FINAL REPORT
December, 1997
Genetic Engineering of Seaweeds

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RATIONALE

Worldwide, seaweeds constitute a major source of food products as well as specialty compounds, e.g., phycocolloids. Because of their high productivity and biomass, they are also an important potential source of energy. Although the United States is the largest producer and consumer of phycocolloids, most seaweeds are imported, and current demand for carrageenan, algin, agar, and agarose far exceeds their production. Accelerated genetic improvement of seaweeds and improved coastal management are key factors helping to meet the increased demand for seaweed products.

Among the targets for genetic improvement in seaweeds are food products, the quality and the quantity of phycocolloids and pharmaceuticals, and increased biomass for energy production. A potentially useful application of seaweed aquaculture would also be their ability to accumulate and sequester heavy metal ions from the surrounding environment. Several attempts at genetic improvement of seaweeds have been made, but in comparison with land plants the efforts and results have been minuscule. Conventional methods of selection by screening wild populations, mutagenesis, and hybridization will, no doubt, continue to play an important role in the improvement of seaweeds. However, these methods are of limited practical value because they involve a recombination of the pre-existing gene pool within a narrow range of sexually compatible genotypes. Recently developed molecular techniques for genetic manipulation (i.e., recombinant DNA and genetic engineering) provide new opportunities for improving commercially useful seaweeds by using specific genes from unrelated organisms.

During the past decade, considerable attention has been focused on the development of reliable transformation systems for higher plant cells. While genetic transformation in some microalgae has been demonstrated and the potential of such techniques for seaweeds has been discussed, no reports of stable genetic transformation of macroalgae have been published. Based upon the perceived need for the application of genetic engineering techniques to the production of genetically improved seaweed varieties, we proposed the following objectives.

OBJECTIVES

1. To optimize the conditions for gene transfer by electroporation and the regeneration of transgenic plants of *Ulva lactuca* and *Porphyra umbilicalis*.

2. To test the effectiveness of the biolistic bombardment technique for transient and stable genetic transformation of *Ulva* and *Porphyra*.

3. To study the photo regulation of a light-inducible SS (small subunit of ribulose bis-phosphate decarboxylase/oxygenase) promoter in *Ulva* and *Porphyra*.

RESULTS AND DISCUSSION

Several techniques are available for transfer of foreign DNA into plant cells. These are: direct uptake, electroporation, microinjection, viral- or bacterial-vector-assisted transformation, and biolistic bombardment. Based upon previous studies from our lab (Primich-Zachwieja and Minocha, 1991), it was concluded that *Agrobacterium* is not a suitable vector for gene transfer in algae. Thus a major objective of the present study was to test the method of electroporation for transformation (Objective 1), and also, to test the feasibility of using biolistic bombardment for transformation of *Ulva lactuca* and several native species of *Porphyra* (Objective 2).

Transformation by Electroporation

Electroporation is a commonly used method of gene transfer into different cell types. In this method, wall-less plant cells (protoplasts) are immersed in a solution of plasmid DNA and treated with an electric pulse of 250-400 V for a fraction of a second during which transient pores are created in the cell membrane, thus allowing the plasmid DNA to be taken up by the cells. The efficiency of gene transfer by electroporation depends upon several electrical and biological parameters, such as voltage, duration of electrical pulse, temperature, ionic strength of the medium in which cells are suspended, DNA concentration and topology (linear vs. supercoiled) of DNA, and the physiological status of the cells. A pre-requisite for transformation by this method is the availability of regeneration protocols from protoplasts and other single cells.

Following limited guidelines from the literature, we developed protocols for regeneration of whole plants from protoplasts of *Ulva lactuca*, and several native species of *Porphyra*. A summary of results on the optimum conditions for protoplasts formation and regeneration for seven species of *Porphyra* are given in Table 1. For different species of *Porphyra*, regeneration from protoplasts varied from 10-20% to as much as 50-60% of the protoplasts depending upon the day of collection, the time of storage before making protoplasts, the temperature of growth, and the enzyme treatments. In the case of *Ulva lactuca* more than 60% of the protoplasts could routinely grow into germlings. Regeneration from swarmers was greater than 70%.

Electroporation was used for transformation with protoplasts and partially digested thallus fragments of *Ulva lactuca*, and protoplasts of six species of *Porphyra*. Detailed analysis of various parameters, such as pulse strength, voltage, time of pulse, etc. were tested for survival and transformation of these cells. The genes that were tested include neomycin phosphotransferase (NPTII) and β-glucuronidase (GUS). For *Ulva*, protocols for regeneration from both swarmers and protoplasts, before and after electroporation, were optimized. More than 50% of the protoplasts regenerated into normal plants (Huang et al. 1996). We tested field strengths ranging from 200-1,000 V/cm and found that 2-3 consecutive pulses of 300-400 V /cm were quite effective in causing transient expression of the GUS gene. **Data from transformation studies indicate that the 35S CaMV promoters is quite active in *Ulva* cells in transient expression assays of the GUS gene.** Two major problems encountered during transformation work with *Ulva* are: (1) the most suitable materials for transformation were thallus explants that were partially

digested with enzymes used for protoplast formation; and (2) cells that easily regenerated into whole plants (i.e. protoplasts and swarmers) were extremely sensitive to electroporation and to reagents used for staining of GUS activity. The inconsistency of results in different experiments could be the result of a low level of expression of the 35S promoter, or a low frequency of transformation, or the physiological status of the plant at the time of collection.

Reliable protocols have been developed for the formation of protoplasts and their regeneration into whole plants, in *Porphyra miniata, P. purpurea, P. amplissima, P. linearis, P. diocia, P. leucosticta and P. umbilicalis* (Kübler, Minocha and Mathieson, 1994; Minocha 1998; Larsen, 1998). The results on protoplast regeneration in different species of *Porphyra* (with the exception of *P. linearis*) are quite similar. The two most significant improvements that we made were (1) the use of commercially available crude enzyme preparations, and (2) the omission of the predigestion incubation with papain. Details of the methods will be a part of the thesis of Jon Larsen and will be published in the near future. The yield of protoplasts was typically 10^6- 10^7 protoplasts per g chopped tissue, with 70-80% of the protoplasts being viable as tested by staining. Protoplasts settled and adhered to cover slips within 24 h. Cell wall material was deposited quickly and the first cell division occurred around 7-10 days. Thereafter, cell divisions occurred more frequently, forming multicellular plantlets. **Another significant modification in the protocols that we have made is that we grow our cultures under non-axenic conditions. This has resulted in a tremendous improvement in the regeneration frequency of the protoplasts.** In most cases the cultures appear clean and free of other organisms, in others we often saw microbial and zooplankton contaminations. Recently a new compound called PPM (Plant Preservative Mixture - Plant Cell Technology, Inc.) has become available for restricting the growth of microorganisms in cell culture media. A sample of this compound was obtained and used to see its effects on the regeneration of protoplasts of different *Porphyra* species. The results shown in Table I indicate that this compound may actually be detrimental to the growth and regeneration of protoplasts of *Porphyra*. Further tests are underway to confirm these results.

The plasmids pBI121 (Clontech, Palo Alto, CA) was initially used for the expression of GUS gene. This plasmid has a 35S CaMV promoter which has been quite successful with a large number of higher plants. A number of variations of this promoter have been suggested that are supposedly more effective in both transient and stable expression assays. We obtained one such variation (pCW122) where a 2x 35S promoter was attached to the GUS gene. **The most effective electroporation conditions were as follows: 1-3 pulses at 100-300 V, from a 330 amp capacitor, exponentially decaying across a 5 mm pathlength. The conditions were optimized to produce 40-50% viability of electroporated cells as determined by vital staining with erythrocine B and trypan blue 1-3 h after treatment. Two days after electroporation, transient expression of the GUS gene was observed at a frequency of 1-15 cells/10,000 cells. No transient expression was observed in the samples electroporated without plasmid DNA.**

In summary, we feel that the 35S promoter of CaMV is quite effective for at least transient expression in the algal cells, and that these algae do not have any residual GUS activity that could interfere with the GUS assays in the transgenic cells.

Means of selection of transformed cells

Since the frequency of transformation by any method is quite low (less than 0.1%), it is imperative that reliable methods of selection of transgenic cells/plants be available. A commonly used approach is the co-transfer of a selectable marker gene such as the one imparting resistance to an antibiotic or an herbicide. Genes for neomycin phosphotransferase (NPT), chloramphenicol acetyltransferase (CAT), hygromycin phosphotransferase (HPT), and dihydrofolate reductase (DHR) have been used to impart resistance to plant cells for selection; NPT being the most commonly used gene. The selection of a selectable marker gene depends upon the degree of sensitivity of the untransformed cells to the toxic agent. This information was not available for any of the algae. **We conducted detailed toxicity analysis for growth of protoplasts of *Ulva lactuca* and several species of *Porphyra* on kanamycin, geneticin, chloramphenicol and hygromycin. The results show that for *Ulva,* lower concentrations of kanamycin or geneticin caused improvement in protoplast regeneration while higher concentrations (0.5 mg/mL or more of kanamycin; 0.05 mg/mL geneticin) caused significant inhibition of growth (Huang et al., 1996). Thus it should be possible to select transformed cells with NPTII gene using these antibiotics. *Porphyra* cells also showed no inherent resistance to kanamycin or hygromycin, therefore, either NPT or HPT gene could be used to select transgenic cells.**

Biolistic bombardment:

Several experiments were conducted with *Porphyra* thallus using the technique of biolistic bombardment using the 'gene gun'. While there was some indication of the development of blue color in some cells, it was extremely difficult to visualize the transformed cells from the thallus surface due to the fact that the cells are very small and they have a very thick cell wall that reflects incident light. Thus it was decided that further experiments with bombardment should be done with conchocelis cells which are quite elongated and would be easy to visualize.

New areas of study/deviation from the stated objectives

Repeated attempts at regeneration of stably-transformed plants from either *Ulva* or any of the species of *Porphyra* did not yield any positive results. This is perhaps due either to the lack of integration of the foreign DNA or silencing of the gene/promoter following integration. Since, sufficient amounts of transformed tissue could be regenerated, it was not possible to experimentally distinguish between these two possibilities. Since the red algae branched off from the other plant groups very early in evolution, it is quite likely that the 35S CaMV promoter, which works quite well in the higher plants, may not be quite effective in the red algae. A real test of this idea will be to work with red algal promoters. Unfortunately, none are available so far. **Realizing the need for such promoters not only to develop the protocols for stable expression of foreign genes but also for future use of the transgenic approach to introduce commercially useful genes in red algae, we initiated a project on the isolation of genes and promoters from *Porphyra*. Current attempts are focused on the isolation of genes and their promoters that are either constitutively expressed or are developmentally regulated in the haploid (thallus) and the diploid (conchocelis) stages of *Porphyra*. In a complementary study,**

we are also attempting to identify and clone genes that determine male and female sex in *P. purpurea*. These genes/promoters will be useful for future genetic manipulation of the reproductive behavior of seaweeds through genetic engineering.

While the progress of research on genetic engineering with marine macroalgae has been slow, these organisms have certain unique aspects of their growth and development that create major impediments to experimentation. These include a specialized cell wall, extremely slow growth from single cells, the need for seawater for growth, lack of callus formation, etc. Based upon published information and personal knowledge of the current work in different laboratories around the world, it is apparent that our laboratory is in a leading position in this research.

FUTURE PROSPECTS

As described above, the successful improvement of any species through genetic engineering depends upon four factors: (1) the availability of commercially useful genes; (2) reliable means of gene transfer to single cells; (3) selection and regeneration of transformed plants; and (4) regulatory sequences (promoters, enhancers, etc.) for controlled expression of foreign gene.

Having established that certain heterologous promoters are functional in driving the transient (short lived) expression of foreign genes in *Ulva* and *Porphyra*, and that isolated single cells in these taxa can be grown into whole plants in the laboratory, it is logical to continue our efforts to optimize the protocols for stable integration and expression of foreign genes and to obtain useful promoters that will allow us to regulate the expression of foreign genes in these organisms . The two specific objectives of the future studies are: (1) to clone and characterize developmentally regulated genes in haploid and diploid stages of *Porphyra*, and (2) to isolate promoters from both constitutively expressed and developmentally regulated genes from *Porphyra*. This will be achieved by first preparing cDNA and genomic DNA libraries from the thallus and conchocelis stages of *Porphyra*, and then using cDNA subtraction and differential display, isolate DNA sequences that are being expressed in the two developmental stages. Significant progress in this direction has already been made in establishing these libraries with support from NOAA under the *Porphyra* aquaculture project.

Publications and Manuscripts to date

1. Primich-Zachwieja, S. and S. C. Minocha. 1991. Induction of virulence response in *Agrobacterium tumefaciens* by tissue explants of various plant species. Plant Cell Rep. 10:545-549.

2. Kübler, J., S. C. Minocha and A.C. Mathieson. 1994. Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyceae). J. Mar. Biotech. 1:165-169.

3. Hinson, T. K., J. C. Weber, A. Porzio and S. C. Minocha. 1993. Transient expression

of the GUS gene in the green alga *Ulva lactuca*. (Abst.) Proc. NE Section Amer. Soc. Plant Physiol. mtg., New London, CT Abs. P4.

4. Porzio, A., T. Hinson, J. C. Weber and S. C. Minocha. 1993. Genetic transformation of *Ulva lactuca*. UROP Symposium, UNH, Durham, NH (Abst.).

5. Weber, J. C., T. K. Hinson and S. C. Minocha. 1992. Protoplast isolation and regeneration in the green alga *Ulva lactuca* L. Proc. NE Section Amer. Soc. Plant Physiol. mtg., Durham, NH (Abst).

6. Huang, X., J.C. Weber, T.K. Hinson, A. C. Mathieson. 1996. Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L. (Chlorophyta). Botanica Marina 39: 467-474.

7. Minocha, S.C. 1997. Genetic engineering in marine algae. 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology, Phuket, Thailand, May 7-10, 1997. Abst.

8. Minocha, S.C. 1997/98. Genetic transformation in protoplasts and partiality digested cells of *Ulva lactuca* (Chlorophyta) and *Porphyra miniata* (Rhodophyta). Proc. 4th International Marine Biotechnology Conference, Sorrento, Italy. September 22-29, 1997 (Invited symposium lecture). In Press.

9. Minocha, S.C. 1998. Genetic engineering of marine algae: *Porphyra* and *Ulva*. Invited symposium lecture at the Aquiculture Algal Biotechnology Symposium, February 15-19, 1998, Las Vegas, Nevada

10. Minocha, S.C. 1998. Advances in the genetic engineering of macroalgae. Invited symposium lecture at the 16th International Seaweed Symposium to be held Cebu City, The Philippines, April 12-17, 1998. (Will be published as Proceedings)

Names of Postdocs and Students Supported/Trained

1. Dr. Xiaohang Huang - Postdoctoral Research Associate
2. Dr. Dennis Mathews - Postdoctoral Research Associate
3. Jon Larsen - Graduate Student, Plant Biology
4. Marc Valyo- Undergraduate Student, Biology
5. Christina Ryan - Undergraduate student, Biology
6. Daria Hahn - Undergraduate student, Biology

Titles of Theses Completed/In Progress: Jon Larsen. Isolation of protoplasts and genetic transformation in *Porphyra spp*. MS Thesis, University of New Hampshire, Durham, NH, Expected completion, May, 1998.