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Novel sporophyte-like plants are regenerated from protoplasts fused between sporophytic and gametophytic protoplasts of *Bryopsis plumosa*

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Abstract Protoplasts of the marine coenocytic macrophyte *Bryopsis plumosa* (Hudson) C. Agardh. [Cauler-pales] can easily be obtained by cutting gametophytes or sporophytes with sharp scissors. When a protoplast isolated from a gametophyte was fused with a protoplast isolated from a sporophyte of this alga, it germinated and developed into either one of two completely different forms. One plant form, named Type G, appeared quite similar to a gametophyte, and the other, named Type S, looked similar to a sporophyte. While the Type G plant contained many small nuclei of gametophyte origin together with a single giant nucleus of sporophyte origin, the Type S plant contained many large nuclei of uniform size. These large nuclei in the Type S plant had metamorphosed from the gametophytic nuclei, and were not formed through division of the giant nucleus of sporophyte origin. Fragments of the Type S plant, each having such a large nucleus, developed into creeping filaments that look very similar to sporophytes. While cell walls of gametophytes and Type G plants were stained by Congo-red, those of the thalli of regenerated Type S plants and sporophytes were not stained by the dye. This indicated that the large nuclei of the Type S plant did not express genes for xylan synthesis, which are characteristic of gametophytes. Two-dimensional gel electrophoretic analysis revealed that most of the proteins synthesized in the Type S plant were identical to those of sporophytes. These results strongly suggest that in the Type S plant, the gametophytic nuclei are transformed into sporophyte-like nuclei by an unknown factor(s) produced by the giant nucleus of sporophyte origin and that the

transformed nuclei express the set of genes characteristic of sporophytes. Despite morphological similarity, however, the regenerated Type S plant could not produce zoospores, because its large nuclei did not divide normally. The transformed large nuclei of gametophyte origin still seemed to be in the haploid state.

Keywords *Bryopsis* · Cell fusion · Gametophyte · Nuclear transformation · Protoplast · Sporophyte

Abbreviations DAPI: 4',6-Diamidino-2-phenylindole · DIC: Differential interference contrast · IEF: Isoelectric focusing · PES: Provasoli's enriched seawater

Introduction

The marine green alga *Bryopsis plumosa* (Hudson) C. Agardh. has a heteromorphic biphasic life cycle, i.e., a macroscopic *Bryopsis* phase (gametophyte) and a microscopic, creeping filamentous phase (sporophyte) (Tatewaki 1973). The body of the gametophyte is composed of a large cylindrical coenocytic thallus with beautiful pinnae. In laboratory conditions, however, such a pinnate gametophyte can only be obtained when it is cultured with gentle aeration (Yamagishi et al. 2003). The dioecious gametophyte of *B. plumosa* contains many small haploid nuclei. When it matures, the pinnae become either male or female gametangia, and a number of male or female gametes are generated within these gametangia. By contrast, a sporophyte is composed of a small, creeping, sparsely branched cell, which contains a single, large diploid nucleus. When maturation of the sporophyte is induced either spontaneously or experimentally, the giant nucleus undergoes meiosis, produces many small nuclei by the following mitosis, and finally forms a number of zoospores (Yamagishi et al. 2003).

Such great differences in morphology and physiology between gametophytes and sporophytes could be a reflection of different sets of genes being expressed

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during the two alternating generations. Analysis of such generation-dependent gene expression seems to be impractical with seed plants, because sporophytic generation occupies most of the life cycle of seed plants and their gametophytes are too greatly reduced. We demonstrate in this article that *B. plumosa* is a very suitable material for such an approach.

It is well known that when the protoplasm of the gametophyte of *B. plumosa* is squeezed out, protoplasts are easily formed, and these easily regenerate cloned gametophytes (Tatewaki and Nagata 1970; Kobayashi and Kanaizuka 1985). In the present study, we applied this phenomenon and the usefulness of *Bryopsis* to the study of nuclear reprogramming. We fused the two protoplasts isolated from a gametophyte and a sporophyte of *B. plumosa* and traced the morphology and fate of the nuclei of the regenerated plants. In this article, we report that some intracellular factors produced by a nucleus can regulate the gene expression of different co-existing nuclei in a fused protoplast. To our knowledge, this is the first attempt at cell fusion between different generations.

Materials and methods

Biological materials

Male and female gametophytes of *Bryopsis plumosa* (Hudson) C. Agardh. were collected from the habitat at Murohama, Miyagi, Japan in July 1998. A unialgal culture of gametophytes was achieved according to a previously described method (Yamagishi et al. 2003). Gametophytes were cultured in Provasoli's enriched seawater medium (PES; Provasoli 1966) at 23°C under continuous white light until use. Since *B. plumosa* is dioecious, male and female gametophytes were cultured separately.

Sexual reproduction of gametophytes was induced by aeration, as previously described (Yamagishi et al. 2003). Liberated male and female gametes were collected and fertilized by mixing them in a Petri dish, which was filled with PES medium. Within several days, zygotes germinated and started to grow as young sporophytes. In suitable culture conditions, i.e., at 23°C under long-day (14 h light:10 h dark regime) exposure to white light at an intensity of about 6 W m⁻², the sporophytes reached a length of about 10 mm 1 month after germination. One-month-old sporophytes were stored at 23°C under dim white light (1 W m⁻², 14 h light:10 h dark regime) until use. Although sporophytes never mature in these conditions, we used sporophytes within 4 months of storage.

Preparation of protoplasts from gametophytes and sporophytes

We prepared gametophytic protoplasts by placing upright thalli of gametophytes in a Petri dish, which was filled with PES medium, and squeezing protoplasm out of the cells. These protoplasmic masses coagulated into spherical protoplasts within several minutes. The protoplasts were divided into two groups according to size, i.e., 148–98 and 62–40 µm in diameter, by filtering through nylon mesh (Kyoushin Riko, Tokyo).

A sporophytic protoplast was obtained by cutting a sporophyte near the single giant nucleus and squeezing out the protoplasm, so that the protoplast always contained one giant nucleus. The sporophytic protoplasts were trimmed with fine tweezers so that they were about 70 µm in diameter. These protoplasts can easily be fused with each other by bringing one into contact with the other (Fig. 1a–c). In the present study, the term fused protoplasts always

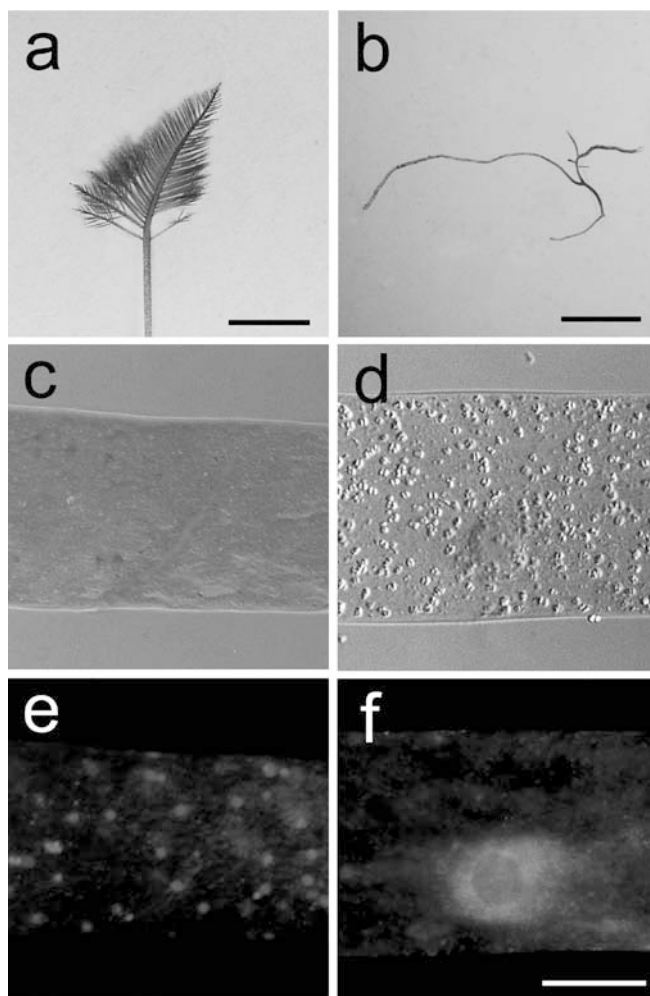


Fig. 1a–f Morphologies of the gametophyte and sporophyte of *Bryopsis plumosa* and those of their nuclei and giant nucleus. **a,c,e** Gametophytes; **b,d,f** sporophytes. DIC (**c,d**) and epifluorescence photomicrographs (**e,f**) are shown. Nuclei of a gametophyte cannot be seen with DIC optics (**c**), while a large spherical giant nucleus of a sporophyte is clearly seen with DIC optics (**d**). Bars = 20 mm (**a**), 15 mm (**b**), 50 µm (**e–f**)

indicates that the protoplasts were the result of fusion between one gametophytic protoplast and one sporophytic protoplast. Fused protoplasts thus produced were cultured in PES medium at 23°C under continuous white light (approx. 6 W m⁻²).

Staining of nuclei with SYBR Green I

Nuclei were visualized with SYBR Green I (Molecular Probes, Eugene, OR, USA) staining, as previously described (Yamagishi et al. 2003). Another well-documented stain 4',6-diamidino-2-phenylindole (DAPI) did not stain the giant nucleus at all. Nuclei were observed with an epifluorescence microscope (Axioplan 2 or Axioskop; Zeiss).

Extraction of proteins and two-dimensional gel electrophoresis

Plant materials (gametophytes, sporophytes or germlings from fused protoplasts) of 500 mg fresh weight were homogenized in a mortar with 2 ml of extraction buffer [50 mM Mes, 5 mM EGTA,

10 mM MgSO₄ (pH 6.8)] on ice. The homogenate was centrifuged at 10,000 *g* for 10 min. Soluble proteins were precipitated with 50% saturated (NH₄)₂SO₄ by keeping them at 0°C for 6 h. The precipitates were collected by centrifugation at 12,000 *g* for 20 min, and the pellet was resolved in a sample buffer [8 M urea, 1% (v/v) Triton X-100, 2% (v/v) Ampholine (pH 3.5–10), 5% (v/v) 2-mercaptoethanol].

Protein samples were analyzed by two-dimensional electrophoresis according to O'Farrell (1975). Isoelectric focusing (IEF)–PAGE was used for first-dimension separation and SDS–PAGE was used for second-dimension separation. The IEF–PAGE gel was composed of 8 M urea, 4% (v/v) polyacrylamide, 2% (v/v) Triton X-100, 1.6% (v/v) Ampholine (pH 3.5–10) and 0.4% (v/v) Ampholine (pH 3.5–5). The IEF–PAGE gels were run stepwise at 50, 100, 150 and 300 V for 1, 4, 9 and 1 h, respectively. The SDS–PAGE gel was composed of a resolving gel [13% acrylamide, 25% 1.5 M Tris–HCl (pH 8.8)] and a stacking gel [0.4% acrylamide, 25% 0.5 M Tris–HCl (pH 6.8)]. The stacking gel was run at 10 mA for 30 min and the resolving gel was run at 20 mA for 1 h. The electrophoresis gels were then stained with silver stain (2D-SILVER STAIN II “DAIICHI”, Daiichi-Kagaku, Tokyo).

Results

Fusion of protoplasts prepared from gametophytes and sporophytes

In a coenocytic tube of the gametophyte of *Bryopsis plumosa* (Fig. 1a), there are many small nuclei 5–6 μm in diameter (Fig. 1e). They cannot be seen, however, with differential interference contrast (DIC) optics (Fig. 1c). In contrast, a small, creeping and sparsely branching sporophyte (Fig. 1b) has only a single giant nucleus of about 20 μm in diameter (Fig. 1d,f). The large nucleus of the sporophyte can clearly be seen with DIC optics because chloroplasts are excluded from the perinuclear region.

When a protoplast from a gametophyte and a protoplast from a sporophyte of similar size were brought into contact with each other, they quickly fused together and became a spherical heterokaryon protoplast within 5 min (Fig. 2). The large giant nucleus and transparent perinuclear region can easily be observed in the fused protoplast (Fig. 2a–e).

Development of germlings

The fused protoplasts germinated and developed into two different types of plant. The majority, i.e., more than 70% of the fused protoplasts, developed into those shown in Fig. 3d,e. A minority of the fused protoplasts looked like a burr or a hedgehog as demonstrated in Fig. 3a–c. We hereafter term the former Type G and the latter Type S, as the former looks very much like a gametophyte, and the latter resembles a sporophyte. The two types of regenerated plant were easily distinguishable from each other at the early germination stage: in the case of Type G, a thick single germ tube arose from the protoplast 2–7 days after the fusion and it grew as an upright straight cylindrical thallus (Fig. 3d,e). The Type G plants also formed pinnae when they were aerated and even formed gametes within

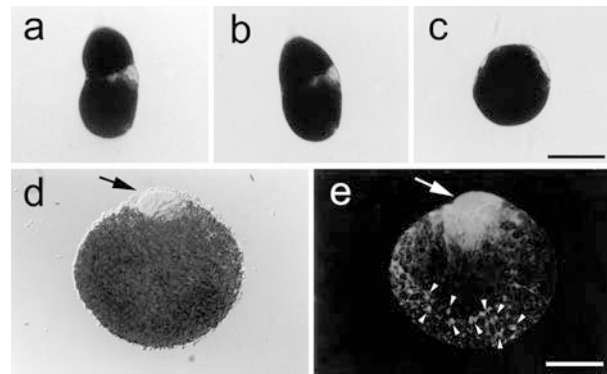


Fig. 2a–e Fusion between gametophytic and sporophytic protoplasts of *B. plumosa*. **a–c** A series of photographs shows the process of cell fusion between the protoplast of a gamete (*upper half*) and that of a sporophyte (*lower half*) 2 min (**a**), 3 min (**b**) and 5 min (**c**) after contact. **d,e** DIC (**d**) and epifluorescence (**e**) photomicrographs of an SYBR Green I-stained fused protoplast (**d** and **e** are the same specimen). *Arrows* indicate the single giant nucleus of sporophyte origin, and *arrowheads* indicate small nuclei of gametophyte origin. Bars = 100 μm (**a–c**), 50 μm (**d,e**)

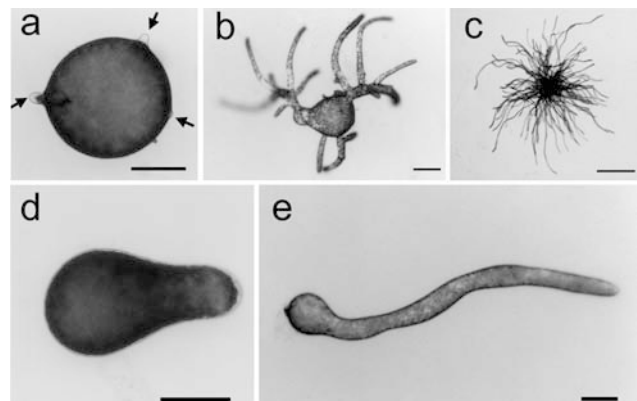


Fig. 3a–e Germination and development from the fused protoplasts of *B. plumosa*. **a–c** Development of Type S plants. Several thin germ tubes (*arrows*) appeared at the surface of the fused protoplast 14 days after the fusion (**a**), and the tubes grew as long as 2–3 mm by 17 days after fusion (**b**). The Type S plant became a burr-shaped spherule of 4–5 mm in diameter 25 days after the fusion (**c**). **d,e** Development of Type G plants. Only one thick germ tube appeared at 4 days (**d**), and it grew as a thallus that resembled a young gametophyte 7 days after the fusion (**e**). Bars = 50 μm (**a,b**), 2 mm (**c**), 100 μm (**d,e**)

their pinnae (data not shown). On the other hand, in the case of Type S, several very small growth points appeared 9–15 days after the fusion at the surface of the protoplast, which then developed into thin germ tubes with sparse branching (Fig. 3a,b). About 3 weeks after germination, the Type S plant had become a characteristic burr-shaped plant (Fig. 3c). Thus, the final morphology is determined at a very early stage and never changes to the other state.

Since gametophytes of *B. plumosa* are dioecious, protoplasts isolated from the gametophytes have either male or female nuclei. As the gender of a gametophyte is not discernible from its morphology until it forms gametangia, there is no morphological difference

between nuclei of male and female gametophytes. To examine whether or not the gender of the gametophyte may control the fate of the fused protoplast, we fused sporophytic protoplasts with either male or female gametophytic protoplasts of the same size. Figure 4a indicates that the gender of the gametophyte does not control the fate of the fused protoplast. Figure 4 also shows that the relative yield of the Type S plant to the total regenerated plants is about 30%.

On the other hand, fusion between large sporophytic protoplasts and small gametophytic protoplasts resulted in a higher yield of Type S plants (Fig. 4b). In this experiment, the size of sporophytic protoplasts was fixed

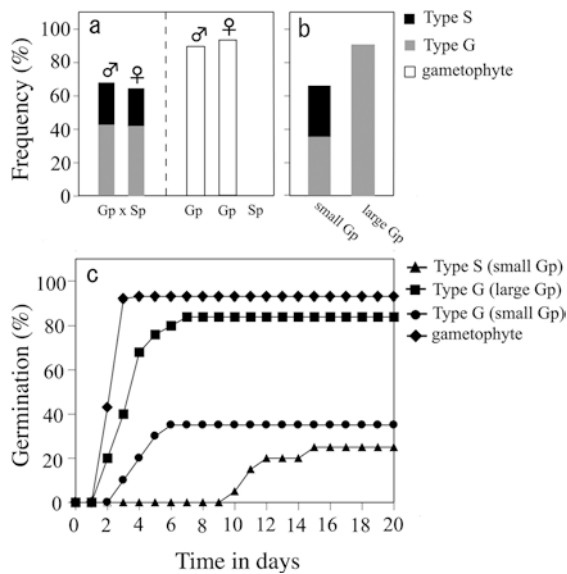


Fig. 4a,b Regeneration of Type S *B. plumosa* plants depends on the size of the gametophytic protoplast relative to that of the sporophytic protoplast and is independent of the gender of the gametophyte. **a** *Left* Yield of either Type S (black column) or Type G plants (gray column) relative to the total number of fusions between male or female gametophytic protoplasts (Gp) and sporophytic protoplasts (Sp). Protoplasts from a male or female gametophyte 70 μ m in diameter were fused with sporophytic protoplasts 70 \pm 1 μ m in diameter. The total germination rate (about 62%) and the relative yield of Type S plants are not significantly different between the two cases when male or female gametophytic protoplasts are fusion partners of sporophytic protoplasts. *Right* Germination rate of male and female gametophytic protoplasts and sporophytic protoplasts without fusion. Gametophytic protoplasts regenerated at a rate higher than 90%, irrespective of their gender, whereas isolated sporophytic protoplasts never germinated. **b** Frequency of Type S and Type G plants regenerated from fusions between sporophytic protoplasts and gametophytic protoplasts of two different size-classes. One-hundred small (small Gp, 40–62 μ m in diameter) or 100 large (large Gp, 98–148 μ m in diameter) gametophytic protoplasts (50 male and 50 female in each case) were fused with sporophytic protoplasts 70 \pm 1 μ m in diameter. The number of germlings was counted 16 days after the fusion. The relative yield of Type S plants increased with increased relative volume of sporophytic protoplast, but the total germination rate decreased. **c** Time course of germination in Type G plants, Type S plants, and gametophytes (data taken from **a** and **b**). Gametophytes and Type G plants germinated quickly, but Type S plants did not germinate before 9 days

at a diameter of about 70 μ m, while that of gametophytic protoplasts was set at a diameter of 40–62 μ m or 98–148 μ m. The volume-ratios of the pairs of protoplasts, i.e., $V_{\text{sporophytic protoplast}}/V_{\text{gametophytic protoplast}}$, were about 2.6 or 0.18, respectively. As shown in Fig. 4b, when sporophytic protoplasts were 2.6 times larger than gametophytic protoplasts (small Gp), the relative yield of Type S plants increased to about 50%, irrespective of the gender of the gametophytic partner. It should be noted, however, that the total regeneration rate decreased when larger sporophytic protoplasts were used.

Another clear difference between Type G and Type S plants is their time course of germination. As Fig. 4c shows, Type G plants germinated within 2 days after fusion. This is almost the same as in case of non-fused gametophytic protoplasts. Type S plants, however, did not germinate sooner than 9 days.

Fate of nuclei in Type G and Type S plants

Type G plants contain small nuclei 5–6 μ m in diameter introduced by a gametophytic protoplast plus a large giant nucleus about 20 μ m in diameter, introduced by a sporophytic protoplast (Fig. 5a,b). The SYBR Green I fluorescence (Fig. 5b) reveals, however, that in the Type G plant, perinuclear cytoplasm that surrounds the giant nucleus is thinner than that in the sporophyte (cf. Fig. 1f). In contrast, in Type S plants there are many large nuclei about 20 μ m in diameter, but no small nuclei (Fig. 6c–e). These large nuclei are surrounded by perinuclear cytoplasm, as in the sporophyte (Fig. 5d).

What are the large nuclei in the Type S plants? Are they enlarged small nuclei of gametophyte origin or are they generated from the giant nucleus of sporophyte origin by nuclear division? To answer this question, nuclei of the young Type S plant were investigated. As Fig. 6 clearly demonstrates, a 7-day-old, Type S plant contains only a single giant nucleus 20 μ m in diameter and several intermediate nuclei, i.e., 8–9 μ m in diameter, but no small nuclei. This strongly suggests that the small nuclei of gametophyte origin in the Type S plant expanded under the influence of the co-existing giant nucleus of sporophyte origin. Division of the single giant nucleus is ruled out, because the giant nucleus remains for quite a long period without becoming emaciated.

Enlarged nuclei of gametophyte origin now express sporophyte-specific genes

To address the role of enlarged nuclei in the Type S plant, we first checked the chemical properties of the cell walls. When young Type S plants were stained with Congo-red, which is known to stain the xylan in the cell wall of the gametophyte of *Bryopsis*, only the first regenerated cell wall of the fused protoplast was stained, whereas those of the extending germ tubes were not stained (Fig. 7a). As already shown in Fig. 6, the Type S

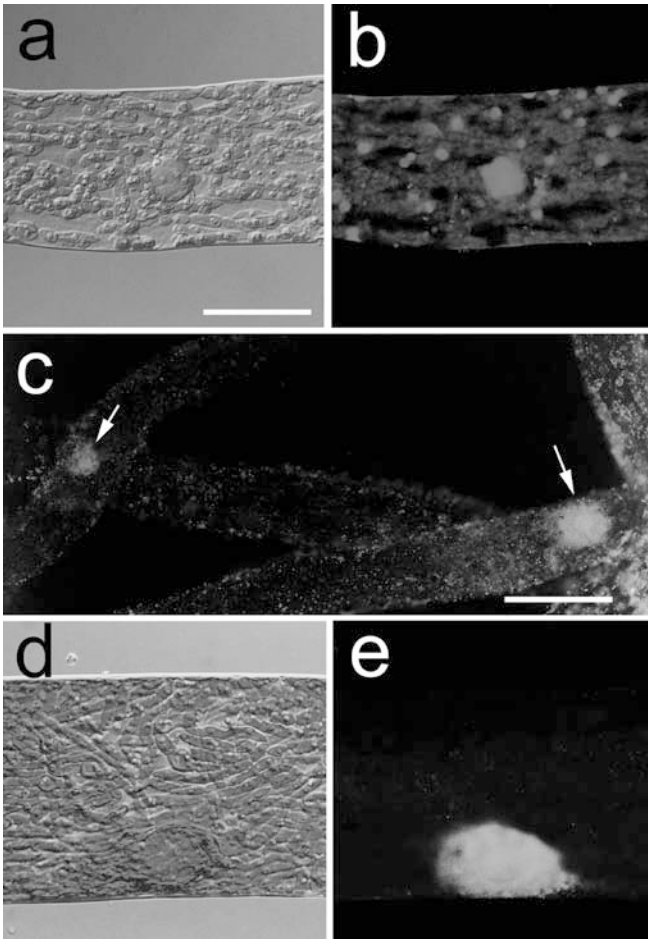


Fig. 5a–e Nuclei of Type G and Type S *B. plumosa* plants. Type G (a,b) and Type S (c,d) plants were fixed about 30 days after germination, stained with SYBR Green I, and observed with DIC optics (a,d) or epifluorescence microscopy (b,c,e). c Two large nuclei are seen in the thallus of a Type S plant. In size and morphology, they resemble the giant nucleus of a sporophyte, not those of a gametophyte. Bars = 50 μm (in a for a,b,d,e), 100 μm (e)

plant at this stage contains one giant nucleus of sporophyte origin and many intermediate-sized nuclei, which are transformed from the gametophytic nuclei. Which nucleus, then, produced the non-xylan part of the thallus cell wall? To answer this question, we removed the influence of this single giant nucleus of sporophyte origin. Several fragments, each having one enlarged nucleus, were excised from the burr-shaped Type S plants, and these fragments were further cultured for 3 weeks. The fragments grew into creeping filaments that closely resemble the sporophytes (Fig. 7b). Congo-red did not stain the cell wall of the sporophyte (Fig. 7d), which is known to be composed of mannan. While the Type G plant was stained well by Congo-red (Fig. 7c), the Type S plant regenerated from a fragment of Type S thallus was not stained (Fig. 7e), as was the case for the sporophyte. This indicates that the enlarged nuclei of gametophyte origin begin to express a different set of genes characteristic of the sporophyte generation.

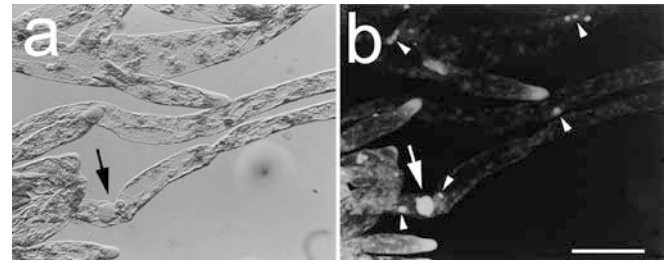


Fig. 6a,b Nuclei of a young Type S *B. plumosa* plant. A Type S plant at early developmental stage, i.e., 7 days after germination, contains one large nucleus of sporophyte origin and many intermediate-sized nuclei. a DIC and b epifluorescence photomicrographs. Arrows indicate a single giant nucleus of sporophyte origin and arrowheads, several intermediate-sized nuclei (8–9 μm in diameter) of gametophyte origin. Bars = 50 μm

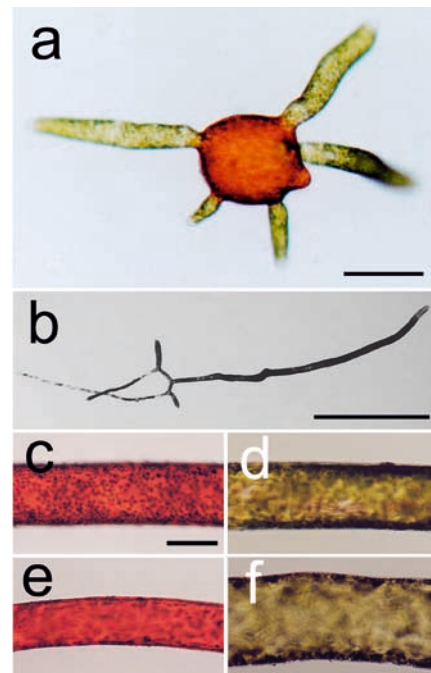
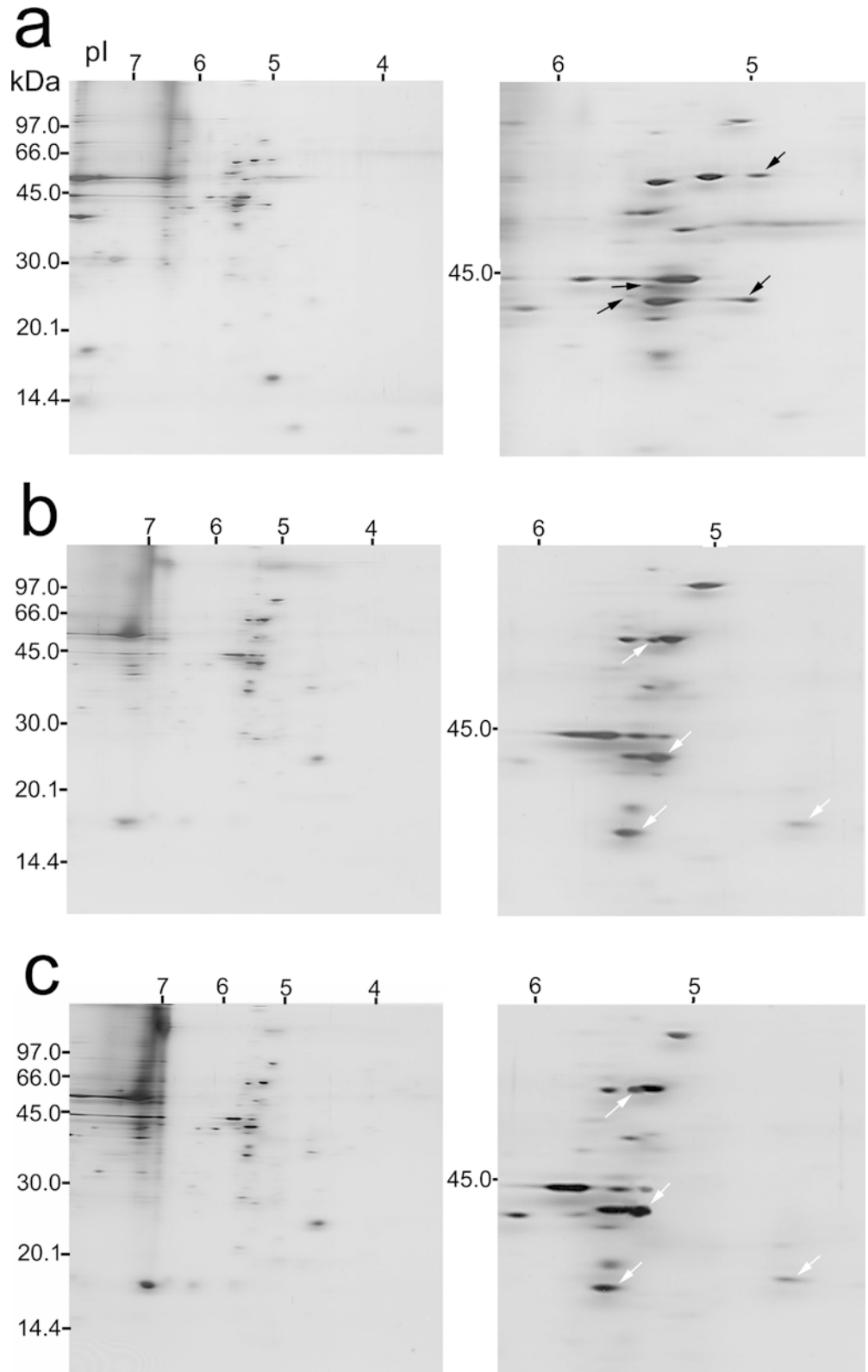


Fig. 7a–f Congo-red stains cell walls of gametophytes and Type G plants of *B. plumosa*, but not sporophytes and Type S plants. a Young Type S plant. The regenerated cell wall of the fused protoplast is stained by Congo-red, whereas spine-like thallus extensions from the protoplast are not stained, indicating that their cell walls do not contain xylan. b A fragment of a Type S plant that contains only one nucleus was cultured for 3 weeks. It greatly resembles the sporophyte. c Gametophyte stained densely by Congo-red; d sporophyte; e Type G plant; f Type S plant. Bars = 100 μm (a), 2 mm (b), 50 μm (in c for c–f)

To further confirm this possibility, soluble proteins of Type S plants were extracted and compared with those of gametophyte and sporophyte by means of two-dimensional gel electrophoresis. Figure 8 demonstrates that the spot pattern of Type S plants almost coincides with that of sporophytes. Looking at a small region of the gel (right-hand panels in Fig. 8a–c), for instance, several gametophyte- and sporophyte-specific spots can easily be detected. Type S plants (Fig. 8c) contain at least four spots common to sporophytes, but four

Fig. 8a–c Two-dimensional gel electrophoresis of proteins extracted from gametophytes, sporophytes and Type S plants of *B. plumosa*. Approximately 10 µg of protein samples extracted from gametophytes (a), sporophytes (b) and Type S plants (c) were separated by two-dimensional gel electrophoresis, in the first-dimension run (left to right) by IEF-PAGE and in the second-dimension run (top to bottom) by SDS-PAGE. Apparent molecular sizes (kDa) and isoelectric points (pI) are indicated on the left side and at the top, respectively. From the magnified photographs on the right, differences in spot pattern can be detected. *Black arrows* indicate spots only present in the gametophyte, and *white arrows* indicate spots only seen in the sporophyte and Type S plants



gametophyte-specific spots are missing. As demonstrated in Table 1, 198, 220 and 223 spots were detected for soluble proteins of gametophytes, sporophytes and Type S plants, respectively. Of the 223 protein spots of

Type S plants, as many as 217 spots are identical to those of sporophytes, whereas only 80 spots correspond to those of gametophytes. Since 78 of 220 sporophytic proteins correspond to those of gametophytes, and since

Table 1 Comparison of the protein spots expressed in Type S plants, gametophytes and sporophytes of *Bryopsis plumosa*, analyzed by two-dimensional gel electrophoresis

Plant type	Total No. of spots	No. of spots in common with gametophyte	No. of spots in common with sporophyte
Gametophyte	198	–	78 ^a
Sporophyte	220	78 ^a	–
Type S	223	80	217

^a78 Spots are common to both sporophytes and gametophytes

the total number of sporophytic proteins (220) is almost the same as that of Type S plants, it seems safe to conclude that Type S plants synthesize almost an entire set of proteins characteristic of sporophytes. Identification of these proteins and the proteomics are, however, not yet possible with *Bryopsis*.

Type S plants cannot produce zoospores

Maturation of the isolated and regenerated Type S plant having a single large nucleus was induced by the two-step culture method developed for sporophyte maturation (Yamagishi et al. 2003). Briefly, the plants were transferred to PES medium supplemented with 5 mM NaHCO₃ and placed under continuous white light (> 6 W m⁻²) for 4–5 days. When an ordinary sporophyte matures, the single giant nucleus starts meiosis and produces numerous small nuclei through successive mitoses (Fig. 9a). These small nuclei finally become zoospores. Figure 9c–e demonstrates the normal course of mitosis observed in a mature sporophyte. When the regenerated Type S plants, each having one transformed large nucleus, were brought to maturation, their large nuclei were also able to divide and produce many small nuclei (Fig. 9b). However, their size and morphology were very irregular and their nuclear division was quite abnormal (Fig. 9f–h): chromosomes were apparently fewer than those of sporophytes and they seemed to be unequally distributed. This can be explained if it is assumed that these large nuclei are still in the haploid stage. In fact, a regenerated Type S plant having metamorphosed nuclei cannot form any zoospores at all. Nevertheless, when an intact Type S plant was brought to maturation, zoospores were formed in and liberated from a single filament, leaving the rest of the filaments in the immature state (data not shown). This indicates that the original sporophytic giant nucleus remained intact in a Type S plant. It is impractical to choose in advance the particular filament having the original sporophytic giant nucleus, because all filaments look very similar.

Discussion

The proportion of Type S plants to total regenerated protoplasts is dependent on the volume ratio of the

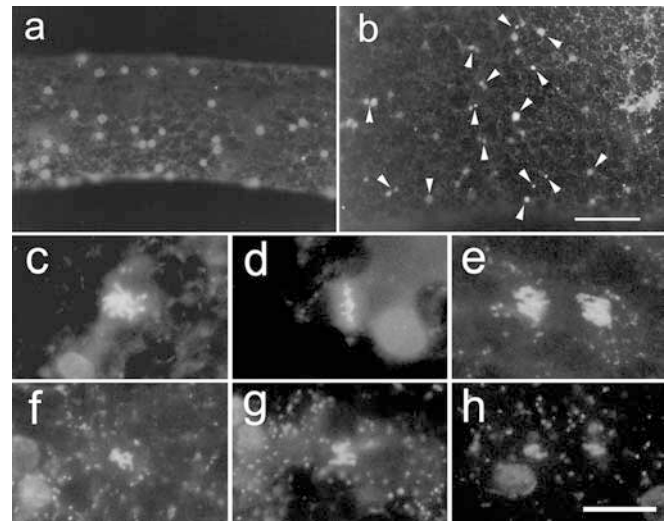


Fig. 9a–h Fate of nuclei in Type S plants of *B. plumosa* after induction of maturation. **a** Mature sporophyte. Uniform small nuclei formed by division of the mature giant nucleus are seen in epifluorescence microscopy after SYBR Green I staining. **b** Mature Type S plant. Sizes of the small nuclei (arrows) formed by division of the large nucleus are not uniform, ranging from 2 to 5 μm in diameter. **c–e** Enlarged images of prophase (**c**), metaphase (**d**) and anaphase (**e**) of the nuclei in mature sporophytes. **f–h** Abnormal division of nuclei in mature Type S plants. Bars = 50 μm (in **b** for **a,b**), 10 μm (in **h** for **c–h**)

sporophytic protoplast to the gametophytic protoplast, but not on the gender of the gametophyte (Fig. 4). Since there must be a clear correlation between number of nuclei and the size of a gametophytic protoplast, there may be a critical number of gametophytic nuclei, lower than that necessary for the development of Type S plants.

During the development of the Type S plant, the small nuclei of gametophyte origin gradually expand. When it is young, e.g., at 7 days after its germination (Fig. 6), the Type S plant contains one large nucleus of sporophyte origin, which is thought to be diploid (2n), and many intermediate-sized (i.e., 8–9 μm in diameter) nuclei. When the Type S plant matures, there are no longer small (5–6 μm in diameter) or intermediate-sized nuclei. Since all nuclei in the mature Type S plant are large and they have a transparent perinuclear cytoplasm, one cannot discriminate, even through SYBR-Green I staining, which is the original giant nucleus introduced from a sporophyte (Fig. 5e). This means that during development of the Type S plant, small nuclei of gametophyte origin (i.e., haploid, n) gradually expand up to the size of the giant nucleus of the sporophyte. The large nuclei in mature Type S plants are not formed through fusion of the small nuclei, because the number of small nuclei in the young Type S plant and that of large nuclei in the mature Type S plant are almost the same (data not shown).

It is well known that the cell wall of the gametophyte of *Bryopsis* is mainly composed of β-1,3-xylan as the skeletal polysaccharide, while that of the sporophyte is

mainly composed of β -1,4-mannan (Huizing et al. 1979). This is also the case in *Derbesia* (Huizing et al. 1979). As Congo-red and chlor-zinc-iodide stain only the cell wall of the gametophyte, the different stainability reflects a difference in cell wall composition. Congo-red does not stain cell walls of the sporophyte or filaments of the Type S plants. Since the Type S plant has many large nuclei introduced from a gametophyte and one giant nucleus of sporophyte origin, the question arises of which nucleus(-i) directs formation of the mannan cell-wall. By using regenerated Type S plants that have only one transformed large nucleus, we were able to confirm that the enlarged nuclei of gametophyte origin stopped expressing the genes for xylan synthesis (Fig. 7f). The cell wall of the regenerated Type S plant is very probably composed of β -1,4-mannan. This strongly suggests that instead of a set of genes characteristic of gametophytic generation, a new set of genes for sporophytic generation is expressed by the enlarged gametophytic nuclei.

This was further confirmed by the protein analysis (Fig. 8). The pattern of proteins synthesized in the Type S plant corresponds fairly well with that in the sporophyte. It should be noted, however, that regenerated Type S plants were not used in the protein analysis of Type S plants because obtaining a sufficient number of regenerated Type S plants was difficult. Nevertheless, this probably does not affect the results.

Our present results are explained in terms of nuclear reprogramming. Regeneration and development of the Type S plant from the protoplast fused between the gametophyte and sporophyte probably result from the activation of genes specific to sporophyte generation and simultaneous silencing of some other genes specific to gametophyte generation through the influence of cytoplasmic factors produced by the co-existing giant sporophytic nucleus. Also, in the Type G plant, gene expression of many gametophytic nuclei may supersede that of the sporophytic giant nucleus, because we found that the larger the gametophytic protoplast, the more Type G plants were regenerated (Fig. 4).

Division of the giant nucleus ($2n$) is the first noticeable sign of the sporophyte's maturation in *Bryopsis plumosa* (Yamagishi et al. 2003). Meiosis of the giant nucleus and subsequent repetitive mitoses of the secondary small nuclei (n) are followed by development of numerous stephanokontic zoospores (Rietma 1971). That the division of the giant nucleus constitutes meiosis was recently confirmed electron microscopically by the detection of a synaptonemal complex (Minamikawa et al. 2002). Meiosis of the large nuclei of the Type S plant was not detected in the present study. Since

abnormal nuclear division should be the result of unequal chromosome segregation during meiosis, the many large nuclei found in a Type S plant, except one true sporophytic nucleus, are considered to be in the haploid (n) stage. Normal meiosis is not accomplished in haploid *Ulva* (Hoxmark and Nordby 1974) and in many other haploid plants produced by anther culture, such as in wheat (Person 1955), oat (Nishiyama and Tabata 1964), pearl millet (Manga and Pantulu 1971) and barley (Sadasivaiah and Kasha 1971). Since chromosomes cannot be paired at pachytene, they are arbitrarily distributed to daughter cell, and hence there is no further growth.

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