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**Photosynthesis-Dependent Extracellular Ca<sup>2+</sup> Influx Triggers an Asexual Reproductive Cycle  
in the Marine Red Macroalga *Porphyra yezoensis***

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## ABSTRACT

*Asexual propagation to increase the number of gametophytic clones via the growth of asexual haploid spores is a unique survival strategy found in marine multicellular algae. However, the mechanisms regulating the asexual life cycle are largely unknown. Here, factors involved in the regulation of production and discharge of asexual spores, so-called monospores, are identified in the marine red macroalga *Porphyra yezoensis*. First, enhanced discharge of monospores was found by incubation of gametophytes in ASPMT1, a modified version of the previously established synthetic medium ASP<sub>12</sub>. Comparison of the compositions of ASPMT1 and our standard medium, ESL, indicated that the Ca<sup>2+</sup> concentration in ASPMT1 was three times lower than that in ESL medium. Thus, we modified ASPMT1 by increasing its Ca<sup>2+</sup> concentration, resulting in reduction of monospore discharge. These findings demonstrate the role of reduced Ca<sup>2+</sup> concentrations in enhancing monospore production and release. Moreover, it was also observed that initiation of asexual life cycle required illumination, was repressed by DCMU, and was induced by a Ca<sup>2+</sup> ionophore in the dark. Taken together, these results indicate that photosynthesis-dependent Ca<sup>2+</sup> influx triggers the asexual life cycle by promoting the production and discharge of monospores in *P. yezoensis*.*

**Keywords:** *asexual life cycle, Bangiophyceae, Ca<sup>2+</sup> influx, monospore, photosynthesis, *Porphyra yezoensis*, Rhodophyta, synthetic medium*

## 1. Introduction

Asexual life cycle occurs in multicellular eukaryotes including algae and fungi [1-3]. In the sexual haploid-diploid life cycle of multicellular plants, meiosis in the diploid sporophyte produces a haploid gametophyte and syngamy (fertilization) of male and female gametes restores the diploid sporophytic genome [3-5]. By contrast, free-living haploid gametophytes of marine macroalgae often produce asexual spores that develop into haploid gametophytic clones by mitotic cell division without ploidy change [6]. Extensive analysis of plant haploid-diploid life cycles has progressed with recent genomic and genetic studies in *Arabidopsis thaliana*, which identified candidate genes involved in the regulation of the sexual life cycle [7-9]. In contrast, despite an accumulation of morphological and cytological observations, mechanisms of regulation of the asexual life cycle are still largely unknown, due to the lack of a model plant for investigation of the asexual life cycle.

*Porphyra yezoensis*, a red macroalga included in Bangiophycideae of Rhodophyta, has recently received considerable attention as a promising model macroalga for physiological and molecular biological studies of marine red algae, which largely depends on the establishment of laboratory cultures in which the haploid-diploid life cycle can be completed in a short period [10]. As for most of Bangiophycideae red algae, asexual spores, so-called monospores, are produced in monosporangia typically occurring at the marginal region of the gametophyte in *P. yezoensis* [11], although a biphasic sexual life cycle, which consists of morphologically distinct macroscopic gametophytic blades in winter and microscopic sporophytic filaments in summer, is predominant [11,12]. Thus, it is important to analyze the production and development of monospores in *P. yezoensis* to elucidate the biological significance and regulatory mechanisms of asexual propagation in multicellular red algae.

In the asexual life cycle of *P. yezoensis*, monospores are released from monosporangia and migrate while undergoing morphological changes, then adhere to the substratum and divide asymmetrically to produce two different vegetative and rhizoid cells [13-17]. The early development of monospores has been extensively studied, revealing the critical involvement of photosynthesis-dependent extracellular  $\text{Ca}^{2+}$  influx and phosphoinositide signaling, including phosphatidylinositol 3-kinase and phospholipase C, in the establishment of cell polarity that directs migration and adherence of monospores [16-19]. In contrast, there is only a few information about monospore production and discharge. Gametophytes may be induced to release monospores by changing water temperature and light conditions [20] and by irradiance with strong light [21]. Also, high yields of monospores from gametophytes treated with allantoin have been reported [22]. A mutant showing high levels of monospore production has also been isolated and characterized [23]; however, this mutant has not yet been analyzed to determine the mechanisms regulating the production and discharge of monospores in *P. yezoensis*. Therefore, knowledge of the mechanisms regulating production and discharge of monospores from gametophytes in *P. yezoensis* is not complete.

Here, we identified factors involved in regulation of the asexual life cycle in *P. yezoensis*. Our establishment of an artificial synthetic medium for the culture of *P. yezoensis* clearly demonstrated that extracellular  $\text{Ca}^{2+}$  influx plays a critical role in the production and discharge of monospores from gametophytes, and that these processes depend completely on photosynthetic activity. These findings could provide new insights into the regulation of the eukaryotic asexual life cycle.

## 2. Materials and Methods

### 2.1. Growth of gametophytes and discharge of monospores

The cultivation of gametophytic blades of *P. yezoensis* strain TU-1 was performed as previously reported [16]. Briefly, the ESL (enriched SEALIFE) medium, which is made by dissolving commercially available SEALIFE powder (Marintech Co. Ltd., Tokyo, Japan) in distilled water (DW) with the addition of ESS<sub>2</sub> (enriched seawater Saga<sub>2</sub>) solution, was renewed weekly until gametophytes were about 3 mm long. Subsequently, gametophytes were grown in ESL or various synthetic media as mentioned in the text. Two pieces of 1 cm polyvinyl alcohol (PVA) monofilaments to which gametophytic blades (ca. 3 mm long) attached were transferred to 100 mL of culture medium. The culture media were continuously bubbled with filter-sterilized air under 60  $\mu\text{mol}/\text{m}^2/\text{s}$  irradiance with a photocycle of 10 h light and 14 h dark at 15°C, with weekly renewal of culture medium. The length of a total of 10 gametophytic blades was measured to calculate their growth rate. Observation of monospore discharge was performed by three different ways as below. First is comparison of accumulation of monospores on the bottom of culture flask, for which flasks were placed on white paper to make photographs taken using a digital camera (Canon PowerShot G10). Second is observation of monospores attached to PVA monofilaments that photographed using the stereomicroscopic (Leica S8AP0) equipped with Nikon DigitalSight (DS-L2). The last is direct counting the number of monospores, for which 6 pieces gametophytes (ca. 3 mm long) were transferred to 35 mm tissue culture dishes (Iwaki Scitech Div., Asahi Techno Glass) containing ESL or various synthetic media and then the number of discharged monospores was counted under the inverted microscopic (Leica DMIL) during 7 days.

## **2.2. Quantification of pigment contents**

Four-week-old gametophytes cultured in ESL or synthetic media were used for calculation of photosynthetic pigments (chlorophyll a, Chl a; phycoerythrin, PE; phycocyanin, PC) and carotenoids (Car). Chl a and Car were measured using a method described previously [24], while extraction and measurement of PE and PC were carried out according to the method Beer and Eshel [25].

## **2.3. Calculation of magnesium, potassium and calcium ion contents**

Concentrations of magnesium, potassium and calcium ions were determined for ESL and ASPMT1 with a polarized Zeeman atomic absorption spectrophotometer (Hitachi Z-6100) according to the manufacture's protocols.

## **2.4. Treatment of gametophytic blades with pharmacological reagents**

The photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 100 mM stock solution. The calcium ion-specific chelator ethylene glycol tetraacetic acid (EGTA) (Dojindo Laboratories, Japan) was dissolved in distilled water (DW) to create a 0.5 M stock solution adjusted to pH 8.0 with NaOH. The calcium ionophore A23187 (Sigma, USA) was dissolved in DW to prepare a 1.0 M stock

solution. The stocks prepared above were added to the each medium to treat gametophytic blades at the working concentrations indicated below. The pH of medium was adjusted at 8.0 after adding of these reagents. The concentration of the solvents DW and DMSO did not exceed 0.4% and 0.1%, respectively, after addition to the media. At the same time, appropriate control experiments were performed with DW at concentration corresponding to the maximum volume of the reagents. For evaluation of the effects of each inhibitor, gametophytes standing-cultured in 35 mm tissue culture dishes were treated with each of pharmacological reagent at working concentrations indicated in the text.

### **3. Results**

#### **3.1. Enhancement of monospore discharge in synthetic medium**

*Porphyra yezoensis* is routinely cultured in our laboratory in ESL [26], which is made with SEALIFE powder whose chemical composition is proprietary. However, it is important to use a culture medium whose chemical compositions are defined for our purpose, so that the medium can be modified experimentally. To date, many kinds of synthetic medium for culturing marine algae have been described, including the ASP series [27,28]. Among these, we selected ASP<sub>12</sub> that was successfully used for the culture of several algae [29,30]. In this study, ASPMT 1 (ASPMT: ASP modified by M. T.) (**Table 1**), in which compositions of ASP<sub>12</sub> were slightly modified, was used as a primary medium.

First, we compared the growth of *P. yezoensis* in ESL and ASPMT1. As shown in **Figure 1**, vegetative cells of ASPMT1-cultured gametophytes assumed a round shape and deep red color

(**Figure 1(b)**), and the growth of gametophytic blades cultured in this medium was significantly slower than that in ESL (**Figure 1(a),(e)**). In addition, when the contents of Chl a, PE, PC and Car were measured for gametophytic blades cultured in ESL or ASPMT1 for 4 weeks, PE content was higher in ASPMT1-cultured gametophytes, whereas Chl a, PC and Car contents showed no significant differences between ESL- and ASPMT1-cultured gametophytes (**Table 2**). A remarkable feature of ASPMT1-cultured gametophytes was an increased discharge of monospores relative to ESL-cultured gametophytes (**Figure 1(f)**). Indeed, a large number of monospores were observed on the bottom of flasks and PVA monofilament in ASPMT1 than ESL (**Figure 1(c),(d)**).

According to these results, we speculated that differences in the chemical composition of ASPMT1 and ESL (see **Table 1**) are responsible for the differences in the degree of red coloration and discharge of monospores.

### **3.2. Nitrogen and phosphorus are not involved in red coloration and monospore discharge**

To identify factors influencing the pigmentation of vegetative cells and discharge of monospores, we changed the composition of ASPMT1 and compared the color and growth of gametophytes cultured in these modified media with those in the ESL. Excess nitrogen and phosphorus are known to be responsible for reduced growth rate and increased pigmentation in *P. yezoensis* [31], and the lack of nitrogen is known to result in growth inhibition and discoloration [32,33]. In addition, phosphorus content in the medium also influences the growth of *P. yezoensis* gametophytes [31,32]. Thus, we first hypothesized that reducing nitrogen and phosphorus concentrations could increase growth rate and reduce strong red coloration occurring in ASPMT1. To test this possibility, we reduced NaNO<sub>3</sub> from 100 mg/L to 60 mg/L in ASPMT1 as is in ESL. The concentration of

Na<sub>2</sub>-glycerophosphate was also reduced to 8 mg/L, and K<sub>3</sub>PO<sub>4</sub> was eliminated from ASPMT1 due to the lack of corresponding information about the K<sub>3</sub>PO<sub>4</sub> concentration in the ESL medium. The modified medium was designated ASPMT2 (**Table 1**). Following culture of *P. yezoensis* gametophytes in ASPMT2 for 4 weeks, a small increase in the growth of gametophytes was observed (**Figures 1(a),(e)**), which was due to a small increase in the size of vegetative cells attributable to an increase in the cytoplasmic chloroplast-free space compared with ASPMT1-cultured gametophytes (**Figure 1(b)**). However, there was no significant change in the degree of monospore discharge (**Figure 1(f)**), because consistently larger quantities of monospores were observed on the bottom of flasks and PVA monofilaments in ASPMT1 or ASPMT2 compared with ESL (**Figure 1(c),(d)**). In addition, culture in ASPMT2 for 4 weeks did not affect red coloration (**Figure 1(b)**) and there were no significant changes in the contents of photosynthetic pigments compared with ASPMT1-cultured gametophytes (**Table 2**). These results indicate that differences in the concentration of nitrogen and phosphorus between ASPMT1 and ESL are not responsible for the red coloration of gametophytes and enhanced discharge of asexual monospores.

### **3.3. Decrease in the extracellular Ca<sup>2+</sup> concentration results in red coloration of vegetative cells and enhancement of monospore discharge**

As shown in **Table 1**, the concentrations of MgCl<sub>2</sub>, MgSO<sub>4</sub>, KCl, K<sub>3</sub>PO<sub>4</sub> and CaCl<sub>2</sub> are unknown in ESL. Thus, the concentration of Mg<sup>2+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> in both ESL and ASPMT1 were measured by determining atomic weight using the polarized Zeeman atomic absorption spectrophotometer. We found that although Mg<sup>+</sup> and K<sup>+</sup> concentrations were similar in these two media, remarkable differences in the Ca<sup>2+</sup> concentration was observed; that is, ESL contains Ca<sup>2+</sup> concentration three

times greater than that in ASPMT1 (**Figure 2**), suggesting that the decreased concentration of  $\text{Ca}^{2+}$  in ASPMT1 and ASPMT2 may be responsible for the observed growth and red pigmentation effects on *P. yezoensis* gametophytes.

To address this possibility, we made two modified versions of ASPMT2 with  $\text{CaCl}_2$  concentrations two times greater (800 mg/L) (ASPMT3) or half (200 mg/L) (ASPMT4) (**Table 1**). Gametophytes cultured in ASPMT3 for 4 weeks exhibited the same rate of growth as those grown in ESL (**Figure 3(a),(e)**), whereas gametophytes grown in ASPMT2 or ASPMT4 (**Figures 1(a) and 3(a)**) exhibited negative growth effects relative to growth in ESL. In addition, although ASPMT4 had no effect on the recovery of color and cell shape, vegetative cells of gametophytes cultured in ASPMT3 showed normal shape and color as those grown in ESL (**Figure 3(b)**). Although cultivation in ASPMT3 resulted in reduced concentration of Chl a and PE compared with ESL-cultured, PE/Chl a, PC/Chl a and PE/PC were similar between ASPMT3 and ESL-cultured gametophytes (**Table 2**). On the other hand, culture in ASPMT4, whose  $\text{Ca}^{2+}$  concentration is approximately 6 times lower than that in ESL (**Table 1 and Figure 2**), Chl a and PE contents were higher than those in ESL-cultured gametophytes (**Table 2**). Moreover, large quantities of discharged monospores were observed in ASPMT4, while the number of monospores discharged from gametophytes grown in ESL and ASPMT3 was similar to each other (**Figure 3(f)**). Furthermore, large numbers of monospores adhered to the bottom of flasks and PVA monofilaments in ASPMT4, while a smaller number of monospores was observed in ESL and ASPMT3 (**Figure 3(c),(d)**).

We therefore concluded that the concentration of  $\text{Ca}^{2+}$  in the medium is an important factor responsible for the negative effects of ASPMT1 on growth, coloration and monospore discharge in

gametophytes, indicating that the extracellular concentration of  $\text{Ca}^{2+}$  has an important influence on the asexual reproductive life cycle in *P. yezoensis*.

### **3.4. Photosynthetic activity regulates discharge of monospores**

Consistent with previous reports showing that discharge of monospores is light-dependent in *P. yezoensis* and *Bangia atropurpurea* [21, 34], strong inhibition of the discharge of monospores was observed under dark conditions (**Figure 4(a)**). To understand the role of the light, we tested whether photosynthetic activity is required for the discharge of monospores using DCMU, an inhibitor of electron transport on the acceptor side of Photosystem II. When gametophytes were treated with 0.1, 1 or 10  $\mu\text{M}$  DCMU for 3 days, after which photosynthesis was gradually inhibited but not showed the cell death (data not shown), the number of discharged monospores decreased in a concentration-dependent manner (**Figure 4(b)**). These results indicate that photosynthesis is another important factor regulating the asexual life cycle in *P. yezoensis*. Thus, enhancement of monospore discharge is regulated by both a decrease in extracellular  $\text{Ca}^{2+}$  concentration and illumination resulting in photosynthesis (**Figures 3 and 4**).

### **3.5. Extracellular $\text{Ca}^{2+}$ influx is required for discharge of monospores**

We next addressed the interaction between extracellular  $\text{Ca}^{2+}$  concentration and photosynthesis. First, extracellular  $\text{Ca}^{2+}$  was reduced by the addition of EGTA to ESL and modified media ASPMT3. After 3 days of treatment of gametophytes with 0.5 or 1.0 mM EGTA, which did not bring cell death (data not shown), the discharge of monospores was enhanced by EGTA in both

ESL and ASPMT3 (**Figure 5(a)**). Thus, it is possible that a decrease in extracellular  $\text{Ca}^{2+}$  may affect the influx of  $\text{Ca}^{2+}$  into vegetative cells. Based on these findings, we then examined the effects of the artificial influx of  $\text{Ca}^{2+}$  on monospore discharge in the dark using the calcium ionophore A23187. As shown in **Figure 5(b)**, although gametophytes cultured in the dark did not produce monospores, as mentioned in **Figure 4(a)**, a number of monospores were released from gametophytes treated with 1  $\mu\text{M}$  A23187 in the absence of illumination, in which no cell death was observed (data not shown), although effects of A23187 treatment on monospore discharge were not observed under the illumination because of strong influence of photosynthesis on monospore discharge as shown **Figure 4**. These above-mentioned results clearly indicate that  $\text{Ca}^{2+}$  influx is photosynthesis-dependent and a critical factor directing the asexual life cycle in *P. yezoensis*.

**Figure 5(b)** shows that the effects of A23187 on monospore discharge were different between ESL and ASPMT3. Since  $\text{Ca}^{2+}$  concentration of ASPMT3 is less than that of ESL (**Table 1** and **Figure 2**), we propose that gametophytes cultured in ASPMT3 may acquire the potential of monospore discharge during cultivation. In addition, it was also observed that extensive deficiency of extracellular  $\text{Ca}^{2+}$  caused by the addition of 2.0 and 5.0 mM EGTA in ASPMT3 and ESL inhibited the discharge of monospores (**Figure 5(a)**). The viability of vegetative cells under these conditions were quite low (data not shown), suggesting that inhibition of monospore discharge by EGTA treatment may be due to cell death before or after the formation of monosporangia.

#### 4. Discussion

The presence of asexual reproduction via monospores is a remarkable strategy for survival in Bangiophycideae red algae because of its absence in land plants. Because the origin of asexual

monospores can be traced back to 1,200 MYA based on fossil records of monospores of an ancient Bangiophycean alga [35], research into the asexual life cycle of modern red algae might provide novel information regarding the origin and evolution of mechanisms regulating the eukaryotic life cycle. To date, it has been known that artificial changes in culture systems provided information about production and discharge of monospores [20,21]; however, regulatory mechanisms of this asexual reproduction mode have not yet been extensively analyzed. In the present study, we demonstrated that photosynthesis-dependent extracellular  $\text{Ca}^{2+}$  influx triggers the production and discharge of monospores from gametophytes of *P. yezoensis* via the establishment of artificial synthetic medium for the laboratory culture of this organism.

The importance of extracellular  $\text{Ca}^{2+}$  influx in the production of monospores (**Figures 3 and 5**) indicates the close relationship between enhanced monospore production and changes in both morphology and color of vegetative cells in gametophytes (**Figures 1 and 3**). We have previously reported that monospore production requires the formation of monosporangia in which vegetative cells become small, red-pigmented monospores [16]. Thus, the characteristics of cells found in gametophytes cultured in ASPMT1 and 2 (see **Figure 1(b)**) resemble those naturally occurring in monosporangia. Accordingly, we proposed that the decrease in extracellular  $\text{Ca}^{2+}$  due to incubation in ASPMT4 resulted in the extensive formation of monosporangia throughout the entire gametophyte followed by enhanced discharge of monospores due to the resulting greater area devoted to the production of monosporangia compared with ESL-cultured gametophytes. Based on this prediction, we hypothesized a mechanism for the formation and discharge of monospores (**Figure 6**). Briefly, the reduction of extracellular  $\text{Ca}^{2+}$  concentration stimulates the formation of monosporangia at the edge of gametophytes in which photosynthesis-dependent extracellular  $\text{Ca}^{2+}$  influx is indispensable. Monosporangia then release monospores autonomously or in a  $\text{Ca}^{2+}$

influx-dependent manner (**Figure 6** upper). Thus, enhanced formation of monosporangia in gametophytes cultured in ASPMT4 (**Figures 3** and **4**) appears to have resulted in the development of nearly all vegetative cells into monospores, causing an extensive discharge of monospores (**Figure 6** lower).

Another significant result of the present study is the establishment of the novel synthetic medium ASPMT3 for *P. yezoensis* culture (**Figure 3**). At present, there are three types of media for red algal cultivation: filter-sterilized natural seawater with addition of a mineral mixture, ESL using commercially released SEALIFE powder with  $ESS_2$  [26], and synthetic medium such as the ASP series [27,28]. The composition of the first medium usually varies greatly depending on location and season, thus it is not fit for physiological studies due to difficulties regarding control conditions and reproducibility. The second medium is a standard in our laboratory; however, the composition of the SEALIFE powder is proprietary, and therefore not obtainable. As mentioned in **Figure 1**, the third synthetic medium, the ASP series, has negative effects on the growth of *P. yezoensis*, although its chemical composition is well known. Considering the previous lack of availability of a good synthetic medium for *P. yezoensis* in physiological experiments, the establishment of ASPMT3 is therefore an important technological improvement for the study of *P. yezoensis*. Because applicability of ASPMT3 to the culture of other Bangiophycean red algae is unknown, it is important to test the growth of *Porphyra* and *Bangia* species of Bangiophycideae in compared with ESL medium to be able to generalize the utility of ASPMT3 for Bangiophycean red algal research.

Our aim is to understand the survival advantage of the asexual life cycle in red algae compared with other algae performing only sexual life cycle. The present study demonstrates the importance of photosynthesis-dependent extracellular  $Ca^{2+}$  influx in monospore production (**Figures 4** and **5**). However, the relative significance of asexual life cycle compared with the sexual life cycle in *P.*

*yezoensis* and other Bangiophycean red algae still remains to be settled. Due to the dominance of the sexual life cycle in red algae, we propose that the advantage of asexual life cycle relates to the relatively low success rate of syngamy between male and female gametes. Red algal cells are aflagellate during their entire life cycle [36,37], thus success of syngamy depends completely on the number of free discharged male gametes compared with the number of female gametes located in gametophytes. The probability of syngamy may be enhanced by increasing the number of free male gametes and increasing the total area producing female gametes in gametophytes. Thus, increasing the density of gametophytic clones produced by asexual propagation of monospores could increase both the number of male gametes released in sea water and the probability of fertilizing female gametes existing in gametophytes. In fact, similar explanation has been given for the tri-phasic life cycle observed in the more advanced red algae, classified as Florideophyceae, in which an extra diploid generation, so-called a tetrasporophyte, is found between the gametophyte and sporophyte phases [38]. The increase in the number of gametophytes via production of haploid tetraspores in tetrasporophytes also makes an advantage for successful syngamy in Florideophyceae. However, regulation mechanism producing spores are completely different between Bangiophyceae and Florideophyceae.

To determine the relative advantages of asexual life cycle against sexual one in *P. yezoensis*, it is necessary to elucidate the molecular mechanisms regulating formation of monosporangia, sporulation of vegetative cells to monospores, and discharge of monospores from gametophytes. Since the importance of extracellular  $\text{Ca}^{2+}$  influx (**Figure 5**) suggested the involvement of pumps and/or transporters of  $\text{Ca}^{2+}$  in monosporangia formation, it is essential to identify such molecules to understand how photosynthesis regulates extracellular  $\text{Ca}^{2+}$  influx for the promotion of monosporangia formation. In addition, it is necessary to elucidate how a decrease of extracellular

$\text{Ca}^{2+}$  concentration stimulates the intracellular  $\text{Ca}^{2+}$  influx, since there is no other example for the promotion of extracellular  $\text{Ca}^{2+}$  influx by decrease in the  $\text{Ca}^{2+}$  concentration outside of cells. Moreover, to understand the effects of the transfer of  $\text{Ca}^{2+}$  into the cytoplasm, it is important to identify targets of  $\text{Ca}^{2+}$  that participate in cellular signal transduction pathways for monosporangia formation, sporulation and monospore discharge.

We have also observed a similar regulatory system in the early development of *P. yezoensis* monospores, in which photosynthesis-dependent extracellular  $\text{Ca}^{2+}$  influx triggers initiation of the directional migration of monospores via the activation of phosphoinositide signaling components such as phosphatidylinositol 3-kinase and phospholipase C [17-19]. Thus, extracellular  $\text{Ca}^{2+}$  influx plays roles in different stages of the early development of monospores in this asexual life cycle. Such complexity related to multiple functions of  $\text{Ca}^{2+}$  should be resolved by focusing on whether extracellular  $\text{Ca}^{2+}$  influx is regulated by the same or different  $\text{Ca}^{2+}$  pumps and/or transporters during the formation of monosporangia and migration of monospores. Therefore, identification of genes involved in the  $\text{Ca}^{2+}$  dependence of the asexual life cycle is necessary for progress in molecular biological and genetic studies on the regulation of the asexual life cycle in eukaryotic red algae.

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## Figure legends

**Figure 1.** Red coloration of gametophytes and enhanced discharge of monospores in *P. yezoensis* cultured in the synthetic media ASPMT1 or ASPMT2. (a-d) Comparison of growth, vegetative cells and monospore discharge among gametophytes cultured in ESL (left), ASPMT1 (center) or ASPMT2 (right). Gametophytes were cultured in the media at 15°C under 60  $\mu\text{mol photons/m}^2/\text{s}$  with photocycle of 10 h light and 14 h dark for 4 weeks. Growth of gametophytes (a), microscopic view of vegetative cells (b), monospore accumulation at the bottom of culture flasks (c) and monospores attached to PVA monofilaments (d) were presented. Bars: (a) 2 cm; (b) 20  $\mu\text{m}$ ; (c) 1 cm; (d) 200  $\mu\text{m}$ . (e) Comparison of gametophytic growth. Cultivation was performed as above in ESL, ASPMT1 or ASPMT2 and the length of gametophytes was measured weekly. Values are the mean  $\pm$  SD ( $n = 10$ ). (f) Comparison of the number of discharged monospores. Gametophytes were cultured 1 week as above in ESL, ASPMT1 or ASPMT2. The number of discharged monospores was counted during 7 days. Values are the mean  $\pm$  SD ( $n = 3$ ).

**Figure 2.** Quantification of magnesium, potassium and calcium ions in ESL and ASPMT1. The concentration of  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  in both ESL and ASPMT1 was measured as atomic weight by the polarized Zeeman atomic absorption spectrophotometer. Columns and vertical bars represent the mean  $\pm$  SD, respectively ( $n = 3$ ).

**Figure 3.** Effects of extracellular  $\text{Ca}^{2+}$  contents on growth, color and monospore discharge. (a-d) Comparison of growth, vegetative cells and monospore discharge among gametophytes cultured in ESL, ASPMT3 or ASPMT4. Gametophytes were cultured in the media at 15°C under 60  $\mu\text{mol photons/m}^2/\text{s}$  with photocycle of 10 h light and 14 h dark for 4 weeks. Growth of gametophytes (a), microscopic view of vegetative cells (b), monospore accumulation at the bottom of culture flasks (c) and monospores attached to PVA monofilaments (d) were presented. Bars: (a) 2 cm; (b) 20  $\mu\text{m}$ ; (c) 1 cm; (d) 200  $\mu\text{m}$ . (e) Comparison of gametophytic growth. Cultivation was performed as above in ESL, ASPMT3 or ASPMT4 and the length of gametophytes was measured weekly. Values are the mean  $\pm$  SD ( $n = 10$ ). (f) Comparison of the number of discharged monospores. Gametophytes were cultured as above in ESL, ASPMT3 or ASPMT4. The number of discharged monospores was counted during 7 days. Values are the mean and SD ( $n = 3$ ).

**Figure 4.** Critical involvement of photosynthetic activity in production and discharge of monospores. (a) Comparison of gametophytic vegetative cells and monospore discharge among gametophytes cultured in ESL (left), ASPMT3 (middle) or ASPMT4 (left). Gametophytes were cultured in media at 15°C under 60  $\mu\text{mol photons/m}^2/\text{s}$  with photocycle of 10 h light and 14 h dark or consecutively dark for 1 week. Under light conditions (upper part), monosporangia were observed only at the edge of the gametophytes in ESL and ASPMT3 but spread to the mid region of gametophytes in ASPMT4. Microscopic view of vegetative cells and monospore accumulation at the bottom of culture flasks were combinationally represented for light and dark conditions. Bars= 20  $\mu\text{m}$  in photos for vegetative cells; 1 cm in photos for bottom of culture flasks. (b) Comparison of the number of discharged monospores. Gametophytes were cultured as above for 3 days in ESL or ASPMT3 with or without DCMU (0.1, 1, 10  $\mu\text{M}$ ), and then the number of discharged monospores was counted. Columns and vertical bars represent the mean  $\pm$  SD, respectively ( $n = 3$ ).

**Figure 5.** Critical involvement of  $\text{Ca}^{2+}$  influx in production and discharge of monospores. (a) Effects of EGTA on discharge of monospores. Gametophytes were cultured in ESL (left), or ASPMT3 (right) with or without EGTA (0.5, 1.0, 2.0, 5.0 mM) at 15°C under 60  $\mu\text{mol photons/m}^2/\text{s}$  with photocycle of 10 h light and 14 h dark for 3 days. Then, the number of discharged monospores was counted. Columns and vertical bars represent the mean  $\pm$  SD, respectively ( $n = 3$ ). (b) Effects of artificial  $\text{Ca}^{2+}$  influx on discharge of monospores. Gametophytes were cultured as above except for under continual dark with or without the calcium ionophore A23187 (1  $\mu\text{M}$ ) and the number of discharged monospores was counted. Columns and vertical bars represent the mean  $\pm$  SD, respectively ( $n = 3$ ).

**Figure 6.** A model of the involvement of  $\text{Ca}^{2+}$  and photosynthesis in production and discharge of asexual monospores in *P. yezoensis* gametophytes. In ESL and ASPMT3, if extracellular  $\text{Ca}^{2+}$  content is decreased, the formation of monosporangia is stimulated at the tip region of gametophytes, followed by discharge of monospores, for which photosynthesis activated by illumination accelerates extracellular  $\text{Ca}^{2+}$  influx to promote the formation of monosporangia (upper part). According to this hypothesis, the strong effects of ASPMT4 on growth of gametophytes and

monospore discharge are explained by enhancement of monosporangium formation throughout the body of gametophytes after severe limitation of extracellular  $\text{Ca}^{2+}$ , resulting in enhanced extracellular  $\text{Ca}^{2+}$  influx and monospore discharge, which reflects growth inhibition (lower part). Area containing monosporangia is schematically indicated by highlighted gray region. Photos show monosporangia in gametophytes cultured in ESL (upper) and ASPMT4 (lower).

**Table 1. Chemical compositions of synthetic culture media used in this study**

	ESL	ASPMT1	ASPMT2	ASPMT3	ASPMT4
HEPES (g/L)	0.1	1.0	1.0	1.0	1.0
NaCl (g /L)	n.d. <sup>+</sup>	28	28	28	28
MgSO <sub>4</sub> · 7H <sub>2</sub> O (g/L)	n.d. <sup>+</sup>	7.0	7.0	7.0	7.0
MgCl <sub>2</sub> · 6H <sub>2</sub> O (g/L)	n.d. <sup>+</sup>	4.0	4.0	4.0	4.0
KCl (mg/L)	n.d. <sup>+</sup>	700	700	700	700
CaCl <sub>2</sub> · 2H <sub>2</sub> O (mg/L)	n.d. <sup>+</sup>	400	400	800	200
NaNO <sub>3</sub> (mg/L)	60	100	60	60	60
K <sub>3</sub> PO <sub>4</sub> (mg/L)	n.d. <sup>+</sup>	10	—	—	—
Na <sub>2</sub> -glycerophosphate (mg/L)	8	10	8	8	8
NaSiO <sub>3</sub> · 9H <sub>2</sub> O (mg/L)	n.d. <sup>+</sup>	150	150	150	150
Fe-EDTA · 3H <sub>2</sub> O (mg/L)	3	—	—	—	—
Na <sub>2</sub> -EDTA (mg/L)	4	10	10	10	10
FeCl <sub>3</sub> (mg/L)	0.196	0.1	0.1	0.1	0.1
H <sub>3</sub> BO <sub>3</sub> (mg/L)	4.56	2.0	2.0	2.0	2.0
MnCl <sub>2</sub> (mg/L)	0.576	0.4	0.4	0.4	0.4
ZnCl <sub>2</sub> (mg/L)	0.0416	0.05	0.05	0.05	0.05
CoCl <sub>2</sub> (mg/L)	0.0161	0.01	0.01	0.01	0.01
Na <sub>2</sub> MoO <sub>4</sub> (mg/L)	n.d. <sup>+</sup>	0.5	0.5	0.5	0.5
KBr (mg/L)	n.d. <sup>+</sup>	10	10	10	10
SrCl (mg/L)	n.d. <sup>+</sup>	2.0	2.0	2.0	2.0
RbCl (mg/L)	n.d. <sup>+</sup>	0.2	0.2	0.2	0.2
LiCl (mg/L)	n.d. <sup>+</sup>	0.2	0.2	0.2	0.2
KI (mg/L)	0.02	0.01	0.01	0.01	0.01
VaCl (mg/L)	n.d. <sup>+</sup>	0.001	0.001	0.001	0.001
Vitamin mix ESS <sub>2</sub> (mL/L) <sup>++</sup>	0.1	0.1	0.1	0.1	0.1
pH	8.0	8.0	8.0	8.0	8.0

<sup>+</sup> n.d., not determined

<sup>++</sup> The composition is 10 µg/L vitamin B<sub>12</sub>, 10 µg/L biotin, 1 mg/L thiamine-HCL, 1 mg/L nicotinic acid, 1 mg/L Ca-pantothenate, 100 µg/L *p*-aminobenzoic acid, 10 mg/L inositol and 1 mg/L thymine

**Table 2. Comparison of concentration of photosynthetic pigments in *P. yezoensis* cultured in ESL and modified media**

	Chl a*	PE*	PC*	Car*	PE/Chl a	PC/Chl a	PE/PC
ESL	1.56±0.11	6.40±1.22	1.5±0.38	0.03±0.01	4.11	0.97	4.26
ASPMT1	1.31±0.24	8.09±0.43	1.41±0.10	0.04±0.02	6.18	1.08	5.73
ASPMT2	1.45±0.07	8.43±0.72	1.70±0.34	0.03±0.01	5.80	1.17	4.96
ASPMT3	1.36±0.12	5.59±1.56	1.28±0.44	0.02±0.01	4.12	0.94	4.38
ASPMT4	1.92±0.35	11.18±0.40	1.47±0.23	0.05±0.01	5.82	0.77	7.60

Concentrations of chlorophyll a (Chl a), phycoerythrin (PE), and phycocyanin (PC) carotenoids (Car) were measured using gametophytes cultured in each medium for 4 weeks.

\*mg/g fresh wt ± SD

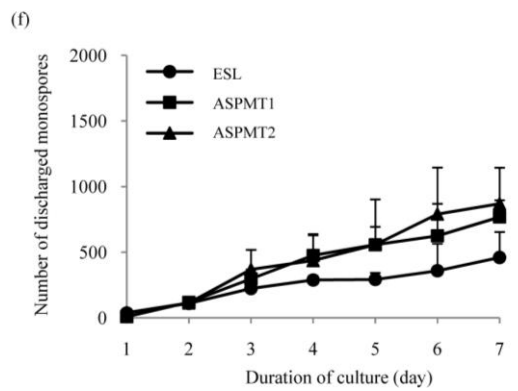
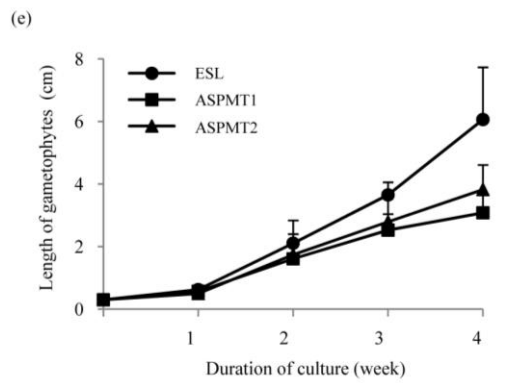
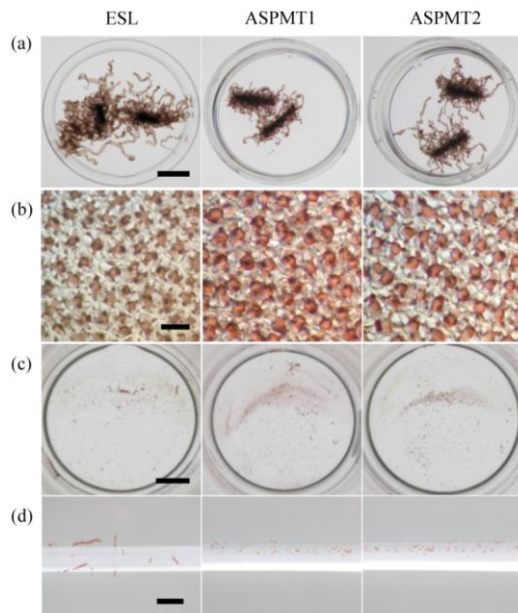


Figure 1

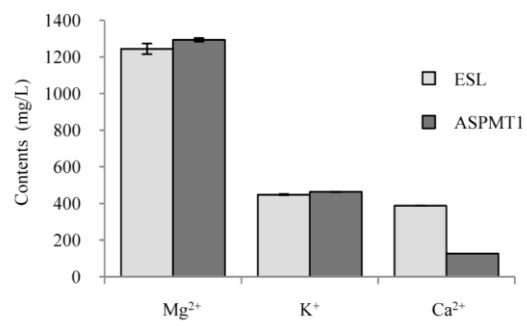


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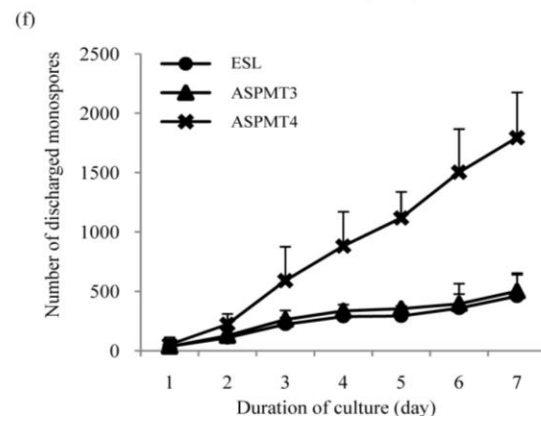
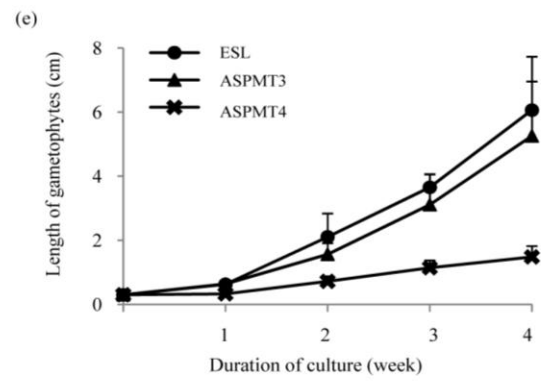
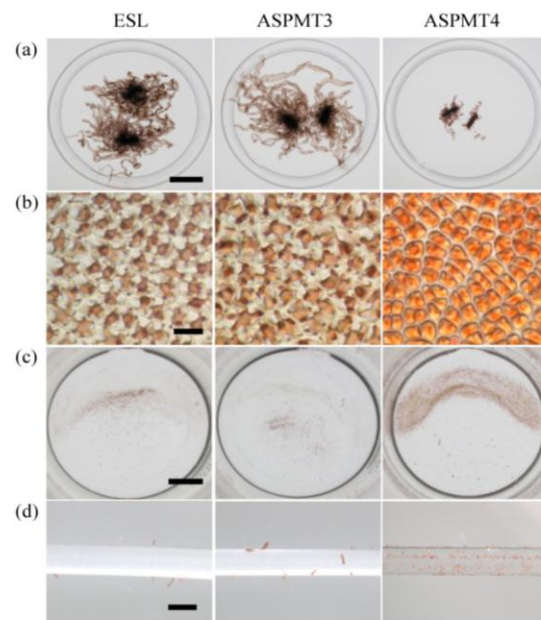


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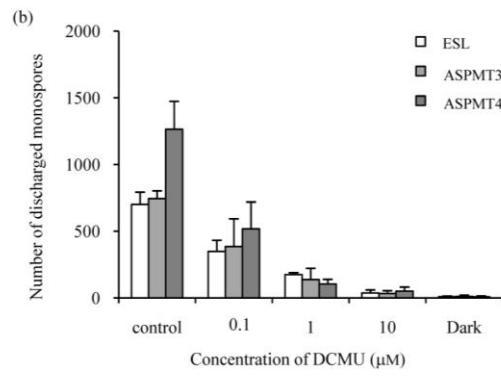
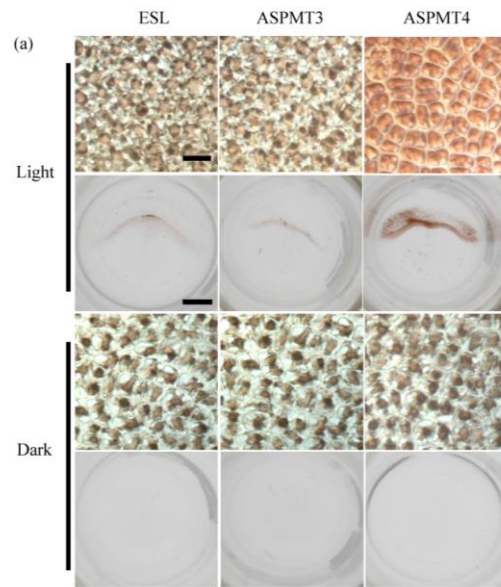


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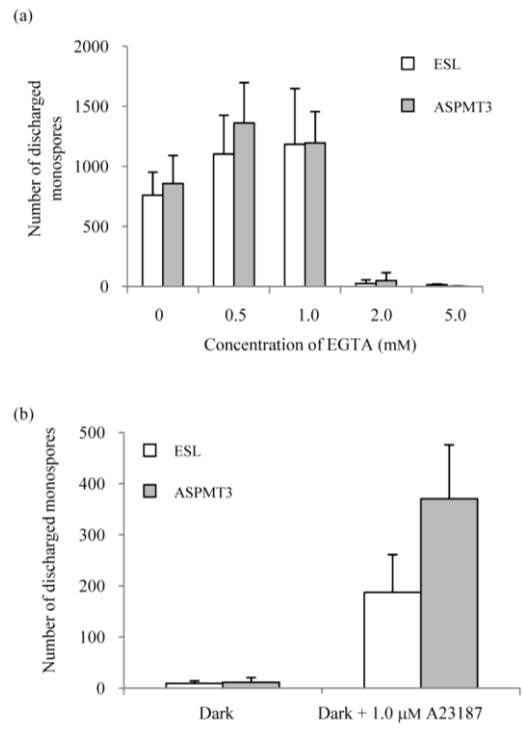


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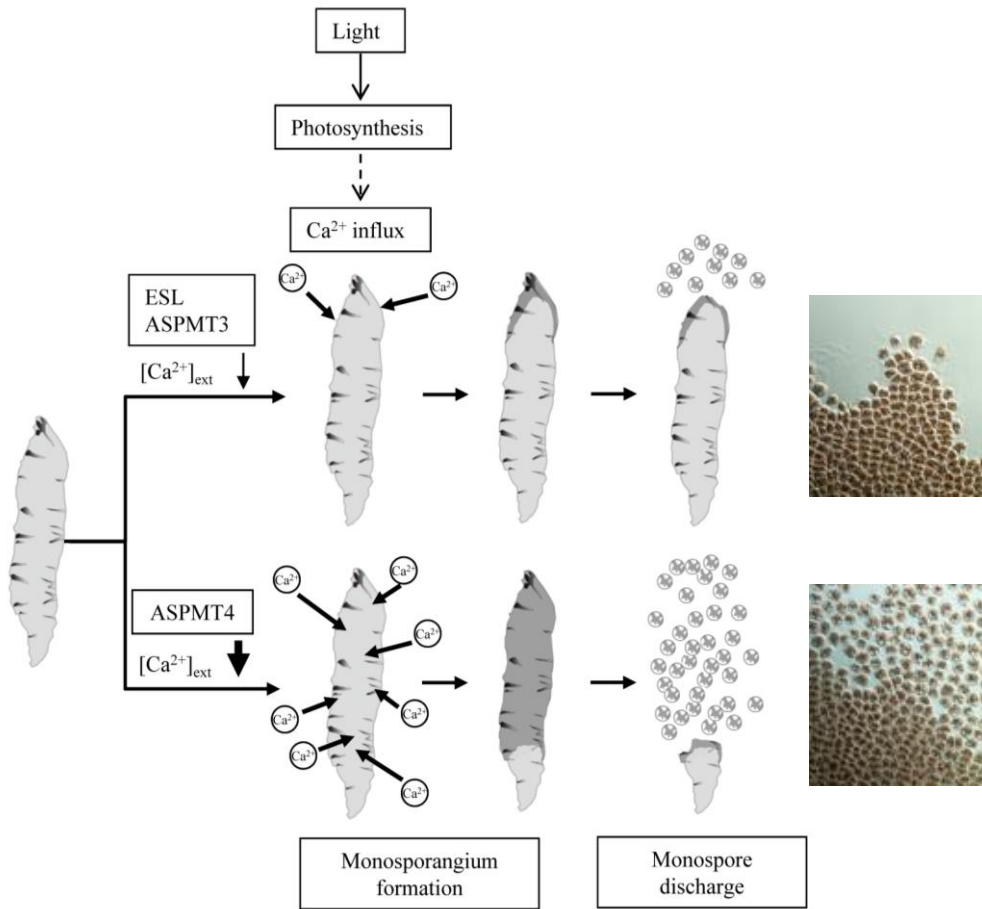


Figure 6