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Chapter 13

Current Advances in Seaweed Transformation

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1. Introduction

Frederick Griffith reported the discovery of transformation in 1928 [1]. Since a harmless strain of *Streptococcus pneumoniae* was altered to a virulent one by exposure to heat-killed virulent strains in mice, Griffice hypothesized that there was a transforming principle in the heat-killed strain. It took sixteen years to indentify the nature of the transforming principle as a DNA fragment released from virulent strains and integrated into the genome of a harmless strain [2]. Such an uptake and incorporation of DNA by bacteria was named transformation. Remarkably, an epoch-making technology in the form of artificial transformation protocol for the model bacterium *Escherichia coli* was established by Mandel and Higa in 1970 [3], which stimulated the development of artificial genetic transformation systems in yeasts, animals and plants. In plants, genetic transformation is a powerful tool for elucidating the functions and regulatory mechanisms of genes involved in various physiological events, and special attention has been paid to plant improvements affecting food security, human health, the environment and conservation of biodiversity. For instance, researchers have focused on the creation of organisms that efficiently produce biofuels and medically functional materials or carry stress tolerance in the face of uncertain environmental conditions [4-6].

Although the first success in the creation of transgenic mouse was carried out by injecting the rat growth hormone gene into a mouse embryo in 1982 [7], the protocol for artificial genetic transformation in plants was established earlier than that in animals. Following the discovery of the soil plant pathogen *Agrobacterium tumefaciens*, which is responsible for producing plant tumors, in 1907 [8], it was found that the tumor-inducing agent is the Ti plasmid containing T-DNA, a particular DNA segment containing tumor-producing genes that are transferred into the nuclear genome of infected cells [9]. By replacing tumor-producing genes by a gene of interest within the T-DNA region, infection of *A. tumefaciens* carrying a modified Ti plasmid results in insertion of a DNA fragment containing the desired genes into the genomes of plants by genetic recombination. Since the report of this protocol in the early 1980s [10,11], transfor-

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mation mediated by *A. tumefaciens* has become the most commonly used method to transmit DNA fragment into higher plants [12].

Since not all plant cells are susceptible to infection by *A. tumefaciens*, other methods were developed and are available in plants. Particle bombardment [13], which is also referred to as microprojectile bombardment, particle gun or biolistics, makes use of DNA-coated gold particles, which enables the transient and stable transformation of almost any type of cell, regardless of rigidity of the cell wall, and is thus extensively used for land plants. For protoplasts, electroporation is well employed, for which a high-voltage electrical pulse temporarily disturbs the phospholipid bilayer of the plasma membrane, allowing cells to take up plasmid DNAs [14,15]. In addition, the polyethylene glycol (PEG)-mediated transformation system is also thought to affect the plasma membrane and induce the uptake of DNAs into cells [15,16] and is almost exclusively applied with the moss *Physcomitrella patens* and liverwort *Marchantia polymorpha* [17,18]. Therefore, several kinds of genetic transformation methods are now available in land green plants.

Seaweeds are photosynthetic macroalgae, the majority of which live in the sea, and are usually divided into green, red and brown algae. Traditionally, all classes of seaweeds are known as human foods especially in Asian countries; for instance, red algae are known as Nori and brown algae are called Konbu and Wakame in Japan. In addition, red and brown algae are utilized as the sources of industrially and medically valuable compounds such as phycoerythrin, n-3 polyunsaturated fatty acids, porphyran, ager and carrageenan from red algae, and fucoxantine, fucoidan and alginate from brown algae [19-22]. Thus, to make new strains carrying advantageous characteristics benefiting industry and medicine, researchers have worked hard since the early 1990s to establish methods of genetic transformation in seaweeds [20,23,24]. However, the process is very difficult, and most of the early studies were reported in conference abstracts without the accompanying manuscript publication [25-28]. This situation has hampered us from gaining an understanding of gene functions in various physiological regulations and also a utilization of seaweeds in biotechnological applications.

Transformation can be divided into genetic (stable) and transient transformations under the control of the genes introduced into cells. In genetic transformation, genes introduced by genetic recombination are maintained in the genome through generations of cells, whereas in transient transformation, rapid loss of introduced foreign genes is usually observed. Establishing the genetic transformation system requires four basal techniques: an efficient gene transfer system, an efficient expression system for foreign genes, an integration and targeting system to deliver the foreign gene into the genome, and a selection system for transformed cells. It is notable that the transient transformation system is completed by the first two of the four required systems. In this respect, the development of an efficient and reproducible transient transformation system is the most critical step to establishing a genetic transformation system in seaweeds.

The current progress in establishing of both transient and genetic transformation systems in macroalgae is reviewed here. Although high-quality review articles for algal transformation have been published previously [20,23,24], I believe addressing the recent activity in seaweed transformation provides valuable information for seaweed molecular biologists and breeding scientists. Since considerable technical improvement was recently made in red seaweeds

[29,30], I focus here on the current progress in red algal transient transformation with summarizing pioneer and recent studies related to seaweed genetic transformation.

2. Transformation in red seaweeds

2.1. Pioneer studies for transient transformation

As far as I know, Donald P. Cheney is the pioneer in researching red algal transformation. He and his colleague performed transient transformation of the red alga *Kappaphycus alvarezii* using particle bombardment [25], which was the first report about the transient transformation of seaweeds (Table 1). In this case, the *Escherichia coli uidA* gene encoding β-glucuronidase (GUS) was expressed as a reporter under direction of the cauliflower mosaic virus (CaMV) 35S promoter (*CaMV 35S-GUS* gene). Since the GUS expression can be visualized as a blue color following treatment with X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) and also be quantified by fluorometric analysis [31,32], this reporter gene is widely used in land green plants having no background of the GUS activity [33,34]. In addition, the *CaMV 35S* promoter is heterologously used in land green plants as a strong constitutive and non-tissue-specific transcriptional regulator [35,36]. Therefore, it is a natural choice for the selection of the *CaMV35S-GUS* gene by pioneers for initial trials of seaweed transformation.

To date, studies have been mainly focused on *Porphyra* species because of their economical values. As shown in Table 1, expression of the *CaMV 35S-GUS* gene was previously observed in *P. miniata*, *P. tenera* and *P. yezoensis* [37-42], all of which were performed by electoroporation using protoplasts. Kuang et al. [38] also tested the particle bombardment of the *CaMV 35S-GUS* gene in *P. yezoensis* and got positive results. Moreover, the availability of mammalian-type simian virus 40 (*SV40*) promoter was reported to express the *E. coli lacZ* reporter gene, encoding β-galactosidase cleaving colorless substrate X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) to produce a blue insoluble product [43], in *P. haitanensis*, *Gracilaria chagii* and *K. alvarezii* by electroporation or particle bombardment [44,45].

2.2. Recent improvement of the transient transformation system in *Porphyra*

As noted above, pioneer experiments of red algal transient transformation were performed using plant viral *CaMV 35S RNA* and animal viral *SV40* promoters in combination with *GUS* and *lacZ* reporter genes (Table 1). 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 [46,47]. However, we have found that the TATA box is not usually found in the core promoters of *P. yezoensis* genes (unpublished observation), and we thus proposed that there were differences in the promoter structure and transcriptional regulation of protein-coding genes between red algae and dicot plants. Indeed, we recently observed quite low activity of the *CaMV 35S* promoter and the *GUS* reporter gene in *P. yezoensis* gametophytec cells [29,30,48]. These observations are completely opposite from the results in previous reports using the *CaMV 35S* promoter [25,37-41]. As a result, the transient transformation system in red seaweeds has recently been improved by resolving this problem.

Species	Status of expression	Gene transfer method	Promoter	Marker or Reporter	Ref.
Kappaphycus alvarezii	transient	particle bombardment	CaMV 35S	GUS	[25]
Porphyra miniata	transient	electroporation	CaMV 35S	GUS	[37]
Porphyra yezoensis	transient	Electroporation particle bombardment	CaMV 35S	GUS	[38]
Porphyra tenera	transient	electroporation	CaMV 35S	GUS	[39]
Porphyra yezoensis	transient	electroporation	rbcS	GUS	[40]
Porphyra yezoensis	transient	electroporation	CaMV 35S	GUS	[41]
Porphyra yezoensis	transient	electroporation	CaMV 35S β-tubulin	GUS	[42]
Gracilaria changii	transient	particle bombardment	SV40	lacZ	[44]
Porphyra haitanensis	transient		SV40	CAT	[128]
Porphyra yezoensis	transient	electriporation	SV40	CAT, GUS	[129]
Porphyra yezoensis	transient	electroporation	Rubisco	GUS, sGFP(S65T)	[130]
Porphyra yezoensis	transient	particle bombardment	CaMV 35S PyGAPDH	PyGUS	[48]
Porphyra yezoensis	transient	particle bombardment	PyAct1	PyGUS	[66]
Porphyra yezoensis	transient	particle bombardment	PyAct1	AmCFP	[70]
Porphyra yezoensis	transient	particle bombardment	PyAct1	AmCFP, ZsGFP, ZsYFP, sGFP(S65T)	[71]
Porphyra tenera Porphyra yezoensis	transient	particle bombardment	PtHSP70 PyGAPDH	PyGUS	[85]
Porphyra species* Bangia fuscopurpurea	transient	particle bombardment	PyAct1	PyGUS sGFP(S65T)	[86]
Porphyra species* Bangia fuscopurpurea	transient	particle bombardment	PtHSP70	PyGUS	[87]
Porphyra yezoensis	stable	*Agrobacterium*-mediated gene transfer	CaMV 35S	GUS	[26]
Porphyra leucostica	stable	ekectroporation	CaMV 35S	lacZ	[27]
Porphyra yezoensis	stable	*Agrobacterium*-mediated gene transfer	(unknown)	(unknown)	[28]
Kappaphycus alvarezii	stable	particle bombardment	SV40	lacZ	[45]
Porphyra haitanensis	stable	glass bead agitation	SV40	lacZ EGFP	[131]
Gracilaria changii	stable	particle bombardment	SV40	lacZ	[91]
Gracilaria gracilis	stable	particle bombardment	SV40	lacZ	[92]

Porphyra species used are *P. yezoensis, P. tenera, P. okamurae, P. onoi, P. variegate* and *P. pseudolinearis.*

Table 1. Transformation in red seaweeds.

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2.2.1. Optimization of codon usage in the reporter gene

Inefficient expression of foreign genes in the green alga *Chlamydomonas reinhardtii* is often due to the incompatibility of the codon usage in the gene's coding regions [49-51]. Expressed sequence tag (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 means the GC content reaches a high of 65.2% [52]. Since bacterial *GUS* and *lacZ* reporter genes have AT-rich codons, the incompatibility of codon usage, which generally inhibits the effective use of transfer RNA by rarely used codons in the host cells, thus decreasing the efficiency of the translation [53], might be responsible for the poor translation efficiency of foreign genes in *P. yezoensis* cells. It is therefore possible that modification of codon usage in the *GUS* gene would enable the efficient expression of this gene in *P. yezoensis* cells.

Accordingly, the codon usage of the *GUS* reporter gene was adjusted to that in the nuclear genes of *P. yezoensis* by introducing silent mutations [48], by which unfavorable or rare codons in the *GUS* reporter gene were exchanged for favorable ones without affecting amino acid sequences. The resultant artificially codon-optimized *GUS* gene was designated *PyGUS*, and its GC content was increased from 52.3% to 66.6% [48]. When the *PyGUS* gene directed by the *CaMV 35S* promoter was introduced into *P. yezoensis* gametophytic cells by particle bombardment, low but significant expression of the *PyGUS* gene was observed by histochemical detection and GUS activity test, indicating enhancement of the expression level of the *GUS* reporter gene [29,30,48]. Optimization of the codon usage of the reporter gene is therefore one of the important factors for successful expression in *P. yezoensis* cells [29,30,48].

2.2.2. Employment of endogenous strong promoters

The *CaMV 35S* promoter has very low activity in cells of green microalgae such as *Dunaliella salina* [54], *Chlorella kessleri* [55] and *Chlorella vulgaris* [56] and no activity in *C. reinhardtii* cells [57-59]. Thus, a low level of *PyGUS* expression under the direction of the *CaMV 35S* promoter is likely to be caused by the low activity of this promoter in *P. yezoensis* cells. A hint to overcoming this problem was that employment of strong endogenous promoters such as the *β-Tub*, *RbcS2* and *Hsp70* promoters results in the efficient expression of foreign genes in microalgae [60-65]. Therefore, it is likely that efficient expression of the *PyGUS* reporter gene in *P. yezoensis* cells is caused by the recruitment of endogenous strong promoters.

By comparison with steady-state expression levels by reverse transcription-polymerase chain reaction (PCR), we found two genes strongly expressed in *P. yezoensis*: genes encoding glyceraldehyde-3-phosphate dehydrogenase (PyGAPDH) and actin 1 (PyAct1) [29]. When the *PyGUS* gene fused with the 5′ upstream regions of these genes were introduced into gametophytic cells by particle bombardment, cells expressing the reporter gene and GUS enzymatic activity were dramatically increased [48,66]. These results indicate that employment of endogenous strong promoters is another important factor necessary for high-level expression of the reporter gene in *P. yezoensis* cells. In addition, the original *GUS* gene was not activated by *PyGAPDH* or *PyAct1* promoter [29,30,48], demonstrating that the *PyGUS* gene and endogenous strong promoter have a synergistic effect on the efficiency of the expression in *P. yezoensis* cells (Figure 1A). Therefore, the combination of endogenous strong promoters with

codon optimized reporter genes is critical for successful transient transformation in *Porphyra* species [29,30]. The established procedure of transient transformation is schematically represented in Figure 2.

2.2.3. Application of the transient transformation for using fluorescent proteins

The *GUS* reporter gene is usually used to monitor gene expression *in planta*; however, visualization of the reporter products requires cell killing. Reporters that function in living cells have also been established to date with fluorescent proteins used most commonly. The green fluorescent protein (GFP) has the advantage over other reporters for monitoring subcellular localization of proteins in living cells, because its fluorescence can be visualized without additional substrates or cofactors [67]. At present, there are GFP variants with non-overlapping emission spectra such as cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and red fluorescent protein, which allows multicolor imaging in cells [68,69].

Until recently, there was no report about the successful expression of fluorescent proteins in seaweeds. However, based on an efficient transient transformation system in *P. yezoensis*, fluorescent reporter systems have recently been established in *P. yezoensis* [29,30,70,71]. The humanized fluorescent protein genes, AmCFP, ZsGFP, and ZsYFP (Clontech) and the plant-adapted GFP(S65T) [72], the GC contents of which are as high as 63.7%, 62.8%, 61.9% and 61.4%, respectively, were strongly expressed in gametophytic cells under the direction of the *PyAct1* promoter using the particle bombardment method [71] (see Figure 1B).

The analysis of subcellular localization of cellular molecules was available using humanized and plant-adapted fluorescent reporters. The first successful attempt at achieving this process was to monitor the plasma membrane localization of phosphoinositides in *P. yezoensis* [70]. Phosphoinositides (PIs), whose inositol ring has hydroxyl groups at positions D3, D4 and D5 for phosphorylation, constitute a family of structurally related lipids, PtdIns-monophosphates [PtdIns3P, PtdIns4P and PtdIns5P], PtdIns-bisphosphates [PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2] and PtdIns-trisphosphate [PtdIns(3,4,5)P_3], all of which are detectable in plants except for PtdIns(3,4,5)P_3 [73,74]. Although the PIs are a minority among membrane phospholipids, they play important roles in regulating multiple processes of development and cell responses to environmental stimuli in land plants and green algae [74,75]. Recently, Li et al. [76,77] demonstrated that PIs are involved in the establishment of cell polarity in *P. yezoensis* monospores. The Pleckstrin homology (PH) domain, a PI-binding module, each part of which has individual substrate specificity, is usually used to monitor PIs *in vivo* by fusion with a fluorescent protein [78-80]. For instance, the PH domains from human phospholipase Cδ1 (PLCδ1) are employed for the detection of PtdIns(4,5)P_2 [81], whereas that from the v-akt murine thymoma viral oncogene homolog 1 (Akt1) has dual specificity in the detection of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [82]. Because of this substrate specificity, we were able to visualize PtdIns(3,4)P_2 and PtdIns(4,5)P_2 at the plasma membrane with humanized AmCFP and ZsGFP fused to the PH domains from PLCδ1 and Akt1 via the direction of the *PyAct1* promoter [70].

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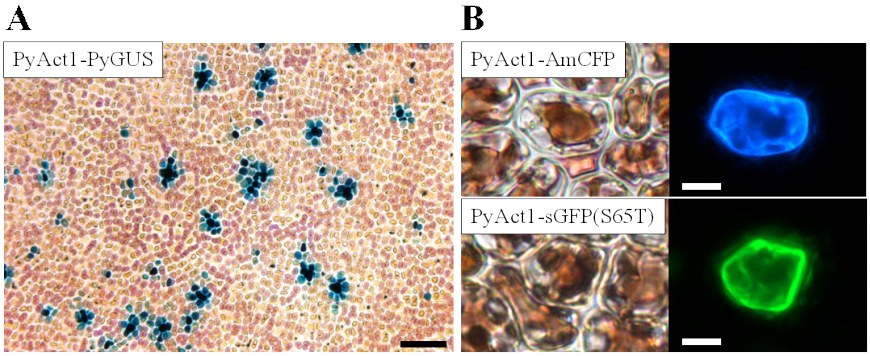

Figure 1. Efficient expression of PyGUS and fluorescent proteins by the transeint transformation with circular expression plasmids in *P. yezoensis* gametophytic cells. (A) Expression of the codon-optimized *PyGUS* reporter gene under the direction of the actin 1 (*PyAct1*) promoter. Blue histochemically stained cells are PyGUS expression cells. Scale bar corresponds to 100 µm. (B) Expression of humanized AmCFP and plant-adapted sGFP(S65T). Gametophytic cells transiently transformed with expression plasminds containng *AmCFP* or *sGFP(S65T)* gene under the control of the *PyAct1* promoter. Left and right panels show bright field and fluorescence images, respectively. Scale bar corresponds to 5 µm.

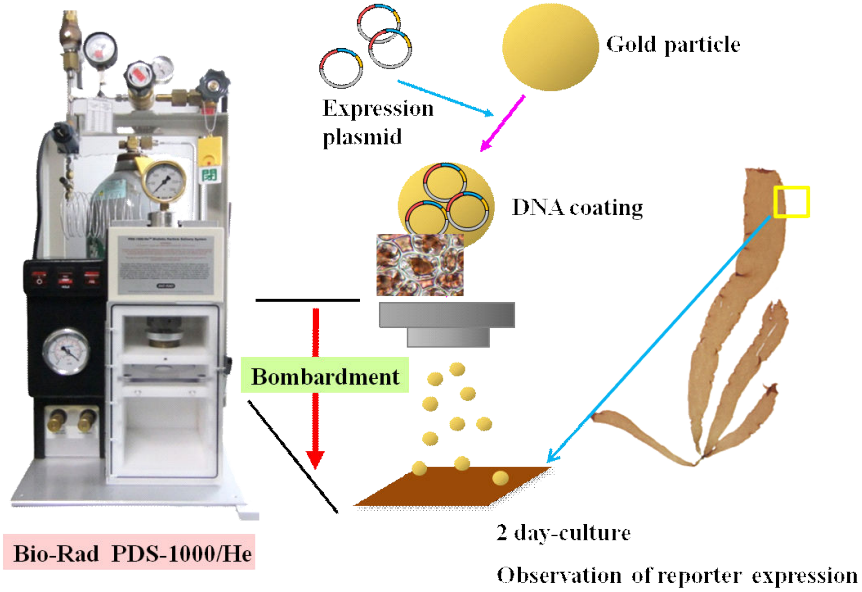

Figure 2. The established procedure of transeient transformation in *P. yezoensis.* A circular expression plasmid is bombarded into *P. yezoensis* gametophytic cells using the Bio-Rad PDS-1000/He after coating of gold particles with the plasmid. Expression of the reporter gene is observed after cultivation of the bombareded gametophyte under dark for two days; for *PyGUS* reporter gene, histochemical staining with X-gluc solution and fluorometric analysis of enzymatic activity are performed; for fluorescent reporter genes, bombarded sanples are examined with fluorescent microscopy.

Moreover, subcellular localization of transcription factors was also visualized in *P. yezoensis*. When complete open reading frames (ORFs) of transcription elongation factor 1 (PyElf1) and multiprotein bridging factor 1 (PyMBF1) from *P. yezoensis* were fused to AmCFP or ZsGFP, nuclear localization of these fusion proteins was observed in gametophytic cells, which was confirmed by overlapping of fluorescent signals with SYBR Gold staining of the nucleus [71]

With the successfull visualization of subcellular localization of cellular molecules, the transient transformation system developed in *P. yezoensis* appearst to be powerful tool to analyze functions of genes and cellular components [29,30].

2.2.4. Applicability of the P. yezoensis transient transformation system in other red seaweeds

As described above, both the adjustment of codon usage of the reporter gene according to algal preference and the employment of the strong endogenous promoters are important for providing highly efficient and reproducible expression of the reporter gene in *P. yezoensis*. In addition to Bangiophyceae like *Porphyra* species, Florideophyceae are also known, including a number of industrially important species such as *Gracilaria* and *Gelidium* as sources of agar and *Chondrus* and *Kappaphycus* as sources of carrageenan. Thus, the establishment of a genetic manipulation system for both Bangiophyceae and Florideophyceae other than *P. yezoensis* is awaited. 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 analysis of the *GAPDH* gene from a Florideophycean alga *Chondrus crispus* showed a high GC content (approximately 60%) in the coding region [83,84], which is consistent with the codon preference in *P. yezoensis*. Since efficient expression of the *GAPDH-PyGUS* gene has recently been confirmed in *P. tenera* [85], the applicability of the *P. yezoensis* transient gene expression system in other red seaweeds is expected. Indeed, using the *PyGUS* and *sGFP(S65T)* reporter genes under the direction of the *PyAct1* promoter, efficient expression of *PyGUS* and *sGFP(S65T)* genes was observed in Bangiophyceae including *P. tenera, P. okamurae, P. psedolinearis* and *Bangia fuscopurpurea*, although the expression efficiency varied among species [86]. Thus, the transient transformation system developed in *P. yezoensis* is widely applicable in Bangiophycean red algae [29,30,86].

No expression of the reporter genes was seen in Florideophyceae [29,30,86]. Since the availability of the *P. yezoensis* promoter is responsible for this deficiency in gene expression, it is important to employ the 5′ upstream region of the suitable endogenous gene from Florideophycean algae. Alternatively, it is possible that the efficiency of plasmid transfer by bombardment parameters is reduced by the cell wall and thus the size of the gold particles, target distance, acceleration pressure and/or amount of DNA per bombardment should be adjusted.

Taken together, *PyGUS* and *sGFP(S65T)* genes act synergistically with the *PyAct1* promoter as a heterologous promoter for transient transformation in Bangiophycean algae. Recently, the same synergistic effect was found in *P. tenera*; that is, Son et al. [85] clearly indicated that the heat shock protein 70 (*PtHSP70*) promoter from *P. tenera* can activate the *PyGUS* gene in gametophytic cells of this alga. Moreover, the *PtHSP70-PyGUS* gene was expressed in *P. yezoensis, P. okamurae, P. psedolinearis* and *B. fuscopurpurea* [85,87]. These findings are consistent

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with the importance of two critical factors for transient transformation in red seaweeds, adjustment of the codon usage in reporter genes and employment of a strong endogenous promoter.

The other important message gleaned from this experimental data is the efficient heterologous activation of *PyGAPDH* and *PtHSP70* promoters in *P. tenera* and *P. yezoensis*, respectively [85, 87]. For the genetic transformation, the target site for recombination is usually determined by the DNA sequence of genes desired for disruption or modification. Thus, it is better to exclude a possibility of homologous recombination at the DNA region corresponding to the promoter sequence used for expression of the reporter gene that is usually sandwiched between two different DNA sequences from the objective gene or its flanking regions. To avoid incorrect recombination at the promoter region, it is critical to employ heterologous promoters, whose sequence has low homology to the genome sequence of the host, to direct the expression of reporter genes. It is therefore possible that *PyGAPDH* and *PtHSP70* promoters are useful for genetic transformation in *P. tenera* and *P. yezoensis*, respectively. The number of promoters acting for heterologous reporter gene expression in red algae must be increased to develop a sophisticated system for red algal genetic transformation.

2.3. Towards genetic transformation in red seaweeds

The successful genetic transformation in red alga has been established only in unicellular algae [20,88]. The first report described chloroplast transformation in the unicellular red alga *Porphyridium* sp. through integration of the gene encoding AHAS(W492S) into the chloroplast genome by homologous recombination, resulting in sulfometuron methyl (SMM) resistance at a high frequency in SMM-resistant colonies [89]. The next report was of stable nuclear transformation in the unicellular red alga *Cyanidioschyzon merolae*, for which the uracil auxotrophic mutant lacking the *URA5.3* gene was used for the genetic background to isolate mutants with uracil prototrophic by employing the wild-type *URA5.3* gene fragment as a selection maker [90].

Table 1 shows preliminary experiments with red seaweeds. The first was by Cheney et al. [26], who introduced the *CaMV 35S-GUS* and *CaMV 35S-GFP* genes in *P. yezoensis* genome via an *Agrobacterium*-mediated transformation system. In addition, they transformed *P. yezoensis* with a bacterial nitroreductase gene via an *Agrobacterium*-mediated method [28] and *P. leucosticte* monospores with an unknown gene by electroporation [27]. However, these reports appeared on conference abstracts and thus details of experimental procedures are unknown. In related work, the genetic transformation of *Gracilaria* species was recently reported [91,92], in which integration of the *SV40-lacZ* gene was checked by PCR using genomic DNAs prepared from particle-bombarded seaweeds; however, selection of transformed cells was not performed. Taken together, these preliminary experiments are not enough to conclude the establishment of genetic transformation in red seaweeds, meaning that the genetic transformation system has not yet been fully established in red macroalgae.

As mentioned above, procedures of integration and targeting of foreign genes into the genome and selection of transformed cells must be developed for establishing the genetic transformation system, although other requirements such as an efficient gene transfer

system and an efficient expression system for foreign genes have been resolved by developing the transient transformation system in Bangiophyceae [29,30]. Regarding the unresolved points, knowledge about the selection of transformed cells is now accumulating. Selection marker genes are required to distinguish between transformed cells and non-transformed cells, since successful integration of a foreign gene into the host genome usually occur in only a small percentage of transfected cells. These genes confer new traits to any transformed target strain of a certain species, thus enabling the transformed cells to survive on medium containing the selective agent, where non-transformed cells die. Genes with resistance to the aminoglycoside antibiotics, which bind to ribosomal subunits and inhibit protein synthesis in bacteria, eukaryotic plastids and mitochondria [93], are generally used as selection markers. For example, the antibiotics hygromycin and geneticin (G418) are frequently used as selection agents with the hygromycin phosphotransferase (*hptII*) gene to inactivate hygromycin via an ATP-dependent phosphorylation [94] and the neomycin phosphotransferase II (*nptII*) gene to detoxify neomycin, G418 and paromomycin [93], respectively. In the green alga *Chlamydomonas reinhardtii,* the hygromycin phosphotransferase (*aph7″*) gene from *Streptomyces hygroscopicus* and the aminoglycoside phosphotransferase *aphVIII* (*aphH*) gene from *S. rimosus* had been reported as selectable marker genes for hygromycin and paromomycin, respectively, with similarity in the codon usage [95-97]. The *aphH* gene from *S. rimosus* is also applicable to the multicellular green alga *Volvox carteri* as a paromomycin-resistance gene [97,98]. In the diatom *Phaeodactylum tricornutum*, the expressed chloramphenicol acetyltransferase gene (*CAT*) detoxifies chloramphenicol [99], and the *nptII* gene confers resistance to the aminoglycoside antibiotic G418 [64]. Likewise, the *nptII* gene gives resistance to the antibiotic G418 in the diatoms *Navicula saprophila* and *Cyclotella cryptica* [100]. However, it is unknown what kinds of antibiotics-based selection marker genes are available for red seaweeds, since red algae usually have strong resistance to antibiotics.

Recently, the sensitivity of *P. yezoensis* gametophytes to ampicillin, kanamycin, hygromycin, geneticin (G418), chloramphenicol and paromomycin was investigated, and lethal effects of these antibiotics on gametophytes were observed at more than 2.0 mg mL^{-1} of hygromycin, chloramphenicol and paromomycin and 1.0 mg mL^{-1} of G418, whereas *P. yezoensis* gametophytes were highly resistant to ampicillin and kanamycin [101]. Although these concentrations are in fact very high in comparison with the cases for the red alga *Griffithsia japonica* and the green alga *C. reinhardtii* that were highly sensitive to 50 μg mL^{-1} and 1.0 μg mL^{-1} of hygromycin [96,102], these four antibiotics and corresponding resistance genes are suitable for the selection of genetically transformed cells from *P. yezoensis* gametophytes. According to these findings, it is necessary to confirm whether *P. yezoensis* gametophytes will obtain antibiotic tolerance by introducing plasmid constructs containing the antibiotic-resistance genes mentioned above. In this case, optimization of codon usage and the employment of strong endogenous promoter are expected for functional expression of the antibiotic resistance genes, according to the knowledge from the transient transformation system [29,30]. Such efforts could effectively contribute to the establishment of the genetic transformation system in red seaweeds in the near future.

3. Transformation in brown seaweeds

According to Qin et al. [103], trials of genetic engineering in brown seaweeds have been started by transient expression of the *GUS* reporter gene under direction of the *CaMV 35S* promoter by particle bombardment in *Laminaria japonica* and *Undaria pinnatifida*, which were first performed in 1994 by them. Descriptions of related experiments were published later [104,105]. Qin et al. then focused on the establishment of genetic transformation in brown seaweeds and provided successful reports of genetic transformation in *L. japonica* [103,106]. Genetic transformation was performed by particle bombardment only and expression of a reporter gene was driven by the *SV40* promoter that is usually used for gene expression in mammalian cells (Table 2). This promoter represented non-tissue and -cell specificity for expression of the *E. coli lacZ* reporter gene [105]. Promoters from maize ubiquitin, algal adenine-methyl transfer enzyme and diatom fucoxanthin chlorophyll a/c-binding protein (*FCP*) genes are also useful for transient expression of the *GUS* gene, and the *FCP* promoter is also employable for the genetic transformation [107]. Interestingly, there has been no successful genetic transformation using the *CaMV 35S* promoter, although this promoter is active in the transient transformation [103].

Despite the reports of successful genetic transformation, there was no experiment using antibiotics-based selection of transformants in brown seaweeds. Although the susceptibility of brown seaweeds to antibiotics has not been well studied, it was reported that *L. japonica* was sensitive to chloramphenicol and hygromycin, but not to ampicillin, streptomycin, kanamycin, neomycin or G418 [103,106]. Since hygromycin is more effective than chloramphenicol [103,106], it is necessary to confirm the utility of the *SV40-hptII* gene for the selection of transformants to fully establish the genetic transformation system in kelp.

Species	Status of expression	Gene transfer method	Promoter	Marker or Reporter	Ref.
Laminaria japonica	transient	particle bombardment	CaMV 35S	GUS	[103]
Laminaria japonica	stable	particle bombardment	SV40	GUS	[105]
Laminaria japonica	transient	particle bombardment	CaMV 35S, UBI, AMT	GUS	[107]
Laminaria japonica	stable	particle bombardment	FCP	GUS	[107]
Laminaria japonica	stable	particle bombardment	SV40	HBsAg	[113]
Laminaria japonica	stable	particle bombardment	SV40	Rt-PA	[114]
Laminaria japonica	stable	particle bombardment	SV40	bar	[114]
Undaria pinnatifida	transient	particle bombardment	CaMV 35S	GUS	[103]
Undaria pinnatifida	transient	particle bombardment	SV40	GUS	[104]

Table 2. Transformation in brown seaweeds.

To date, stably transformed microalgae have been employed to produce recombinant antibodies, vaccines or bio-hydrogen as well as to analyze the gene functions targeted for engineering [108-111]. Based on the success in genetic transformation, *L. japonica* is now proposed as a marine bioreactor in combination with the *SV40* promoter [112]. Indeed, the integration of human hepatitis B surface antigen (HBsAg) and recombinant human tissue-type plasminogen (*rt-PA*) genes into the *L. japonica* genome resulted in the efficient expression of these genes under the direction of the *SV40* promoter [113,114]. Therefore, *L. japonica* promises to be useful as the bioreactor for vaccine and other medical agents, although it is necessary to continually check the safety and value of its use by oral application.

There is no competitor against the Chinease group in the field of using brown algal genetic transformation at present [103,106,115], meaning there is currently no way to confirm the replicability of the experiments. It is necessary to re-examine the effective use of the non-plant *SV40* promoter and bacterial *lacZ* gene in brown algal genetic transformation, which is also important for the evaluation of genetic transformation in red seaweeds *Gracilaria* species, for which the *SV40-lacZ* gene was used such as transgene, as described above [91,92].

4. Transformation in green seaweeds

The first successfull genetic transformation in green algae was reported in the unicellular green alga *Chlamydomonas reinhardtii* for which the particle bombardment and glass-bead abrasion techniques were employed [116,117]. The availability of electoroporation was then confirmed in *C. reinhardtii* and *Chlorella saccharophila* [118,119]. These methods produce physical cellular damage, allowing DNA to be introduced into the cells. Moreover, particle bombardment was confirmed to be useful for a diverse range of species, including transient transformation in the unicellular *Haematococcus pluvialis* [120] and genetic transformation in the multicellular *Volvox carteri* and *Gonium pectoral* [97,120-122]. *Agrobacterium*-mediated transformation was also reported in *H. pluvialis* [123]. Thus, all methods employed in land green plants are applicable for green microalgae [88] (see Table 3).

In contrast, there is no report about genetic transformation in green seaweeds (Table 3). To date, only two examples of transient transformation have been reported in green seaweeds, *Ulva lactura* by electroporation and *U. pertusa* by particle bombardment [124,125]. As shown in Table 3, some of the experiments with micro- and macro-green algae used the promoter of the *CaMV 35S* gene and the coding region of the *E. coli GUS* gene. Although functionality of the *CaMV 35S* promoter and bacterial *GUS* coding region is the same in land green plants, the expression of the *GUS* reporter gene seems to be very low in the green seaweed *U. lactuca* [124]. In fact, codon-optimization is critical for the expression of reporters like the *GFP* gene and antibiotic-resistance genes in *C. reinhardtii* [47,90,115,126]. Moreover, the *HSP70A* promoter was employed to increase the expression level of the reporter genes [47,115]. Therefore, it is possible that changes in codon usage in the reporter gene and promoter region could result in increased reporter gene expression in transient transformation of green seaweeds. Recently, the Rubisco small subunit (*rbsS*) promoter was used for expression of the *EGFP* reporter gene

in transient transformation of *U. pertusa* by particle bombardment [125]; however, it is still unclear whether the *rbsS* promoters and the *EGFP* gene work well in cells in comparison with the *CaMV 35S* promoter and codon-optimized *EGFP* gene.

Species	Status of expression	Gene transfer method	Promoter	Marker or Reporter	Ref.
Microalga					
Chlamidominas reinhardtii	stable	particle bombardment			[116]
Chlamidominas reinhardtii	stable	glass bead agitation	Nitrate reductase	Nitrate reductase	[117]
Chlamidominas reinhardtii	stable	electroporation	CaMV 35S	CAT	[118]
Chlamidominas reinhardtii	stable	glass bead agitation	rbcS2	aphVIII	[95]
Chlamidominas reinhardtii	stable	glass bead agitation	β2-tubulin	Aph7″	[96]
Chlorella saccharophila	transient	electroporation	CaMV 35S	GUS	[119]
Haematococcus pluvialis	transient	particle bombardment	SV40	lacZ	[120]
Haematococcus pluvialis	stable	*Agrobacterium*-mediated gene transfer	CaMV 35S	GUS,GFP, hptII	[123]
Volvox Carteri	stable	particle bombardment	β2-tubulin	arylsulfatase	[121]
Volvox Carteri	stable	particle bombardment glass bead agitation	Hsp70A-rbcS2 fusion	aphVIII	[98]
Volvox Carteri	stable	particle bombardment	β-tubulin, Hsp70A	aphH	[97]
Gonium pectoral	stable	particle bombardment	VcHsp70A	aphVIII	[122]
Seaweed					
Ulva lactuca	transient	electroporation	CaMV 35S	GUS	[124]
Ulva pertusa	transient	particle bombardment	UprbcS	EGFP	[125]

Table 3. Transformation in green algae.

If the *rbsS-EGFP* gene is useful as a reporter gene for genetic transformation in green seaweeds, the remaining problems to be settled are methods for foreign gene integration into the genome and selection of transformed cells, which is the same as the situation with red seaweeds. Reddy et al. [24] commented on the antibiotic sensitivity of green seaweeds, indicating the considerable resistance of protoplast from *Ulva* and *Monostroma* to hygromycin and kanamycin.

Insensitivity to hygromycin is inconsistent with the case for red and brown seaweeds [101-103,106]. It is therefore necessary to check the sensitivity of green seaweed cells to other antibiotics to identify the genes employable for selection of transformed cells, which could stimulate the development of the genetic transformation system in green seaweeds.

5. Conclusion

It is nearly 20 years since the first transient transformation of a red seaweed with a circular expression plasmid [25], and many efforts have been made to develop a system for transient and stable expression of foreign genes in many kinds of seaweeds; however, a seaweed transformation system has still not been developed. The main problem is the employment of the *CaMV 35S-GUS* gene in the pioneer attempts at system development as shown in Tables 1, 2 and 3. This problem was recently resolved through the development of an efficient transient transformation system in *P. yezoensis* [29,30]. It is clear that the *CaMV 35S* promoter and the *GUS* gene are not active in seaweed cells [48], which is supported by knowledge from green microalgae [54-65]. These findings strongly indicate that defects in the transfer and expression of foreign genes were resolved by knowledge about two critical factors required for reproducibility and efficiency of transient gene expression, namely, the optimization of codon usage of coding regions and the employment of endogenous strong promoters [29,30]. However, these significant improvements are not enough to allow the establishment of a genetic transformation system in seaweeds.

At present, genetic transformation is reported in red and brown seaweeds using the *SV40* promoter (Tables 1 and 2) [91,92,103,105-107,113,114]; however, isolation of transgenic clone lines produced from distinct single transformed cells, which is the final goal of the genetic transformation of seaweeds as a tool, has not been reported, and seaweed genetic transformation is thus not fully developed. Therefore, the next step is to develop the gene targeting system via integration of a foreign gene into the genome and the system for selection of transformed cells. Since candidates of antibiotic agents for selection of transformed algal cells were mentioned recently [101-103,106], it is necessary to confirm the possibility of stable integration of a plasmid or a DNA fragment containing the selection maker gene into the seaweed genome. Once a positive result is obtained, it could lead us to establish the gene targeting method via the homologous recombination using an appropriate antibiotics resistance gene, if possible, with the heterologous promoter. To this end, we must reevaluate the availability of the methods for gene transfer such as electroporation and *Agrobacteriumu* infection.

Due to the problems with efficient genetic transformation systems, the molecular biological studies of seaweeds are currently progressing more slowly than are the studies of land green plants. Since a genetic transformation system would allow us to perform genetic analysis of gene function via inactivation and knock-down of gene expression by RNAi and antisense RNA supression, its establishment will enhance both our biological understanding and genetical engineering for the sustainable production of seaweeds and also for the use of seaweeds as bioreactors.

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