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Transient Transformation of Red Algal Cells: Breakthrough Toward Genetic Transformation of Marine Crop *Porphyra* Species

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1. Introduction

Genetic transformation is a powerful tool not only for elucidating the functions and regulatory mechanisms of genes involved in various physiological events but also for establishing organisms that efficiently produce biofuels and medically functional materials or carry stress tolerance in face of uncertain environmental conditions (Griesbeck et al., 2006; Torney et al., 2007; Bhatnagar-Mathur et al., 2008). Eukaryotic algae classified into microalgae and macroalgae (seaweeds) are highly diverse photosynthetic plants that are utilized as human food and animal feed as well as sources of valuable compounds such as fatty acids, pigments, vitamins and polysaccharides (Hallmann, 2007; Sugawara et al, 2011). Because of their importance in ecology and industry, algae are now considered promising organisms for economical and industrial applications and are thus a target of genetic transformation (Walker et al., 2005; Hallmann, 2007; Blouin et al., 2011). To date, genetic transformation has succeeded in microalgae; thus, stably transformed microalgae are now employed to produce recombinant antibodies, vaccines, or bio-hydrogen as well as to analyze the gene functions targeted for engineering (Sun et al., 2003; Zorin et al., 2009; Specht et al., 2010; Wu et al., 2010). However, it has proven difficult to establish transgenic macroalgae, which has hampered understanding their gene functions in various physiological regulations and also their utilization in biotechnological applications.

The red macroalga *Porphyra yezoensis* is the most popular sea crop in Japan with economical and pharmaceutical importance as the source of foods such as "nori" and pharmacological regents such as the sulfated polysaccharide "porphyran", which has anti-tumor and anti-allergic activities (Noda et al., 1990; Zemke-White & Ohno, 1999; Ishihara et al., 2005). Recently, non-beneficial climate change due to global warming has decreased the quality and yield of *P. yezoensis* at algal farms by enhancing discoloration and red rot disease caused by fungal infection (Kakinuma et al., 2008; Park et al., 2000). Although breeding of *P. yezoensis* by traditional selection and crossing methods has progressed to obtain strains showing high growth rates and economically valuable characteristics, these methods have limitations in terms of the isolation of strains carrying heat-stress tolerance or disease

resistance. Development of a molecular breeding method based on genetic transformation is expected to resolve this problem using knowledge about gene functions in *P. yezoensis*.

Despite the difficulty of genetic transformation, genetic information about *P. yezoensis* is now accumulating by a collection of ESTs from leafy gametophytes and filamentous sporophytes (Nikaido et al., 2000; Asamizu et al., 2003) and also by ongoing full-length cDNA and whole genome analyses. Genetic information provides a sophisticated way to isolate *P. yezoensis* genes of interest; however, utilization of this knowledge – in both basic biological research and molecular breeding of *P. yezoensis* – is hindered by the lack of a genetic transformation system.

Given this situation, it is clear that the development of a genetic transformation system for *P. yezoensis* is critical for sustainable production as well as the enhancement of biological study. In our laboratory much effort has been expended on the establishment of a genetic transformation system; as a result we have identified several factors preventing the establishment of a genetic transformation system in macroalgae: for example, the lack of efficient methods for foreign gene transfer and expression systems and the absence of valid selectable markers for isolation of genetically transformed cells. Thus, it is considered worthwhile to develop a transient gene expression system, because some of the above inhibiting factors, such as defects in the transfer and expression of foreign genes, could be removed by using non-integrated plasmids containing genes for expression. Since we have recently succeeded in developing an efficient transient gene expression system in Bangiophyceae including *Porphyra* species, the establishment and successful application of this system in a molecular biological study are summarized here.

2. Development of a transient gene expression system in *P. yezoensis* cells

2.1 Problems in previously published experiments

Expression of foreign genes in macroalgae has already been attempted using bacterial *lacZ* (β-galactosidase) and *uidA* (β-glucronidase, *GUS*) genes under direction by promoters of the cauliflower mosaic virus 35S RNA (*CaMV 35S*) and simian virus 40 (*SV40*) genes (Kübler et al., 1994; Kuang et al., 1998; Huang et al., 1996; Gan et al., 2003; Jiang et al., 2003). The *CaMV 35S* and *SV40* promoters are typical eukaryotic class II promoters with a TATA box and thus are generally employed to drive transgenes in dicot plant and animal cells, respectively (Kang & An, 2005; Funabashi et al., 2010). However, we have observed a quite low activity of the *CaMV 35S* promoter in *P. yezoensis* cells as shown in Figs. 1 and 2 (Fukuda et al., 2008), which is strongly supported by the fact that the TATA box is not found in the core promoters of *P. yezoensis* genes (unpublished observation), suggesting differences in the promoter structure and transcriptional regulation of protein-coding genes between red algae and dicot plants. We believe that the same could be predicted for the SV40 promoter. Thus, it seems to be necessary to reconfirm the results of reporter expression using the *CaMV 35S* and *SV40* promoters in red algae like *P. miniata*, *P. tenera*, *Kappaphycus alvarezii* and *Gracilaria changii* in previously published literatures (Kurtzman & Cheney, 1991; Kübler et al., 1993; Okauchi & Mizukami, 1999; Cheney et al., 2001; Gan et al., 2003).

In addition, the use of the *GUS* reporter gene is also a problem, since we have already confirmed that the *GUS* reporter gene is not functional in red algal cells by histochemical analysis and enzymatic activity test (see below). This is supported by previous reports indicating background levels of the GUS enzymatic activity in red and brown macroalgal cells when the GUS reporter gene was driven by *CaMV 35S* and endogenous beta-tubulin promoters (Liu et al., 2003; Gong et al., 2005).

According to the above findings, previous reports of successful expression of the *GUS* reporter gene under the direction of the *CaMV 35S* and *SV40* promoters are not likely to be replicated. Thus, an important step is to enable the effective expression of the *GUS* reporter genes in *P. yezoensis* cells, which should eventually lead to the establishment of a transient gene expression system via the development of procedures for introduction and expression of foreign genes as described below.

2.2 Codon-optimization of the reporter gene

Inefficient expression of foreign genes in algal cells is often due to the incompatibility of the codon usage in their coding regions, inhibiting the effective use of transfer RNA by rarely used codons in the host cells and thus decreasing the efficiency of the translation (Mayfield & Kindle, 1990). In this respect, successful development of foreign gene expression in green alga *Chlamydomonas reinhardtii* may be an important clue to overcoming the deficiency of the GUS reporter gene in *P. yezoensis* cells. For example, a low expression level of the gene encoding a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, whose codon usage is rich in AT residues, was dramatically increased by adjustment of the GC content of the coding region corresponding to the codon usage in *C. reinhardtii* nuclear genes (Fuhrmann et al., 1999). In addition, the synthetic luciferase gene whose codon usage was adapted to that in nuclear genes from *C. reinhardtii* was also expressed efficiently, although the original luciferase gene had less efficiency in *C. reinhardtii* cells (Fuhrmann et al., 2004; Ruecker et al., 2008; Shao & Bock, 2008). In fact, EST analysis of *P. yezoensis* reveals that the codons in *P. yezoensis* nuclear genes frequently contain G and C residues especially in their third letters, by which the GC content reaches a high of 65.2% (Nikaido et al., 2000). Since bacterial *GUS* and *lacZ* reporter genes have AT-rich codons, the incompatibility of codon usage might be responsible for the poor translation efficiency of foreign genes in *P. yezoensis* cells. We therefore postulated that modification of codon usage in the *GUS* gene would enable the efficient expression of this gene in *P. yezoensis* cells. This possibility led us to modify the codon usage of the GUS reporter gene to that in the nuclear genes of *P. yezoensis* by introducing silent mutations in the GUS coding region, by which unfavorable or rare codons in the GUS reporter gene were exchanged for favorable ones without affecting amino acid sequences. In the resultant artificially codon-optimized GUS gene, designated *PyGUS*, the GC content was increased from 52.3% to 66.6%.

To transfer the plasmid into cells, we employed particle bombardment, also referred to as micro-projectile bombardment, particle guns, or biolistics, which are among the most popular techniques for achieving nuclear and organelle transformation not only for algae but also for plants, fungi, insects, animals and even bacteria. In this method, DNA-coated heavy-metals, usually gold or tungsten particles, are introduced into cells by helium pressure, which enables the introduction of foreign DNA regardless of whether the cell has a cell wall. This method has been successfully applied in many algal species such as *C. reinhardtii* (Kindle et al., 1989), *Volvox carteri* (Schiedlmeier et al., 1994), *Dunaliella salina* (Tan et al., 2005), and *Phaeodactylum tricornutum* (Apt et al., 1996).

When the *PyGUS* gene directed by the *CaMV 35S* promoter was introduced into *P. yezoensis* gametophytic cells by particle bombardment, very low but significant expression of the *PyGUS* gene was observed by histochemical detection and GUS activity test (Figs. 1 and 2), indicating the enhancement of the expression level of the *GUS* reporter gene. Optimization of the codon usage of the reporter gene is therefore one of the important factors for successful expression in *P. yezoensis* cells (Fukuda et al., 2008; Mikami et al., 2009; Takahashi et al., 2010; Uji et al., 2010; Hirata et al., 2011).

2.3 Employment of an endogenous strong promoter

A low level of *PyGUS* expression under the direction of the *CaMV 35S* promoter was thought to be caused by the low activity of this promoter in *P. yezoensis* cells. Although the *CaMV 35S* promoter is widely used as a heterologous promoter because of its strong, constitutive and non-tissue-specific transcriptional function in dicot plant cells (Louis et al., 2010; Wally & Punja, 2010), it is well known that this promoter has very low activity in cells of green algae such as *Dunaliella salina* (Tan et al., 2005), *Chlorella kessleri* (El-Sheekh, 1999), and *Chlorella vulgaris* (Chow & Tung, 1999) and no activity in *C. reinhardtii* cells (Day et al., 1990; Blakenship & Kindle, 1992; Lumbreras et al., 1998). However, employment of strong endogenous promoters such as the *β-Tub*, *RbcS2* and *Hsp70* promoters has achieved efficient expression of foreign genes in these algae (Davies et al., 1992; Stevens et al., 1996; Schroda et al., 2000; Walker et al., 2004). In addition, many endogenous promoters have been developed and successfully used for foreign gene expression in cells of some microalgae, such as the diatom *Phaeodactylum tricornutum* and the Chlorarachniophyte *Lotharella amoebiformis* (Zaslavskaia et al., 2000; Hirakawa et al., 2008). Therefore, it is possible that recruitment of suitable strong promoters could result in efficient expression of the *PyGUS* reporter gene in *P. yezoensis* cells.

Since we found strong expression of *P. yezoensis* genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin 1 (Act1) by reverse transcription-PCR (RT-PCR) (see Fig. 5), the 5′ upstream regions of these genes were isolated from the *P. yezoensis* genome and fused with the *PyGUS* gene to construct the expression plasmids pPyGAPDH-PyGUS and pPyAct1-PyGUS in addition to the pPyGAPDH-GUS and pPyAct1-GUS plasmids. When pPyGAPDH-GUS and pPyGAPDH-PyGUS were introduced into gametophytic cells by particle bombardment, cells expressing the reporter gene and GUS enzymatic activity were dramatically increased using the *PyGUS* gene as a reporter, although the original *GUS* gene was not efficiently expressed (Figs. 1 and 2). The same results were obtained using pPyAct1-PyGUS and pPyAct1-GUS plasmids (Figs. 1 and 2). Thus, the combination of endogenous strong promoters with codon optimization brings a synergistic effect on the efficiency of the expression of the reporter gene. These results indicate that employment of an endogenous strong promoter is another important factor necessary for high-level expression of the reporter gene in *P. yezoensis* cells (Fukuda et al., 2008).

It is noteworthy that significant expression of PyGUS under the direction of the *PyAct1* and *PyGAPDH* promoters was also observed in sporophytic cells in *P. yezoensis*, when the expression plasmid was introduced using particle bombardment (Fig. 3A). However, the PyGUS enzymatic activity was much lower than those in gametophytic cells (Fig. 3B). This difference was thought to be mainly due to the difference in DNA-transferring efficiency based on the difficulty of targeting the cell for gene transfer in sporophytes of thin filamentous form by particle bombardment.

Our transient gene expression system with the above methodological imorovement is represented schematically in Fig. 4.

2.4 Evaluation of gene transfer methods

Our initial development of transient expression of the *PyGUS* reporter gene in *P. yezoensis* cells depended on the use of particle bombardment, in which the gene transfer rate was usually 10^3 per μg of DNA. However, it is necessary to increase the efficiency of the foreign gene expression to 10^6 to 10^7 per μg of DNA, because the establishment of a genetic
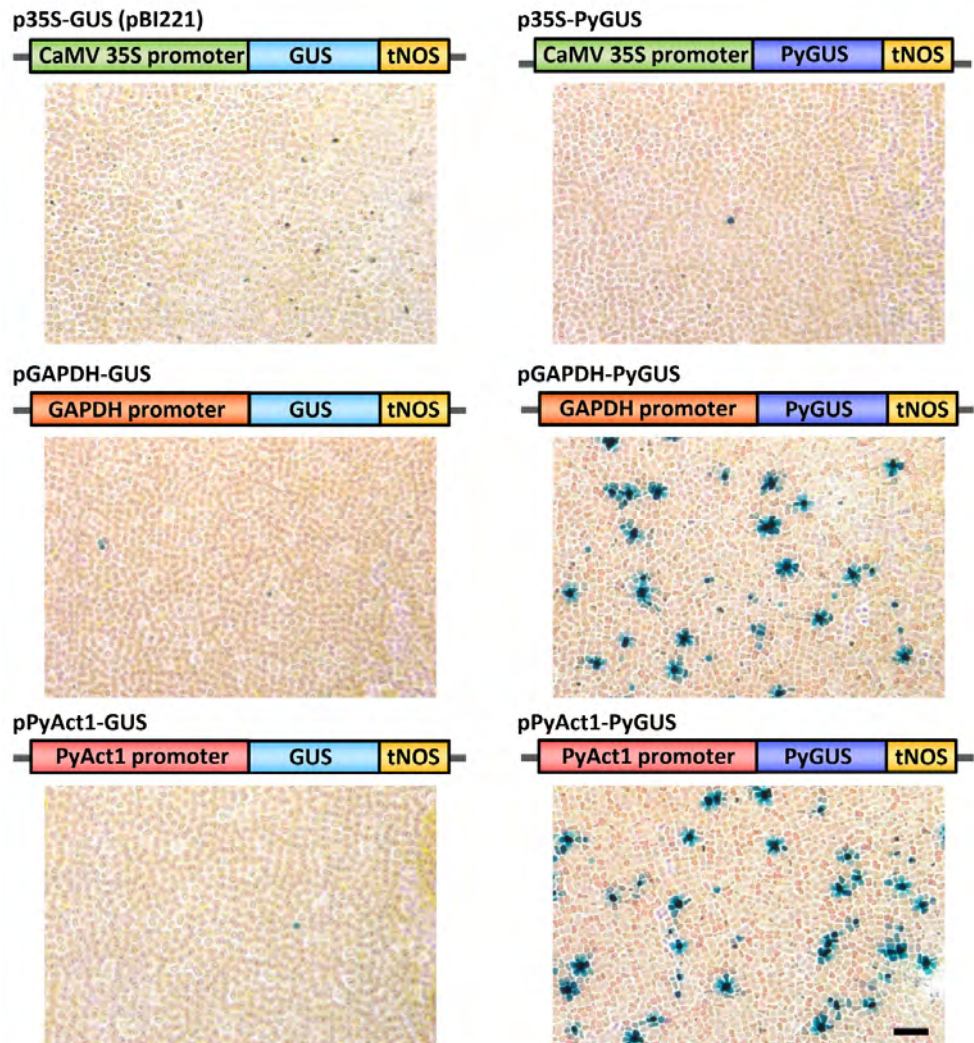
Fig. 1. Effcts of the strength of promoters and codon optimization of the reporter gene on the efficiency of the foreign gene expression in *P. yezoensis* gametophytic cells. The 835 bp *CaMV 35S* (green), 2.3 kb *PyGAPDH* (orange) or 3.0 kb *PyAct1* (red) promoter was fused to the coding region of native (sky blue) or codon-optimized PyGUS (blue), which resulted in the construction of p35S-GUS, p35S-PyGUS, pPyGAPDH-GUS, pPyGAPDH-PyGUS, pPyAct1-GUS and pPyAct1-PyGUS. The tNOS (yellow) indicates the *nos* gene terminator (253 bp). Gametophytic cells of *P. yezoensis* were transiently transformed with each expression plasmid by particle bombardment and then examined by histochemical staining 48 h after transient transformation. Scale bar corresponds to 100 µm.
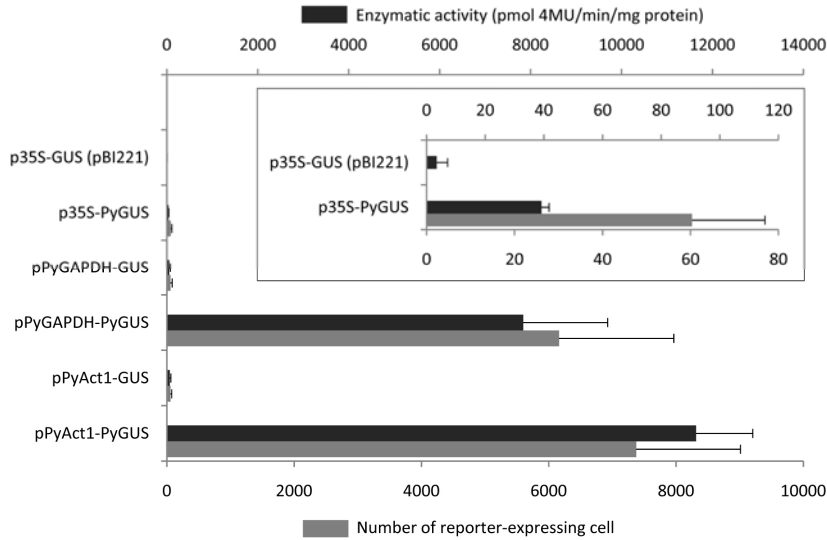
Fig. 2. Confirmation of the synergistic effects of the use of strong promoters and codon optimization on the efficiency of foreign gene expression in *P. yezoensis* gametophytic cells. The GUS or PyGUS enzymatic activity (dark gray) or the number of GUS- or PyGUS-expressing cells (light gray) in gametophytic cells of *P. yezoensis* were compared. The inner box shows a magnified figure of p35S-GUS (pBI221) and p35S-PyGUS.
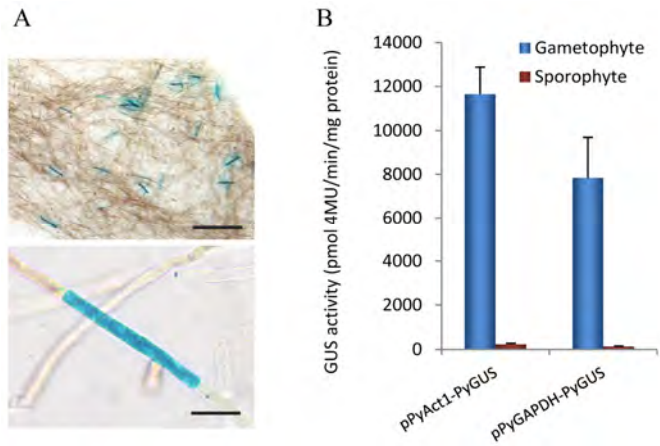

Fig. 3. Availability of PyGUS for transient gene expression in sporophytic cells of *P. yezoensis*. (A) Sporophytic cells expressing PyGUS by transient transformation with pPyAct1–PyGUS and stained with X-gluc solution after 48 h of bombardment. Upper and lower panels show low- and high-magnification images, respectively. Scale bar corresponds to 100 μm (upper) and 10 μm (lower). (B) Comparison of GUS activity between gametophytic and sporophytic cells of *P. yezoensis* when pPyAct1-PyGUS or pPyGAPDH-PyGUS was introduced.
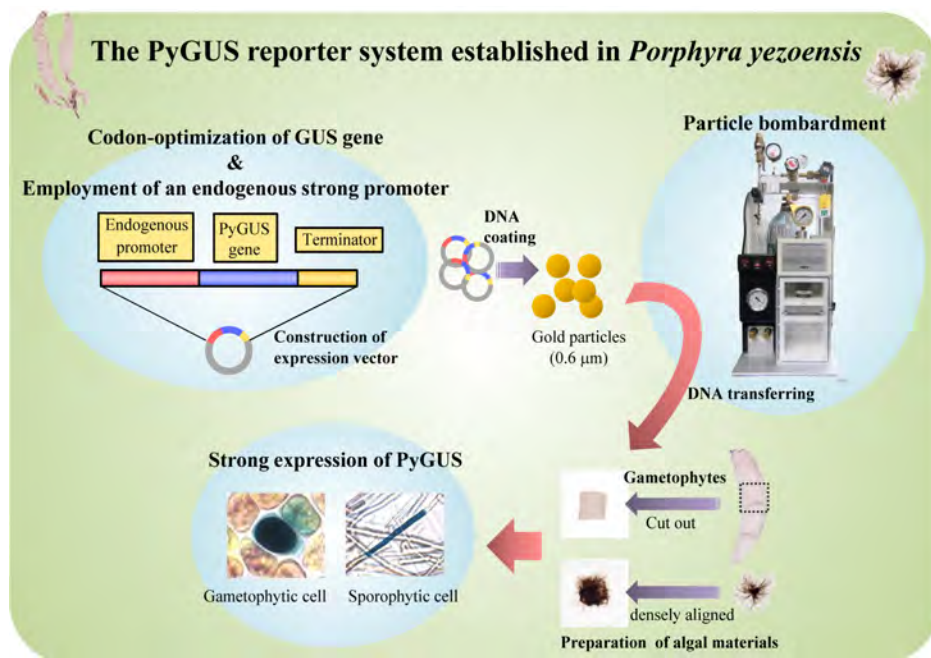
Fig. 4. Representation of the transient gene expression system established in *P. yezoensis*.

transformation system requires a large number of gene-transferred cells, a small portion of which are stably transformed by integration of the foreign gene into the genome. Thus, it is important to develop an efficient method of gene transfer into *P. yezoensis* cells toward the establishment of a stable transformation system via genetic recombination. However, our preliminary experiments with the glass beads method (Kindle, 1990; Feng et al., 2009), PEG method (Ohnuma et al., 2008) and magnetofection (Plank et al., 2003; Svingen et al., 2009) resulted in failure to introduce foreign genes into *P. yezoensis*. Thus, particle bombardment appears to be the only method available for transferring foreign genes into *P. yezoensis* cells at present, and further development of a gene transfer method to acheive very high efficient expression of the *PyGUS* reporter gene is required.

In fact, there have been several reports on DNA transfer into *Porphyra* cells using electroporation (Kübler et al., 1994) and *Agrobacterium*-mediated transformation (Cheney et al., 2001). Although the experiments in these reports had problems in the efficiency of the reporter gene expression based on the use of the *CaMV 35S* promoter and original *GUS* reporter gene, they are valuable as points of comparison for the increase in efficiency of gene transfer and expression by using, for example, the *PyAct1-PyGUS* or *PyGAPDH-PyGUS* reporter gene.

3. Application of the transient gene expression

3.1 Promoter analysis by PyGUS reporter system

Elucidation of the regulatory mechanisms of gene expression is crucial to understanding the molecular mechanisms governing plant stress responses, leading to enhancement of stress tolerance in plants through genetic transformation (Shinozaki & Yamaguchi-Shinozaki,

2007; Bhatnagar-Mathur et al., 2008). The transient gene expression system has been successfully applied to analyze promoters of stress-inducible genes to identify stress-responsible *cis*-regulatory elements in higher plants. For example, cold- and drought-responsible elements have been identified using the GUS reporter system in barley and *Arabidopsis thaliana* (Brown et al., 2001; Sakamoto et al., 2004). These successes are based on the quantitative correlation between promoter strength and reporter gene expression. Confirmation of this point in the transient gene expression system is indispensable for further analysis of stress-inducible genes in *P. yezoensis*.

To check the correlation between the promoter strength and expression level of the PyGUS reporter in *P. yezoensis* cells, mRNA expression levels of the actin 1 (*PyAct1*), GAPDH (*PyGAPDH*), Na$^+$-ATPase (*PyATP1A*) and transcription elongation factor 1 (*PyElf1*) genes were compared with PyGUS enzymatic activities derived from expression plasmids of the *PyGUS* gene under the direction of these promoters. The result indicated a strong correlation between the mRNA levels of these genes analyzed by RT-PCR and GUS enzymatic activities via expression of the PyGUS reporter gene directed by the corresponding promoter (Fig. 5), demonstrating the applicability of our transient gene expression system with the *PyGUS* gene to promoter analyses of *P. yezoensis* genes. Identification of *cis*-regulatory elements by promoter analysis is now underway for stress-inducible genes using the transient PyGUS reporter expression system.
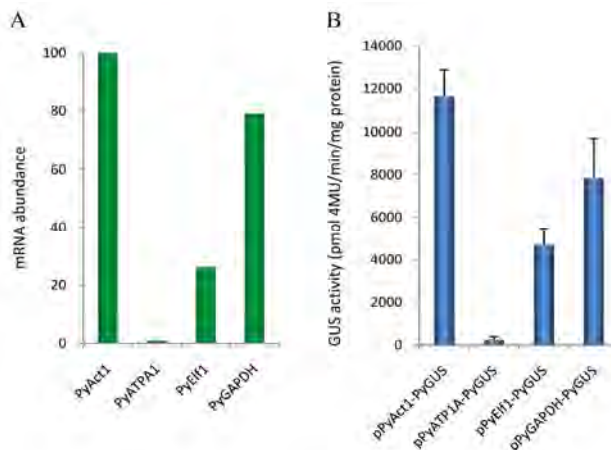

Fig. 5. Correlation of mRNA abundance and the PyGUS enzymatic activity in transient gene expression in *P. yezoensis* cells. (A) Comparison of mRNA abundance for *PyAct1*, *PyATP1A*, *PyElf1* and *PyGAPDH* genes in gametophytic cells. RNA samples were separately prepared from gametophytic cells transiently transformed by these genes and used for quantitative RT-PCR with gene-specific primer sets. The relative abundance of each mRNA is presented, with the abundance of the *PyAct1* mRNA set as 100. (B) Comparison of the GUS enzymatic activities under the direction of different endogenous promoters. Gametophytic cells were transiently transformed with expression plasmids of the *PyGUS* gene under the direction of the *PyAct1*, *PyATP1A, PyElf1* and *PyGAPDH* promoters by particle bombardment. The GUS enzymatic activities were then examined 48 h after transient transformation. Data shown are means ± SD from three independent experiments.

3.2 Development of fluorescent protein reporter system

Since histochemical and quantitative enzymatic analyses in the PyGUS reporter system require cell killing, monitoring of the reporter expression during development or response to environmental stress in living cells is impossible to analyze. However, living cell imaging to monitor gene expression is possible using fluorescent proteins without additional substrates and cofactors (Ehrhardt, 2003). Indeed, fluorescent proteins are usually employed as useful tools to visualize protein localization in living cells in land plants (Buschmann et al., 2010; Kokkirala et al., 2010; Meng et al., 2010; Pribat et al., 2010).

We first examined the usability of fluorescent proteins as reporters in *P. yezoensis* cells, since the expression of fluorescent protein has not yet been demonstrated in red algal cells. Table 1 lists fluorescent protein genes such as green fluorescent protein (GFP) and its variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) from various organisms including the jellyfish *Aequorea victoria*, reef coral *Zoanthus* sp. and copepoda *Pontellina plumata*, whose codon usage is biased to be GC-rich by humanization. When these genes were expressed in *P. yezoensis* cells under the direction of the *PyAct1* promoter, a fluorescent signal was clearly observed in every transiently transformed cell. Fig. 6A represents the expression of AmCyan and ZsGreen as examples. Interestingly, the gene encoding plant-adapted GFP(S65T) (Niwa et al., 1999) under the direction of the *PyAct1* promoter also produced the GFP signal (Fig. 6A). Since the GC contents of humanized fluorescent proteins and sGFP(S65T) are as high as 60% (Table 1), a GC content of 60% appears to be sufficient for efficient expression of foreign genes in *P. yezoensis* cells.

Applicability of the fluorescent protein system to visualize protein localization in *P. yezoensis* cells has already been demonstrated. For example, as shown in Fig. 6B, plasma membrane localization of Pleckstrin homology (PH) domains from human phospholipase Cδ1 was demonstrated using AmCyan (Mikami et al., 2009) and nuclear localization of *P. yezoensis* transcription factor PyElf1 was also confirmed using AmCyan and ZsGreen (Uji et al., 2009). Thus, these applications will provide new opportunities for analyzing subcellular localization of various genes from *P. yezoensis*.

Color	Name	Max (Ex[1])	Max (Em[2])	Structure	GC content (%)	Origin	Acquisition
Humanized fluorescent protein							
cyan	AmCyan	458	489	tetramer	63.3	*Anemonia majano*	Clontech
green	mWasabi	493	509	monomer	59.5	*Clavularia* sp.	Allele biotech
	TurboGFP	482	502	monomer	63.8	*Pontellina plumata*	Evrogen
	ZsGreen	493	505	tetramer	63.2	*Zoanthus* sp.	Clontech
yellow	PhiYFP	525	537	monomer	60.9	*Phialidium* sp.	Evrogen
	ZsYellow	529	539	tetramer	62.3	*Zoanthus* sp.	Clontech
Plant-adapted fluorescent protein							
green	sGFP(S65T)	490	510	monomer	61.4	*Aequorea victoria*	Niwa et al. 1999

[1] Excitation. [2] Emission.

Table 1. Properties of humanized and plant-adapted fluorescent proteins whose expression was confirmed in *P. yezoensis*.
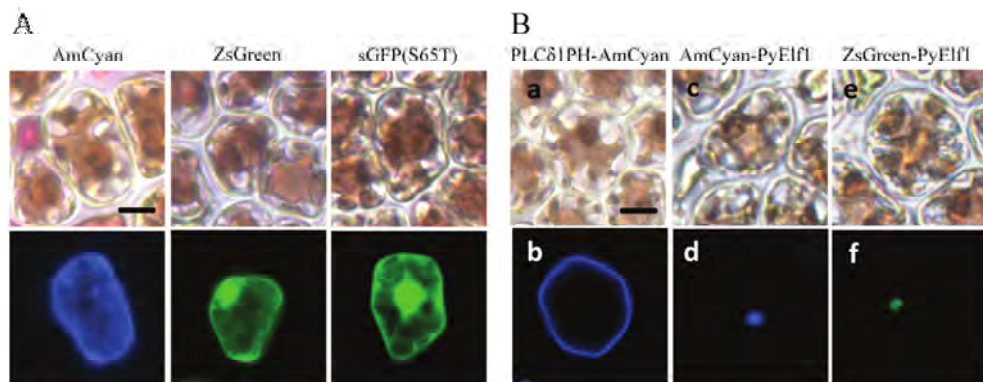
Fig. 6. Successful expression of fluorescent proteins in *P. yezoensis* gametophytic cells. (A) Expression of humanized fluorescent proteins and plant-adapted sGFP(S65T). Gametophytic cells were transiently transformed with expression plasmids containing the *AmCyan*, *ZsGreen* or *sGFP(S65T)* gene under the control of the *PyAct1* promoter by particle bombardment, 48 h after which cells were examined by fluorescent microscopy. Upper and lower panels show bright field and fluorescence images, respectively. The scale bar corresponds to 5 µm. (B) Visualization of subcellular localization with humanized fluorescent proteins in *P. yezoensis* cells. Expression plasmids pPyAct1-PLCδ1PH-AmCyan (a and b), pPyAct1-AmCyan-PyElf1 (c and d) and pPyAct1-ZsGreen-PyElf1 (e and f) were introduced into gametophytic cells of *P. yezoensis* by particle bombardment. Gametophytes were examined by fluorescent microscopy after 48 h of transient transformation. Plasma membrane localization of AmCyan fused with the PH domain from human phospholipase δ1 in gametophytic cells (a, bright field image; b, fluorescent image) and nuclear localization of AmCyan and ZsGreen fused with PyElf1 in gametophytic cells (c and e, bright field images; d and f, fluorescent images) are presented. The scale bar corresponds to 5 µm.

3.3 Application of PyGUS and sGFP(S65T) reporter systems in other red macroalgae

Since red macroalgae include a number of industrially important species such as *Porphyra* as the source of the food known as Nori, *Gracilaria* and *Gelidium* as sources of agar and *Chondrus* and *Kappaphycus* as sources of carrageenan, the establishment of a genetic manipulation system for these algae is eagerly anticipated. However, a transient gene expression system has not yet been developed in these red macroalgae other than *P. yezoensis*. In fact, EST analysis of *P. haitanensis* revealed that the GC content of the ORFs in this alga was as high as that in *P. yezoensis*, and the GC content at the third position of the triplets was significantly higher than those at the other two positions, which is consistent with the result reported in *P. yezoensis* (Fan et al., 2007). Similarly, analysis of the *GAPDH* gene from *Chondrus crispus* showed a high GC content (approximately 60%) in the cording region (Liaud et al., 1993). Thus, it is possible that the *PyGUS* gene could be commonly functional in red algae as a reporter of the gene expression. Indeed, applicability of the *PyGUS* gene in *P. tenera* has recently been confirmed by another laboratory (Son et al., 2011). The applicability of the *P. yezoensis* transient gene expression system was examined using the PyGUS and sGFP(S65T) reporter genes under the direction of the *PyAct1* promoter in the red macroalgae listed in Table 2. Good expression of PyGUS and sGFP(S65T) was observed

in Bangiophyceae including genus *Porphyra* and *Bangia*, whereas no expression of the reporter genes was seen in Florideophyceae (Table 2). Thus, it was concluded that the transient gene expression system developed in *P. yezoensis* is widely applicable in Bangiophycean red algae (Hirata et al., 2011).

The number of reporter-expressing cells varies among species (Table 2; Hirata et al., 2011), suggesting the possibility of the improvement of the gene transfer and expression systems in each species using respective endogenous strong promoters to drive the expression of the PyGUS and the sGFP(S65T) reporter genes. Similarly, expression of these reporter genes in Florideophycean algae seems to be possible if suitable endogenous promoters can be employed. Establishment of an efficient transient gene expression system will stimulate future development of stable transformation systems in various red macroalgae, enabling more effective utilization of them for industrial purposes.

Species	Number of reporter-expressing cells	
	PyGUS	sGFP(S65T)
Bangiophyceae		
Laboratory cultured strain		
Porphyra yezoensis strain TU-1[*1]	2535 $\pm$ 506	152 $\pm$ 32
Porphyra tenera strain JTW[*1]	2565 $\pm$ 600	140 $\pm$ 42
Naturally corrected species		
Porphyra yezoensis	721 $\pm$ 129	27 $\pm$ 8
Porphyra okamurae	290 $\pm$ 93	27 $\pm$ 9
Porphyra onoi	293 $\pm$ 97	20 $\pm$ 11
Porphyra variegata	333 $\pm$ 76	9 $\pm$ 3
Porphyra pseudolinearis (female)	455 $\pm$ 124	24 $\pm$ 6
Porphyra pseudolinearis (male)	9 $\pm$ 4	2 $\pm$ 1
Bnagia fuscopurpurea	118 $\pm$ 55	3 $\pm$ 1
Floredeophyceae		
Naturally corrected species		
Chondrus ocellatus	0	0
Gloiopeltis furcata	0	0
Gracilaria vermiculophylla	0	0
Mazzaella japonica	0	0

[*1] Laboratory-cultured strain

Table 2. Comparison of the number of reporter-expressing cells among red macroalgae after transfection of the pAct1-PyGUS or pAct1-sGFP(S65T) plasmid. The data shown are means ± S.D. from three independent experiments.

4. Conclusion

Genetic transformation is indispensable both to elucidation of gene function and to molecular breeding of crop plants. Our major goal is to establish a stable transformation system to produce *Porphyra* species carrying biotic and abiotic stress tolerances. For this purpose, our recent development of a transient gene expression system for Bangiophycean algae, by which the lack of transfer and expression systems for foreign genes was resolved, is the first breakthrough toward the establishment of a genetic transformation method in red macroalgae. We now know that the construction of an expression plasmid requires both optimization of codon usage in coding regions of the reporter gene and recruitment of endogenous strong promoters; also that particle bombardment is the proven method of gene transfer into red algal cells. The next steps are therefore development of targeted or random integration systems of foreign genes into the genome and selection of marker genes useful for isolation of transformed cells. Resolution of these methodological problems could contribute to accelerating progress in molecular biological research on physiological regulations that will be useful for the industrial production of transgenic red macroalgae.

5. Acknowledgements

6. References

Apt, K. E., Kroth-Pancic, P. G. & Grossman, A. R. (1996). Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Molecular and General Genetics*, Vol.252, No.5, pp. 572-579.

Asamizu, E., Nakajima, M., Kitade, Y., Saga, N., Nakamura, Y. & Tabata, S. (2003). Comparison of RNA expression profiles between the two generations of *Porphyra yezoensis* (Rhodophyta), based on expressed sequence tag frequency analysis. *Journal of Phycology*, Vol.39, No.5, pp. 923-930.

Bhatnagar-Mathur, P., Vadez, V. & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. *Plant Cell Reports*, Vol.27, No.3, pp. 411-424.

Blankenship, J. E. & Kindle, K. L. (1992). Expression of chimeric genes by the light-regulated cabII-1 promoter in *Chlamydomonas reinhardtii*: a cabII-1/nit 1 gene functions as a dominant selectable marker in a nit1-nit2-strain. *Molecular and Cell Biology*, Vol.12, No.11, pp. 5268-5279.

Blouin, N. A., Brodie, J. A., Grossman, A. C., Xu, P. & Brawley, S. H. (2011) *Porphyra*: a marine crop shaped by stress. *Trends in Plant Science*, Vol.16 , No.1 , pp. 29-37. .

Brown, A. P. C., Dunn, M. A., Goddard, N. J. & Hughes, M. A. (2001). Identification of a novel low-temperature-response element in the promoter of the barley (Hordeum vulgare L) gene *blt101.1. Planta* Vol.213, No.5, pp. 770-780.

Buschmann, H., Green, P., Sambade, A., Doonan, J. H. & Lloyd, C. W. (2010). Cytoskeletal dynamics in interphase, mitosis and cytokinesis analysed through *Agrobacterium*-mediated transient transformation of tobacco BY-2 cells. *New Phytologist*, doi: 10.1111/j.1469-8137.2010.03587.x.

Cheney, D., Metz, B. & Stiller, J. (2001). *Agrobacterium*-mediated genetic ransformation in the macroscopic marine red alga *Porphyra yezoensis*. *Journal of Phycology*, Vol.37 (Supplement S3), pp.11–12.

Chow, K. C. & Tung, W. L. (1999). Electrotransformation of *Chlorella vulgaris*. *Plant Cell Reports*, Vol.18, No.9, pp. 778-780.

Davies, J. P., Weeks, D. P. & Grossman, A. R. (1992). Expression of the arylsulfatase gene from the beta 2-tubulin promoter in *Chlamydomonas reinhardtii*. *Nucleic Acids Research*, Vol.20, No.12, pp. 2959-2965.

Day, A., Debuchy, R., Dillewijn, J., Purton, S. & Rochaix, J. D. (1990). Studies on the maintenance and expression of cloned DNA fragments in the nuclear genome of the green alga *Chlamydomonas reinhardtii*. *Physiologia Plantarum*, Vol.78, No .2, pp. 254-260.

Ehrhardt, D. (2003). GFP technology for live cell imaging. *Current Opinion in Plant Biology*, Vol.6, No.6, pp. 622-628.

El-Sheekh, M. M. (1999). Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. *Biologia Plantarum*, Vol.42, No.2, pp. 209-216.

Fan, X. L., Fang, Y. J., Hu, S. N. & Wang, G. C. (2007). Generation and analysis of 5318 expressed sequence tags from the filamentous sporophyte of *Porphyra haitanensis* (Rhodophyta). *Journal of Phycology,* Vol.43, No.6, pp. 1287–1294.

Feng, S. Y., Xue, L. X., Liu, H. T. & Lu, P. J. (2009). Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Molecular Biology Reports*, Vol.36, No.6, pp. 1433-1439.

Fuhrmann, M., Oertel, W. & Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant Journal*, Vol.19, No.3, pp. 353–361.

Fuhrmann, M., Hausherr, A., Ferbitz, L., Schödl, T., Heitzer, M. & Hegemann, P. (2004). Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. *Plant Molecular Biology*, Vol.55, No.6, pp. 869-881.

Fukuda, S., Mikami, K., Uji, T., Park, E. J., Ohba, T., Asada, K., Kitade, Y., Endo, H., Kato, I. & Saga, N. (2008). Factors influencing efficiency of transient gene expression in the red macrophyte *Porphyra yezoensis*. *Plant Science*, Vol.174, No.3, pp. 329-339.

Funabashi, H., Takatsu, M., Saito, M. & Matsuoka, H. (2010). Sox2 regulatory region 2 sequence works as a DNA nuclear targeting sequence enhancing the efficiency of an exogenous gene expression in ES cells. *Biochemical and Biophysical Research Communications*, Vol.400, No.4, pp. 554-558.

Gan, S. Y., Qin, S., Othman, R. Y., Yu, D. & Phang, S. M. (2003). Transient expression of *lacZ* in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). *Journal of Applied Phycology.* Vol.15, No.4, pp. 351–353.

Gong, Q., Yu, W., Dai, J., Liu, H., Xu, R., Guan, H. & Pan, K. (2005). Efficient *gusA* transient expression in *Porphyra yezoensis* protoplasts mediated by endogenous beta-tubulin flanking sequences. *Journal of Ocean University of China*, Vol.6, No.1, pp. 21-25.

Griesbeck, C., Kobl, I., & Heitzer, M. (2006). *Chlamydomonas reinhardtii*. A protein expression system for pharmaceutical and biotechnological proteins *Molecular Biotechnology*, Vol.34, No.2, pp. 213-223.

Hallmann, A. (2007). Algal Transgenics and Biotechnology. *Transgenic Plant Journal*, Vol.1, No.1, pp. 81-98.

Hirakawa, Y., Kofuji, R. & Ishida, K. (2008). Transient transformation of a chlorarachniophyte alga, *Lotharella amoebiformis* (chlorarachniophyceae), with *uidA* and *egfp* reporter genes. *Journal of Phycology*, Vol.44, No.3, pp. 814-820.

Hirata, R., Takahashi, M., Saga, N. & Mikami, K. (2011). Transient gene expression system established in *Porphyra yezoensis* is widely applicable in Bangiophycean algae. *Marine Biotechnology*, in press (doi: 10.1007/s10126-011-9367-6).

Huang, X., Weber, J. C., Hinson, T. K., Mathieson, A. C. & Minocha, S. C. (1996). Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L (Chlorophyta). *Botanica Marina*, Vol.39, No.5, pp. 467-474.

Ishihara, K., Oyamada, C., Matsushima, R., Murata, M. & Muraoka, T. (2005). Inhibitory effect of porphyran, prepared from dried "Nori", on contact hypersensitivity in mice. *Bioscience Biotechnology and Biochemistry*, Vol.69, No.10, pp. 1824-1830.

Jiang, P., Qin, S. & Tseng, C. K. (2003). Expression of the *lacZ* reporter gene in sporophytes of the seaweed *Laminaria japonica* (Phaeophyceae) by gametophyte-targeted transformation. *Plant Cell Reports*, Vol.21, No.12, pp. 1211-1216.

Kakinuma, M., Coury, D., Nakamoto, C., Sakaguchi, K. & Amano, H. (2008). Molecular analysis of physiological responses to changes in nitrogen in a marine macroalga, *Porphyra yezoensis* (Rhodophyta). *Cell Biology and Toxicology*, Vol.24, No.6, pp. 629-639.

Kang, H. G. & An, G. H. (2005). Morphological alterations by ectopic expression of the rice *OsMADS4* gene in tobacco plants. *Plant Cell Reports*, Vol.24, No.2, pp. 120-126.

Kindle, K. L., Schnell, R. A., Fernandez, E. & Lefebvre, P. A. (1989). Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *The Journal of Cell Biology*, Vol.109, No.6, pp. 2589–2601.

Kindle, K. L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.87, No.3, pp. 1228-1232.

Kokkirala, V. R., Yonggang, P., Abbagani, S., Zhu, Z. & Umate, P. (2010). Subcellular localization of proteins of *Oryza sativa* L. in the model tobacco and tomato plants. *Plant Signaling & Behavior*. Vol.5, No.11, pp. 1336-1341.

Kuang, M., Wang, S. J., Li,Y., Shen, D. L. & Zeng, C.K. (1998). Transient expression of exogenous GUS gene in *Porphyra yezoensis* (Rhodophyta). *Chinese Journal of Oceanology and Limnology*, Vol.16 (Supplement), pp. 56–61.

Kübler, J. E., Minocha, S. C. & Mathieson, A.C. (1994). Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyta). *Journal of Marine Biotechnology,* Vol.1, pp. 165–169.

Kurtzman, A. M. & Cheney, D. P. (1991). Direct gene transfer and transient expression in a marine red alga using the biolistic method. *Journal of Phycology*, Vol.27 (Suppliment S3), pp. 42

Liaud, M. F., Valentin, C., Brandt, U., Bouget, F. Y., Kloareg, B. & Cerff, R. (1993). The GAPDH gene system of the red alga *Chondrus crispus*: promoter structures, intron/exon organization, genomic complexity and differential expression of genes. *Plant Molecular Biology*, Vol.23, No.5, pp. 981–994.

Liu, H. Q., Yu, W. G., Dai, J. X., Gong, Q. H., Yang, K. F. & Zhang, Y. P. (2003). Increasing the transient expression of *GUS* gene in *Porphyra yezoensis* by 18S rDNA targeted homologous recombination. *Journal of Applied Phycology*, Vol.15, No.5, pp. 371-377.

Louis, J., Lorenc-Kukula, K., Singh, V., Reese, J., Jander, G. & Shah, J. (2010). Antibiosis against the green peach aphid requires the *Arabidopsis thaliana MYZUS PERSICAE-INDUCED LIPASE1* gene. *Plant Journal*, Vol.64, No.5, pp. 800-811.

Lumbreras, V., Stevens, D. R. & Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant Journal,* Vol.14, No.4, pp. 441-447.

Mayfield, S. P. & Kindle, K. L. (1990). Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.87, No.6, pp. 2087-2091.

Meng, Q. T., Du, J. Z., Li, J. J., Lu, X. M., Zeng, X. A., Yuan, M. & Mao, T. L. (2010). Tobacco microtubule-associated protein, MAP65-1c, bundles and stabilizes microtubules. *Plant Molecular Biology*, Vol.74, No.6, pp. 537-547.

Mikami, K., Uji, T., Li, L., Takahashi, M., Yasui, H. & Saga, N. (2009). Visualization of Phosphoinositides via the development of the transient expression system of a cyan fluorescent protein in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, Vol.11, No.5, pp. 563-569.

Nikaido, I., Asamizu, E., Nakajima, M., Nakamura, Y., Saga, N. & Tabata, S. (2000). Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga, *Porphyra yezoensis. DNA Research,* Vol.7, No.3, pp. 223-227.

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. & Kobayashi, H. (1999). Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant Journal*, Vol.18, No.4, pp. 455-463

Noda, H., Amano, H., Arashima, K. & Nisizawa, K. (1990). Antitumor activity of marine algae. *Hydrobiologia*, Vol.204-205, No.1, pp. 577-584.

Ohnuma, M., Yokoyama, T., Inouye, T., Sekine, Y. & Tanaka, K. (2008). Polyethylene glycol (PEG)-mediated transient gene expression in a red alga, *Cyanidioschyzon merolae* 10D. *Plant and Cell Physiology*, Vol.49, No.1, pp. 117-120.

Okauchi, M. & Mizukami, Y. (1999). Transient β-Glucuronidase (GUS) gene expression under control of *CaMV 35S* promoter in *Porphyra tenera* (Rhodophyta). *Bulletin of National Research Institute of Aquaculture*, Vol.1, pp. 13-18.

Park, C. S., Sakaguchi, K., Kakinuma, M. & Amano, H. (2000). Comparison of the morphological and physiological features of the red rot disease fungus *Pythium* sp isolated from *Porphyra yezoensis* from Korea and Japan. *Fisheries Science*, Vol.66, No.2, pp. 261-269.

Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J. & Rosenecker, J. (2003). The magnetofection method: Using magnetic force to enhance gene delivery. *Biological Chemistry*, Vol.384, No.5, pp. 737-747.

Pribat, A., Noiriel, A., Morse, A. M., Davis, J. M., Fouquet, R., Loizeau, K., Ravanel, S., Frank, W., Haas, R., Reski, R., Bedair, M., Sumner, L. W. & Hanson, A. D. (2010). Nonflowering plants possess a unique folate-dependent phenylalanine hydroxylase that is localized in chloroplasts. *Plant Cell*, Vol.22, No.10, pp. 3410-3422.

Ruecker, O., Zillner, K., Groebner-Ferreira, R. & Heitzer, M. (2008). Gaussia-luciferase as a sensitive reporter gene for monitoring promoter activity in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Molecular Genetics and Genomics*, Vol.280, No.2, pp. 153-162.

Sakamoto, H., Maruyama, K., Sakuma, Y., Meshi, T., Iwabuchi, M., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2004). Arabidopsis Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiology*, Vol.136, No.1, pp. 2734-2746.

Schiedlmeier, B., Schmitt, R., Muller, W., Kirk, M. M., Gruber, H., Mages, W. & Kirk, D. L. (1994). Nuclear transformation of *Volvox carteri*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.91, No.11, pp. 5080-5084.

Schroda, M., Blöcker, D. & Beck, C.F. (2000). The *HSP70A* promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. *Plant Journal*, Vol.21, No.2, pp. 121-131.

Shao, N. & Bock, R. 2008. (2008). A codon-optimized luciferase from *Gaussia princeps* facilitates the in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*. *Current Genetics*, Vol.53, No.6, pp. 381-388.

Shinozaki, K. & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, Vol.58, No.2, pp. 221-227.

Son, S. H., Ahn, J.-W., Uji, T., Choi, D.-W., Park, E.-J., Hwang, M. S., Liu, J. R., Choi, D., Mikami, K. & Jeong, W.-J. (2011) Development of an expression system using the heat shock protein 70 promoter in the red macroalga, *Porphyra tenera*. *Journal of Applied Phycology*, in press (doi: 10.1007/s10811-011-9652-9).

Specht, E., Miyake-Stoner, S. & Mayfesqaxeld, S. (2010). Micro-algae come of age as a platform for recombinant protein production. *Biotechnology Letters*, Vol.32 No.10, pp. 1373-1383.

Stevens, D. R., Rochaix, J. D. & Purton, S. (1996). The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Molecular & General Genetics*, Vol.251, No.1, pp. 23-30.

Sugawara, T., Sakai, S. & Hirata, T. (2011). Nutritional and physiological functions of *Porphyra*. In: Porphyra yezoensis: *Frontiers in Physiological and Molecular Biological Research*, K. Mikami, (Ed.), in press, Nova Science Publishers, New York, U.S.A.

Sun, M., Qian, K. X., Su, N., Chang, H. Y., Liu, J. X. & Chen, G. F. (2003). Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. *Biotechnology Letters*, Vol.25, No.13, pp. 1087-1092.

Svingen, T., Wilhelm, D., Combes, A. N., Hosking, B., Harley, V. R., Sinclair, A. H. & Koopman, P. (2009). Ex vivo magnetofection: A novel strategy for the study of gene function in mouse organogenesis. *Developmental Dynamics*, Vol.238, No.4, pp. 956-964.

Takahashi, M., Uji, T., Saga, N. & Mikami K (2010). Isolation and regeneration of transiently transformed protoplasts from gametophytic blades of the marine red alga *Porphyra yezoensis*. *Electronic Journal of Biotechnology*, Vol.13, No.2, http://www.ejbiotechnology.cl/content/vol13/issue2/full/7/index.html.

Tan, D., Qin, S., Zhang, Q., Jiang, P. & Zhao, F. (2005). Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *The Journal of Microbiology*, Vol.43, No.4, pp. 361-365.

Torney, F., Moeller, L., Scarpa, A. & Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from maize. *Current Opinion in Biotechnology*, Vol.18, No.3, pp. 193-199.

Uji, T., Takahashi, M., Saga, N. & Mikami, K. (2010). Visualization of nuclear localization of transcription factors with cyan and green fluorescent proteins in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, Vol.12, No.2, pp. 150-159.

Walker, T. L., Becker, D. K. & Collet, C. C. (2004). Characterisation of the Dunaliella tertiolecta RbcS genes snd their promoter activity in *Chlamydomonas reinhardtii*. *Plant Cell Reports*, Vol.23, No.10-11, pp. 727-735.

Walker, T. L., Collet, C. & Purton, S. (2005). Algal transgenics in the genomic ERA. *Journal of Phycology*, Vol.41, No.6, pp. 1077-1093.

Wally, O. & Punja, Z. K. (2010). Enhanced disease resistance in transgenic carrot (*Daucus carota* L.) plants over-expressing a rice cationic peroxidase. *Planta*, Vol.232, No.5, pp. 1229-1239.

Wu, S. X., Huang, R., Xu, L. L., Yan, G. Y. & Wang, Q. X. (2010). Improved hydrogen production with expression of *hemH* and *lba* genes in chloroplast of *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, Vol.146, No.3, pp. 120-125.

Zaslavskaia, L. A., Lippmeier, J. C., Kroth, P. G., Grossman, A. R. & Apt, K. E. (2000). Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. *Journal of Phycology*, Vol.36, No.2, pp. 379-386.

Zemke-White, W. L. & Ohno, M. (1999). World seaweed utilisation: An end-of-century summary. *Journal of Applied Phycology*, Vol.11, No.4, pp. 369-376.

Zorin, B., Lu, Y. H., Sizova, I. & Hegemann, P. (2009). Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the *PHOT* gene. *Gene*, Vol.432, No.1-2, pp. 91-96.