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Transient expression of foreign genes in electroporated *Porphyra yezoensis* protoplasts

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Abstract The introduction and transient gene expression of foreign genes in protoplasts of *Porphyra yezoensis* was examined. Expression vectors were constructed ligating the ribulose-bisphosphate-carboxylase / oxygenase (Rubisco) gene promoter at the 5'-flanking site of the ß-glucuronidase (GUS) gene of vector pBI 221. When these vectors were electroporated into protoplasts, blue cells stained by indigotin were observed after the histochemical GUS assay. The GUS activity was also detected by quantitative assays with a chemiluminescent substrate. The effective expressions of the GUS gene were obtained under the electroporation conditions of 200 to 300 V field strength, 47 ms pulse length and 0.03 μg/ml vector DNA concentration. Relatively high expression rates of introduced genes were observed at 3 to 5 days after the electroporations. When the firefly luciferase (LUC) gene was used as the reported gene instead of the GUS gene, LUC activity was also detected reproducibly although the activity levels were considerably lower. These results show some of the technical conditions for the gene introduction and transient expression of foreign genes in *P. yezoensis*.

Key words: *Porphyra yezoensis*, transient expression, GUS gene, LUC gene

Introduction

The red marine macroalga, *Porphyra yezoensis*, is an economically important seaweed and is extensively cultured in eastern Asia, mainly in Japan. This alga is called "nori" and widely used as a food material in Japan. Therefore, a great deal of effort has been put into the improvement of cultured stocks and the culture techniques for this alga. Few studies have reported on foreign gene introduction and expression in red macroalgae (Kübler *et al.*, 1994; Okauchi and Mizukami, 1999). The difficulties of such studies on red macroalgae are thought to arise from the lack of effective promoter sequences for foreign gene expression and appropriate methods for the introduction of foreign genes into the nuclei of algal cells. The CaMV35S (35S) promoter sequence is known to show high transient gene expression of foreign genes and have a wide host range in land plant cells (Odell *et al.*, 1985; Jefferson *et al.*, 1987). The 35S promoter has also been used in the foreign gene expression of unicellular green algae (Chlorophyta) (Jarvis and Brown, 1991; Hawkins and Nakamura, 1999; Kim *et al.*, 2002). However, there are few reports of the use of the 35S promoter in the transient gene expression in brown (Phaeophyta) and red (Rhodophyta) macroalgae cells. We previously examined the effect of the 35S promoter on gene introduction and expression in *P. yezoensis* cells (Okauchi and Mizukami, 1999). In that study, we used the vector pUC19-El_2-CaMV35S -Ω, which contained the enhancer and promoter sequences of 35S RNA (Kosugi *et al.*, 1991), ß-glucuronidase (GUS) gene and Nos terminator (Jefferson *et al.*, 1986), and could detect GUS gene expression. However, these gene expressions were difficult to carry out and had a low reproducibility. In addition, the widely used plant vector pBI 221, which contains the 35S promoter and GUS reporter gene (Jefferson *et al.*, 1987) did not show any signal of gene ex-

pression at all in *Porphyra* protoplasts.

Previously unpublished data showed that *Porphyra* was not susceptible to *Agrobacterium* infection. Therefore, electroporation was considered as one possible method for gene transfer into *Porphyra* protoplasts. However, a cell of *Porphyra* contains very large chloroplasts and a relatively small nucleus, and is largely occupied by the large lamella structures of the chloroplast (Gibbs, 1962). We doubted whether foreign genes introduced into the cell of *Porphyra* by electroporation could reach the nucleus and transcribe mRNAs in it. Several workers have reported that the foreign genes could be expressed in chloroplasts of a green alga (Blowers *et al.*, 1990) and land plants (Svab and Maliga, 1993). These studies have shown that foreign genes can be expressed in chloroplasts of algal cells even if they can not reach the nuclei.

In the present study, we constructed vectors using the promoter sequence of the ribulose-1.5-bisphosphate-carboxylase / oxygenase (Rubisco) gene of *Porphyra yezoensis* chloroplast and the GUS and luciferase (LUC) genes (Ow *et al.*, 1986; Schneider *et al.*, 1990; Millar *et al.*, 1992) as reporter genes. These vectors were introduced into protoplasts by electroporation and the expressions of the reporter genes were examined.

Materials and methods

Alga

Protoplasts were prepared from the thalli of the *Porphyra yezoensis* cultivar "Shin-Saga", which was supplied by the Saga prefectural Ariake Research and Development Center. This alga was cultured for about a month at seafarms in Ariake Bay, Japan and sun-dried for about 3 h just after harvest and stored at −20℃.

Protoplast isolation and culture

The methods for isolation of protoplasts from *P. yezoensis* have been reported previously (Mizukami *et al.*, 1992). In brief, the number of isolated protoplasts was counted and the protoplast concentration was adjusted to about 5×10^6 ml^{-1} with 1 mM HEPES (pH 7.0), 0.8 M mannitol. Protoplasts were cultured after electroporation in a 0.8% agarose SWM-III or liquid SWM-III medium containing streptomycin and penicillin G (each 50 μg ml^{-1}) in a 12 or 24 well plate at 15℃ under light : dark conditions of 12 h : 12 h before being collected for enzyme assays.

Electroporation

Protoplast suspensions were kept on ice for 10 min after being added to various concentrations of plasmid DNA. Sonicated salmon DNA was used instead of the plasmid DNA as a control. The 0.25 to 0.5 ml of these protoplast suspensions were placed in 1 ml disposable electroporation chambers with a 0.4 cm space between electrodes and subjected to electroporation at various capacitances, field strengths and pulse durations at room temperature with a BRL CELL-PORATOR Electroporation System (BRL Life Technologies Inc., MD)

Plasmids

The plasmid pBI 221 was purchased from Clonetech Laboratories Inc. (Palo Alto., CA), which contained the GUS gene with CaMV 35S promoter and Nos poly A site from the *Agrobacterium tumefaciens* Ti plasmid (Jefferson *et al.*, 1987). The plasmid pYez-Rub (4, 5 and 6)-GUS were constructed from the plasmid pBI 221 by the replacement of the 35S promoter sequence with approximately 350 bp DNA fragments which contained 5'-flanking sequence of the Rubisco gene coding region in *P. yezoensis*. These DNA fragments were generated from the total DNA of *P. yezoensis* by PCR with primers 5'-GGCATCTGTAACTTGAGATACTGA-3', 5'-GAGACATGTATTCCCTCCTTGA-3', 5'-TGAGACATGTATTCCCTCCTTG-3' and 5'-TTGAGACATGTATTCCCTCCTTG-3' designed referring to the sequence data of *Porphyra purpurea* chloroplast DNA (Reith and Munholland, 1995). The plasmids pYez-Rub (4, 5 and 6)-LUC were constructed from the plasmid pGL3 (Promega Co., WI) by the insertion of the same approximately 350 bp DNA fragments as those described above at the multi-cloning site flanking with the LUC gene in the plasmid pGL3.

Histochemical GUS assay and quantification of GUS expression

Histochemical GUS assay was performed according to the method of Jefferson (1987) with slight modifications. The substrate of GUS, 5-bromo-4-chloro-3 indolyl-ß-D-glucuronide (X-

Gluc) was dissolved in DMSO and added to the culture medium of protoplasts to give a final concentration of 0.5 mg/ml at one or two days of culture. The incubation of protoplasts was continued for more than 2 days after the addition of the substrate. When the GUS assay was performed using the agarose culture of protoplasts, warm (35℃) low temperature melting agarose solution and X-Gluc were added to the liquid culture medium at two or three days after the initiation of protoplast culture to give final concentrations of 0.8% agarose and 0.5 mg/ml X-Gluc. After being left to stand for 30 min, the agarose plates were incubated for more than 2 days at 15℃ until quantification of the expression cells. Quantification of the GUS stained cells was done under an inverted microscope and only dark blue protoplasts were counted as GUS expression cells. Frequencies of transient GUS expression were calculated on a percentage basis of the total number of protoplasts. For the chemiluminescent detection of GUS activity, the Aurora TM GUS chemiluminescent reporter gene assay system (ICN Biomedicals Inc., CA) was used. The cell extraction and GUS reaction was carried out according to the methods given in the manuals of the manufacturer of this system. The chemiluminescent signal intensity was counted with a Turner Designs Luninometer TD-20 / 20 (Promega Co., WI). Protein concentration in the cell extract was measured with the Bradford method using a Protein assay kit (Bio-Rad). All experiments were repeated at least twice.

Luciferase assay

Approximately 1×10^7 protoplasts were electroporated in the presence of vectors pYez-Rub (4, 5 and 6)-LUC and cultured in liquid medium for 4 days at 15℃. After the culture, protoplasts were assayed for luciferase activity using the Luciferase assay reagent (Promega). The cell extracts of these protoplasts were prepared in the same manner as described above. 20 μl of cell extract was used for the assay of luciferase activity according to the manufacturer's protocols. Chemiluminescent intensity was measured by with the Turner Designs Luminometer.

Survival rate of protoplasts

Protoplasts were cultured in two-layer agarose plates (Mizukami *et al.*, 1992) after being electporated under various electrical conditions and plasmid DNA concentrations. The number of live cells was counted on the initial and on 4 or 5 days after the initiation of culture. Survival rates were calculated as the rates of live cells detected after 4 or 5 days of culture to those detected on the initial day of culture. Live cells could be distinguished from dead cells in agarose plates by the color of cells; the former were brown (red) and the latter were green or had no pigment.

Results

Rubisco promoter and the GUS gene activity

The nucleotide sequences of the Rubisco gene promoter region of *P. yezoensis* were used as promoters of *P. yezoensis* expression vectors in the present study. Figure 1 (a) shows nucleotide sequences of the promoter region of the Rubisco gene in *P. yezoensis*. The putative ribosomal binding site (rbs) is detected at -12 to -9, and putative -35 and -10 promoter sequences are seen at -46 to -40 and -20 to -15 positions respectively in the nucleotide sequences shown in Fig. 1. On the base of nucleotide sequences of the promoter region, three kinds of DNA fragments were generated by PCR using the PCR primers indicated as underlined sequences in Fig. 1 (a). These DNA fragments were different by one or two bases in length from each other and used as promoters. Three kinds of vectors, pYez-Rub4-GUS, pYez-Rub5-GUS and pYez-Rub6-GUS, were constructed from the higher plant vector pBI 221, which contains the CaMV-35S promoter, GUS gene and Nos terminator (Jefferson *et al.*, 1987), by the replacement of the CaMV-35S promoter with each promoter fragment described above. These vectors were electroporated into protoplasts and the expression of the GUS gene was examined. Figure 1 (b) shows the results of the histochemical assays of the GUS gene expression after electroporation of these vectors. It was observed that GUS genes were significantly expressed in protoplasts when the vectors pYez-Rub4-GUS and pYez-Rub5-GUS were used for the electroporation and the former had the highest expression rate compared to the other vectors. Therefore, vector pYez-Rub4-GUS was used in the further studies on the foreign gene expression.

In the case of higher land plants, GUS activi-

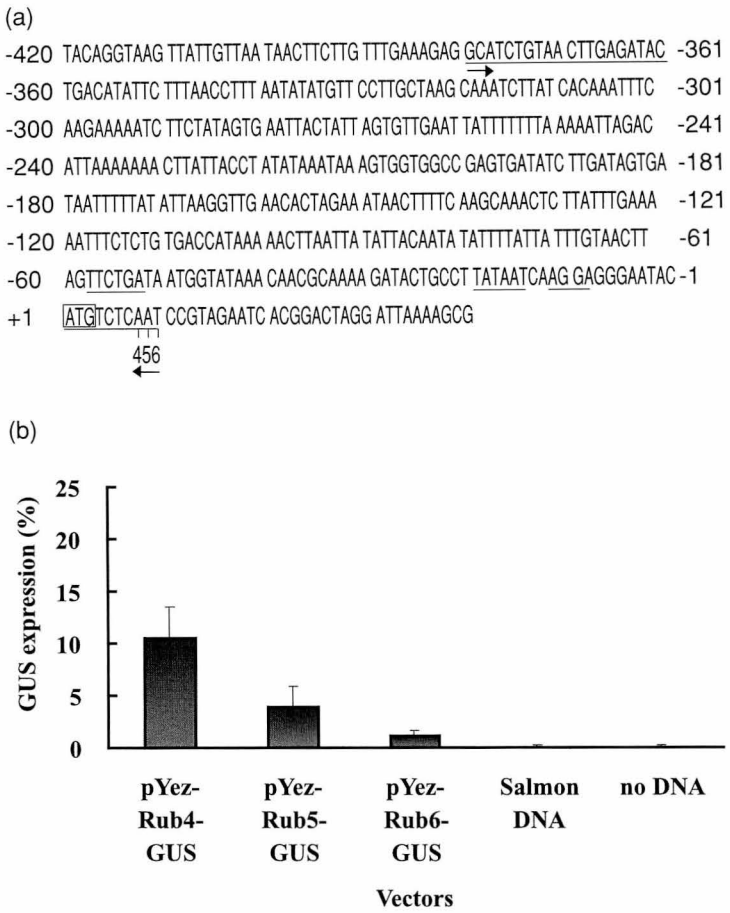

Figure 1. Nucleotide sequence of the promoter region of the Rubisco gene and comparison of GUS gene expression among vectors. (a) : Nucleotide sequence of the promoter region. The framed ATG shows the initiation codon of the Rubisco coding region. The underlined AGGA, TATAAT and TTCTGA indicate the putative rbs, -10 and -35 promoter sequences, respectively. The underlined sequences with arrows show the nucleotide sequences used for the construction of promoter DNA fragments as PCR primers. The figures 4, 5 and 6 indicate nucleotide sequences used as primers for the construction of promoters Rub4, Rub5 and Rub6 which were different by one or two bases in length from each other and used as promoters of the expression vectors shown in (b). +1 indicates the first nucleotide of the Rubisco coding sequence. (b) : Comparison of the GUS gene expression rates. Three vectors, pYez-Rub4-GUS, pYez-Rub5-GUS and pYez-Rub6-GUS had promoter sequences Rub4, Rub5 and Rub6, respectively (shown in (a)), and GUS genes as reporter genes. The salmon DNA was also introduced as a control. The vertical bars indicate the standard deviations.

ties have been assayed histochemically at 37℃ (Jefferson, 1987). However, in the present study with red algal protoplasts, only weak GUS expression signals could be detected at 37℃. Instead, clear signals of gene expression could be observed when protoplasts electroporated with the GUS gene were assayed histochemically at 15℃ for more than 2 days. Figure 2 (a) and (b) shows protoplasts electropulsed in the presence of the vector pYez-Rub4-GUS or salmon DNA, respectively, and then assayed histochemically for GUS activity at 15℃. Blue cells are seen in Fig. 2 (a) but not in (b). It has been shown that the GUS gene expression results in the production of indigotin which is blue, insoluble in water and accumulates in cells in the presence of X-Gluc as a substrate (Lojda, 1971). The results shown in Fig. 2, therefore, suggests the GUS gene expression in the electroporated protoplasts.

The GUS gene activity was also assayed by the enzyme reaction of cell extracts in the

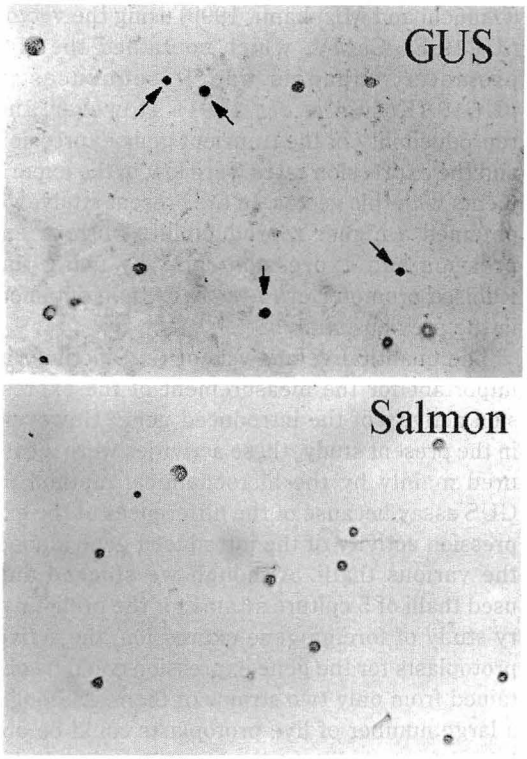

Figure 2. Photographs of GUS expression cells and control cells. Protoplasts were electroporated in the presence of vector pYez-Rub4-GUS (GUS) or salmon DNA (Salmon) and cultured in the liquid medium consisting of sea water. After 1 day of the culture, X-Gluc and liquid agarose were added to the culture medium to be 0.5 mg/ml and 0.8%, respectively, and culture was further continued for 3 days. The arrows indicate GUS expression cells. Scale bar = 50 μm.

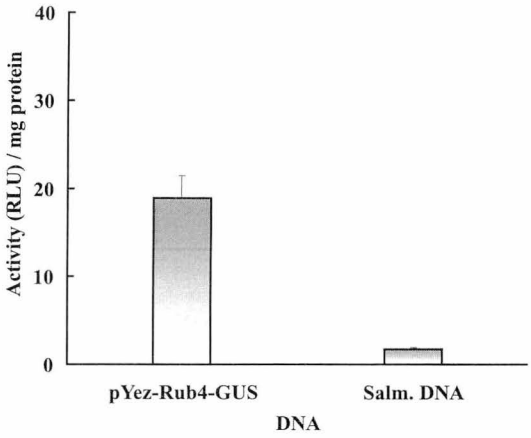

Figure 3. GUS activity of protoplasts introduced by the pYez-Rub4-GUS. The protoplasts were introduced by the pYez-Rub4-GUS and the salmon DNA(as a Control). The GUS activities were assayed by the enzyme reaction using fluoresein GUS substrates and measured with a luminometer.

presence of a chemiluminescent GUS substrate. Figure 3 shows the results of the same experiment with those shown in Fig. 2 but the GUS activity was assayed by the enzyme reaction. Significant GUS activity was detected only when the vector pYez-Rub4-GUS was used for the electroporation. This result confirmed that the GUS gene was expressed in these protoplasts.

Effects of pulse voltage, pulse length and vector DNA concentration

Figure 4 shows the effects of pulse voltage, pulse length and vector DNA concentration of the electroporation on the expression rates of the GUS gene. Relatively high expression rates were obtained under the conditions of 200 and 300V field strength, 47 ms pulse length and 0.03 μg/ml DNA concentration. The survival rates of protoplasts were hardly influenced by these electrical conditions and the DNA concentration, suggesting that the differences in the expression rates appeared in Fig. 4 were not caused by the differences of survival rates of the electroporated protoplasts.

Figure 5 shows that GUS gene expression was observed from 2 days after the introduction of the gene. The number of GUS expression cells increased gradually until Day 4 of culture and reached a constant level on Day 5 of culture. None of the indigotin-blue cells were detected with the histochemical GUS analysis of cell cultured for 10 and 20 days after the GUS gene introduction.

Luc gene activity

Three DNA fragments containing the Rubisco promoter sequences shown in Fig. 1 were ligated respectively at the 5'-flanking sites of the luciferase gene (LUC) in the vector pGL3. These vectors were introduced into protoplasts in the same manner as those described in the above section and these protoplasts were cultured for 3 days. Figure 6 shows the results of the LUC assay of these protoplasts. Although the vector pGL3 and the LUC gene used were developed for animal cells, low, but significant LUC activity was reproducibly detected with the protoplasts electroporated in the presence

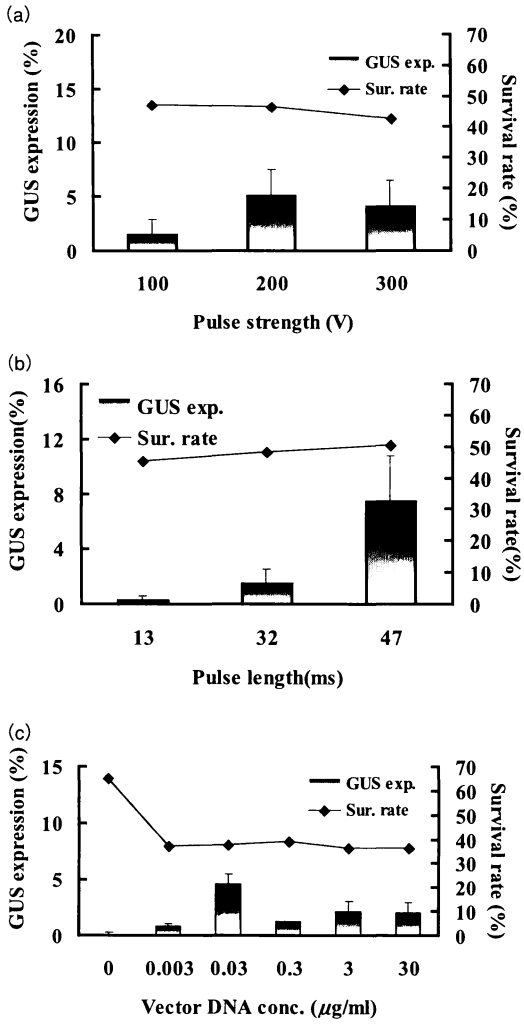

Figure 4. Effects of pulse strength, pulse length and vector DNA concentration on the expression rates of the GUS gene and the survival rate of protoplasts. Protoplasts were electroporated with the pYez-Rub4-GUS under the electrical conditions and DNA concentrations indicated. The GUS activities were measured as shown in Fig. 2 and the expression rates were calculated. The survival of cells was counted at 4 days after being electroporated and the survival rates were calculated as shown by the line graphs. (a) : Effect of pulse strength, (b): Effect of pulse length, (c): Effect of vector DNA concentration. The vertical bars indicate the standard deviations.

of vector pYez-Rub6-LUC.

Discussion

We previously observed the transient expression of a foreign gene in *P. yezoensis* protoplasts (Okauchi and Mizukami, 1999) using the vector pUC19-El_2-CaMV, which contained the 35S promoter, enhancer and Ω sequences in pUC19 (Kosugi *et al.*, 1991). However, the reproducibility of the transient gene expression and the expression rates were low in the experiments with this vector. In the present study, we obtained a higher reproducibility of gene expression and expression rates by using the Rubisco promoter of *P. yezoensis* than obtained in the previous study.

The quantitative analysis of GUS activity is important for the measurement of the expression activity of the introduced gene. However, in the present study, these activities were measured mainly by the histochemical method of GUS assay because of the differences of the expression activity of the introduced gene among the various thalli. Although we stocked and used thalli of 5 culture strains for the preliminary study of foreign gene expression, the active protoplasts for the gene expression could be obtained from only two strains of them. Although a large number of live protoplasts could be obtained from thalli of remaining 3 strains, these protoplasts did not show significant expression activity of the introduced gene. The reason why the activity of foreign gene expression was different among various strains is now under investigation. However, a large number of thalli which release protoplasts readily and show high expression activity of the introduced gene are necessary to be stocked for the quantitative measurement of introduced gene expression.

In the present study, the expression rates of the reporter GUS gene were calculated from the proportion of indigotin blue cells to the total cells. Therefore, these expression rates indicate the frequency of cells in which the GUS gene is expressed. For further study of gene transfer, it is important to analyze the gene expression activities by quantitative experiments and compare them among various culture strains.

For higher plants, the GUS gene activity was histochemically assayed at 37℃ for more than 20h in the presence of GUS substrate. In the case of the agarose culture of *Porphyra* protoplasts, we could not detect any clear GUS activity after electroporated protoplasts were incubated at 37℃ with GUS substrate. Instead, we could detect clear blue cells indicating GUS activity after these protoplasts were incubated

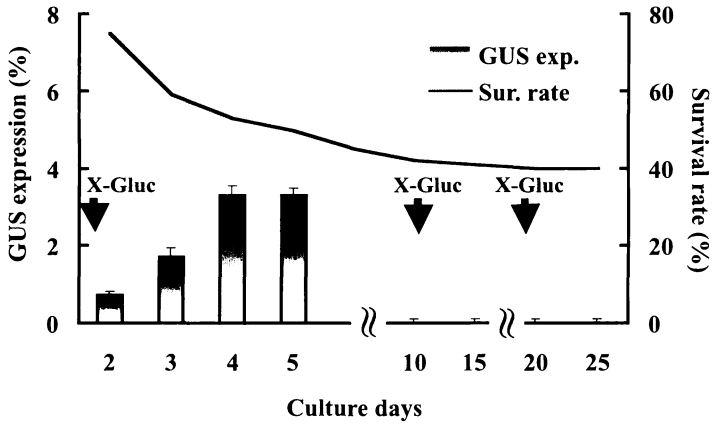

Figure 5. Time course of the GUS gene expression. Protoplasts were electroporated with the vector pYez-Rub4-GUS and cultured in a medium of sea water. After one, 10 and 20 days of culture, the culture medium were mixed with an equal volume of liquid 1.6% agarose (35° C) and X-Gluc (final concentration, 0.5 mg/ml). The total and blue-stained cells in each well were counted every day after the initiation of the agarose culture. The vertical bars indicate the standard deviations.

at 15℃ for more than two days with GUS substrate. The reason why the GUS activity in the electroporated protoplasts could not be detected at 37℃ remains unconfirmed. However, it is noteworthy that the protoplasts are easily analyzed at 37℃ and the sea water which was used as the culture medium contains high concentration of sodium chloride.

The optimal electrical conditions for the introduction of vectors into *Porphyra* protoplasts were 200 to 300 V field strength and 47 ms pulse length. These electrical conditions were very similar to those reported for higher plants (From *et al.*, 1985; From *et al.*, 1986). On the other hand, the optimal concentration of vector DNA for the electroporation was 0.03 μg/ml, which is a considerably lower concentration than those reported for higher plants (From *et al.*, 1985). The GUS gene expression occurred mainly during 3 to 5 days after their introduction into the protoplasts. The transient expression pattern of the GUS gene described above was very similar to those of higher plants.

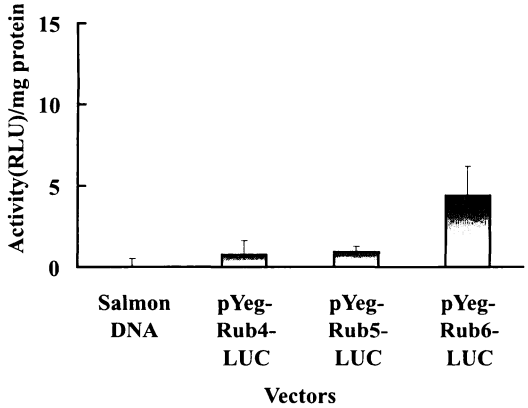

Figure 6. LUC activities of protoplasts introduced with vectors pYez-Rub4-LUC, pYez-Rub5-LUC and pYez-Rub6-LUC. The "Salmon DNA" indicates control protoplasts which were electroporated with only salmon DNA. The vertical bars indicate the standard deviations.

The results obtained in the present study showed that the Rubisco promoter of *P. yezoensis* could function as a promoter of foreign gene expression in the protoplasts. In *Porphyra*, the Rubisco gene consisted of a promoter, large subunit, spacer and small subunit, all of which were involved in the plastid genome (Reith and Munholland, 1995; Brodie *et al.*, 1998). Therefore, it is likely that the expression of introduced foreign genes occurred in the plastids. The transformation of the plastid genome and the foreign gene expression in plastids have been reported for higher plants (Svab and Maliga, 1993) and a green alga (Blowers *et al.*, 1990).

The present study has reported information on the introduction of foreign genes into *P. yezoensis* cells. The next step of this study is expected to integrate foreign genes into the plastid or nuclear genome and produce transgenic algae for the molecular breeding of *P. yezoensis*.

Acknowledgments

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アマノリプロトプラストへの外来遺伝子導入と一過性発現

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藻類（真核生物藻類）のうち，緑藻，特にクラミドモナス，クロレラ等の淡水性単細胞緑藻では遺伝子導入研究が活発に行なわれ，遺伝子組み換え体作出についてすでに多くの報告がなされている。一方，多細胞性の紅・褐藻類における遺伝子導入研究は，2，3の予備的な研究を除いて，殆ど報告がない。養殖ノリ(スサビノリ)，コンブ等産業上の重要な種についても，この分野での研究は著しく遅れている。

本研究ではスサビノリプロトプラストへ外来遺伝子を導入し，導入条件および導入遺伝子の一過性発現について検討した。植物ベクターpBI 221を用いて，β-グルクロニダーゼ（GUS）遺伝子上流にスサビノリのリブロースニリン酸カルボキシラーゼ遺伝子のプロモーターを繋いでベクターpYez-Rub-GUSを構築し，プロトプラストへ電気穿孔法で遺伝子導入した。組織化学的分析および蛍光標識基質を用いた酵素反応によって遺伝子発現活性を調べたところ，遺伝子導入した細胞にGUS活性が認められた。比較的高いGUS活性は，電圧200～300V，パルス長17 ms，ベクター濃度0.03 μg/mlの遺伝子導入条件下で，遺伝子導入後3～5日間培養した細胞に観察された。GUS遺伝子に代えて，ホタルルシフェラーゼ（LUC）遺伝子を用いてGUS遺伝子の場合と同様にベクターを構築し，プロトプラストへ遺伝子導入した。これらの細胞では，活性が幾分低かったものの，ルミノメーター測定法によってLUC活性が検出された。以上の結果から，スサビノリプロトプラストへの遺伝子導入および一過性発現に関するいくつかの知見を得ることができた。