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Ultrastructural and molecular evidence for monospore formation in the thallus of *Porphyra yezoensis*

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Abstract: It is well known that *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta) can release a kind of asexual spore, e.g. monospore, in its life history. Monospores have a significant value in cultivation of this seaweed. In order to ascertain the molecular mechanism in the course of monospore formation from blades, *P. yezoensis* thallus is divided into three areas that contained vegetative cells, spore mother cells, and unreleased monospores, based on their morphological feature. The light and electron microscopic observations on these tissues of each distinct region and the released monospores provided the evidence of gradual development from the vegetative cells to monospores. The color of cells alters from dark-green to golden-yellow, and the diameter decreases from 15 - 30μm to 10 - 13 μm, when the cell shapes become spherical from polygonal. During monosporogenesis, the vacuoles come to decrease and even disappear, and the chloroplast condenses gradually and gathers to the center of a cell, while the floridean starches and fibrillar vesicles come to emerge. The isolation of total RNA from the unicells or protoplasts that came from the nori tissues after enzymatic dissociation is developed. The ratios of A_{260} to A_{280} from vegetative cells, spore mother cells, and unreleased monospores are 2.046, 2.058 and 2.103, respectively. It suggests that this extraction method of total RNA is simple, efficient, and convenient. Complementary DNA is synthesized through reverse transcription PCR. It is found that the unreleased monospores lack 1 kb band that differs from the other samples on agarose gel of the amplified cDNA products. These results show that there is divergence during the differentiation and development of vegetative cells to monospores in *P. yezoensis*, but the molecular mechanism remains to be further probed into thoroughly.

Key words: *Porphyra yezoensis*; cDNA; mRNA; monospore; thallus; ultrastructure

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1 Introduction

Porphyra is a red alga genus that diverged early in red algal phylogenetic tree. It maintains several primitive features such as asexual reproduction through generation of monospores. *P. yezoensis* Ueda is one of the species

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in the genus that is able to generate monospores. Monospores develop directly from vegetative cells in foliose thallus, thus their genomes are haploid. A new nori individual with rhizoid and blade can be generated directly from a released monospore. In the light of this life history, monospores can also be regarded as a seedstock resource for *P. yezoensis* cultivation in addition to the conchospores. As early as in the 1960s, Wang et al.[1] have ever undertaken the experiment of nori seedling attachment with monospores released from *P. yezoensis* in Zhoushan, Zhejiang Province, China. Li[2] concluded that the growth of monosporelings was more rapid than that of conchosporelings, and the percentage of germination of monospores (86%) was higher than that of conchospores (31%) under the same conditions after comparative studies. Up to now, it is obscure whether the species produces monospores mainly by the specific environmental conditions or genetically. So it is necessary to know how to produce monospores, and how to regulate monospore formation in this red alga.

Kurogi[3] described several species of *Porphyra* and compared them for the selection of the best candidates for cultivation. In his descriptions of the thalli, three types of cells were mentioned: namely, the unique cells of the holdfast (with their long tapered extension), the sexually differentiated cells, and the vegetative cells. After developmental observations on the regeneration from the dissociated tissues of *Porphyra perforata* J. Agardh, Polne-Fuller & Gibor[4] mapped four areas containing different cell morphologies on *Porphyra* blades and identified five different cell types in them. Continuous studies on the electrophoresis of glycoproteins in differentiated regions on this red alga thallus, Kaska *et al*.[5] thought that the morphologically distinct regions of the thallus also differed biochemically. Polne-Fuller & Gibor[6] thought that the efficiency of protoplast isolation from thalli of four *Porphyra species* and the developmental patterns of the regenerating protoplasts depended on the types of tissues from which they were isolated. Using subtracted cDNA libraries, Liu *et al*.[7] obtained 8 unique clones for the sporophyte of Porphyra purpurea and 7 for the gametophyte. After confirmation of their phase-specificities by hybridization to sporophyte and gametophyte mRNA, these 15 phase-specific cDNAs were sequenced, two proteins encoded by the sporophyte-specific cDNAs and two by the gametophyte-specific cDNAs were identified by their similarity to databank entries. Afterwards, also in the same red alga, Liu *et al*.[8] found another gametophyte cell wall protein, which might play a role as a cell wall structural protein involved in cross-linking polysaccharides, and another sporophyte cell wall protein that was a novel member of the chymotrypsin family of serine proteases. In the meanwhile, Hong *et al*.[9] identified various tissue-specific markers in differentiated regions of the *P. perforata* thallus by comparing the differential display derived from RNA-PCR with arbitrary primers. All these suggested that the tissue specificity did exist in *Porphyra* thallus, and morphological differentiation might be manipulated spatially and temporally by specific genes. So it is reasonable to assume that the monospore developed from the vegetative cell in a nori blade is also regulated by specific genes. All these questions, such as what the molecular mechanism is, and how or why they can do it, are still in suspense.

With these in mind, we proposed the present experiments hoping to compare the differences during the formation of monospores from vegetative cells in *P. yezoensis* thalli. In addition to the light and electron microscopic observations, the basic molecular evidence would be provided. This was greatly beneficial for us to further understand the basic mechanisms of monosporogenesis in this red alga.

2 Materials and Methods

2.1 Algal sample collection and pre-treatment

P. yezoensis Ueda thalli were collected at sea in Ganyu County, Jiangsu Province on December 10, 1998, the best time just here in a year for monospore production. The collected samples were stored at −20 ℃ after

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being air dried. The stored samples were recovered and incubated in seawater for three days[10]. The healthy nori thalli were chosen and wiped clean with 5% NaOCl (v/v) in sterilized seawater, and then rinsed sequentially in five separate beakers of sterilized seawater. The clean blade was divided into three areas(Fig.1), e.g. vegetative cells, spore mother cells or monosporangia, and unreleased monospores, depending on the morphological feature.

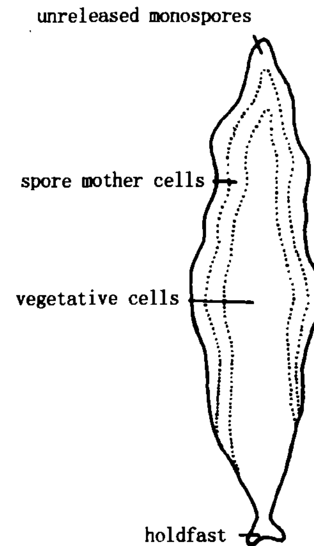

Fig.1 Diagram of *P. yezoensis* thallus showing the areas of vegetative cells, monospore mother cells, and unliberated monospores

2.2 Unicell isolation from nori thallus

About 0.5 g of the recovered and dissected *Porphyra yezoensis* thallus tissues were cut into pieces with a knife, and digested with 1% sea snail enzyme (Qingdao Oceanology University, Shandong, China) at 26℃ for one hour. The enzyme mixture was prepared in sterile seawater (salinity 1.020 – 1.022) supplemented with 0.8 $mmol \cdot L^{-1}$ mannitol, 10 $mmol \cdot L^{-1}$ $CaCl_2$, 10 $mmol \cdot L^{-1}$ $MgSO_4$, and the pH was adjusted to 5.8 – 6.2 with HCl. The isolated cells were passed through a nylon net (60μm in diameter) and washed with sterilized seawater for 3 – 4 times, then the cells were collected by centrifugation at 1500 $r \cdot min^{-1}$ for 3 min[10].

2.3 Preparation for light and electron microscopic observations

During the recovery of these clean and maturated blades in seawater, the liberation of monospore might often take place. After some physicochemical stimulation, the monospores could be released to a huge amount and they were collected after centrifugation at 5000 $r \cdot min^{-1}$ for 10 min at room temperature.

Some of the enzymatically dissociated unicells and the collected monospores were fixed immediately in 1% osmium tetroxide (w/v) in 0.2 $mol \cdot L^{-1}$ phosphate buffer (pH 7.3), and the ultrastructural examination was processed as described in detail by Wang & He[10] and Wang[11]. The recovered nori thalli could be directly used for light microscopic observation.

2.4 RNA extraction

Some of the isolated unicells from *P. yezoensis* thallus were transferred to a porcelain mortar and ground in liquid nitrogen. The cell powder was poured together with liquid nitrogen to a 15 mL polypropylene tube, and let the liquid nitrogen to evaporate. Then 1 mL of Trizol reagent (GIBCO BRL, Rockville, MD) was added, and the mixture was transferred to a 1.5 mL Eppendorf tube. Total RNA was extracted with Trizol reagent following the manufacturer's protocol.

For isolation of RNA from *P. yezoensis* monospores, the Eppendorf tube containing monospores was brought from liquid nitrogen, thawed quickly between fingers, and 200μL of Trizol reagent was added immediately. After being replaced in liquid nitrogen, the monospores could be broken in Trizol reagent by pipeting up and down. Then the RNA extracts were combined to a new 1.5 mL tube, and extracted as described above.

After being air dried, the total RNA pellet was re-suspended in 20 μL of DEPC-treated TE and incubated at 65 ℃ for 5 min. An aliquot of 1 μL sample was diluted in 99 μL of water for a spectrophotometric assay at A_{260} and A_{280} to estimate the quantity. An aliquot of 1 – 2μg of RNA was loaded in 1.1% formaldehyde-denatured agarose gel following the procedures described by Sambrook *et al*.[12] to examine the quality.

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2.5 PCR - based synthesis of cDNA and electrophoresis

SMART™ PCR cDNA synthesis kit (Clontech Laboratories, Inc.) was used to reverse transcribe mRNA from the total RNA of *P. yezoensis* with a poly-d(T) linker primer, and subsequently to amplify the whole cDNA with primers following the manufacturer's protocol included in the kit.

About 7μL PCR amplified products were loaded in 1.2% agarose gel containing $0.5\mu g \cdot mL^{-1}$ ethidium bromide for electrophoresis, and run for 1.5h at 110 V in 1 × TAB buffer (40 $mmol \cdot L^{-1}$ Tris-acetate, pH 8.0, 1 $mmol \cdot L^{-1}$ EDTA).

3 Results

3.1 Light microscopic observation at different stages during the formation of monospore

In view of the monospore formation in *Porphyra yezoensis* thallus, except for the released monospores, one thallus could be divided into three areas (Fig. 1). The tissues or cells of these areas could represent characteristic features of different developmental stages. Although the boundary among these areas wasn't very sharp due to the existence of transitional cells, the typical patches of cells could be identified in view of different size, color and morphology (Plate - 1, 2, 3, 4).

Plate - 2 showed the morphological features of cells just above and near the holdfast. These cells were typically vegetative. The diameter of cells was about 15 - 30μm. It was rectangular or polygonal in outline. Many cells contained a large vacuole located towards the holdfast. The color of cells was dark green or greyish green. The chloroplast appeared asteriate in the cell.

The cells located on the top or near the margin of the blade were spore mother ones or monosporangia (Plate - 3). They were slightly smaller than vegetative cells in size. They were polygonal in outline and with a diameter of about 15 - 25μm. Small or no vacuoles were found in the cells. Although the color of cell was brownish red, the color of the periphery was lighter because chloroplast began to condense to a thicker lump in the center.

The margin of the upper part in a blade was occupied by the unliberated monospores (Plate - 4). At the stage of monospore formation, cell wall existed, but the gap among the cells increased well. The cells were nearly spherical in outline and about 10μm in diameter. The color was reddish yellow or golden. No vacuoles were found and the outline of the chloroplast was similar to the monospore mother cells. The refractivity of cells was very strong. Plate - 5 showed the monospores were being released. The cells assumed yellow or golden color and were about 10 - 13μm in diameter. The chloroplast was condensed further in the center and had a stronger refractivity.

The cellular morphology at the stages during the course of monospore formation was summarized in Table1.

Tab.1 Morphological features of the cells from *P. yezoensis* thallus at a light microscope in the course of monospore formation

	vacuole	color	diameter of cell (μm)	cell shape	chloroplast	refractivity	space in cells
vegetative cell	big	dark green	15 - 30	polygonal	asteriate, big	no	small
monospore mother cell	small or no	red-brown	15 - 20	polygonal	concentrated to the center, indistinctly asteriate	little	small
unreleased monospore cell	no	red or golden-yellow	10	spherical	much more concentrated to the center	strong	big
monospore	no	golden-yellow or -red	10 - 13	spherical	much more concentrated to the center	strong	cells separated

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3.2 Ultrastructure observation on monosporogenesis

The vegetative cells after enzymatic dissociation were nearly spherical (Plate – 5). Each cell possessed a stellate chloroplast with long or short arms varying in size and radiating from the center. The chloroplast was so large that it almost occupied the whole volume of cell. There was a large pyrenoid in the center of chloroplast, and its diameter was up to 7μm. Several thylakoids were concentrically parallel to the chloroplast envelope and around the pyrenoid. The nucleus was located laterally and between the membranes of cytoplasma and chloroplast. It varied in shape. Mitochondrion dispersed near the end of chloroplast arms. Many vacuoles varied in size and outline occurred in the cells. There were few or none of floridean starch granules.

Many floridean starch granules were formed out in the cells (Plate – 6). A lot of small vesicles containing a large amount of electron-dense fibrillar materials appeared among the arms of the chloroplast. Sometimes several fibrillar vesicles could be fused into large ones. The chloroplast was smaller in size than that of the vegetative cells. The nuclear pores appeared to be more concentrated in certain areas of the envelope. There was an increase in the number of mitochondria during the development. Mitochondria often appeared in the vicinity of floridean starch granules.

In an unliberated monospore, floridean starch grains were still increasing in number (Plate – 7). Large quantities of fibrillar vesicles aggregated around the chloroplast. Fibrillar materials were also increased, and they formed one or several electron-dense particles in the vesicles. The volume of a chloroplast was decreased and the arms became shorter and thicker. The nucleus was obvious and the nucleolus was bigger.

The monospore had a low electron-density (Plate – 8). There is no cell wall but only a layer of membrane surrounded the cells. The chloroplast was condensed into the center and the arms were shortened in length. Its volume was much smaller than that of vegetative cells. The cells were filled with small or large vesicles so that it was hard to distinguish other organelles, except mitochondria. Floridean starch grains dispersed between vesicles and small vacuoles. During the liberation of monospores, the vesicles could migrate toward the periphery of the cell and subsequently, sometimes expelled their fibrillar contents.

Table 2 showed the ultrastructural changes during the course of monospore formation.

Tab.2 Ultrastructure changes of *P. yezoensis* developmental cells in the course of monospore formation

	chloroplast	vacuole	floridean starch	fibrillar vesicle
vegetative cell	large	many	few or no	no
monospore mother cell	less large	many	a few	a few
unreleased monospore	reduced and gathered	reduced	many	many
monospore	small and concentrated to the center	almost disappeared	many	much more

3.3 Molecular evidence in the monosporogenesis

As we know, the genomic DNA itself does not show clearly the direct differences during the development of monospores in the red alga, but RNAs, especially the message RNA (mRNA) is sure to provide the molecular evidence. After enzymatic dissociation, the unicells from the red algal blade tissues could be easily used for total RNA extraction. Plate – 9 illustrated the electrophoretic pattern of total RNA from the red algal tissues and released monospores. Four bright bands of the total RNA represented 28S, 23S, 18S, and 16S rRNA, respectively. The purity of extracted RNA with this approach is high qualitative according to the ratio of A_{260}/A_{280}, which is usually above 2.0 (Table 3). About 20 – 40μg of total RNA were obtained from one gram of thalli. The mRNA was further purified and the corresponding cDNA was synthesized. Plate – 10 presented the pattern of every tissue and monospore cDNA.

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It was of interest to find that the cDNA from the unliberated monospores lacked 1 kilobase (kb) band. This result suggested that morphological and ultrastructural changes during the formation of monospores would be related to the expression of specific genes. That is to say, monosporogenesis is regulated spatially and temporally as well.

Tab.3 Yield and purity of total RNA extracted from the monospores and unicells of *P. yezoensis* thalli after enzymatic treatment

	A_{260}/A_{280}	yield ($\mu g \cdot g^{-1}$)
vegetative cells	2.046	16.8
spore mother cells	2.058	25.3
unreleased monospores	2.103	26
monospores	2.076	10.4

4 Discussion

Developmental cells during the course of monospore formation can be obtained from different parts of a monospore-releasing thallus. Although the delimitation of these areas with cells different in morphology and structure exists actually, the boundary among them is not very obvious, showing the development course from the vegetative cells to monospores is progressive. New organelles, such as small or large vesicles and floridean starch granules, appear and copious fibrous materials accumulate during the developmental course. Cell outline and color, mitochondrion number, chloroplast size and distortion also have a change accompanying the formation of monospores.

The most obvious observation of monosporogenesis from vegetative cells is the cell color and chloroplast changes at a light microscope level. The latter change will also be observed under the electron microscope. During the course, the chloroplast contracts progressively towards the center of cell. Possibly as a result of the varied contents or compositions of pigments, the color becomes golden or red progressively from green. This will be further confirmed by extra experimental data.

The other most characteristic feature of *P. yezoensis* monosporangia is the prolific production of small and large fibrillar vesicles that appear to be formed primarily by dictyosome activity. This is in agreement with the results of other ultrastructural investigations of monosporogenesis in the Bangiophyceae[13-16]. The fibrillar vesicles also appear in carpospores of *P. variegata*[16] and *P. yezoensis*[11], and also in other sexual or asexual spores[13]. So it is generally thought that the fibrillar vesicles play a role in spore (or gamete) release and subsequent adhesion to the substrate. In addition, Wang[11] thought that the aggregation of small or large vesicles in monospores prepared for the formation cell wall after settlement and germination.

McBride & Cole[15, 16] noted an increase in amount of endoplasmic reticulum and starch granules during the formation and germination of monospores in Smithora naiadum. In *P. yezoensis* only the latter was noted in the present research, which was in agreement with the observations in *P. gardneri* by Hawkes[13]. As well in the cytological study on *P. yezoensis*, Kito[14] did not describe the appearance of endoplasmic reticulums in monospores and vegetative cells. The floridean starch granules, however, are observed in all these genera during monosporogenesis. It is conceivably inferred that it may be associated with material and energy storage for monospore germination because that there is a marked loss of floridean starch granules noted in *Smithora naiadum* with this process[16] and in carpospore germination of *P. variegata*[17]. Floridean starch granules are present closely to the chloroplast in monospores, and often accumulate around mitochondria, which is helpful and effective to storage or recycled metabolism of material and energy.

Of course, there must be other variations unknown only under light or electron microscopy during the monospore formation in *P. yezoensis*. But from the phenomenon observed, it is concluded clearly that the development of vegetative cells into monospores is a progressive process from quantitative to qualitative. This will also draw our attention to some researches at gene levels on the inner intrinsic changes.

Molecular biology studies of development now use two general approaches for the isolation of tissue- and developmental stage-specific mRNA sequences. These methods do not require any prior knowledge of the target

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genes or their products[7]. Here we report the preliminary results of RNA extraction and cDNA variation during monosporogenesis in *P. yezoensis*.

Initially, we have tried to isolate total RNA directly from the thallus of Porphyra yezoensis using Trizol reagents. The extracted RNA pellet, however, is difficult to be re-suspended in TE buffer. The dissolved RNA solution was very sticky, possibly due to prolific amounts of polysaccharides. So the enzymatic dissociation of tissues was decided to be processed prior to the extraction of total RNA. The dissociated cells from *P. yezoensis* blades can be successfully used for total RNA isolation, and the monospores can be directly used for this purpose without any enzymatic treatment. The quality of obtained RNA with this method is good enough for the following research. Such an electrophoresis pattern of total RNA is similar to that of wheat[18].

After a PCR-based cDNA synthesis, as shown in Plate - 10, the cDNA size is between 0.3 - 0.5 kilobase (kb). In the electrophoresis pattern, several bright bands representing the highly expressed genes appear, as seen in human placenta cDNA in control. The cDNA patterns for the four RNA samples are highly reproducible suggesting that the total RNA samples of monospore and three monosporeforming cells in thalli of *P. yezoensis* are in good quality to be reverse-transcribed into cDNA. The electrophoresis pattern of cDNA also shows that there is no 1 kb band occurring in the unliberated monospores hinting that the genetic materials have a hand in the development from vegetative cells to monospores in *P. yezoensis*. It is thought that environments are regarded only as the stimuli but inherent genetic materials of cells play a leading role in this process. This may be one of reasons why distromatis species and species with two chloroplasts in a cell do not produce monospores, and why some monostromatic species produce monospores while others do not. What the specific genes are during the monosporogenesis remains to be further studied.

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Explanation of Plate

1. Polygonal and dark green vegetative cells with asteriate chloroplasts of *Porphyra yezoensis* thallus (× 600); 2. Irregular and red-brown monospore mother cells with an indistinctly asteriate chloroplasts of *P. yezoensis* thallus (× 600); 3. Approximately round and red- or gold-yellow chloroplasts are concentrated and occupied at the center of the cells from *P. yezoensis* thallus (× 600); 4. Arrows show that round and gold-yellow or -red monospores are released from the edge of *P. yezoensis* thallus (× 600); 5. Ultrastructure of vegetative cells isolated from enzymatically dissociated tissues of *P. yezoensis* thalli, showing chloroplast (c), mitochondrion (m), nucleus (n), nucleolus (nu), pyrenoid (p), and vacuole (v); 6. Ultrastructure of monospore mother cell isolated from *P. yezoensis* thalli by enzymatic dissociation, showing chloroplast (c), floridean starch (fs), mitochondrion (m), nucleus (n), nucleolus (nu), pyrenoid (p), and vacuole (v). Arrow shows the nuclear pores; 7. Ultrastructure of unreleased monospore isolated from *P. yezoensis* thallus by enzymatic dissociation, showing chloroplast (c), floridean starch (fs), mitochondrion (m), nucleus (n), nucleolus (nu), and vacuole (v); 8. Ultrastructure of monospore of *P. yezoensis*, showing chloroplast (c), floridean starch (fs), fibrillar vesicle (fv), mitochondrion (m), nucleus (n), and pyrenoid (p). Arrow shows the release of contents in one fibrillar vesicle; 9. *P. yezoensis* total RNA in 1.1% formaldehyde-denatured agarose gel from monospores (lane 1), unreleased monospores (lane 2), monospore mother cells (lane 3), and vegetative cells (lane 4).; 10. Electrophoresis pattern of cDNA synthesized by a PCR-based method from the RNA samples as shown in Plate - 9, human placenta total RNA was used as a control for reverse transcription and PCR reactions (lane 6). Lanes 1 is marker of 100 bp ladder, 2 vegetative cells, 3 spore mother cells, 4 unliberated monospores, 5 monospores, and 7 marker of λDNA *Hind* III/ *EcoR* I

条斑紫菜单孢子形成的超微结构及分子生物学证据

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摘要:条斑紫菜具有放散单孢子的特性,其在紫菜苗种生产中具有极其重要的意义。为了探索条斑紫菜营养细胞分化发育成单孢子的机理,以条斑紫菜叶状体为研究材料,依据单孢子的形成过程,将其划分为营养细胞、单孢子母细胞(或单孢子囊)及未释放的单孢子等3个区域,同时收集刚刚放散的单孢子,相应地代表了单孢子发生过程中的四个阶段。显微及超微结构观察结果表明,随着营养细胞向单孢子的分化,细胞颜色由深绿色变成金黄色,大小由营养细胞的15~30μm变成单孢子的10~13μm,形状由多角形变成圆球形,细胞内的液泡逐渐变小以至消失,星状叶绿体逐渐浓缩并居细胞中央,红藻淀粉和大小纤维囊泡大量出现,线粒体数目显著增加等等。对上述3个区域的组织进行酶解获得单细胞,然后提取RNA,其产率为每g细胞获得RNA 20~40μg,A_{260}/A_{280}的比值分别为营养细胞2.046、单孢子母细胞2.058及未释放的单孢子2.103,显示这种提取RNA的方法是简便、有效、可行。对mRNA进一步纯化后通过反转录PCR合成cDNA,发现4个阶段的cDNA电泳图谱有些变化,特别是未释放的单孢子明显缺少1 kb条带,说明在单孢子形成过程中遗传信息的表达发生变化。但导致单孢子产生的分子机理仍有待进一步研究。

关键词:条斑紫菜; cDNA; mRNA;单孢子;叶状体;超微结构
中图分类号:S917;Q248 **文献标识码**:A

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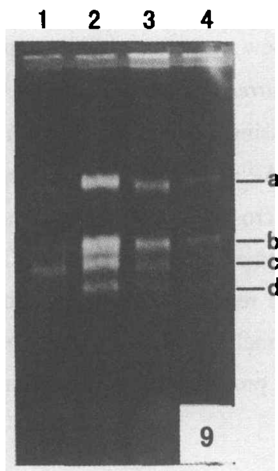

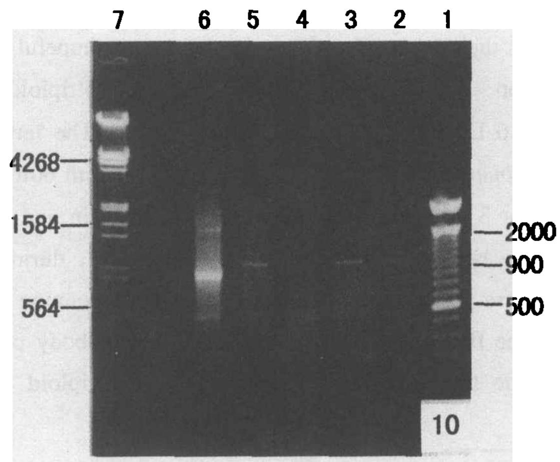

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