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A codon-optimized bacterial antibiotic gene used as selection marker for stable nuclear transformation in the marine red alga *Pyropia yezoensis*

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Running title: Stable nuclear transformation in *Pyropia*

Abstract

Marine macroalgae play an important role in marine coastal ecosystems and are widely used as sea vegetation foodstuffs and for industrial purposes. Therefore, there have been increased demands for useful species and varieties of these macroalgae. However, genetic transformation in macroalgae has not yet been established. We have developed a dominant selection marker for stable nuclear transformation in the red macroalga *Pyropia yezoensis*. We engineered the coding region of the aminoglycoside phosphotransferase gene *aph7"* from *Streptomyces hygroscopicus* to adapt codon usage of the nuclear genes of *Pyropia yezoensis*. We designated this codon-optimized *aph7"* gene *PyAph7*. After bombarding *P. yezoensis* cells with plasmids containing *PyAph7* under the control of their endogenous promoter, 1.9 thalli (or individuals) of hygromycin-resistant strains were isolated from a 10 mm square piece of the bombarded thallus. These transformants were stably maintained throughout the asexual life cycle. Stable expression of *PyAph7*was verified using Southern blot analysis, genomic PCR and RT-PCR analyses. *PyAph7* proved to be a new, versatile tool for stable nuclear transformation in *P. yezoensis*.

Key words: *Pyropia yezoensis*; red alga; selection marker; stable transformation

Introduction

The marine red macroalga *Pyropia yezoensis* (*nori* in Japanese) is one of the most important marine crops. It is widely cultivated in eastern Asian countries, including Japan, Korea and China, and generates US $1.3 billion per year (Blouin et al. 2010). In addition, *P. yezoensis* has attracted considerable interest as a model for physiological and genetic studies of marine red algae (Saga and Kitade 2002, Waaland et al. 2004). To date, several studies have been performed to make this alga a sophisticated model organism. For example, a laboratory culturing system in which the life cycle of *P. yezoensis* could be completed within a few months was established (Kuwano et al. 1996). In addition, a database for expressed sequenced tags (EST) analysis is now available (Nikaido et al. 2000, Asamizu et al. 2003), and recently the draft data of whole genome sequence has been analysed by next generation sequencing (Nakamura et al. 2013). However, a stable transformation system, a powerful tool both for elucidating gene functions and conferring valuable characteristics to an organism, has not yet been established for *P. yezoensis* or other marine macroalgae.

As an initial step in establishing stable transformation, we previously developed a transient gene expression system to monitor gene expression in *P. yezoensis* cells using particle bombardment. Because *P. yezoensis* genes have a strong GC bias in the third nucleotide of their codons, it is important to adapt codon usage of foreign genes to the nuclear genes of *P. yezoensis* for their efficient expression. In fact, codon-optimized β-glucuronidase (PyGUS) and GC-rich fluorescent proteins, such as AmCFP and sGFP(S65T), have been expressed in *P. yezoensis* cells under the control of an endogenous promoter (Fukuda et al. 2008, Mikami et al. 2009, Uji et al. 2010).

In addition to developing an efficient expression system, a reliable method to select

and isolate transformed cells is required to establish stable transformation in macroalgae. Recently, we have revealed that *P. yezoensis* cells are sensitive to several aminoglycoside antibiotics, including hygromycin B, paromomycin and geneticin (Takahashi et al. 2011). Thus, these antibiotics are possible candidate selection agents for stable *P. yezoensis* transformation.

Regarding a selection marker, the aminoglycoside phosphotransferase gene *aph7"* from *Streptomyces hygroscopicus*, which confers resistance against hygromycin B, should be available for hygromycin-based stable transformant selection because the GC content in its coding region is as high as 70.94% (Zalacain et al. 1986). The *aph7"* gene has been successfully used for the transformation of green microalgae, such as *Chlamydomonas reinhardtii* (Berthold et al. 2002), whose codons are also rich in GC residues. However, several codons that are rarely used in *P. yezoensis* nuclear genes are found in the *aph7"* gene, especially in its N-terminal region. These mismatches in codon usage would be predicted to inhibit efficient translation in *P. yezoensis* cells.

Thus, in the present study, we synthesized a codon-optimized *aph7"* gene and examined its utility as a selection marker for stable nuclear transformation in *P. yezoensis*.

Materials and methods

Culturing of P. yezoensis

Gametophytes of *P. yezoensis* strain TU-1 and transformants were cultured in enriched sea life (ESL) medium under conditions described by Fukuda et al. (2008).

Plasmid construction

To construct a pEA7 plasmid, a fragment containing the ORF of *PyAph7* and 3′ UTR of *CrRbcS2* was amplified using pHyg4 as a template and a pair of primers, XbaI-PyAph7-F (5′-GCTCTAGAATGACGCAGGAGTCCCTGCTGCTGCTC-3′) and EcoRI-CrRbcS2-R (5′-GGAATTCTTCCATGGGATGACGGGCCCGG-3′). The amplified PCR product was digested with *Xba*I and *Eco*RI and subsequently inserted into *Xba*I–*Eco*RI-digested p35S-PyGUS (Fukuda et al. 2008), which was designated p35S-PyAph7. To replace the CaMV 35S promoter with an endogenous promoter, the 5′ upstream region of *PyElf1* was amplified using pPyElf1-PyGUS (Mikami et al. 2011) as a template and the following primers: HindIII-PyElf1-F/XbaI-PyElf1-R (5′ -CCCAAGCTTCCAGACCCGTGGAAAGTACCATC-3′ /5′ GCTCTAGACTTGCCCATGGTGGGGGGG-3′). The PCR product was digested with *Hin*dIII and *Xba*I and subsequently inserted into the *Hin*dIII–*Xba*I site of p35S-PyAph7. This resulted in pEA7 construction.

Particle bombardment

Expression plasmids were purified from 100 mL of *E. coli* culture using a NucleoBond Xtra Midi (MACHEREY-NAGEL, Germany). For particle bombardment, a gametophytic thallus with monosporangia covering a wide range of the thallus (>10 mm in width) was cut into 10-mm-square pieces, which were subsequently placed on a filter paper. After removing excess fluid, the expression plasmids were introduced into the gametophytic cells using PDS-1000/He particle bombardment under the conditions described previously (Hirata et al. 2011).

Isolation of hygromycin-resistant transformants

The bombarded algal pieces were cultured in a 100-mL glass flask (Iwaki Sci Tech Div., Asahi Techno Glass, Japan) in 50 mL of ESL medium under non-selective conditions for 1 week. Subsequently, the medium was replaced with the ESL medium containing hygromycin

B (final concentration of 1 mg mL^{-1}), and the medium was renewed weekly. After incubation for 6–8 weeks in the antibiotic-containing medium, visible hygromycin-resistant transformants regenerated from the pieces of bombarded thalli were individually isolated in another culture flask and continuously cultured in ESL medium with or without 1 mg mL^{-1} hygromycin B.

Assay for hygromycin resistance

To prepare individuals for hygromycin resistance assay, gametophytes isolated as hygromycin-resistant transformants were clonally propagated for 3 weeks in different culture flasks containing ESL medium via monospores. Gametophytes of transformants or wild type strains (ca. 20 mm in length) cultured were respectively transferred into a 6-well plate (3 individuals/well) (Iwaki Sci Tech Div., Asahi Techno Glass, Japan) containing 5 mL of ESL medium with 0, 1.0, 2.5, 5.0, 7.5 or 10.0 mg mL^{-1} hygromycin B and incubated under shaking culture for 2 weeks at 15°C. The medium was renewed weekly. After culture, gametophyte viability was estimated by staining using 0.01% erythrosine (Wako Pure Chemical Industries, Japan) in ESL medium according to a previous report (Takahashi et al. 2011).

Genomic PCR and RT-PCR

Genomic DNA was extracted from gametophytes of transformants cultured for 4 weeks in ESL medium without hygromycin B or wild type strains for genomic PCR as described by Hwang et al. (2010) and purified using a phenol–chloroform extraction and ethanol precipitation. The precipitate was resuspended in 50 μL of TE buffer, and 2 μL of this suspension was used as a template for genomic PCR. RNA extraction and cDNA synthesis for RT-PCR were performed as described by Uji et al. (2012). Genomic PCR and RT-PCR

analyses were conducted using *TaKaRa LA Taq* with GC Buffer (TaKaRa-Bio). The primer pairs PyAph7-RT-F/R (5′-CATTGACTCGGACGACTCCTACGCGAG-3′/5′-AAGTCGTGCAGGAAGGTGAAG-3′) and PyElf1-RT-F/R (5′-AAGGCCAAGGCACCCAAGCTG-3′/5′-ACCACACCAAGAGCGTCCAATC-3′) were used to amplify the fragments of *PyAph7* (864 bp) and *PyElf1* (734 bp), respectively. The amplified PCR products were examined on a 1.3% agarose gel.

Southern blotting

Genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method from 1.0 g (FW) of wild-type and transformants that had been cultured for more than three months after isolation. Extracted DNA was further purified by ultracentrifugation as follows: 3.1 g of cesium chloride (CsCl) and 15 µL of ethidium bromide (EtBr) were added to 3.0 mL of the DNA solution and centrifuged at 400,000 *g* for 24 h at 20°C. The DNA band was visualised under UV light and collected. EtBr was removed by three extractions with an equal volume of 1-butanol. CsCl was removed by ethanol precipitation three times. Purified DNA (2.0 µg) was digested with *Pst*I, run on agarose gel and transferred to a nylon membrane. The pEA7 plasmid was digested with *Xba*I and *Sal*I and a 730 bp fragment of *PyAph7* was collected. This fragment was labelled by random 32P priming and used as a probe (see Fig. 1a).

Results and discussion

To optimize the codon usage of the *aph7*'' coding region to that of *P. yezoensis*, we employed site-directed mutagenesis using a pHyg4 plasmid that contained the *aph7''* gene (Berthold et al. 2002) and a KOD-Plus-Mutagenesis Kit (TOYOBO, Japan) with the oligonucleotides shown in Table S1. The synthetic *aph7*'' gene was designated *PyAph7* (Fig.

S1). Subsequently, the protein coding region of *PyAph7* was fused with the endogenous *PyElf1* promoter (Mikami et al. 2011). The resulting plasmid was designed pEA7 (Fig. 1a). The pEA7 expression plasmids containing *PyAph7* were introduced into *P. yezoensis* gametophytes by particle bombardment. When gametophytic thalli with the introduced pEA7 were cultured in a non-selective ESL medium for 1 week (Fig. 1b), some monospores (asexual spores) were released from the bombarded thalli and adhered to the bottom of the culture flask (Fig. 1c). The medium was subsequently replaced with selective ESL medium containing 1.0 mg mL^{-1} hygromycin B, which effectively kills wild-type cells or, at least, completely inhibits their growth (Takahashi et al. 2011). After 6–8 weeks of culture in the selective medium, hygromycin-resistant thalli from them released monospores or from vegetative cells of bombarded blades were approximately 5–10 mm long (Fig. 1d, e). These transformants were transferred into separate culture vessels to identify the homogeneous lines and cultured further in a non-selective ESL medium (Fig 1b). Consequently, an average of 1.9 thalli (or individuals) of the hygromycin-resistant strains were obtained from a piece of the bombarded thallus (29 transformants per 15 bombardments). These transformants were successfully maintained over more than 5 generations as independent lines through the asexual life cycle via monospores. Subsequently, we examined the hygromycin B tolerance of six isolated transformants: EA1–EA6. As shown in Figs. 2 and S2, when the gametophytes were cultured in an ESL medium containing 1.0 mg mL^{-1} hygromycin B, all wild-type gametophytes were dead after 2 weeks of culture. In contrast, all transformants survived and grew in this medium. Transformants EA2, EA3 and EA4 survived even in the presence of 2.5–10.0 mg mL^{-1} hygromycin B (Figs. 2 and S2).

Finally, to verify whether the exogenous *PyAph7* gene had been successfully introduced and expressed in these isolated transformants, genomic PCR and RT-PCR analyses were performed on four transformant strains (EA1–EA4). Using primers specific for the *PyAph7*

gene sequence, a DNA fragment of the expected size was amplified for all of the examined strains, whereas this fragment was not observed in a wild-type strain (Fig. 3a). This indicated that *PyAph7* had been successfully introduced and expressed in these transformants. We further analysed the stable integration of *PyAph7* into the genome by Southern blot analysis. The southern blot analysis revealed that multiple DNA fragments could be detected in all of the examined transformants, which indicated that *PyAph7* had multiplied and randomly integrated into the genome (Fig. 3b). Strong signals of an approximately 4.2 kbp DNA fragment that corresponded to the full-length of the pEA7 vector were commonly detected in all transformed strains. The results intimate the possibility that a part of introduced pEA7 vector might be stably maintained as entire circular plasmids in the transformed cells through the cell divisions and propagation. Interestingly, there have been several reports on plasmid DNA isolation from some red macroalgae, including *Porphyra tenera*, a closely related species of *P. yezoensis* (Goff and Coleman 1990, Choi et al. 2000; 2001). Thus, we need to investigate on mechanisms for maintaining plasmid DNA in *P. yezoensis* cells.

Taken together, the codon-optimized *PyAph7* introduced by particle bombardment was stably maintained and expressed in *P. yezoensis* and conferred resistance to hygromycin B. Our results demonstrate that *PyAph7* is useful as an efficient selection marker for stable nuclear transformation of *P. yezoensis*. This is the first report of a bacterial antibiotic gene used as a selection marker for stable transformation in marine macroalgae. Further development of this stable transformation system in *P. yezoensis* will overcome some of the barriers in molecular biological studies of marine red algae.

Acknowledgments

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Figure legends

Fig. 1 Isolation of hygromicin-resistant transformants in *P. yezoensis*

(a) Schematic diagram of the hygromycin selective vector pEA7. The coding region of *PyAph7* is fused in-frame to 5′ PyElf1 (promoter, 5′-untranslated region of *PyElf1* and the initiation codon). 3′ CrRbcS2 indicates the 3′ untranslated region of the *RbcS2* gene from *Chlamydomonas reinhardtii*. The position and length of the DNA fragment amplified by genomic PCR or RT-PCR are indicated. The position of the probe used in Southern blotting is indicated (probe). (b) Time-line for isolating hygromicin-resistant transformants.(hyg B, hygromycin B). (c) Macroscopic view of the bottom of the culture flask on which monospores released from bombarded thalli were attached (arrowheads). Scale bar = 10 mm. (d) Hygromycin-resistant thalli regenerated from monospores attached to the bottom of a culture flask (arrowheads). Scale bar = 10 mm. (e) Hygromycin-resistant thalli regenerated from the vegetative cells of a bombarded thallus (arrowheads). Scale bar = 5 mm.

Fig. 2 Analysis of hygromycin B resistance for wild type and transgenic *P. yezoensis* strains Survival rates of wild type and six lines of the hygromycin-resistant strains (EA1–EA6) when cultured with varying concentrations of hygromycin B (1.0–10.0 mg mL^{-1}). The survival rate was calculated by counting viable and dead gametophytes during 2 weeks culture in ESL medium containing hygromycin B. Values are means ± SDs (n = 30).

Fig. 3 PCR and Southern blot analyses of hygromicin-resistant transformants

(a) Expression of the exogenous *PyAph7* gene in hygromycin-resistant transformants was detected by genomic PCR and RT-PCR. PCR was performed using primers specific for the *PyAph7* gene sequence (Fig. 1) and genomic DNA or total RNA from a wild type strain and

the transformants EA1–EA4. *PyElf1* was used as the internal control gene in *P. yezoensis*. Only transformants were expected to yield an 864 bp fragment of *PyAph7*. (b) Southern blot analysis of hygromycin-resistant transformants. Genomic DNA from a wild type strain and the transformants EA1–EA4 were digested with *Pst* I, separated on agarose gel, transferred to nylon membrane and hybridized with a labelled probe corresponding to the *PyAph7* fragment (Fig.1). Lane M, molecular weight marker.

Fig. S1 Comparison of the original aph7" and synthetic PyAph7 coding regions

Differences between original *aph7"*and synthetic *PyAph7* are noted below the original aph7"sequence in red letters. Optimized codons were based on codon usage indicated in: http://www.kazusa.or.jp/codon/cgibin/showcodon.cgi?species=Porphyra+yezoensis+[gbpln]. The amino acid sequence is shown below the nucleotide sequence by the single letter code. The nucleotide numbers are indicated on both the left and right hand sides.

Fig. S2 Hygromycin B resistance in wild type and transgenic *P. yezoensis* strains

Wild type and six hygromycin-resistant strains (EA1–EA6) were cultured in ESL medium containing hygromycin B (1.0–10.0 mg mL^{-1}) for 2 weeks. Scale bar =50 μm.

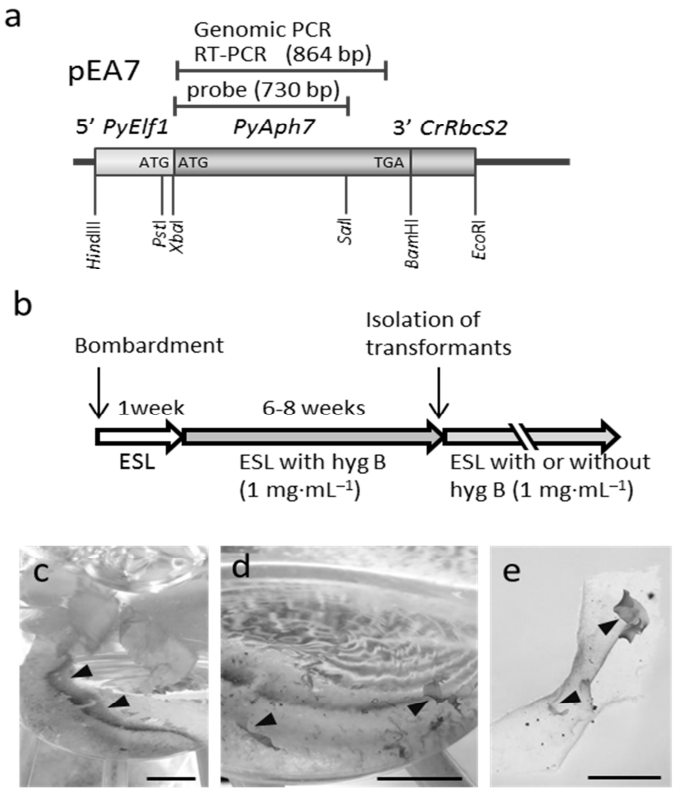

a
Genomic PCR
RT-PCR (864 bp)
pEA7
probe (730 bp)
5' PyElf1
PyAph7
3' CrRbcS2
ATG
ATG
TGA
HindIII
PstI
XbaI
SalI
BamHI
EcoRI
b
Bombardment
Isolation of
transformants
1week
6-8 weeks
ESL
ESL with hyg B
(1 mg·mL−1)
ESL with or without
hyg B (1 mg·mL−1)
c
d
e

Fig. 1

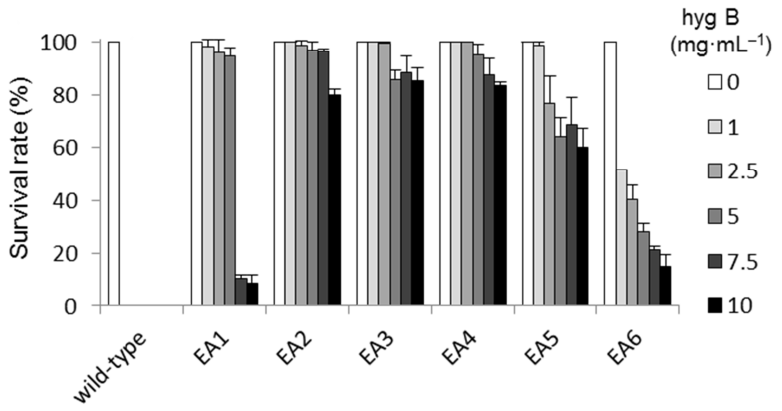

Survival rate (%)
100
80
60
40
20
0
wild-type
EA1
EA2
EA3
EA4
EA5
EA6
hyg B
(mg·mL⁻¹)
0
1
2.5
5
7.5
10

Fig. 2

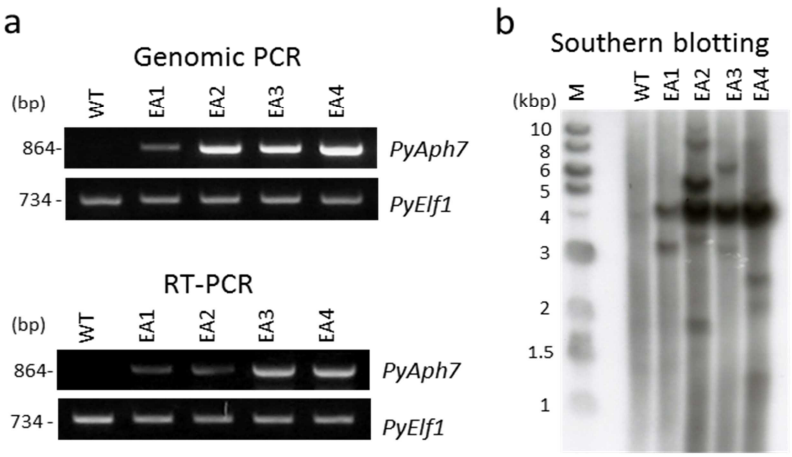

a
Genomic PCR
(bp)
WT
EA1
EA2
EA3
EA4
864-
PyAph7
734 -
PyElf1
RT-PCR
(bp)
WT
EA1
EA2
EA3
EA4
864-
PyAph7
734 -
PyElf1
b
Southern blotting
(kbp)
M
WT
EA1
EA2
EA3
EA4
10
8
6
5
4
3
2
1.5
1

Fig. 3