Planta (1990) 181:522–528

Planta

Protoplast isolation and regeneration in the marine red alga *Porphyra nereocystis**

J. Robert Waaland, Leal G. Dickson, and Bruce A. Watson**

Department of Botany, University of Washington, Seattle, WA 98195, USA

Abstract. We have developed a method for isolating viable protoplasts from the blade phase of the epiphytic marine red alga *Porphyra nereocystis* Anderson, using a two-step enzymatic digestion with commercially available enzymes. The first step uses papain, the second step uses abalone acetone powder. The method is rapid and gives a high yield of viable protoplasts. In liquid culture in enriched seawater medium, the protoplasts can undergo regeneration along three pathways: they directly form filaments resembling the conchocelis phase of *Porphyra*; they form calli with relatively thick-walled, pigmented cells; and they indirectly form blades from the edges of these calli. *Porphyra nereocystis* protoplasts also may serve as an alternative propagation method in aquaculture and be useful for studies of cell-wall formation, cell division, and thallus differentiation. They may also be used in somatic selection, somatic hybridization and gene-transfection experiments.

Key words: Protoplast (*Porphyra*) – *Porphyra* – Rhodophyta

Introduction

Protoplasts, i.e., plant cells stripped of their walls, are useful for studies of cell-wall regeneration, cell division, development of multicellularity, and differentiation in plants. Uses of protoplasts also include somatic selection, cell fusion and hybridization, genetic engineering, and use of regenerates as substitutes for spores, zygotes or seeds. Few such studies have been done with algae. The chemical composition of algal cell walls differs so much from that of vascular plants that wall-digesting enzymes used for obtaining protoplasts of vascular plants do not work on algal tissues. Berliner (1981, 1983), Cheney (1986), Polne-Fuller et al. (1986) and Saga et al. (1986) have summarized much of the work to date on protoplast production and regeneration in eukaryotic algae. Only a small number of seaweeds have yielded protoplasts which could regenerate multicellular calli or thalli (e.g. Kloareg and Quatrano 1987; Ducreux and Kloareg 1988). Such success has been obtained in only two genera of multicellular red algae, *Porphyra* (Tang 1982; Polne-Fuller and Gibor 1984; Polne-Fuller et al. 1984; Saga and Sakai 1984; Fujita and Migita 1985; Chen 1987) and *Gracilaria* (Cheney et al. 1986).

In this paper we describe the isolation and regeneration of protoplasts from gametophytes of *Porphyra nereocystis* Anderson (Rhodophyta, Bangiales), a large (up to 3 m long), monostromatic species which in nature occurs as an epiphyte on the large, canopy-forming brown alga, *Nereocystis luetkeana* (Mertens) Postels et Ruprecht. While, in nature, *P. nereocystis* is always an epiphyte on *N. luetkeana*, it is not an obligate epiphyte and can be grown in the laboratory independently (Dickson and Waaland 1985). It has an heteromorphic life cycle in which a small, filamentous phase, called the conchocelis, alternates with the macroscopic blade. In this and most other *Porphyra* species, the blades are haploid and the conchocelis phase diploid (Hawkes 1978; Ma and Miura 1984; Burzycki and Waaland 1987; Ohme and Miura 1988; Mitman 1988). *Porphyra nereocystis* has an excellent flavor and has received serious consideration for use as the edible product called "nori" (Woessner 1977) for which other *Porphyra* species are now being cultivated in Washington State (Merrill and Waaland 1988).

Of the protoplast-preparation methods for *Porphyra* used by other workers some are very time consuming, some result in relatively low yield, and others require extraction of enzymes from particular microorganisms or algivorous molluscs. Our goals in this work were: (i) to develop a simple, rapid and efficient procedure

* This paper is dedicated to the memory of the late Dr. Munenao Kurogi (1921–1988), Professor Emeritus of Hokkaido University

** *Present address:* Department of Oenological Chemistry, Columbia Winery, 14030 NE 14th St., Woodinville, WA 98072, USA

Abbreviations: AAP = abalone acetone powder; PAP = papain; FDA = fluorescein diacetate

using commercially available enzymes, and (ii) to obtain protoplasts that would regenerate.

Material and methods

Plant material. Porphyra nereocystis was collected from stipes of *Nereocystis luetkeana* growing attached at Turn Rock east of Turn Island and Pt. George, Shaw Island, or drifting near Friday Harbor, San Juan Island in the San Juan Island Archipelago of northern Washington. The plants were kept on ice and returned to the laboratory where they were temporarily maintained in illuminated (25 μmol photons $\cdot m^{-2} \cdot s^{-1}$) aquarium tanks (10° C). Pieces of tissue (17-mm-diameter disks) were excised for short- or long-term laboratory culture and grown in aerated flasks (f/2 medium, McLachlan 1973; 10° C, 25 μmol photons $\cdot m^{-2} \cdot s^{-1}$) or were used within 24–48 h for protoplast isolation. In all experiments great care was taken to insure that any reproductive cells were excluded from the tissue selected for protoplast isolation; for most experiments tissue was taken from regions just adjacent to the axis of bilateral symmetry of a blade.

Enzyme preparation. The enzyme solutions for protoplast isolation were papain (P3375, Type II Crude Powder; Sigma Chemical Co., St. Louis, Mo., USA) which was used first at 10% w/v; and of abalone acetone powder (A7514; Sigma) which was used after the papain treatment at 2% w/v, dissolved in sterile seawater. In addition the enzyme solutions contained 0.5% potassium dextran sulfate (CalBiochem, San Diego, Cal., USA), 0.5 M mannitol (in the 2% abalone enzyme solution only) and were buffered at pH 6.0 (0.05 M 2-(N-morpholino)ethanesulfonic acid; Sigma). This mixture was homogenized by hand for 30 s, centrifuged 8 min at 17000 $\cdot g$, and filtered through an 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass., USA). The enzyme solutions were used within 30 min of preparation or frozen at −70° C for future use.

Protoplast isolation and viability tests. In our typical successful protoplast isolation protocol, thallus pieces (4–5 cm^2) were cleaned by treatment with 1% "Betadine" (Povidone-iodine; Purdue Frederick Co., Norwalk, Conn., USA) for 90 s, rinsed three times in sterile seawater, and 17-mm-diameter disks were cut out with a cork-borer. The disks were chopped to 1–2 mm^2 pieces, rapidly rinsed three more times in sterile seawater, and then incubated in the first enzyme solution. We have generally used a two-step enzyme treatment based on that of Fujita and Migita (1985). The first incubation was in 10% papain (PAP) at 10° C (a typical temperature for seawater around the San Juan Islands) for 0.5–2 h until some "softening" or distortion of the tissue was observable at ×50 with a stereo microscope or at ×100 with an inverted microscope. During incubation the tissue pieces were gently swirled on a rotary shaker at 100 rpm. They were then transferred to the 2% abalone enzyme (AAP) solution and incubated for 0.5–2 (up to 18) h at 10° C, 100 rpm on the rotary shaker. For some trials, 1% Onozuka Cellulase RS (Yakult Honsha Co., Tokyo, Japan) was added to the abalone enzyme solution, but it was not routinely used. Microscopic observation was used to ascertain protoplast release. Protoplasts were removed from the enzyme solution by filtration through a 35-μm nylon mesh followed by centrifugation at 500 rpm in 15-ml tubes in a benchtop clinical centrifuge for 4–5 min. They were washed and recentrifuged three times using sterile f/2 medium (pH 8.0) with 0.5 M mannitol. The final pellet was resuspended in 1–3 ml of f/2 with 0.5 or 0.25 M mannitol. To determine yield, samples were removed from this suspension and counted using a haemocytometer. The suspension was then dispensed into 10-ml of medium in 60-mm-diameter, 15-mm-high polystyrene Petri dishes at a density of 10^4 protoplasts per dish.

Absence of a cell wall on the protoplasts was confirmed by the change from the "rectangular" cross section of cells in tissue to the spherical shape of naked, wall-less cells, by the osmotic swelling and lysibility of such spherical, wall-less cells in distilled water, by the absence of a cell wall when examined by transmission electron microscopy, and by lack of fluorescence after staining with 0.01% w/v Calcofluor White ST (American Cyanamid, Bound Brook, N.J., USA) which tests for presence or absence of wall material (see Waaland and Waaland 1975). Fluorescein diacetate ("FDA" F7378; Sigma) uptake was used to assay protoplast viability (Larkin 1976). We also examined the chloroplasts of these protoplasts for both chlorophyll and phycoerythrin fluorescence.

Electron microscopy. Freshly released and regenerating protoplasts were fixed 1–2 h with 2% (w/v) glutaraldehyde, post-fixed 1–2 h with 2% (w/v) osmium tetroxide, embedded in Epon, sectioned with a diamond knife, and examined in a JEOL (Tokyo) 100 electron microscope (Scott et al. 1980). A cacodylate buffer was used during fixation (Hepler 1981).

Protoplast culture and regeneration. Culture of isolated protoplasts was initiated by decreasing the osmoticum from 0.5 M mannitol to mannitol-free f/2 in a three-step dilution as follows: the first dilution was done by resuspending the protoplasts immediately after isolation in equal parts of 0.5 M mannitol f/2 and mannitol-free f/2; the second dilution was done by adding more f/2 at 2–4 h; finally, 18–20 h after isolation all the medium was replaced by mannitol-free f/2. Regenerating protoplasts were maintained at 10° C and a 16 h light:8 h dark regime with 10–25 μmol photons $\cdot m^{-2} \cdot s^{-1}$, in 60×15-mm polystyrene Petri dishes. Protoplast density varied from several hundred $\cdot ml^{-1}$ to approx. $3 \cdot 10^3$ ml^{-1}. An antibiotic mixture (streptomycin sulfate and kanamycin sulfate, Sigma, both at 50 μg $\cdot ml^{-1}$) was sometimes added to retard bacterial growth. The quality and condition of the protoplasts and their subsequent development were assessed using light microscopy.

Results and discussion

Isolation of protoplasts. Vegetative cells in intact blades of *P. nereocystis* are more or less rectangular in cross-section and have a single stellate chloroplast (Fig. 1). During the enzyme treatments, the cells become spherical as the cell wall softens. As the enzyme treatment progresses, protoplasts are liberated from the disintegrating "cuticle" and cell-wall matrix (Fig. 2). Freshly prepared protoplasts are 18–20 μm in diameter and have a conspicuous centrally or laterally positioned chloroplast (Fig. 3). Protoplast release often begins as early as 30–40 min after transfer from the PAP into the AAP enzyme solution and continues for up to 2 h. Longer incubation does not increase yields significantly. We have found the two-step treatment to be essential: a PAP-only or AAP-enzyme-only treatment does not yield useful quantities of protoplasts. Furthermore, it is essential to perform the PAP treatment first, followed by the AAP enzyme treatment. Very few protoplasts are produced if the order of enzymes is reversed, or if they are used simultaneously. Protoplast yields of 30–50% of the cells in the original tissue sample are typical, with some yields as high as 70%. A piece of tissue of 16 mg fresh weight (2.4 mg dry weight) and 17 mm diameter (227 mm^2) contains $0.65 \cdot 10^6$ cells and yields $0.2 \cdot 10^6$–$0.3 \cdot 10^6$ protoplasts. Curiously, commercial purified papain (Sigma P4762) was ineffective in preparing cells for the AAP-enzyme treatment.

Factors affecting yield. Protoplast isolation and yield are highly variable. Yield is affected by the age and "quali-

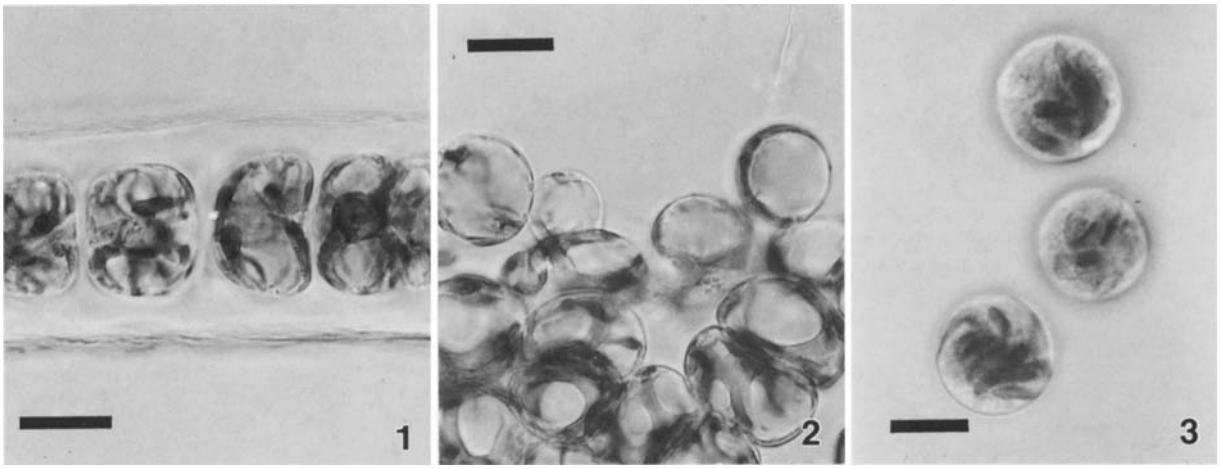

Fig. 1. Cross-section of intact *P. nereocystis* thallus showing rectangular cross-section of cells within cell wall, and hyaline "cuticle". Bar = 20 μm; × 640

Fig. 2. Swelling and dissolution of "cuticle" and cell wall of *P. nereocystis* near end of abalone-enzyme treatment as cells are released from dissolving wall. Bar = 20 μm; × 580

Fig. 3. Isolated protoplasts of *P. nereocystis* after washing and free of wall debris. Bar = 10 μm; × 1030

ty" or physiological state of the starting material. We have obtained protoplasts from plants taken directly from the field as well as from plants maintained in the laboratory for up to five weeks. Plants in laboratory culture for longer times rarely yielded protoplasts. Protoplast yield also depended on the portion of the blade used. Samples from the basal, central and terminal third of blades were tested for protoplast yield. The lowest yield (< 2%) was from the basal part, good yield (10%–30%) was from the middle third, and the best yield (30%–70%) was from the terminal third of the blade. Some tested blades failed to yield protoplasts; we have been unable to correlate such results with any identifiable property of the specimen, its cultural history, or the enzymes used. Such variability in yield from a given donor material is a common experience in plant protoplast research (Evans and Bravo 1983).

Approximately 40%–50% of field-collected plants will yield protoplasts if isolation is attempted within 24–48 h after collection. Approximately 30% of the plants which do not release protoplasts initially will do so if they are cultured in the laboratory for 7–25 d. Such changes in enzyme susceptibility most likely reflect a change in cell-wall composition during laboratory culture; however, preliminary data from field and cultured *P. nereocystis* have so far demonstrated only slight differences in the degree of sulfation of the porphyran component (Gretz and Waaland 1989). This is an area of research which merits further investigation.

A major difference between our protoplast-production method and that of others is its rapidity. With the exception of Chen's (1987) work with *P. leucosticta*, other *Porphyra* protoplast-production protocols use enzyme incubation times ranging from 5 to 24 h (e.g. Chen 1986; Polne-Fuller and Gibor 1984). Furthermore, even after long incubation, protoplast release may still require mechanical tissue homogenization as in *P. perforata* (Polne-Fuller and Gibor 1984).

Evidence of wall removal and viability. Evidence that we routinely produced true, wall-less protoplasts came from three sources: osmotic lysis, absence of Calcofluor fluorescence, and electron microscopy (Fig. 4). Evidence of viability was obtained by FDA staining which resulted in a bright yellow-green fluorescence localized in the cytoplasm. In addition, fluorescence microscopy showed that the chloroplasts of protoplasts produced a dull red fluorescence indicative of an intact photosynthetic apparatus. In contrast, the chloroplasts of unhealthy or dying protoplasts produced a very bright orange fluorescence as phycoerythrin was uncoupled from the photosynthetic pigment-antenna system.

Protoplast regeneration. Isolated protoplasts were observed periodically for features such as cell-wall formation and cell division which would be harbingers of regeneration. Transmission electron microscopy demonstrated occurrence of substantial cell-wall regeneration as early as 20 h after release from the original blade (Fig. 5). Calcofluor-positive cell-wall material was present in detectable amounts 48–72 h after isolation. We have observed earliest (5–7 days after isolation) regeneration (cell division) in protoplasts which were transferred as rapidly as possible from the isolation medium containing 0.5 M mannitol to mannitol-free medium so that the protoplasts were in mannitol-free medium 18–20 h after isolation. It is possible that removal of the osmoticum induces or permits cell-wall formation. Protoplasts maintained in the mannitol-containing osmoticum for extended periods (several days) do not divide and are often overgrown by bacteria. Although initially we prepare our protoplasts under sterile conditions, once we have isolated protoplasts, for longer-term culture experiments we ordinarily maintain the cultures as unialgal cultures in enriched seawater medium. Many multicellu-

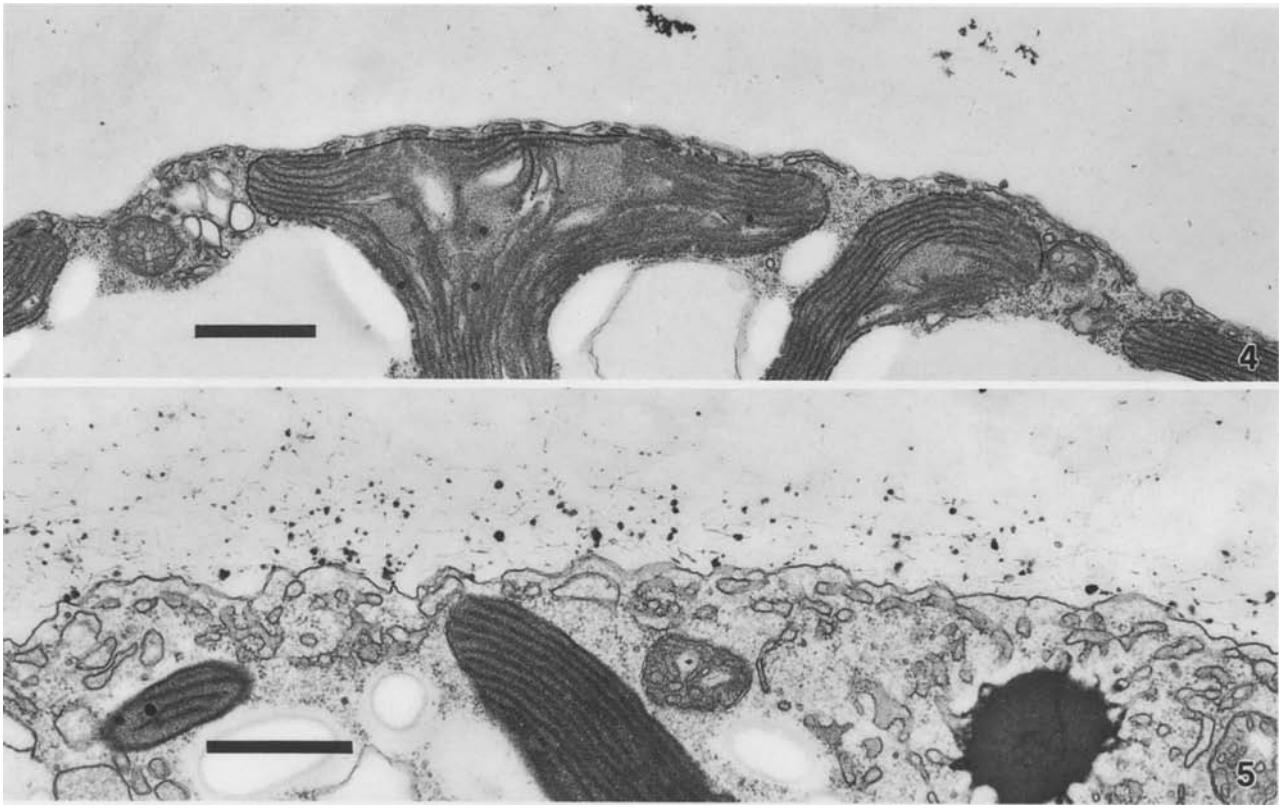

Fig. 4. Transmission electron micrograph of a freshly released protoplast of *P. nereocystis*; the cell wall is completely absent. The cytoplasm shows part of a chloroplast, mitochondria, starch grains and other cytoplasmic elements. Bar = 1 μm; × 16150

Fig. 5. Transmission electron micrograph of a 20-h protoplast of *P. nereocystis* showing fibrillar elements of the newly synthesized cell wall. Bar = 1 μm; × 20000

lar marine algae do not develop normally in axenic culture but require a bacterial microflora for expression of normal morphology (Provasoli and Carlucci 1974; Buggeln 1981).

We have observed three major regeneration pathways in protoplasts of *P. nereocystis*: a direct filamentous pathway, a direct callus-forming pathway, and an indirect blade-forming pathway. In each of these three pathways, the regeneration product comes from cells from the central portion of the *Porphyra* blade. This differs from the findings of Polne-Fuller and Gibor (1984) who used *P. perforata* and obtained different protoplast-regeneration pathways depending on what part of a parental blade was used as protoplast source.

Filamentous regeneration. The first evidence of filamentous regeneration occurred as outgrowths from the now-walled, former protoplasts approx. 10–14 d after protoplast isolation (Fig. 6). This type of regeneration occurred about 75% of the time. These outgrowths closely resembled the germination tubes observed during carpospore germination. In this case, however, multiple germination tubes (two to five) were typically produced instead of the usual single germ tube produced by a germinating carpospore. The size and overall appearance of the filaments resembled typical filamentous conchocelis-phase material of *P. nereocystis*. It is interesting that removal of the wall from a haploid vegetative cell from a *Porphyra* blade is sufficient to permit that cell to develop a morphology which normally occurs only after sexual reproduction has produced diploid cells (carpospores). In other species of *Porphyra* (Mukai et al. 1981; Frei and Preston 1964) and in a close relative *Bangia* (Frei and Preston 1964; Gretz et al. 1980) the thallus-phase (blade in *Porphyra*; cylinder in *Bangia*) has a cell wall rich in xylan while the conchocelis-phase has a cellulosic wall; there is also a marked difference in protein composition in the species investigated. Thus not only is a significant morphological change occurring during filamentous regeneration, there is also likely a substantial change in cell-wall composition. Another important feature observed in the filamentous regenerates is that they have pit connections between adjacent cells. Pit connections are complex structures in which a precisely formed and positioned aperture in the transverse septum is occluded by a pit plug and associated structures (Pueschel 1987). Such structures are absent in the haploid blades, but are present in normal, diploid conchocelis filaments.

When oyster shells were inoculated with the filamentous regenerates, they did penetrate the calcareous shell and grew in it as does carpospore-derived conchocelis. Such filamentous regenerates also produced groups of

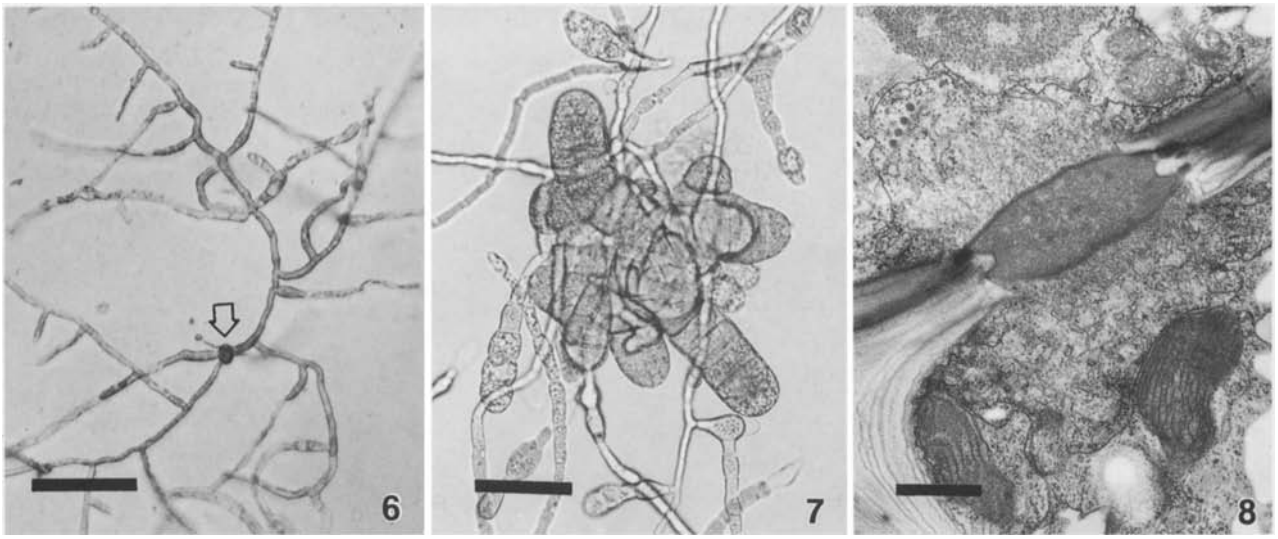

Fig. 6. Germinated protoplast of *P. nereocystis* (*arrow*) which has produced multiple germination tubes; subsequent development has produced conchocelis-like filaments. Bar = 100 μm; × 150

Fig. 7. Conchosporangia (enlarged cells or "fertile cell rows") produced by protoplast-derived conchocelis-like filaments of *P. nereocystis*; these subsequently released conchospores which germinated to produce blades. Bar = 50 μm; × 275

Fig. 8. Transmission electron micrograph of a callus of *P. nereocystis* showing a septal plug in a pit connection between callus cells. Pit connections have not been reported from *Porphyra* blade cells, but do occur between cells of the conchocelis phase. Bar = 1 μm; × 11 250

cells resembling "fertile cell rows" or conchosporangia of *P. nereocystis* (Fig. 7). When subjected to the dual-daylength photoperiod which induces conchospore release in *P. nereocystis* (Dickson and Waaland 1985), they released "conchospores". Germination of these spores was bipolar, typical of conchospores, and small blades were produced. At a size of several dozen cells, however, the small blades aborted. Unless this problem is overcome, the usefulness of this filamentous protoplast-regeneration pathway for gene-transfer experiments might be limited. Although we have not yet determined the ploidy level of the nuclei of these filamentous regenerates, our working hypothesis is that they are haploid since we saw no evidence of protoplast fusion (e.g. more than one chloroplast per cell or cells of double the normal volume) and because the regenerates originated from haploid blade cells. That haploid conchocelis-like morphology, complete with pit connections and "conchosporangia" which release "conchospores," can occur in haploid cells raises interesting questions about the relationship between ploidy and morphology in plants with heteromorphic life-history phases. However, other such cases are known in marine macroalgae (Nakahara and Nakamura 1973; Wynne and Loiseaux 1976; Kapraun and Freshwater 1987; van der Meer 1988). Several species of *Porphyra* have been reported to have the same chromosome numbers in both the blade and conchocelis phases (Mumford and Cole 1977), but in *P. nereocystis* Hawkes (1978) observed a haploid number of 3 in blades and a diploid number of 6 in conchocelis.

Callus formation. Callus formation from protoplast regeneration occurred by more than one pathway. Some callus masses developed directly by cell divisions; such calli were first recognizable three to four weeks after protoplast isolation. This occurred about 25% of the time. The cells of such calli had well-developed chloroplasts and pigmention. Their cell walls were relatively thick (2.5 μm in calli versus 0.25 μm in filaments). Typical calli reached 3–4 mm in diameter and large ones were up to 8–9 mm in diameter. Large calli may fragment to produce several smaller ones. Callus, which certainly lacks the "linear" organization of filamentous conchocelis, nevertheless produces pit connections and pit plugs (Fig. 8). Furthermore, the callus can produce blade outgrowths which appear to be free of pit connections. Other multicellular red algae traditionally classified in the subclass Florideophycidae have pit connections in both diploid and haploid life-history phases. It would be interesting to know what governs expression of pit connections in *Porphyra* which is traditionally classified in the subclass Bangiophycidae.

In addition to direct callus production by regenerating protoplasts in about 30% of the filamentous regenerates, we have also observed callus formation in the middle and at or near the edges of conchocelis-like filament masses and from thickened regions of sheets of cells formed by regenerating protoplasts. We have also observed production of small monospores from about 70% of the calli. Upon germination, these spores produced more callus. In other *Porphyra* species, such spores have been observed but have produced blades upon germination (see below).

Blade formation. Blade formation has occasionally been observed as outgrowths from about 1%–2% of the *P.*

nereocystis calli but not directly from individual, regenerating protoplasts. Formation of blades by regenerating protoplast tissue has been observed in other *Porphyra* species. For example, Polne-Fuller and Gibor (1984) reported that *P. perforata* calli growing on solid medium have developed foliose morphology when flooded with liquid medium. In other species of *Porphyra* small cells (sometimes referred to as monospores) produced by calli have germinated to produce blades (Fujita and Migita 1985; Chen 1986, 1987).

Conclusions. We have succeeded in developing a rapid and efficient method for producing large numbers of viable protoplasts from the marine red alga *P. nereocystis*. This method results in protoplasts capable of cell-wall formation, sustained cell division, and differentiation. Under uniform culture conditions, regenerates from these protoplasts have exhibited three different developmental pathways. Thus *P. nereocystis* protoplasts offer a useful and promising material in which to explore alternative propagation methods for nori, and to pursue studies of cell-wall formation, cell division, and development and differentiation of multicellular thalli. Furthermore, this material should undoubtedly prove useful in somatic selection, somatic hybridization, and gene-transfection experiments in this species and possibly other marine algae.

This research was supported by the Washington Sea Grant Program (National Oceanic and Atmospheric Administration). We thank Professor Y. Fujita (Nagasaki University, Japan), Professor S.-J. Wang (Shanghai University of Fisheries, P.R. China) and Dr. H. Kito (Seikai Regional Fisheries Research Laboratory, Nagasaki, Japan) for sharing their experience with *Porphyra* protoplast production with us. We thank J.S. Charleston for expert technical assistance in preparation of the electron-microscopy specimens. We also thank Dr. S.K. Herbert and John Carrier (Friday Harbor Laboratories) and Dr. John Merrill and D. Gillingham (American Sea Vegetable Co. and Applied Algal Research, Seattle) for collections of *P. nereocystis*.

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Received 2 February; accepted 12 Febuary 1990