TECHNICAL NOTE

Isolation and regeneration of transiently transformed protoplasts from gametophytic blades of the marine red alga *Porphyra yezoensis*

Short running title: Isolation of transformed red algal protoplasts

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Financial support: This study was supported in part by a grant from the Sumitomo Foundation (to KM) and by Grants-in-Aid for Scientific Research (C; no. 21580213) and a National Project "Knowledge Cluster Initiative" (Innovative Stage, "Hakodate Marine Bio Industrial Cluster-Green Innovation of UMI") from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to NS)

Keywords: allantoin, protoplast, *P. yezoensis*, regeneration, transient gene expression

Abbreviations: AmCFP: humanized cyan fluorescent protein from *Anemonia majano*

AMX: emzyme mix containing β-agarase, β-1,4-mannanase and β-1,3-xylanase

ESL: enriched sealife

GUS: β-glucuronidase

MES: 2-(*N*-Morpholino)ethanesulfonic acid

PH: Pleckstrin homology

PyAct1: *P. yezoensis* actin1

PyGUS: *P. yezoensis*-adapted β-glucuronidase

ABSTRACT

Despite the recent progress of transient gene expression systems in a red alga *Porphyra yezoensis* by particle bombardment, a stable transformation system has yet to establish in any marine red macrophytes. One of the reasons of the difficulty in genetic transformation in red algae is the lack of systems to select and isolate transformed cells from gametophytic blades. Thus, toward the establishment of the stable transformation system in *P. yezoensis*, we have developed a procedure by which transiently transformed gametophytic cells were prepared from particle bombarded-gametophytic blade as regeneratable protoplasts. Using mixture of marine bacterial enzymes, yield of protoplasts was high as reported elsewhere; however, these protoplasts did not develop. In contrast, protoplasts prepared from gametophytes treated with allantoin were normally developed, in which the overexpression of a β-glucuronidase reporter gene had no effect on the regeneration of protoplasts. Therefore, the use of allantoin in protoplast preparation sheds a new light on the realization of an efficient isolation and selection of study transformed cells from gametophytic blades.

INTRODUCTION

Porphyra yezoensis is recently received a great attention as a most promising model macrophyte for physiological and molecular biological studies in marine red algae (Saga and Kitade, 2000; Waaland, 2004). Physiological study of *P. yezoensis* has been enhanced by our establishment of the laboratory culture system of the *P. yezoensis* strain TU-1 (Kuwano et al. 1996). For example, the ability of constant harvestigation of monospores derived from gametophytic blades enabled us to examine how cell polarity is determined during migration and following development in monospores (Li et al. 2008; Li et al. 2009).

In contrast, until recently, experimental systems for analyzing regulation and function of genes has yet to establish in any marine macrophyte, which prevented the progress of molecular biological studies in multicellular algae. However, we have recently succeeded in the efficient expression of the modified β-glucuronidase (*PyGUS*) gene, whose codon usage had been adapted to that of *P. yezoensis*, and humanized cyan and green fluorescent protein (*AmCFP* and *ZsGFP*) genes by transient transformation of gametophytic blade using particle bombardment (Fukuda et al. 2008; Mikami et al. 2009; Uji et al. 2009). Such a technical development is expected to contribute to progress studies on subcellular localization of protein and regulation of the gene expression in *P. yezoensis*. Indeed, a plasma membrane localization of Pleckstrin homology (PH) domains from human proteins was correctly observed using PH

domain-AmCFP fusion gene (Mikami et al. 2009) and nuclear localization of *P. yezoensis* transcription factors was also confirmed with AmCFP and ZsGFP (Uji et al. 2009).

Despite the development of transient gene expression systems in *P. yezoensis*, the fine analysis of gene function requires methods for manipulation of genome via the stable transformation. To develop the stable transformation procedure in *P. yezoensis*, efficient isolation and selection systems of transformed gametophytic cells are indispensable. In the present study, we developed a method to isolate transiently transformed gametophytic cells as regeneratable protoplasts from particle bombarded-gametophytic blades. Our results indicate that allantoin treatment, which was originally reported by Mizuta et al. (2003), is useful for preparation of transformed protoplasts from gametophytes. This is the first report of the regeneration of transiently transformed protoplasts in red algae.

MATERIALS AND METHODS

Cultivation of gametophyte of *P. yezoensis* in the ESL (enriched sealife) medium was performed as described previously (Li et al. 2008). Protoplasts were prepared by two different methods. The first is a treatment of 5.0 mg gametophytic blade with 1.0 ml of AMX solution containing 20 mM MES (pH 7.5), 0.5 M mannitol, 4 mg of β-agarase, 4 mg of β-1,4-mannanase and 4 mg of β-1,3-xylanase (all of which parchased for Yakult

Co Ltd, Tokyo, Japan), essentially according to Araki et al. (1994) with modification as using gametophytic blades pre-treated with 1.0 ml of 5% papain solution [the ESL medium containing 20 mM MES (pH 7.5) and 0.5 M mannitol] for 60 min. The second employs allantoin as described previously (Mizuta et al. 2003) expect for culture in the ESL medium with 60 μmol m^{-2} s^{-1} irradiance (10 L: 14 D cycle). After cultivation in allantoin-containing ESL medium for 3 weeks, gametophytic blades were homogenized in Downce type homogenizer to release protoplasts. For both methods, resultant protoplasts were filtered through 50 μm of nylon mesh and centrifuged at 1,000 rpm for 5 min, then resolved in 10 ml of the ESL medium. The total number of collected protoplasts was counted three times with a Thoma hemacytometer.

To construct a plasmid to express the *PyGUS* reporter gene, the Cauliflower mosaic virus 35S RNA promoter was released from p35S-PyGUS (Fukuda et al. 2008) by digestion with HindIII and BamHI and then a 5' upstream region of the *actin1* gene from *P. yezoensis* (*PyAct1*), which amplified with two primers, such as HindIII-PyACT1-F1 (5'-CCCAAGCTTCCACGCTCAGAGGGTTGAAG-3') and BamHI-PyACT1-R1 (5'-CGCGGATCCGGGCTTGCTCATGGTGGC-3'), by polymerase chain reaction whose conditions were 98℃ for 10 s followed by 30 cycles of 94℃ for 10 s and 60℃ for 30 s with Prime STAR HS DNA polymerase (TaKaRa, Tokyo, Japan), was inserted into the promoter-less vector after digestion with HindIII and BamHI. Resultant plasmid designated pPyAct1-PyGUS were used for transient transformation of *P. yezoensis* gametophytes by particle bombardment as described

previously (Mikami et al. 2009). Histochemical staining of bombarded blades with 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) was performed according to Fukuda et al. (2008) for GUS assays.

RESULTS AND DISCUSSION

We first test the usability of two methods for preparation of protoplasts by treatment of *P. yezoensis* gametophytic blades with AMX solution and allantoin. Number of protoplasts isolated from 5 mg of gametophytic blades by treatment with AMX solution was 0.7 x 10^6 cells. However, the regeneration of released protoplasts was not observed (Figure 1A). On the other hand, the recovery of protoplasts isolated from gametophytes treated with 10 mM allantoin through homogenization was 1 x 10^6 cells per 0.1 g of gametophytic blades, 70 % of which was able to regenerate as shown in Figure 1 B and C. Therefore, allantoin treatment is considered to be useful for preparation of protoplasts from transformed gametophytic blades, although recovery is 14-fold less than the method with AMX solution.

Next, we examined the isolation of transiently transformed protoplasts from allantoin-treated gametophytic blades. As shown in Figure 2A and B, allantoin treatment did not affect on the efficiency of transient transformation of gametophytic cell; that is, the number of PyAct1-PyGUS expressing cells was 700 in average in pieces of gametophytes containing 3.0 x 10^5 cells from both control and 10 mM

allantoin-treated blades. Importantly, PyAct1-PyGUS expressing protoplasts prepared from particle bombarded gametophytic blades were regeneratable (Figure 2C-M). However, the number of PyGUS expressing cells was 40 cells per particle bombarded blade, meaning that the recovery rate of transformed protoplasts was only 6%.

In the present study, we have succeeded to isolate transiently transformed cells from particle bombarded-gametophytic blade as regeneratable protoplasts. We have already test polyethylene glycol and glass beads methods to introduce the *PyGUS* gene into a large amount of protoplasts from allantoin-treated blades, which had succeeded in other alga (Kindle, 1990; Ohnuma et al. 2008) as well as a method using Magnetofection (OZ biosciences); however, any protoplast expressing *PyGUS* reporter gene was not observed (date not shown). These findings indicated that the use of the particle bombardment is an only way to transform gametophytic cells in *P. yezoensis* at present. Therefore, our procedure using allantoin-treated gametophytic blades is only one way to isolate transiently transformed protoplasts from particle bombarded gametophytic blades. However, there is a problem in the recovery rate of protoplasts after homogenization of particle bombarded-gametophyte. We proposed that low efficiency of the recovery is probably due to the death of PyGUS-expressing cells during homogenization and/or the disappearance during centrifugation. Thus, it is necessary to improve the recovery rate of transformed cells from gametophytes, by which the method with allantoin is expected to contribute to the establishment of the stable transformation system in *P. yezoensis* in future.

ACKNOWLEDGMENTS

We are grateful to Dr. Hiroyuki Mizuta (Hokkaido University) for helpful and valuable comments for the allantoin method.

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Figure legends

Figure 1. Comparison of the ability in regeneration of *P. yezoensis* protoplasts prepared by the treatment with AMX solution and allantoin.

(a) Protoplast prepared from gametophytic blades by treatment with AMX solution. No regeneration was observed.

(b) Protoplast prepared from gametophytic blades which were cultured in the ESL medium containing 10 mM allantoin for 3 weeks.

(c) Ability of regeneration in protoplasts prepared from allantoin-treated gametophytic blade.

Scale bars: 20 μm.

Figure 2. Expression of the *PyGUS* reporter gene in gametophytes and protoplasts prepared from allantoin-treated gametophytic blades.

(a and b) GUS expression of control and 10 mM allantoin-treated gametophytic blades, respectively, for which the number of PyGUS expressing cells was equal.

(c-m) Normal regeneration of transiently transformed protoplasts in which the *PyGUS* gene was overexpressed under the direction of the *PyAct1* promoter. PyGUS expression was observed in protoplast prepared from particle-bomberded gametophytic blades (c) and during regeneration of these protoplasts (d-m).

Scale bars: a and b, 100 μm; c-m, 20 μm.

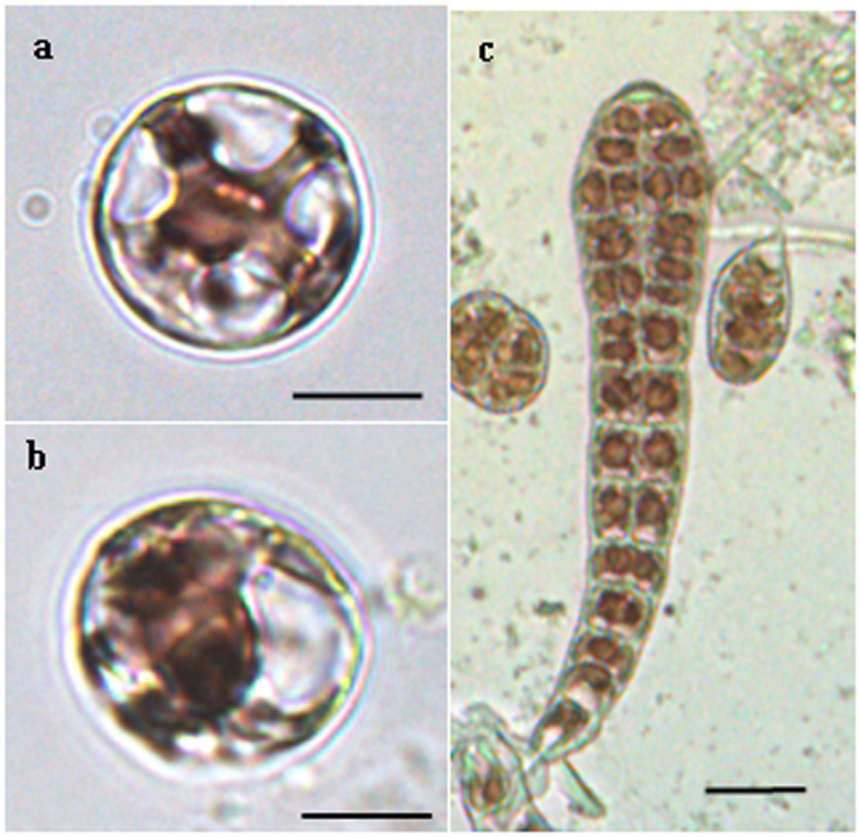

Figure 1

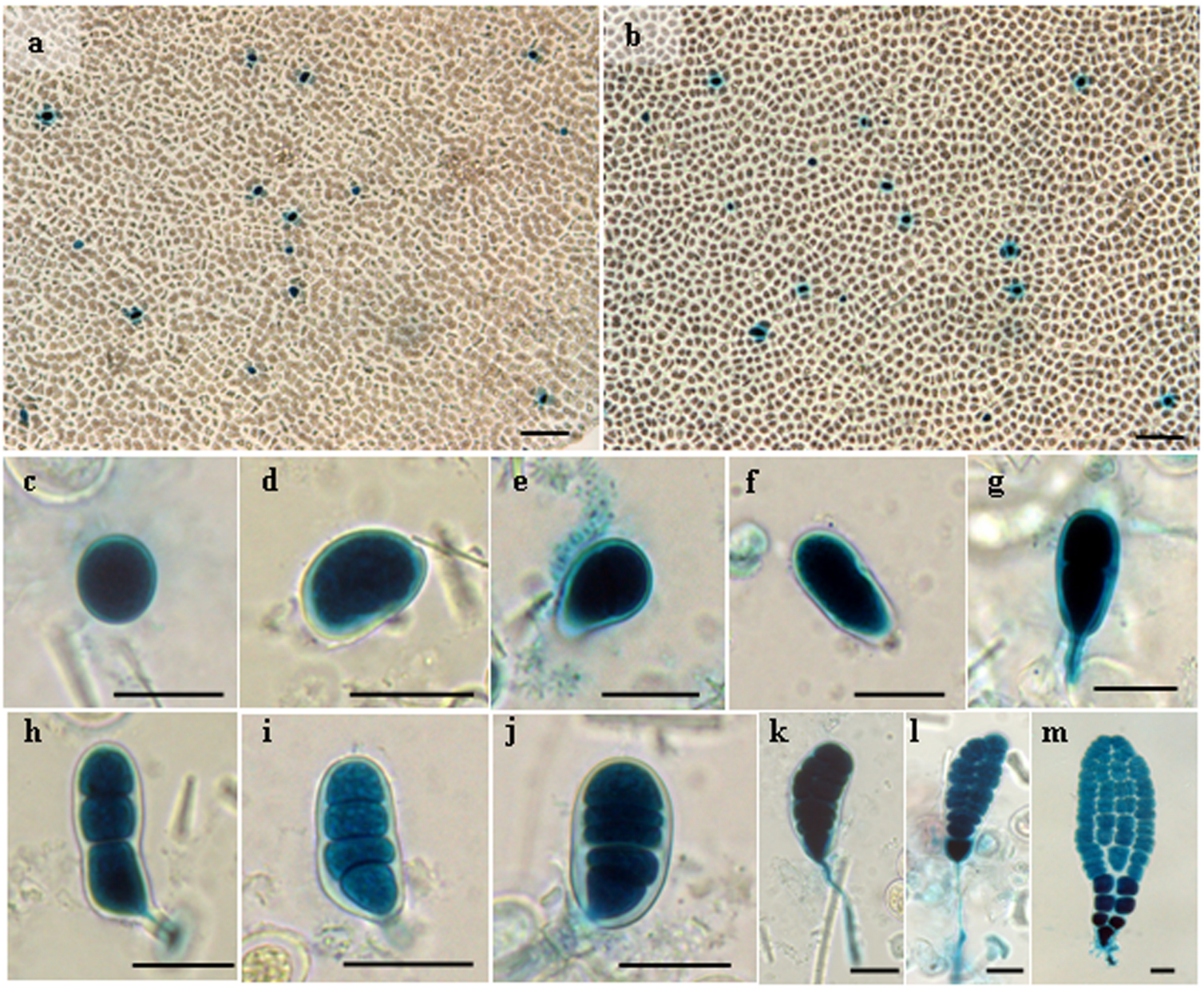

Figure 2