to retain *bar* activity and the stable expression of the non-selectable gene may therefore indicate that the two plasmids were integrated together at the same site in the genome, as most likely occurs in the moss *Physcomitrella patens* (Kammerer and Cove, 1996) and *Phaeodactylum tricornutum* (Falciatore et al., 1999). After being co-transformed by electroporation, *E. huxleyi* cells were incubated under 14 h∶10 h light-dark cycling cultivation at 16 °C for 24 h and the *gfp*

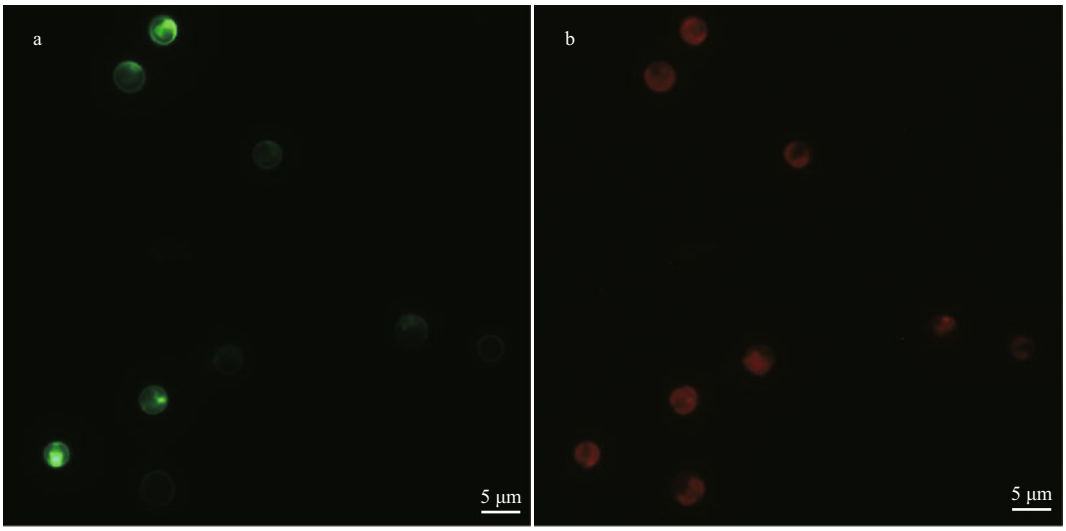

Fig.2 Microscopic images of *Emiliania huxleyi* BOF 92 strain cells transformed with pEhux-I-*spt* and pEhux-II vectors by electroporation and cultured for 24 h

a. *Gfp* fluorescent images of GFP expression; b. autofluorescence of chloroplast.

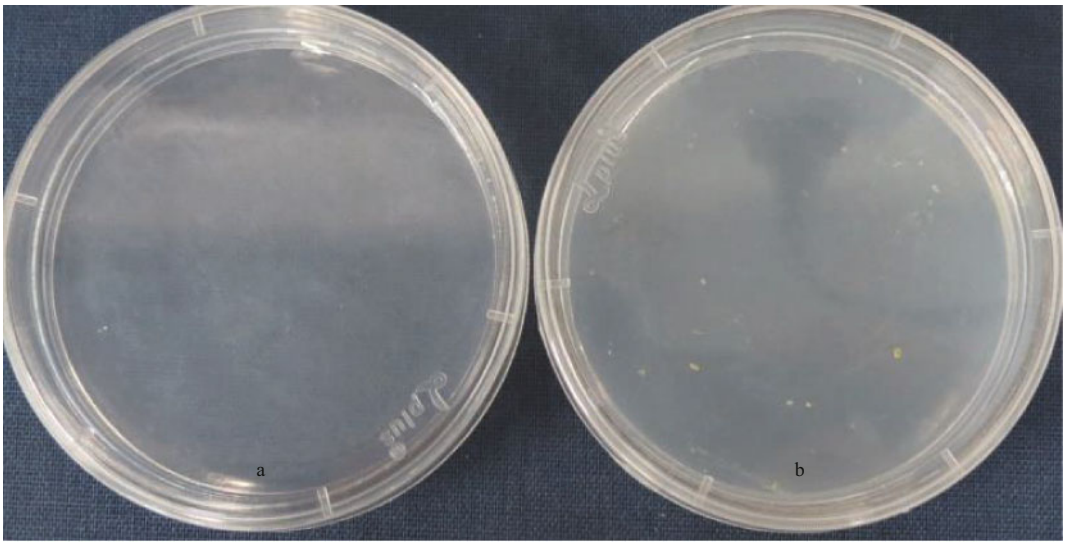

Fig.3 Cultures of wild-type (a) and transgenic (b) *Emiliania huxleyi* BOF 92 cells streaked on the glufosinate-ammonium containing medium (f/50 medium) cultured for 20 days after electroporation

gene could be expressed in transformed *E. huxleyi* cells via fluorescence microscopy (Fig.2a & b). The culture was then supplemented carefully with 50 μg/mL of PPT and continued to culture. 25 μg/mL of PPT was sufficient to completely inhibit the growth of *E. huxleyi*. Hence, 50-μg/mL PPT was used for selection of transformants. The negative controls were selected in the same fashion. After one week of culturing, the transformed *E. huxleyi* cells were allowed to grow on PPT selective medium, whereas none of the negative controls survived.

3.3 Stability of the transformed phenotype

Transformants cultures in liquid selective medium were collected by centrifugation and then screened on f/50 selective plates containing 50-μg/mL PPT. After for 4–5 days cultivated, some very small single colonies could be observed and the pigmented colonies appeared after extended incubation about three weeks (Fig.3a & b).

The survived cells on selection plates were scraped off using disposable plastic inoculating rings and transferred into liquid f/2-Si selective medium, meanwhile untransformed cells inoculated in parallel. After one-week cultivation, transformed cells from solid media re-grown slowly, while untransformed cells inoculated in paralleled could not grow even after 2 weeks, indicating that *bar* was successfully

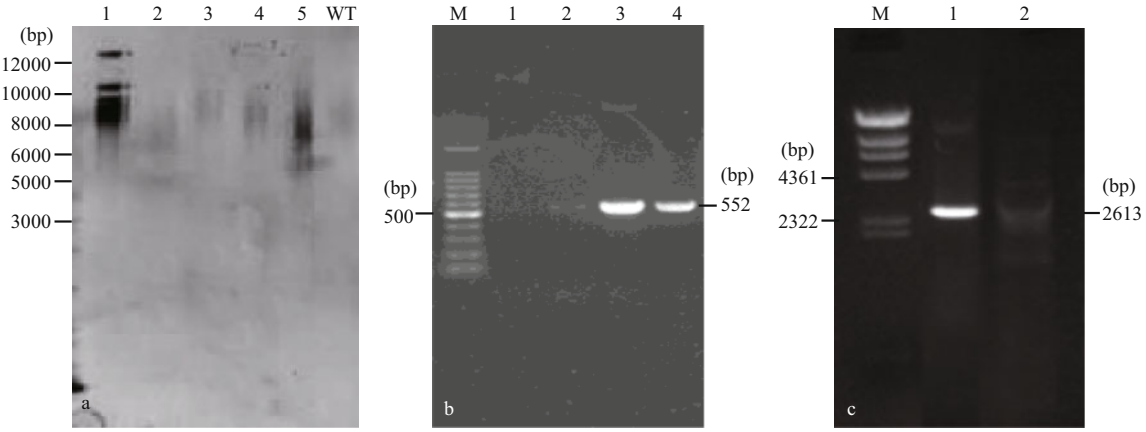

Fig.4 Southern blot and genome PCR analysis of wild-type and transformants of *Emiliania huxleyi* BOF92

a. Southern blot. Lanes 1–5, genomic DNA from five strains digested with *Bgl* II; WT: wild-type genome DNA digested with *Bgl* II. Molecular marker bands are on the left; b: genome PCR for *bar* gene. Lane M: 100-bp DNA ladder marker; Lane 1: untransformed control; Lanes 2–4: transgenic cell line showing a 552-bp *bar* band; c. genome PCR for *spt* gene. Lane M: *Hind* III digest DNA Marker; Lane 1: transgenic cells showing a 2 613-bp *spt* band; Lane 2: untransformed control.

expressed and resulted in PPT resistance, indicating that a nuclear transformation system has been developed for *E. huxleyi* BOF92 using electroporation to introduce the selectable marker *bar* gene into cells. Transformants were obtained when the *fcp* promoter was used to drive *bar* expression and a numbers of transformants were generated (3.8 colonies/10^6 cells). It showed that cultures have to be adapted to permanent light for an extended period as done with *P. tricornutum* since gene expression is apparently regulated in a circadian rhythm (Oeltjen et al., 2004). Therefore, we could infer that *fcp* promoter has higher efficiency under continuous light conditions. The pulsed *E. huxleyi* were kept in nonselective medium for 24 h to allow recovery before spreading on selection plates. In the present study, the transformation efficiency (3.8 colonies/10^6 cells) was lower than those of the results reported for *P. tricornutum* transformation systems by using electroporation (1 colony/10^5 cells) (Niu et al., 2012) and microparticle bombardment (6.5 colonies/10^6 cells) (Miyagawa et al., 2009). The first stable nuclear transformation of coccolithophore, *Pleurochrysis carterae* was established by means of polyethylene glycol (PEG)-mediated transfer and acquired a transformation efficiency approximately of 9.5 colonies/10^6 cells (Endo et al., 2016). Our experiment was conducted on constructs containing larger fragments of the *fcpA* promoters FAP (600 bp)/FBP (300 bp) and the *fcpA* terminator FAT1/FAT2 (a 600-bp region from the end of the coding region), though it seemed that the use of these constructs did not acquire the higher transformation efficiency. Electroporation commonly results in highly variable integrated transgene copy numbers and low copy transformants. In electroporation, electric pulses are varied in intensity, duration and number to control the efficiency of exogenous DNA delivery (Qin et al., 2012). In short, the results of this study demonstrate that electroporation might be an effective and a convenient method for the transformation of coccolithophore *E. huxleyi*, though further analysis should be performed to confirm a clear relationship between transformation efficiencies and electroporation conditions in *E. huxleyi*. The transformation method has to be optimized to attain higher transformation efficiencies.

3.4 Analysis of the stable co-transformants

The stable integration of the *spt* into the genomic DNA of *E. huxleyi* was demonstrated by Southern blot and genome PCR. Southern hybridization was performed with a probe with a 625-bp fragment of *spt*. The genomic DNA isolated from a transgenic strain was digested with *Bgl* II. Southern hybridization indicated that approximately 3–4 *spt* sequences were integrated into the genomic DNA of two transformed cells (Fig.4a). PCR screening acquired a 552-bp *bar* gene (Fig.4b) and an expected 2 613-bp band of *spt* gene (Fig.4c) in the transgenic cell lines using primers *spt*-F_1/*spt*-R_1 and *bar*-F/*bar*-R, respectively, while absent in wild-type. Fragment obtained from transformants were sequenced to confirm that the fragment was amplified from the introduced gene. Based on these results, we concluded that relatively stable transformation of *spt* was achieved.

The copy number of the EhV-99B1-*spt* gene was

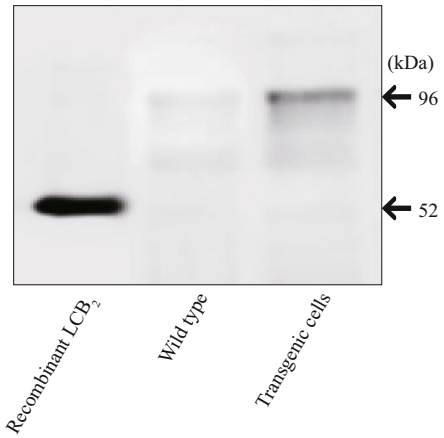

Fig.5 Western blot analysis

Equal amount of each protein from the wild-type and transgenic cells were loaded; the cross-reacting EhV-99B1-LCB_2 bands (arrowhead) were detected in transgenic cells and purified recombinant LCB_2 protein while not in wild-type.

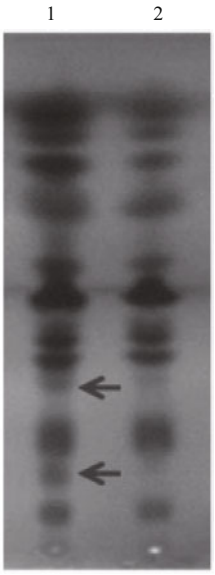

Fig.6 Thin-layer chromatography (TLC) of the total lipids in *Emiliania huxleyi* BOF 92 exposing the plates to copper acetate (3%)

Lane 1: transformed *E. huxleyi* cells; Lane 2: wild *E. huxleyi* cells. Arrows indicated the different lipid components.

evaluated by absolute qRT-PCR assay. A DNA melt curve analysis was implemented with specific primers *spt*-f_2/*spt*-r_2 and *E. huxleyi* cDNA. The pEhux-I-*spt* as detection template was carried out to verify amplification specificity. In order to detect the quantity of RNA in transformation group and wild type *E. huxleyi* cells, decimal dilutions of pEhux-I-*spt* vector were tested, and their C_t values linearly related to the logarithm of the starting cDNA copy number were plotted as the standard curve of the reaction (Supplementary Fig.S4). The parameters obtained for the SYBR Green I qRT-PCR: the standard curve y= -3.427 67x+39.468 41, correlation coefficient (R^2) 0.998 and percentage efficiency (EFF) 95.7%. In the transformation group *E. huxleyi*, the absolute expression level of *spt* gene was analyzed based on the amplification curves and melt plots and obtained 28.21 of C_t value. By calculation, copy number of the *spt* gene was approximately 2.06 copies/cell in the transgenic *E. huxleyi* cells, suggesting that these genes had been integrated successfully into the genome of *E. huxleyi* and were transcribed in transformed cells under the control of the endogenous *fcp* putative promoter. In transgenic organisms, the transgene copy number can greatly influence the expression level and genetic stability of the target gene, and thus the estimation of transgene copy number is most important. Comparative experiments were also conducted on constructs containing only medium size fragments of the *fcp A* promoter (484 bp), which resulted in the lower transgene copy number of 1.67 copies/cell (data not shown). Use of the larger size both of the *fcp* promoter/terminator fragments might be needed to acquire stable insertion into genome.

The Western blot analyses were performed to evaluate expression of the *spt* gene in *E. huxleyi* transgenic cells using polyclonal antibodies specific for EhV-99B1-LCB_2. As determined by immunoblots, the LCB_2 protein (~52.0 kDa) was present in the transformed cell lines examined while absent in wild-type cells (Fig.5). In this work, a successful growth of transformants could be kept and growing on solid selective media and the integration of *spt* and *bar* genes into the nuclear genome of *E. huxleyi* appeared to be stable.

3.5 Changes in total lipid by TLC analysis

SPT is the key enzyme in sphingolipid biosynthesis and it is considered to be a heterodimer of two subunits of $Sptlc_1$ (LCB_1) and $Sptlc_2$ (LCB_2) (Hanada et al., 2000). Interestingly, EhV-99B1-*spt* presented a single open reading frame (ORF), in which its N-terminal domain most closely resembled the LCB_2 subunit and the C-terminal domain most closely resembled the LCB_1 subunit of eukaryotic SPT (Wilson et al., 2005; Liu et al., 2012). To evaluate the possible function and activity of EhV-99B1-*spt*, we developed the new vector system for heterologous gene expression in *E. huxleyi*, producing transformants with EhV-*spt* gene. TLC analysis result clearly showed that a significant change of total lipid

compositions in transformed *E. huxleyi* cells (Fig.6), indicating that the EhV-*spt* had certain catalytic activity.

4 CONCLUSION

We have successfully transformed the constructs incorporating a tandem cassette containing *bar*, *spt*, and MCS driven by the *fcpA* promoter/*fcpA* terminator of *E. huxleyi* BOF92. The *spt* gene was integrated successfully into the nuclear genome and expressed in the *E. huxleyi* cells, which revealed the effectiveness of general transformation vector. For a functional identification, EhV-99B1-*spt* gene expression resulted in a clearly change of total lipid compositions in transformed *E. huxleyi* cells. The creation of a transformation system for *E. huxleyi* provided additional genetic resource with potential for exploring basic biological questions such as *E. huxleyi* virus-host interaction and also might make the organism a potential bioreactor of bioactive metabolites.

5 DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed in this study are available from the corresponding author upon reasonable request.

6 ACKNOWLEDGMENT

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Electronic supplementary material

Supplementary material (Supplementary Figs.S1–S4) is available in the online version of this article at https://doi.org/10.1007/s00343-020-9325-0.

Fig.S1 Agarose gel electrophoresis of the digest products of the recombinant plasmids pEhux- I (a–b).

Fig.S2 Agarose gel electrophoresis of the digest products of the recombinant plasmids pEhux- II.

Fig.S3 Agarose gel electrophoresis of the digest product of the recombinant plasmids pEhux- I-*spt*.

Fig.S4 Expression level of viral *spt* gene in transformed *Emiliania huxleyi* cells (a–d).

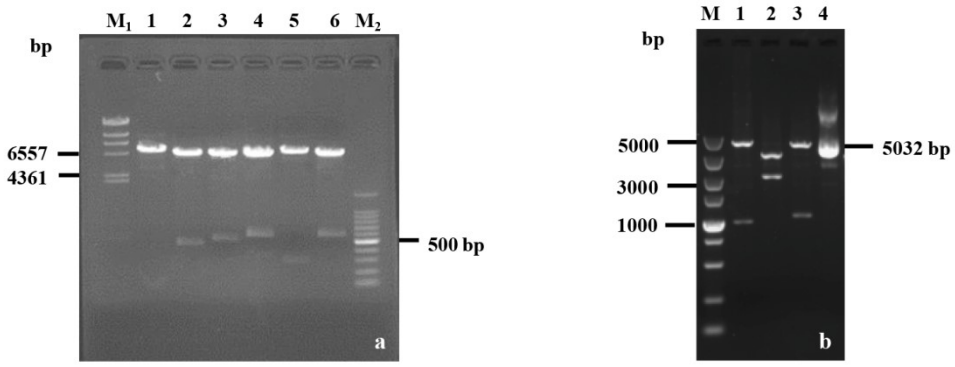

Fig.S1 Agarose gel electrophoresis of the digest products of the recombinant plasmids pEhux- I (a–b). (a) M_1 represents λ-Hind III digested DNA Marker; Lane 1, recombinant plasmid pEhux- I (5032 bp); Lane 2~6, the recombinant plasmid digested by *Bgl* II / *Spe* I for FAP gene (484 bp), *Spe* I / *Mlu* I for bar gene (552 bp), *Hind* III / *Xho* I for FAT1 gene (600 bp), *Not* I / *Eco*R I for FBP gene (300 bp) and *Mlu* I / *Not* I for FAT2 gene (600 bp); M_2 represents 100bp DNA Ladder Marker. (b) M_1 represents λ-HindIII digested DNA Marker; Lane 1 ~ 3, the recombinant plasmid digested by *Bgl* II / *Mlu* I for FAP - bar (1036 bp), *Bgl* II / *Eco*R I for FAP-Bar-FAT1-FBP (2536bp); and *Bgl* II /*Not* I for FAP-Bar-FAT1 (1600bp) ; Lane 4, recombinant plasmid pEhux-I (5032 bp).

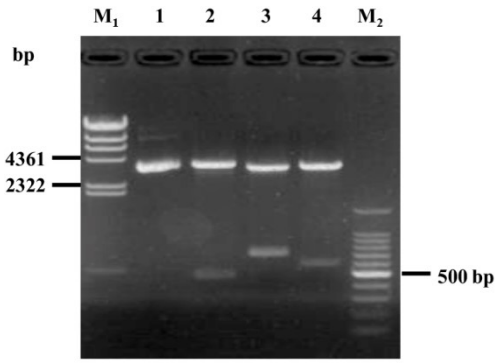

Fig.S2 Agarose gel electrophoresis of the digest products of the recombinant plasmids pEhux- II. M_1 represents λ-*Hind* III digest DNA Marker; Lane 1, recombinant plasmid pEhux-II (4265 bp); Lane 2 ~ 4, the recombinant plasmid digested by *Bgl* II / *Spe* I for FAP gene (484 bp), *Spe* I / *Eco*R I for gfp gene (717 bp) and *Mlu* I / *Not* I for FAT2 gene (600 bp); M_2 represents 100bp DNA Ladder Marker

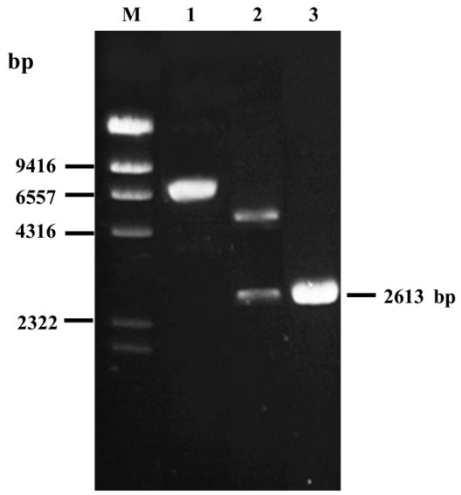

Fig.S3 Agarose gel electrophoresis of the digest product of the recombinant plasmids pEhux- I-*spt*. M represents λ-*Hind* III digest DNA Marker; Lane 1, recombinant plasmid pEhux-II (7570 bp); Lane 2, the recombinant plasmid digested by *Sma* I / *Hind* III for spt gene (2613 bp), Lane 3, PCR product of recombinant plasmids pEhux- I-*spt* (2613 bp)

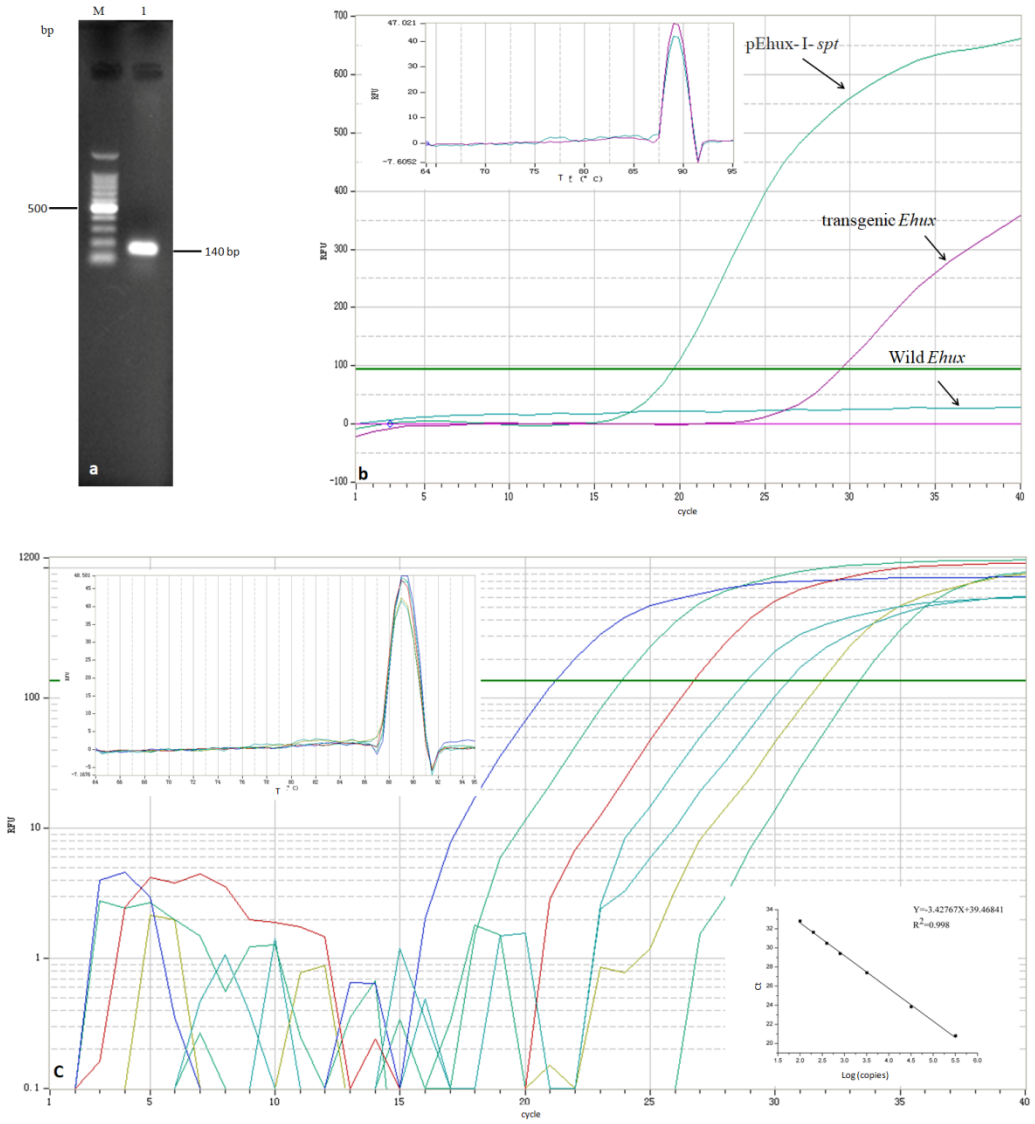

Fig.S4 Expression level of viral *spt* gene in transformed *Emiliania huxleyi* cells (a–d). (a) Agarose gel electrophoresis of *spt* gene qRT-PCR in the transformed *E. huxleyi*. **(b)** qRT-PCR Amp/Cycle Graph and Melt Curve Graph of *spt* gene in the transformed *E. huxleyi*. **(c)** Decimal dilutions of the pEhux-I-*spt* vector were tested. The parameters obtained for the SYBR Green I qRT-PCR: the standard curve Y=-3.42767x+39.46841, correlation coefficient (R_2) 0.998, percentage efficiency (EFF) 95.7%; Amplification curves and melt plots analyzed the expression of the *spt* gene, and obtained 28.21 of Ct value. By calculation, copy number of the *spt* gene was about 2.06 copies / cell in the transformed *E. huxleyi* cells.