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Transient expression of the enhanced green fluorescent protein (*egfp*) gene in *Sargassum horneri**

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Abstract *Sargassum horneri* is a macroalga widespread in North Asia-Pacific region, and these years its bloom has caused huge damage to the environment and the economic in China. To make up the blank on genetic engineering research, a transient transformation system for the multicellular marine brown alga *S. horneri* was established in this research. The algae used in this research were collected from the Yellow Sea of China and verified as a same species *S. horneri* with analysis of molecular markers. The *S. horneri* parietal leaves were transformed with the enhanced green fluorescent gene as the reporter by micro-particle bombardment. The results show that green fluorescent protein (GFP) is an effective transgene reporter for *S. horneri* and that particle bombardment is a suitable method for transformation of *S. horneri*. Through selection of four different promoters for EGFP and six groups' bombardment characters, the highest transformation efficiency approximately 1.31% was got with the vector pEGFP-N1 at bombardment characters 900 spi and 6 cm distance. This research paves a way for the further research and application of *S. horneri*.

Keyword: green fluorescent protein (GFP); particle bombardment; *Sargassum horneri*; transgenesis

1 INTRODUCTION

Sargassum horneri (Turner) C. Agardh is a dioecious brown marine macroalga growing up to 3–7 m long. The species forms underwater forests along the coast of the North Pacific Ocean and is mainly distributed in China, Japan, and Korea (Yoshida, 1983; Tseng and Lu, 2000). This alga *S. horneri* has important influences on marine ecosystems and huge economical value for aquaculture. For example *S. horneri* provides habitat and spawning grounds for marine invertebrates and fish (Choi et al., 2008), and its extracts for example the high content of oligosaccharides, have development potential in biofertilizers (Wang et al., 2016) and pharmaceutical industries (Ma et al., 2014; Wen et al., 2016; Sanjeewa et al., 2017). These years the "floating golden tide" caused by *S. horneri* has been brought into focus in North Asia-Pacific region, which has caused ¥ 5 billon RMBs' economic damage to aquaculture in Jiangsu Province in China (Yi et al., 2009; Xing et al., 2017). It is urgent to know the mechanism of *S. horneri* huge biomass occurrence within a short time, and meanwhile the treatments and amplifications millions' of algae fronts. But at present, no reports on genetic engineering of *S. horneri* were published, which has hindered the basic research and application of *S. horneri* by genetic engineering and biotechnological techniques. To make up the blank on genetic engineering, a transient transformation system for the multicellular marine brown alga *S. horneri* was established in this research.

The researches on macroalgae genetic engineering

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are much fewer than the microalgae transformation for macroalgal complicated constructs and life history (Mikami, 2013, 2014). Till now, there are just several researches about macroalgal transient systems such as *Porphyra yezoensis* (Hirata et al., 2011) and *Kappaphycus alvarezii* (Wang et al., 2010), and stable transformation system of *P. yezoensis* (Hirata et al., 2014), *Ulva mutabilis* (Oertel et al., 2015) and *Laminaria japonica* gametophyte (Deng et al., 2009; Li et al., 2009). But the successful transformation systems on *P. yezoensis*, *U. mutabilis* and *L. japonica* were not suitable for *S. horneri* directly.

The particle bombardment method and the *egfp* gene were adopted in our research for their universal high efficiency. More than 20 different strains of marine algae have been transformed successfully using a variety of transformation methods (Qin et al., 2012). Direct gene transfer by means of a biolistic method (micro-particle bombardment) has been proven to be the most efficient method and is highly reproducible for delivery of exogenous DNA into algal cells. Biolistic transformation remains the most useful tool for transgenic studies of marine macroalgae regardless of the cell wall composition and life cycle. On the other way, the *Aequorea victoria* green fluorescent protein (GFP) shows a number of desirable traits as a universal reporter (Heim et al., 1994; Ormö et al., 1996), and has been shown to function as a reporter in a wide range of species including algae (Lechtreck et al., 2002; Pöggeler et al., 2003).

To get high transformation efficiency, the bombardment characters and four vectors with different commonly used promotors were employed and compared by the GFP fluorescence in transformed *S. horneri*. This is the first research on the genetic engineering of *S. horneri* and lays the foundation for further research and the algal amplification.

2 MATERIAL AND METHOD

2.1 Strain

Nine *Sargassum horneri* samples were collected from the Yellow Sea of China, nearby Qingyutan village in Shandong Province, and incubated at 10°C under a 12 h:12 h (light:dark) photoperiod and light intensity of 40–50 μmol photons/(m^2·s). The seawater for *S. horneri* culture was filtered through a membrane of pore size 0.22 μm and was changed every two days. Parietal leaves were removed from the *S. horneri* samples and cultured in a plastic tank (60 cm× 40 cm) preparing for transformation.

Table 1 Information of primers sequences

Marker	Primer (5′→3′)
18S rDNA	F: TACCTGGTTGATCCTGCCAG R: CCTTCCGCAGGTTCACCTAC
cox3	F: ATGTTTACTTGGTGRAGRGA R: CCCCACCARTAWATNGTNAG
rbcL	F: TATGATTGATTTAGTGGTTGG R: GTTCGTCACTTAAATCTGGTA

2.2 Analyses of multiple molecular markers

Total DNA was extracted from approximately 100 mg fresh *S. horneri* ground in liquid nitrogen using the Plant Genomic DNA Kit (TIANGEN Biotech Co., Beijing, China), in accordance with the manufacturers' instructions, then dissolved in 100 μL TE buffer. The extracted DNA was stored at -20°C and used to amplify the molecular markers *18S* rDNA, *cox3* and *rbcL*.

The primer pair used in this research was listed in Table 1. PCR amplifications were performed in a thermal cycler (Eppendorf, Germany) with the following conditions for *18S* rDNA: initial denaturation at 95°C for 5 min; 28 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. PCR routines for *cox3* and *rbcL* were carried out as *18S* rDNA except that the annealing temperature was 45°C for PCR of the *cox3* and the annealing temperature was 55°C for PCR of the *rbcL*. The PCR products were purified using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, Dalian, China) and sequenced in Sangon Biotech Company (Sangon Biotech, Shanghai, China).

The resulted sequences were aligned using BioEdit software (http://www.brothersoft.com/bioedit-490363.html). An additional 14 sequences of *18S* rDNA, 11 sequences of *cox3* and 8 sequences of *rbcL* (specified information in the following resulted figures) from algae were downloaded from the National Center for Biotechnology Information (NCBI). Phylogenetic trees were constructed by the neighbor-joining (NJ) method using MEGA 5.0. The NJ trees were constructed using Kimura 2-parameter distance (Kimura, 1980). A bootstrap analysis with 1 000 replicates was performed to assess bootstrap support for the NJ tree topology. For *18S* rDNA analysis, the sequence *Ectocarpus siliculosus* L43062.1 was used as outgroup. For *cox3* analysis, the sequence *Carpophyllum angustifolium* JN637984.1

was used as outgroup. For *rbcL* analysis, the sequence *Coccophora langsdorfii* KY432510.1 was used as outgroup.

2.3 Transformation by particle bombardment

Four commercial vectors with the egfp gene as reporters were adopted in this research. The vectors contained different universal efficient promotors for the egfp gene, such as the CMV promoter in the pEGFP-N1 vector (Clontech Laboratories, Inc., Palo Alto, CA, USA), the SV40 promotor in the PCMS-EGFP vector (Clontech Laboratories, Inc., Palo Alto, CA, USA), the CaMV35S promotor in the pBi221-EGFP vector (Youbio, China) and the NOS promotor in the pEGFP-1-NOS vector (Youbio, China). The vectors were amplified in *Escherichia coli* Top10 strain. Plasmids were isolated from *E. coli* cells with a TIANpre mini plasmid Kit (TIANGEN, Beijing, China) and the plasmids DNA final concentration were not less than 1 μg/μL.

Healthy blades from *S. horneri* samples were selected and placed in a petri dish. Wet gauze was placed between the blades and the fresh seawater medium as used in incubation step to prevent the blades from withering. Plasmid DNA (6 μL) was coated onto gold carrier particles following the method of Cui et al. (2010). The blade tissue was bombarded with six sets characters: 450 psi at 6 cm distance and 9 cm distance, 650 psi at 6 cm distance and 9 cm distance, 900 psi at 6 cm distance and 9 cm distance. After bombardment, the *S. horneri* blades were incubated in the dark for 8 h. Blades bombarded with particles without plasmids were used as control. All experiments were performed in triplicate.

2.4 GFP Detection

From the 4th to 7th days after transformation, the *S. horneri* was examined by a Nikon Eclipse 50i microscope (Nikon Corp., Tokyo, Japan) with blue-light excitation (470–500 nm) from an apochromatic mercury lamp (100 W) and the time lapse set as 1 min. The objective lens (NA 0.5) with 20 times magnification was adopted and the wave length of emission fluorescence was set 570–700 nm. The pictures were taken at a 5-s exposure time, by OLYMPUS DP 72 digital imaging device with single chip color CCD camera and Peltier cooling system, which photosensitivity (ISO) is 200–1 600. And for transformation rates evaluation, 5 microscope fields of view were selected randomly.

3 RESULT

3.1 The molecular markers in the collected samples showed high similarities which certified the samples belonging to *S. horneri*

The collected *S. horneri* samples were named as 1, 2, 3 and to 9 in order. Three molecular markers *18S* rDNA, *cox3* and *rbcL* were cloned from the nine *S. horneri* samples, and the resulted *18S* rDNA sequences were named as S1, S2 and to S9 in order ranging from 1 762 to 1 780 bp, the *cox3* sequences named as R1, R2 and to R9 with 555 bp, the *rbcL* sequences named as A1, A2 and to A9 with 859 bp. The similarities among nine *18S* rDNA sequences S1 to S9, nine *cox3* sequences R1 to R9, nine *rbcL* sequences A1 to A9 were all higher than 99.9%. And in cladogram, the sequences from the collected samples were placed in the same clade with the relevant known *S. horneri* sequences (*18S* rDNA to AB011429.1 as in Fig.1, *cox3* to JF461005.1 as in Fig.2, *rbcL* to KF281799.1 as in Fig.3). In the cladogram of *18S* rDNA, the nine sequenced samples and the known *S. horneri* AB011429.1 formed a large clade (representing members of the Fucales) with three additional brown algal species (Fig.1). These results showed all samples collected in the present study showed conserved molecular markers sequences and certified their same clarification to *S. horneri* species.

3.2 The *egfp* gene could be expressed in *S. horneri* with high efficiency

After particle bombardment with all the four vectors at 650 spi pressure and 6 cm distance, part of cells in *S. horneri* blade exhibited green-yellow fluorescence under blue-light (450–520 nm) excitation, whereas the controls showed the characteristic red chlorophyll fluorescence (Fig.4). The activity of GFP was observed from the 4th to 7th days after transformation, and GFP activity decreased 15 days later. In Fig.4a, the cells with green-yellow fluorescence were regarded as the positive cells, and the cells with red fluorescence were regarded as the wild cells. No positive cells were observed among the controls. To get a high efficiency, the bombardment characters were optimized, and the resulted *S. horneri* transformation efficiencies were listed in Table 2. The highest rate 1.31% was obtained by with the vector pEGFP-N1 at bombardment characters 900 spi and 6 cm distance. Among the four vectors, the pEGFP-N1 and the pBi221-EGFP showed higher rates ($>10^{-3}$) than the pCMS-EGFP and the pEGFP-1-NOS at the same treatments.

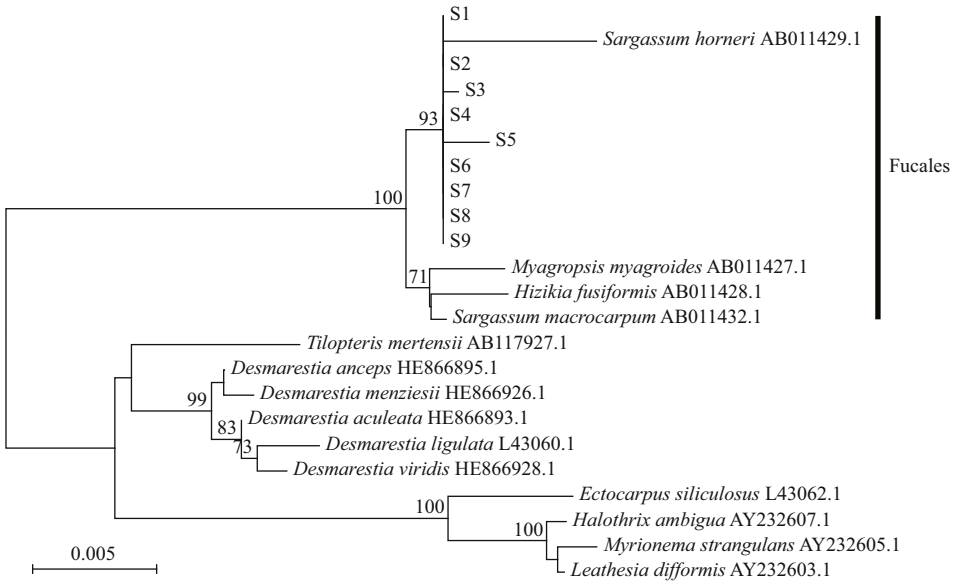

Fig.1 Neighbor-joining (NJ) tree constructed for *18S* rDNA sequences

Nine sequences from the collected samples were named as S1, S2 to S9, and the other 14 sequences were downloaded from NCBI. The numbers under the branches represent full heuristic bootstrap support values (1 000 replicates) greater than 70%. Branch lengths are proportional to the extent of sequence change.

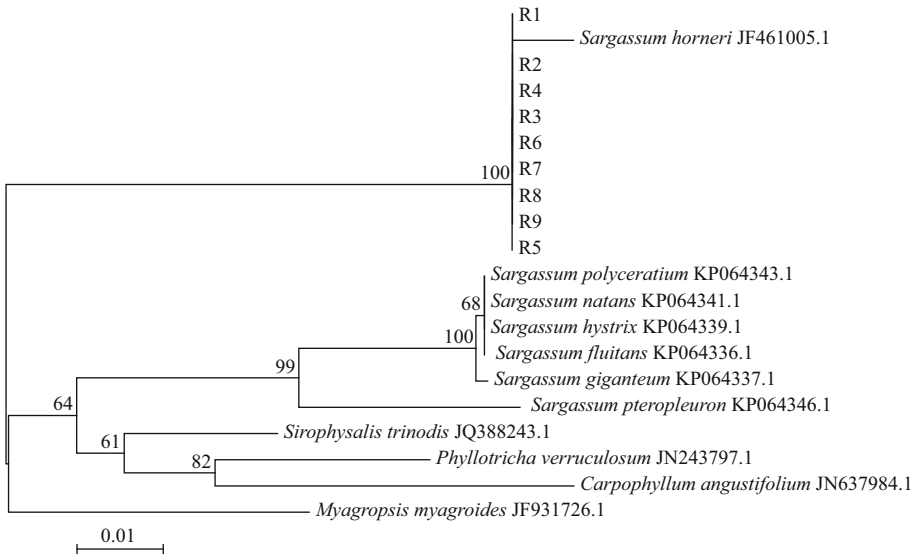

Fig.2 Neighbor-joining (NJ) tree constructed for *cox3* sequences

Nine sequences of the collected samples were named as R1, R2, to R9, and the other 11 sequences downloaded from NCBI. The numbers under the branches represent full heuristic bootstrap support values (1 000 replicates) greater than 50%. Branch lengths are proportional to the extent of sequence change.

Table 2 The transformation rate of *S. horneri* with different characters

Bombardment characters	pEGFP-N1	pBi221-EGFP	pCMS-EGFP	pEGFP-1-NOS
450 psi+6 cm	$(3.92\pm0.23)\times10^{-4}$	$(2.33\pm0.03)\times10^{-4}$	-	-
450 psi+9 cm	-	-	-	-
650 psi+6 cm	$(7.12\pm0.43)\times10^{-4}$	$(6.25\pm0.65)\times10^{-4}$	$(6.43\pm0.58)\times10^{-4}$	$(3.97\pm0.25)\times10^{-4}$
650 psi+9 cm	$(4.51\pm0.18)\times10^{-3}$	$(4.74\pm0.25)\times10^{-3}$	-	-
900 psi+6 cm	$(1.31\pm0.02)\times10^{-2}$	$(8.65\pm0.94)\times10^{-3}$	$(9.68\pm0.95)\times10^{-4}$	$(1.13\pm0.01)\times10^{-3}$
900 psi+9 cm	$(7.54\pm0.74)\times10^{-3}$	$(5.65\pm0.74)\times10^{-3}$	$(4.27\pm0.07)\times10^{-4}$	$(5.86\pm0.02)\times10^{-4}$

- means no data.

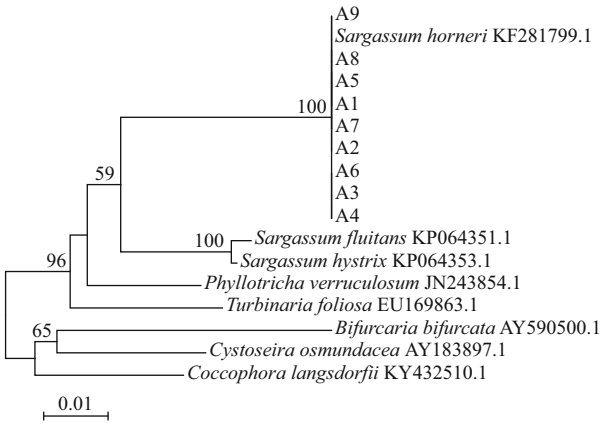

Fig.3 Neighbor-joining (NJ) tree constructed for *rbcL* sequences

Nine sequences from the collected samples was named as A1, A2, to A9. And the other 8 sequences downloaded from NCBI. The numbers under the branches represent full heuristic bootstrap support values (1 000 replicates) greater than 50%. Branch lengths are proportional to the extent of sequence change.

4 DISCUSSION

The *S. horneri* samples collected from Yellow Sea of China, at Qingyutan village of Shandong province where there was a large number of floating *S. horneri* from April to June these years. The samples were tested by molecular markers as *S. horneri* species and were treated as the same one in the further genetic engineering manipulation. There were no significant differences on the transformation rates around these samples in this research.

For macroalgae genetic engineering, among the commonly used methods for genes transferring such as the electric shock, glass bead agitation and so on, the particle bombardment is an effective one for many macroalgae. In the present study, *S. horneri* was successfully transformed with the EGFP reporter by a micro-particle bombardment method. As expected, the EGFP fluorescence could be examined in the brown alga *S. horneri* without background interference. The positive *S. horneri* look like a lot of lumps as shown in Fig.4a, which may be related to the concentrated bombardment range and the limited growth of *S. horneri* in 7 days after transformation.

In the construct of plasmids, the promotor is a critical character for the foreign genes' expression, and the endogenous promoters show higher efficiency than the heterologous. Without genomic sequences information and enough biological researches in *S. horneri*, the four universal heterologous promoters used in algal genetic engineering were adopted in this research. The four promoters were all efficient in *S. horneri* and CMV promoter was indicated as the most

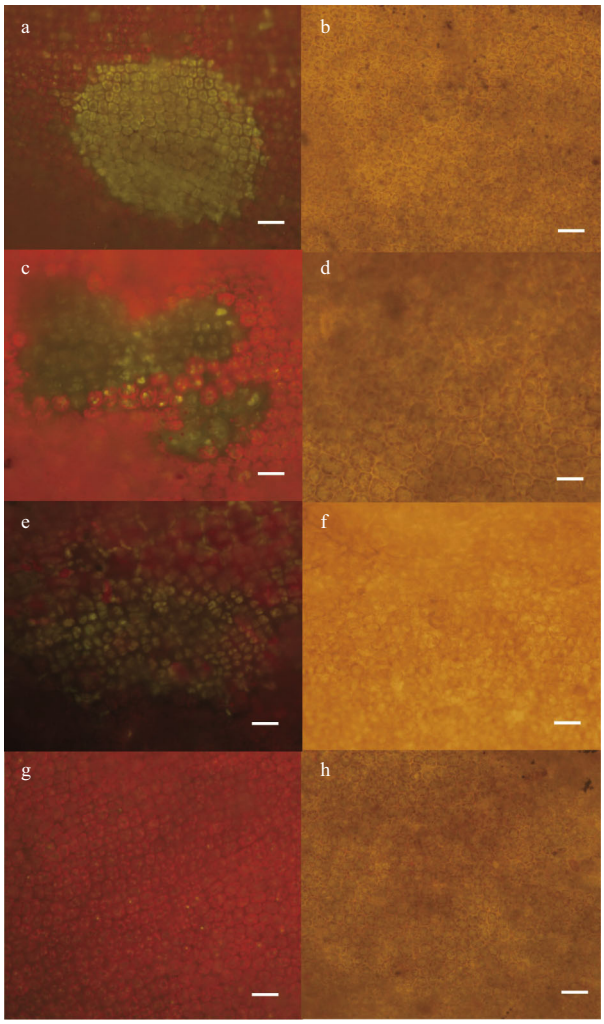

Fig.4 EGFP fluorescence examination in *S. horneri*

The activity of GFP was observed from 4th to 7th days after transformation. a, c and e. bombarded *S. horneri* illuminated with excitation fluorescence; g. control bombarded with blank particle; b, d and f. bombarded *S. horneri* illuminated with white light; h. control illuminated with white light. All blades were viewed with a Nikon Eclipse 50i microscope (Nikon, Japan) under blue light (470–500 nm) excitation. Bars=100 μm.

effective one. With different bombardments the transformation rates of the same vector showed obvious differences, and the set of 900 psi with 6 cm was more suitable than the others in this research. These results may indicate more power with higher pressure and shorter distance facilitates for higher rates, and the further research will be conducted in this fact. The transient transformation system is just a first step of *S. horneri* genetic engineering research, and the stable transformation system will be established in the further research, such as the antibiotic and the relevant selective markers for selecting genetically transformed *S. horneri*, integration of the expression constructs into the genome or the over expression of homologous genes, and the inheritance of the mutants.

5 CONCLUSION

This is the first research on *S. horneri* genetic engineering and more works are needed for as intact effective transformation system, such as the selection strategies and the stable inheritance at its dioecious heredity. Anyhow this report lays the foundation for *S. horneri* genetic engineering and will facilitate its basic research and application of the high amount fronts.

6 DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available in NCBI.

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