Molecular Characterization of Seaweeds Using RAPD and Differential Display

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A rapid and economical method of simultaneous extraction of DNA and RNA from seaweeds has been developed by the use of lithium chloride. Lithium chloride facilitates the softening of cell walls resulting in a decrease in both compressive and tensile modulus of elasticity. The DNA was characterized by high molecular weight larger than 27 kb and a relative lack of carbohydrate and protein contamination. The DNA and RNA extracted by the method from many seaweeds were of sufficient quality to be used as a template for PCR amplification with a plant intergenic gene primer set, for RAPD analysis with arbitrary primers, and for differential display with arbitrary primers in the morphologically distinct regions of the matured *Porphyra* thallus. The cDNA polymorphism indicated that the reproductive tissue types (male, female, patch) had a relatively high degree of similarity; the vegetative tissue types (dividing, non-dividing) also showed a similar pattern with respect to each other. Holdfast tissue had very low similarity with the other tissues, but appeared most similar to vegetative non-dividing tissue type.

Key words : differential display, LiCl, PCR, RAPD, seaweed

Inrtoduction

Polymerase chain reaction (PCR) technique allows the specific amplification of discrete fragments of DNA and cDNA to detect and characterize DNA and RNA that are initially present in the cell in picogram quantities (Saiki et al., 1985; Liang and Pardee, 1992). Usually, acidic polysaccharides contaminated in the extraction from plant tissues have been shown to be inhibitory for PCR amplification (Demeke and Adams, 1992) and restriction digestion (Do and Adams, 1991). The seaweed tissue is composed of cells embedded in a cell wall and intercellular matrix of complex polysaccharides. In red and brown seaweeds, sulfated and carboxylic polysaccharides are the major carbohydrates (Bold and Wynne, 1978; Ragan, 1981). They are more water-soluble than the neutral polysaccharides of land plants and also their solutions are highly viscous. The composition of cell walls from different seaweed species, from different tissue parts, or from different growth stages, showes differences on the properties of the seaweed tissue (Dring, 1982). Thus, the extraction of DNA from seaweed tissues that are heavily embedded in viscous polysaccharides is complicated and time-consuming (Fain et al., 1988; Mayes et al., 1992). Lithium chloride (LiCl) is used extensively to soften seaweed tissues facilitating easier squashing for chromosome observations (Evans, 1963; Yabu, 1979). It has also been used to extract a spinach carboxylase (Sato et al., 1990), bacterial nickase (Fujii et al., 1987), and mammalian RNA and DNA (Raha et al., 1990). From these facts, LiCl is suspected to have a similar effect on the softening of various seaweed tissues and release of nucleic acids through the loosened cell wall. We have recently established a quick and simple protocol for DNA and RNA extraction from the red alga *Porphyra* by LiCl treatment (Hong et al., 1992; Hong et al., 1995a; Hong et al., 1995b). This pa-

per describes the effects of LiCl to extract DNA and RNA from several seaweeds and their quality to be used as a PCR template with a specific primer set or arbitrary primers. We have also measured the compressive and tensile properties of the seaweed *Porphyra* blades to understand the effect of LiCl on the tissue. Finally, we have identified and compared the tissue-specific genes from the various thallus regions of *Porphyra* for a potential use as genetic markers for studies on the regulation of development and differentiation.

Materials and Methods

Plant material

Leafy thalli of seaweeds were collected at Korean coast or at Point Piedras Blancas, California. Tissues were dried under forced air of an electric fan for at least 2 hr to approximately 30% moisture content and stored at -70℃ for the convenient use of samples. Stored tissues were used successfully for DNA and RNA extraction over a year.

Nucleic acid extraction

For DNA extraction, 0.1 g of the partially dried tissue was cut randomly with scissors to about 0.25 cm^2 and heated in 4 ml of extraction solution (0.8 M LiCl, 0.6% sarcosyl, 10 mM EDTA, 0.2% PVPP, 5% β-mercaptoethanol, pH 9.0) for ten min at 55℃ (Hong et al., 1995a). For RNA extraction, 4 M guanidium thiocyanate is added to the extraction solution (Hong et al., 1995b). Then, the tissue was shaken gently at 4℃ for one hr. The released nucleic acid was precipitated directly by addition of 0.1 vol of 3 M sodium acetate, pH 5.4 and 2 vol of 100% ethanol. The precipitate was resuspended in 300 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for stock solution and then diluted ten-fold in TE again for assays. Finally, the nucleic acid solution was spun for 10 min in a microfuge, and the supernatant was used for the determinations of nucleic acid amount, PCR, and impurities.

DNA and RNA quantification

DNA was quantified using a Hoefer Mini-Fluorometer (Model TKO 100) against standard concentrations of salmon sperm DNA (Calbiochem) and calculated as total DNA (μg) against one gram of partially dried tissue. To determine RNA concentration, the nucleic acid pellet was incubated with 4 μl of RNase-free DNase I (1 u $\cdot$ μl^{-1}) (Ausubel et al., 1987). A reading of 1.0 at 260 nm was calculated to be equal to 40 μg $\cdot$ ml^{-1} for RNA (Sambrook et al., 1989).

Carbohydrate quantification

Total carbohydrate was determined by the phenol-sulfuric acid method (Kochert, 1978), and expressed with respect to a glucose standard in mM.

Protein impurity

The protein impurity was represented by the ratio between absorbance readings at 260 nm and 280 nm (Sambrook et al., 1989).

PCR amplification

PCR amplification was carried out using the Perkin-Elmer Cetus DNA thermal cycler. The primer set was the plant universal primers c and d for the intron of the *trnL* gene in chloroplast (Taberlet et al., 1991) or arbitrary primers of the Operon's 10-mer purchased from Operon Technologies, Inc. The cycling parameters included an initial incubation at 94℃ for 5 min followed by 30 cycles of 1 min-denaturation at 94℃, 2 min-annealing at 55℃, and 3 min-extension at 72℃ with the plant universal primer set. The thermal cycling with arbitrary primers was programmed by 45 cycles to denature at 94℃ for 5 sec, anneal at 36℃ for 1 min, and extend at 72℃ for 2 min in a reaction solution (Yu and Pauls, 1992).

Agarose gel electrophoresis

A 10 μl sample of nucleic acid extract, mixed with

0.1 vol of loading dye (20% Ficoll 400, 0.025% BPB), was loaded on a 0.5% agarose gel containing 0.5 μg/ml of ethidium bromide and run for 1 hr at 50 V in 0.5X TAE buffer (Sambrook et al., 1989). The 10 μl of PCR product was subjected to electrophoresis on a 2% agarose gel.

Compression testing

Compression testing was performed with an Instron Capillary Rheometer System (Model 3211) at room temperature. Tissues of 0.2 cm^2 were compressed at a rate of cross-head speed of 0.06 cm/min with a compressive loading strength of 20 kg. The compressive modulus of elasticity is expressed as stress divided by strain below the proportional limit of a material (ASTM, 1991a).

Tension testing

Tensile properties were measured with an Instron Tensile Tester (Model 1122). Tissue samples, approximately 4 mm wide and 20 mm long, were drawn at a rate of 5 mm/min (cross-head speed) with a tensile loading strength of 8 Newtons. The percentage elongation at break, the tensile strength, and the tensile modulus of elasticity are calculated (ASTM, 1991b).

cDNA synthesis

Five μl of total RNA (2.5 μg) was used as a cDNA template in 20 μl of reaction mixture, and followed by the Invitrogen's cDNA protocol.

Similarity matrix

A similarity matrix is prepared based on the presence or absence of individual prominent bands generated by each primer set, and all pairwise comparisons were calculated using the Jaccard's equation, $J_{ij}=C_{ij}/(n_i+n_j-C_{ij})$, where C_{ij} is the number of common bands for a pair of tissues, and ni and nj are the numbers of bands in i and j members of the pair, respectively (Sneath and Sokol, 1973). A dendrogram was constructed based on the similarity matrix data by applying cluster analysis (Nei, 1987).

Results

Nucleic acid extraction

A rapid and economical method of DNA extraction from a red seaweed *Porphyra* has been developed by the use of LiCl (Hong et al., 1995a). The large amount and relatively pure DNA were obtained using the optimized compositions of extraction solution and heating at 55℃ for 10 min. By increasing the temperature and heating time, more DNA was released with more contamination of carbohydrate and protein. With the preheated extraction solution at 55℃, the extaction time of 5 min at 55℃ was a critical condition to get an optimal balance for the highest DNA yield and the lowest amount of impurities. Meanwhile, the heating time of 10 min at 55℃ was generally acceptable when the DNA is extracted with the extraction solution maintained at room temperature. The optimized condition for the extraction of DNA from *Porphyra* has been used for the extraction of DNA and RNA from various seaweed tissues. Size of the most DNAs is estimated to have an average of larger than 27 kb. The LiCl method is very suitable to extract nucleic acids from *Enteromorpha*, *Monostroma*, and Ulva among Chlorophyta seaweeds (Hong et al., 1996). The LiCl method is also suitable to extract nucleic acids from *Ceramiopsis, Chondrus, Corallina, Gigartina, Gloiopeltis, Grateloupia, Gymnogongrus, Phacelocarpus, Porphyra,* and *Symphyocladia* among Rhodophyta seaweeds. The method is also good to extract nucleic acids from *Dictyota, Myelophycus,* some *Sargassum*, and *Undaria* among Phaeophyta seaweeds. The DNA amounts released by the LiCl method have had no direct relationship with the algal classification. Total DNA yield was approximately 8 to 243 $\mu g \cdot g^{-1}$ of partially dried tissue. Total RNA is also significantly extracted simultaneously. The quality of the DNA was relatively not much pure with respect to protein contamination cal-

culated as the $A_{260/280}$ ratio in the range of 0.8 to 1.6, but total carbohydrate was contained as low as approximately 1 to 9 mM in assay solution from 1 g of partially dried tissue. The extracted DNA was then tested for its ability to function as a template for PCR amplification. The DNAs from *Ceramiopsis, Ceramium, Chondria, Chondrus, Corallina, Dictyota, Enteromorpha, Gigartina, Gloiopeltis, Gymnogongrus, Hizikia, Laminaria, Monostroma, Phacelocarpus, Phachymeniopsis, Porphyra, Ulva*, and *Undaria* worked as a good quality for PCR template. When the plant universal primer set is used to obtain PCR amplification, the prominent reaction product mostly from 1 μl of the LiCl-extracted DNA in assay solution has been represented by a single or a few bands of 200 to 600 bp. Otherwise, variable DNA concentrations of 1 μl to 1 pl have been available as a template for RAPD assay with arbitrary primers. Because different seaweed tissues produce DNA of widely different amount and purity, it may be necessary to optimize the amount of DNA used in the RAPD assay to achieve reproducibility and strong bands. The LiCl-extracted DNA was digested directly with *Rsa* I, *Hind* III, *Eco* RI, and *Bam* HI for 18 h. Restriction enzymes such as *Rsa* I and *Hind* III that require low and medium salt buffers digested DNA almost completely. However, restriction enzymes such as *Bam* HI and *Eco* RI that require a high salt buffer could not digest this DNA. None of pretreatments such as CTAB treatment, phenol extraction, PVP treatment, Millipore VS filter dialysis, Spermidine, and potassium-glutamate buffer was available to facilitate restriction digest of *Bam* HI in the high salt buffer (unpubl. data).

Compressive and tensile properties

The DNA extraction solution softened the *Porphyra perforata* tissue by decreasing the compressive and tensile modulus. The tissue was processed through the LiCl method used for DNA extraction described previously. For the mechanical effect of LiCl treatment, tissues treated with or without LiCl were then compared to tissues treated with seawater. The LiCl-treated tissue showed a 87% decrease in compressive modulus of elasticity, a 63% decrease in tensile strength, and a 54% decrease in tensile modulus of elasticity. The LiCl-untreated tissue, which underwent the same DNA extraction procedure without LiCl, had a slight decrease in tensile properties, but its compressive modulus of elasticity did not change (unpubl. data). Thus, the addition of LiCl in the DNA extraction solution clearly effected the tissue, resulting in tissue softening and fragility with decreased compressive modulus of elasticity and tensile properties. The LiCl treatment caused tissue changes enough to permit DNA release through the softened cellular structure.

RAPD identification

For the stock identification, random amplified polymorphic DNAs (RAPD) have been applied with arbitrary primers. Using the technique, we have investigated genetic characteristics and the degree of similarity between local individuals of *P. yezoensis* and *P. kuniedai* (Kim et al., 1994) and between local individuals of *Hizikia fusiformis* (Park et al., 1995). The species-specific markers can be used for identification of inter-species and discriminating between populations drived from transplanted and original strain at DNA level. It can also be a very accurate way to screen variants in a population study in combination with sequencing or RFLP analysis.

Differential display

The red seaweed *P. perforata* has unique properties which make it a suitable system for the study of cellular differentiation. Six tissue regions of the differentiated thallus were processed through the LiCl-guanidinium method for the RNA extraction. The differential display assay was applied using *in vitro* amplification of cDNA by the PCR technique. We have developed genetic markers of the specific thallus regions for more studies on the regulation of development and

Table 1. Similarity matrix based on Jaccard's equation. The total number of amplified products revealed in each tissue is shown in the diagonal. Letters of M, F, P, Vd, Vn, and Hf are represented as male, female, patch, vegetative dividing, vegetative non-dividing, and holdfast tissues

	M	F	P	Vd	Vn	Hf
M	25					
F	0.54	18				
P	0.45	0.46	17			
Vd	0.34	0.28	0.20	37		
Vn	0.29	0.28	0.26	0.54	37	
Hf	0.14	0.13	0.17	0.15	0.29	17

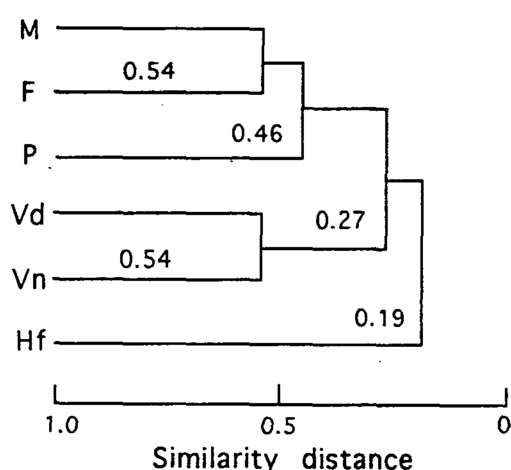

Fig. 1. Dendrogram based on the similarity matrix data for clustering the differentiated tissues of *P. perforata*. Values above the lines are similarity distances between tissues, namely values of 1 and 0 indicate an identical match and complete dissimilarity, respectively. Letters of M, F, P, Vd, Vn, and Hf are represented as male, female, patch, vegetative dividing, vegetative non-dividing, and holdfast tissues.

differentiation at gene expression level. Fifteen different arbitrary primers and 80 combination sets of two primers were screened for primers to amplify cDNA from differentiated tissue. Depending on the cDNA-primer combination, 2 to 10 DNA segments were amplified, ranging in size from approximately 200 bp to 1500 bp. Amplified polymorphic patterns or tissue-specific markers were observed between the six differentiated tissues with the OPA 3 primer and with the OPA 1 and OPA 10 primer sets (Hong et al., 1995b). cDNA polymorphism indicates that differential display markers can be used to show similarities between the differentiated tissues (Table 1). In the use of Jaccard's equation to calculate similarity coefficients, values of 1 and 0 indicate an identical match and complete dissimilarity, respectively. The result is summarized in a cluster analysis dendrogram (Fig. 1). A group of male, female, and patch tissues had relatively high degrees of similarity, as evidenced by similarity coefficients of 0.46 or greater. Thus, it can be concluded that the patch tissue has a similar pool of total RNA with the reproductive tissues. Another group of vegetative dividing and non-dividing tissues showed relatively high similarity coefficients of 0.54. Holdfast tissue had as low as 0.19 of similarity with all other tissues. However, the holdfast tissue had 0.29 of similarity with vegetative non-dividing tissue, suggesting that the holdfast tissue may be derived from the vegetative non-dividing tissue by further differentiation processes.

Discussion

In vitro, LiCl affects tissue softening in seaweeds (Evans, 1963), morphological changes of mammalian cells (Tyobeka and Becker, 1990), and release of cellular components from animal (Raha et al., 1990), plant (Sato et al., 1990) and bacterial cells (Fujii et al., 1987). Tissues of some seaweeds are very tough and difficult to spread by squashing under a coverglass af-

ter chromosome staining. The most suitable softening technique as a pretreatment to the chromosome staining on *Fucus* was found to be immersion of small pieces of fixed tissue in a 1 M solution of a monovalent LiCl for 15 minutes (Evans, 1963). At first, it was suggested that the marked softening effect of monovalent metal on *Fucus* tissue resulted from the greater solubility of the alginates. Divalent metal ions can form ionic cross linkages between the carboxyl groups of adjacent molecules of alginic acid, thus linking the alginate chains into a semirigid and insoluble framework. Thus in seawater, with its relatively high calcium content, the alginic acid will be fixed in the cell wall as calcium alginate. If the divalent ions are replaced by monovalent ion, these cross-linkages would be eliminated and alginate chains loosened from each other, thus rendering the molecules soluble. As a result, the cell wall when treated with excess LiCl loses much of its rigidity so that slight pressure would cause the walls to be deformed and the cells to be separated. Secondly, LiCl might swell the cellulose component of the cell wall and thereby cause a general disarticulation of cell masses and softening of individual walls (Evans, 1963). For the extraction of total cellular RNA and DNA from cultured mammalian cells, a rapid procedure is described by Raha et al. (1990). This method combines the simultaneous disruption of cells and extraction of nucleic acids in a single step with the use of phenol and a buffer containing 100 mM LiCl. This procedure also yielded high molecular weight of DNA, which was a suitable substrate for restriction endonucleases. From these facts, LiCl is suspected to have a similar effect on the softening of seaweed tissues and release of DNA through the loosened cell wall and cell membrane. Thus, LiCl which affects tissue softening in seaweeds has been applied to extract DNA and RNA simultaneously from seaweed tissues. LiCl eliminated the problems of viscous polysaccharides released by grinding tissue in liquid nitrogen. The extraction procedure using LiCl appeared as a simple and efficent method to extract DNA and RNA directly. This rapid and convenient procedure works on both fresh wet tissue and dried tissue stored up to two years at −70℃. Differentiated tissue types of *Porphra* thalli varied in their mechanical properties probably reflecting chemical differences in their composition. When compared with gel strength of 1.5% Bacto-agar, *P. perforata* tissue has a 2~4 fold higher compressive modulus of elasticity. In addition, its tensile properties are comparable to the low values of a low density polyethylene (Brandrup and Immergut, 1989). Adding LiCl to the DNA extraction solution caused a marked decrese in tensile strength and tensile modulus of elasticity with some decrease of compressive modulus of elasticity. Very little decrease occured when LiCl was not present. The weakening of the tissues by LiCl hardly changed the elongation capacity, but did decrease tensile strength, requiring 62% less energy per unit volume to break apart the tissue. LiCl also reduced the tensile modulus of elasticity by 54%. Therefore, LiCl had a major role in tissue softening in the DNA extraction method.

Total RNA is also significantly extracted simultaneously by this LiCl extraction procedure by adding guanidinium thiocyanate in the extraction solution. Transcriptional levels within a cell change in response to a wide variety of signals that occur during cell development, differentiation, disease, and other physiological function. (Siebert, 1991). Application of the differential display technique provides an extremely sensitive and rapid method to detect specific mRNAs. Usually, 1 μg of cytoplasmic RNA is sufficient for amplification of rare mRNA sequences such as those occurring in 1 to 10 copies per cell (Kawasaki, 1990). First-strand cDNA synthesis may be accomplished by extension with random hexamers, the downstream primer, or oligo (dT). Among them, the random hexamer reaction has been known as the most efficient, containing the highest amount of final amplified product (Siebert, 1991). The aim is to use a population of oligonucleotides whose sequence diversity is so large that at least some individual oligonucleotides

will anneal to the template and serve as primers for reverse transcriptase (Sambrook et al., 1989). Because different oligonucleotides will bind to different sequences, a large proportion of the sequences of the template will be copied by the enzyme, and if all of the primers are present at equal concentrations, all sequences of the template should be copied at equal frequencies. Amplified cDNA polymorphic patterns were observed in the analysis of RNA transcripts of six tissue-regions of differentiated thallus with some arbitrary primers. Minor bands produced by the differential display analysis were much more variable and thus harder to score since some of the minor bands may be products of nonspecific priming. It is probably better to ignore such weak bands and use only the most prominent ones as genetic markers or as data for calculation of similarity coefficients. For the most part, only slight polymorphisms were produced in reproductive tissues, whereas they were clearly distinguishable from those of the vegetative and holdfast tissues. A few PCR amplified segments were specific to the tissue types, and some PCR amplified segments were common in all six tissue regions of differentiated thallus. Such differential display markers can then be used to identify the matured tissue types or compare similarities of gene expression as RNA phenotyping followed by the tissue differentiation.

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