

# Effect of temperature on the development of *Saccharina japonica* gametophytes

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**Abstract** Spores (collected at  $10\pm 1$  °C, 2 h after releasing) and young gametophytes (newly generated from spores cultured at  $10\pm 1$  °C for 8 days) of *Saccharina japonica* were first cultured at  $15\pm 1$ ,  $19\pm 1$ , and  $23\pm 1$  °C for various times (2, 5, and 8 days) and then at  $10\pm 1$  °C (culturing patterns S and G, respectively). Spores were also cultured at a constant of  $10\pm 1$  °C (pattern C) and used as the control. The length and percentage of young gametophytes, size and percentage of gametophytes, and ratio of female to male gametophytes were measured in order to determine the effect of temperature on the development of gametophytes. Temperature and exposure time of spores and young gametophytes at the first culturing temperature significantly affected the development of gametophytes as were indicated by all biological parameters except the ratio of female to male gametophytes. The spores were more sensitive to temperature than young gametophytes. Gametophytes developed from the spores that survived temperature stress can recover their growth. High temperature selection at the early developmental stages of gametophytes was effective for screening gametophytes applicable for breeding high temperature-resistant varieties and hybrids.

**Keywords** Gametophyte · Temperature · *Saccharina japonica* · Gametophyte selection

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## Introduction

*Saccharina japonica* is indigenous to Japan, but has been cultivated in China, Japan, Russia, France, and Korea. With the development and implementation of summer sporeling raising and floating frame (currently, rope) culturing methods (Li 1990; Tseng et al. 1955), its cultivation in China evolved rapidly into the largest mariculture industry worldwide (Tseng 2001). As *S. japonica* originally inhabits cold seawater, temperature is one of the most important environmental factors affecting its cultivation. Blades may decay or even drop away from floating ropes because seawater temperature in summer is high and annually increasing due to global warming (Li 2008). Use of high temperature-resistant varieties could reduce the cost of raising seedlings, shorten staying time of seedlings in water-conditioned raising house, and extend sporophyte-growing time in seawater. Besides high yield, kelp breeding is always oriented to the development of high temperature-resistant varieties and hybrids.

Traditional approaches of breeding high temperature-resistant kelps (e.g., 1170 and Haiqing no. 1 varieties) are either pedigree selection or sporophytic hybridization in combination with continuous self-crossing and selection (Fang et al. 1962; Li 2008). With the development of gametophyte cloning and gametophyte clone hybridization methods, staying time of seedlings in raising house could be sharply shortened (Li et al. 1999) but unfortunately, sporophyte-growing time in seawater is seasonally restricted. In the last decade, both normal varieties (e.g., 90-1) (Zhang et al. 2007) and hybrid kelps (e.g., Dongfang nos. 2 and 3) (Li et al. 2007, 2008) which are both high yielding and relatively high temperature-resistant have been developed by the gametophyte clone hybridization method; however, such a finite resistance was

blindly bred. Selection of high temperature-resistant gametophytes and their application in the development of high temperature-resistant varieties and hybrids are greatly needed.

Gametophyte selection plays an important role in evolution and genetic differentiation and has been used as a breeding tool in higher plants (Darakov 1995; Hormaza and Herrero 1996; Koval 2000; Robert et al. 1992; Seoighe et al. 2005). At different developmental stages of gametophytes, temperature stress can be used to select gametophytes with high temperature-resistant genotypes (Mulcahy 1979). Such a strategy has been tried previously (Bajaj et al. 1992; Clarke et al. 2004; Frova et al. 1995) and proved to be effective in angiosperms (Chang et al. 2010; Domínguez et al. 2005; Zamir et al. 1982; Zamir and Gadish 1987). For *Saccharina*, the gametophytes can be preserved and cultivated using the gametophyte cloning method. Therefore, this strategy is applicable for species of *Saccharina*. The effect of temperature on the development of widely cultured *S. japonica* was determined in this study which contributes to understanding the stage differential responses of gametophytes to temperature and the efficient selection of high temperature-resistant gametophytes for hybrid breeding.

## Materials and methods

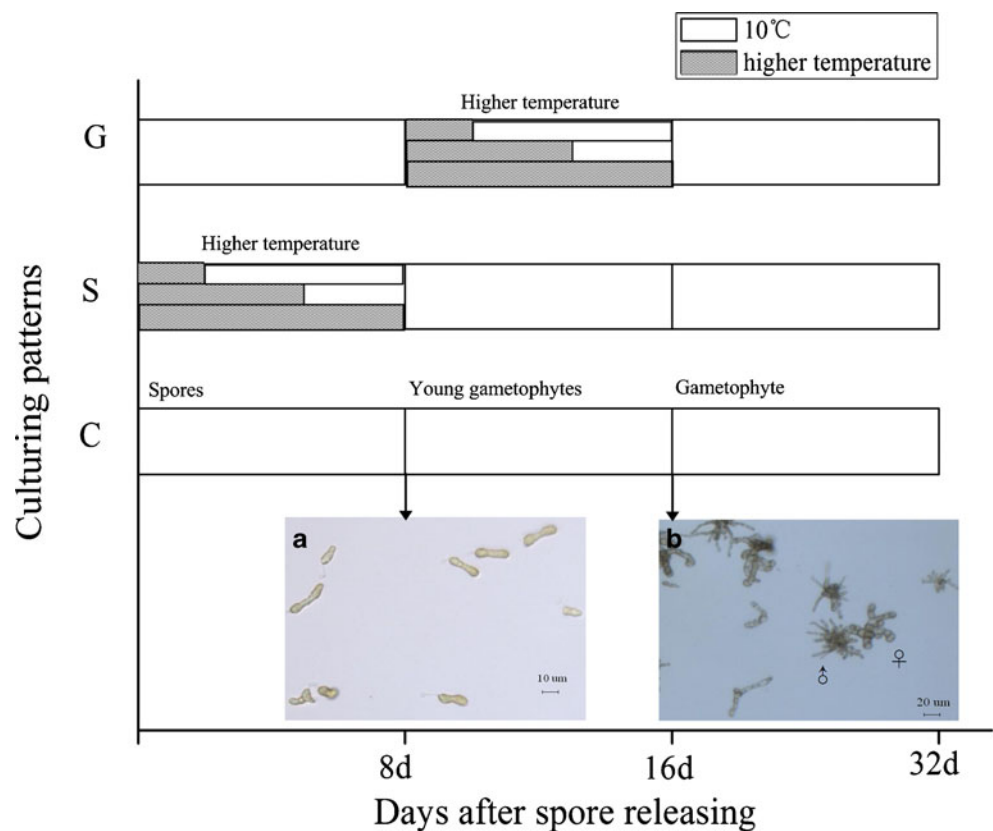
### Spore collection

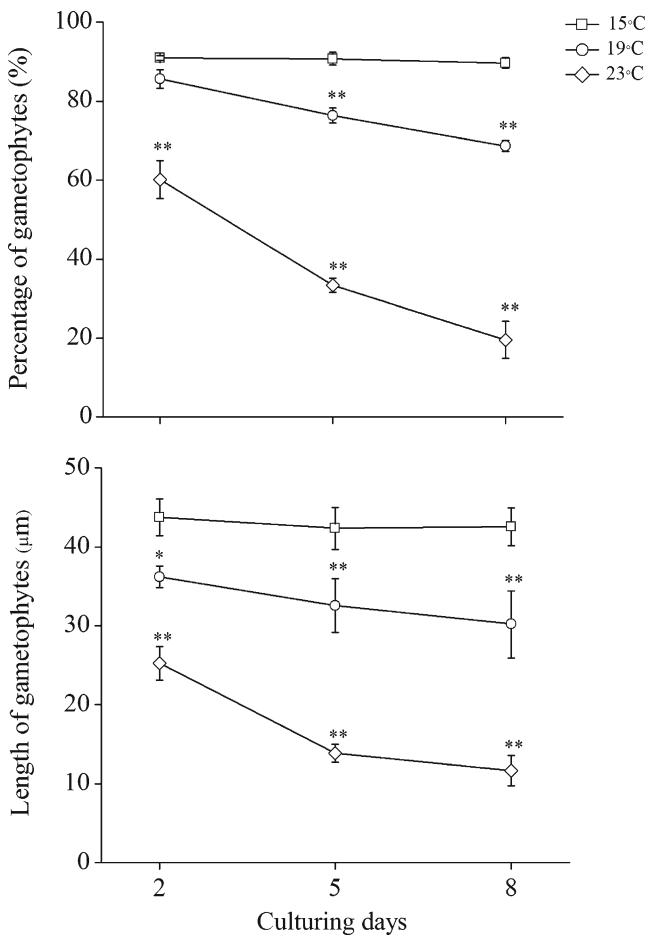
Mature sporophytes of *Saccharina japonica* were collected in Chudao Island Experimental Field of National Algal Engineering and Technique Research Center (37° 01' N, 122°33' E) in July, 2011. The fertile blade with sori was scrubbed with absorbent cotton, soaked in 1.5 % KI, and washed with sterile seawater. After being air-dried for 2 h, the blade was placed in sterile seawater (10±1 °C) to release spores. The spores released in 2 h were diluted to approximately 10,000 spores mL<sup>-1</sup> and dispensed into Petri dishes (9 cm in diameter) and then incubated at 10±1 °C and under a constant irradiance of 40–60 μmol photons m<sup>-2</sup> s<sup>-1</sup>.

### Spore and young gametophyte culture

As illustrated in Fig. 1, three culturing patterns were used. In culturing pattern C (control), spores were cultured at a constant of 10±1 °C. In culturing pattern S and G, spores (2 h after releasing) and young gametophytes (newly generated from spores cultured at 10±1 °C for 8 days) were first cultured at three different

**Fig. 1** Three culturing patterns of spores and young gametophytes. *C* spores were cultured constantly at 10±1 °C; *S* spores (2 h after releasing) were cultured at one of the preset temperatures for various times (2, 5, and 8 d) and then constantly at 10±1 °C; *G* young gametophytes were cultured at one of the preset temperatures for various times (2, 5, and 8 d) and then constantly at 10±1 °C. *a* Young gametophytes observed on day 8 after spore releasing. *b* Newly generated gametophytes observed on day 16 after spore releasing. Patterns *S* and *G* were applicable for all the preset temperatures (15±1, 19±1, and 23±1 °C)





**Fig. 2** Percentage and length of young gametophytes in culturing pattern S observed on day 8. Asterisks denote significant level. These two parameters in pattern C (control) were  $91.1 \pm 1.6\%$  and  $44.7 \pm 2.3\ \mu\text{m}$ , respectively. Data are means  $\pm$  SD ( $n=3$ )

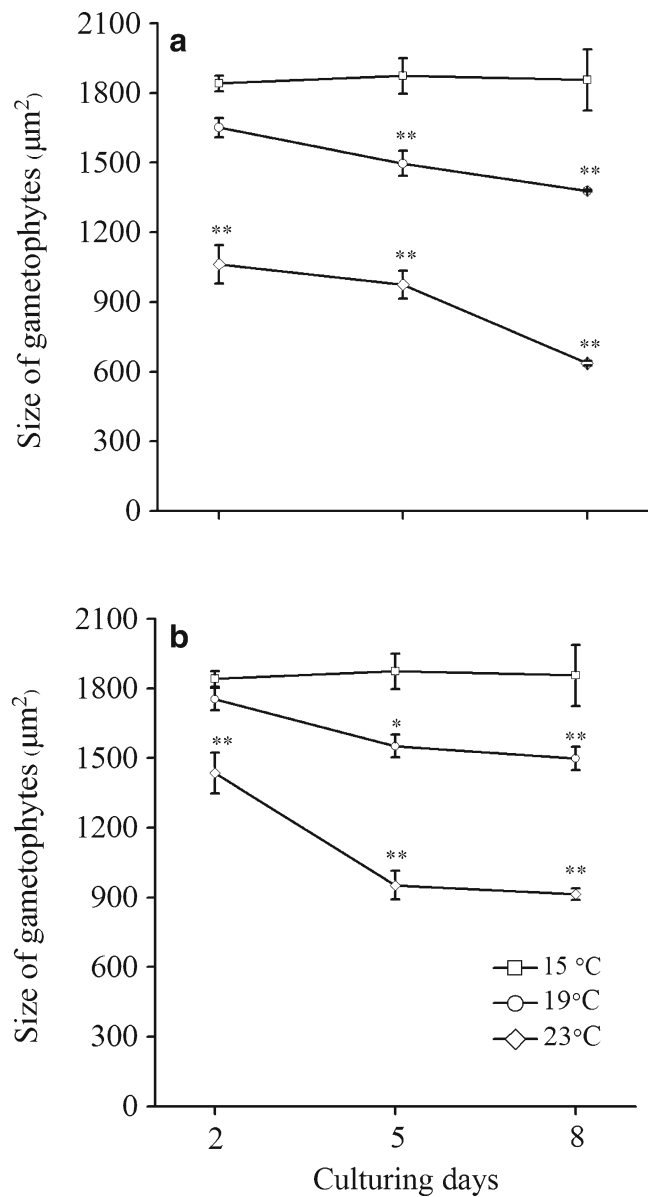
temperatures ( $15 \pm 1$ ,  $19 \pm 1$ , and  $23 \pm 1\ \text{°C}$ ) for various times (2, 5, and 8 days) and then at  $10 \pm 1\ \text{°C}$ . Three replicates were used for each temperature for each first culturing time and in each culturing pattern.

**Determination of temperature effect**

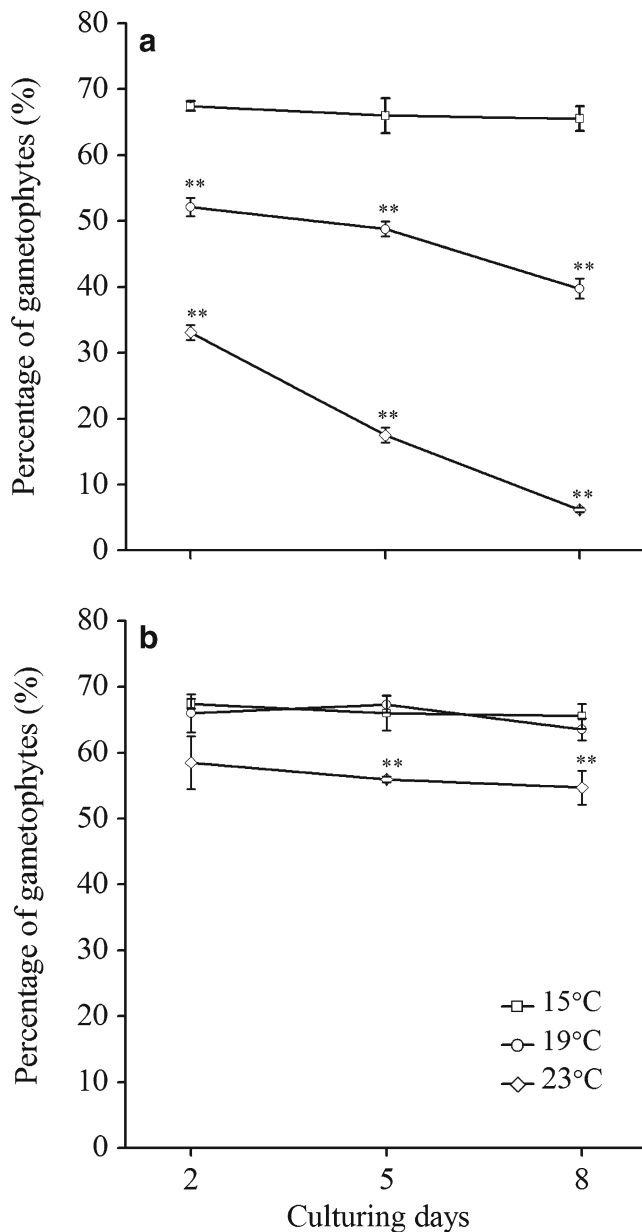
The effect of temperature was determined by measuring the length and the percentage of young gametophytes, the size and the percentage of gametophytes, and the ratio of female to male gametophytes, which were calculated according to either the counts in randomly selected fields under an inverted phase contrast microscope or the length and size measured with Qcapture and Image-Pro Plus 5.0 from images captured with the same microscope. The length and the percentage of young gametophytes were observed on day 8 in culturing pattern S. The size and the percentage of gametophytes and the ratio of female to male gametophytes were observed on day 16 and day 32 in both culturing patterns.

**Statistical analysis**

The data were analyzed with the three-way ANOVA (SPSS 15.0). Developmental stage was set as the first factor and temperature the second and exposing time the third. The normality and homoscedasticity of the percentages were calculated with square-root arcsine transformation. A post hoc Dunnett test was applied in finding the significance of effect difference from that of control (Sokal and Rohlf 1995). Significance level was set at  $P < 0.05$  (\*, significant) and  $P < 0.01$  (\*\*, extremely significant).



**Fig. 3** Size of gametophytes in culturing patterns S (a) and G (b). The size was measured on day 16 after spore releasing. Asterisks denote significant level. The size of gametophytes in pattern C (control) was  $1861.8 \pm 76.3\ \mu\text{m}^2$ . Data are means  $\pm$  SD ( $n=3$ )



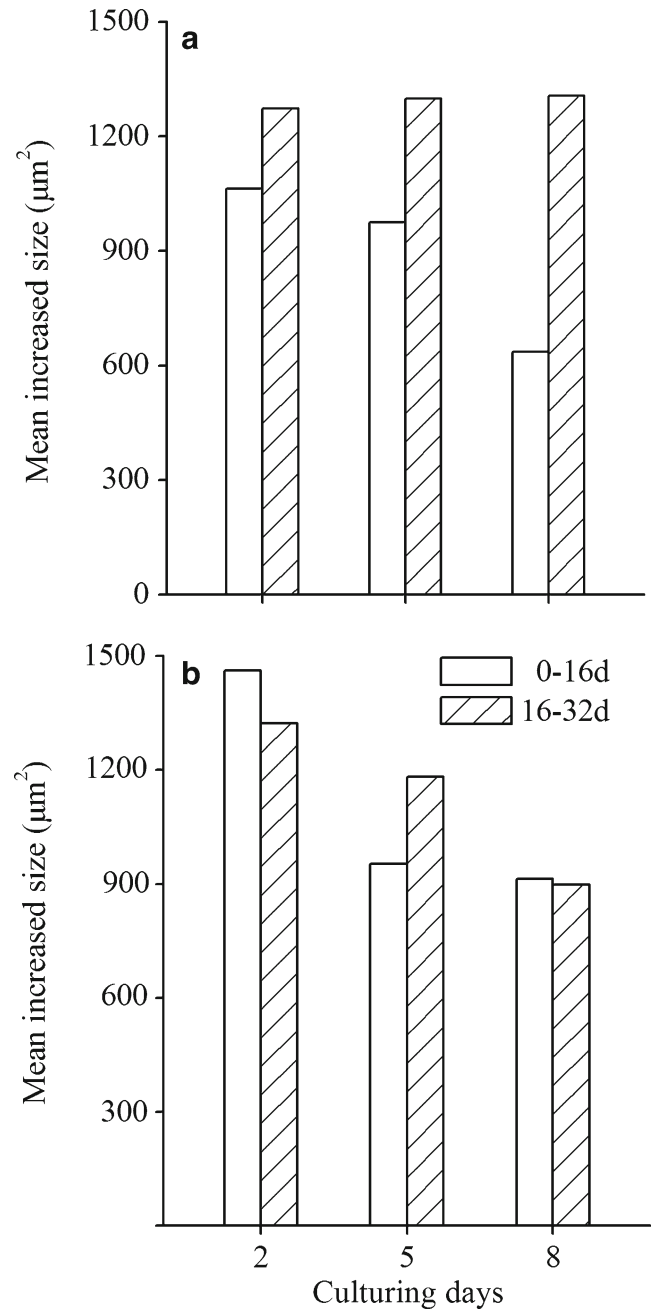
**Fig. 4** Percentage of gametophytes in culturing pattern S (a) and G (b). The percentage was calculated on day 16 after spore releasing. Asterisks denote significant level. The percentage of gametophytes in pattern C (control) was  $72.4 \pm 2.1$  %. Data are means  $\pm$  SD ( $n=3$ )

## Results

At a constant of  $10 \pm 1$  °C and under continuous  $40\text{--}60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  irradiance, young gametophytes with a single cell or simple branches were developed on day 8 (Fig. 1a). Discernible female and male gametophytes with complex branches were observed on day 16 (Fig. 1b). Female gametophytes were identified by fewer branches of larger cells and males by more branches of smaller cells.

In culturing pattern S, the length and the percentage of young gametophytes were observed on day 8 after spore releasing

(Fig. 2). It was found that the length and the percentage of young gametophytes were similar to those of young gametophytes in control (pattern C) when spores were first cultured at  $15 \pm 1$  °C and then at  $10 \pm 1$  °C. With the increases of the first culturing temperature and the exposure time in the first culturing temperature, the length and the percentage of young gametophytes decreased. When the first temperature was  $19 \pm 1$  °C and exposure time at this temperature was either 5 or 8 days, the



**Fig. 5** Comparison of the growth of gametophyte between 0–16 and 16–32 days in culturing patterns S and G. Either spores (2 h after releasing) or young gametophytes (newly generated in 8 days after spore releasing) were first cultured at  $23 \pm 1$  °C for all exposure times (2, 5, and 8 d) and then  $10$  °C. The size of gametophytes was measured on days 16 and 32

length and the percentage of young gametophytes were significantly lower than those of the control ( $P < 0.05$ ). When the first temperature was  $23 \pm 1$  °C, the length and the percentage of young gametophytes were also significantly lower than those of the control, reaching the shortest  $11.67 \pm 1.91$  µm and the lowest  $19.61 \pm 4.72$  %, respectively (Fig. 2).

In both culturing patterns (S and G), the size and the percentage of gametophytes were observed on day 16 after spore releasing. In culturing pattern S, the size of gametophytes decreased when spores were first cultured at  $19 \pm 1$  and  $23 \pm 1$  °C and then at  $10 \pm 1$  °C (Fig. 3a). A reduction of the size of gametophytes was also found in culturing pattern G. The higher the first temperature and the longer the spores and young gametophytes were exposed to the first temperature, the smaller was the size of the gametophytes (Fig. 3b). In culturing pattern S, the percentage of gametophytes followed the decreasing trend in the size of gametophytes. It decreased significantly when the first temperature was  $19 \pm 1$  and  $23 \pm 1$  °C, for all exposure times ( $P < 0.01$ ; Fig. 4a). When the first temperature was  $23 \pm 1$  °C and exposure time at this temperature was 5 and 8 days, the percentage of gametophytes was  $17.49 \pm 1.16$  and  $6.17 \pm 0.35$  %, respectively. In culturing pattern G, a significant decrease was not found when the first temperature was lower than or equal to  $19 \pm 1$  °C. Significant, but not very obvious, decreases in the percentage of gametophytes were observed when the first temperature was  $23 \pm 1$  °C (Fig. 4b).

At the first temperature was  $23 \pm 1$  °C, the size of gametophytes for all exposure time in both culturing patterns was measured on days 16 and 32. On day 16, the mean size decreased with the increase of exposure time when spores were cultured first at this temperature (in culturing pattern S). It was interesting that the mean increase in size was similar from day 16 to day 32 (Fig. 5a). When young gametophytes were cultured first at  $23 \pm 1$  °C (in culturing pattern G) for 2, 5, and 8 days, and then at  $10 \pm 1$  °C, a continued decrease of the mean size of gametophytes was observed. These results indicate that, although spores exposed to the highest temperature were affected by the exposure time during the first 16 days of growth, they were not for the second.

In culturing pattern S, when spores were cultured at  $23 \pm 1$  °C for 5 and 8 days, the ratio of female to male gametophytes significantly increased as was observed on day 16 ( $P < 0.05$ ). However, such a ratio was similar to that of the control in both culturing patterns as was observed on day 32 (data not shown).

## Discussion

The *Saccharina* gametophyte cloning method was developed at the end of the 1970s and implemented in breeding in the 1990s. Gametophytes can maintain vegetative growth at low temperature and under continuous weak light (Fang et al.

1978). It has been proposed that the temperatures optimal for the vegetative growth of gametophytes and suitable for the survival of gametophytes were related to species distribution (Bartsch et al. 2008; Wang 1987). In this study, spores were collected from Chudao Island ( $37^{\circ}01' N$ ,  $122^{\circ}33' E$ ). The gametophytes are usually cultured at temperatures ranging from 10 to 15 °C. It has been found that gametophytes are not able to survive at  $25 \pm 1$  °C (Li et al. 2004). According to these findings,  $15 \pm 1$ ,  $19 \pm 1$ , and  $23 \pm 1$  °C were selected for determining the effect of temperature on the development of gametophytes of *S. japonica*.

Comparison of the effect of temperature in two culturing patterns showed that spores were more sensitive to and intolerant of high temperature than young gametophytes. It has been reported that the tolerance of *Lessonia* to UVR irradiation increases with its ontogenetic development (Véliz et al. 2006), and seaweeds at early developmental stages are more susceptible to diverse environmental perturbations (Coelho et al. 2000). The detrimental effect of high temperature on the ultrastructure of *Laminaria* spores has been reported by Steinhoff et al. (2008). At high temperature, the maximum ultrastructural disturbances including appearance of mottling in nucleoplasm and formation of small spheroidal plastoglobuli in chloroplasts were detected. Besides cell structure, physiological functions could also be affected detrimentally (Machalek et al. 1996; Voskoboinikov and Kamnev 1991). There are different stages during the formation of gametophytes, including zoospores, settled spores, and finally gametophytes. Increases of cell size, formation of well-developed cell wall, division of chloroplasts, and extrusion of adhesion vesicles have been reported in this process (Henry and Cole 1982; Oliveira et al. 1980). Therefore, the cell structure of gametophytes is more complete and the function of gametophyte cells is more stable in comparison with spores.

High temperature not only caused death of a portion of spores and gametophytes but also inhibited the growth of survived spores and gametophytes. Such an inhibition intensified obviously with the elongation of exposure time in the first culturing temperature. High temperature changes pigment content, PSII reaction center densities, efficiency of light harvesting and electron transporting systems, and activity of related enzymes of photosynthesis, finally reducing photosynthetic efficiency (Davison 1987; Davison et al. 1991; Gerard 1997; Machalek et al. 1996). It also increases reactive oxygen species formation and inhibits antioxidant proteins (Wang 2003). However, the mechanism underlining the growth reduction and mortality of *Saccharina* gametophytes is not fully understood. When spores were cultured at  $23 \pm 1$  °C in culturing pattern S, the mean increased size of gametophytes for 8 days exposure was the smallest from day 0 to day 16. However, the mean increased size for 8-day exposure was almost the same as that for 2-day exposure and 5-day

exposure from day 16 to day 32, suggesting that the spores that survived  $23 \pm 1$  °C recovered their growth at  $10 \pm$  °C. It has been reported that nonlethal UV-irradiated spores recovered their growth at PAR conditions and developed into gametophytes with sporophyte-yielding ability (Roleda et al. 2005; Tala et al. 2007; Véliz et al. 2006). The biomass of the gametophytes obtained in both culturing patterns is accumulating in order to prove their sporophyte-yielding abilities.

Although varied during culture, the ratios of female to male gametophytes in both culturing patterns (S and G) calculated on day 32 were similar to that of the control (pattern C), which was different from the results on day 16. Generally, the female and male gametophytes should be distinguishable each other on day 16 after spore releasing. Female gametophytes have more branches and larger cells than males. However, high temperature delayed the growth and development of spores, and a portion of spores appeared as expanded single cells on day 16 after releasing. In contrast, these two types of gametophytes were well developed 32 days after spore releasing, and accordingly, the ratio of female to male gametophytes calculated on day 32 was more creditable than that calculated on day 16. Several previous studies showed that temperature affects the ratio of female to male gametophytes of *Saccharina* more or less. Male gametophytes were prevalent at higher temperature in *Laminaria saccharina* (now known as *S. latissima*) (Lee and Brinkhuis 1988) and lower temperature in *Laminaria ochroleuca* (Izquierdo et al. 2002). However, fewer male gametophytes at both higher and lower temperature in *S. religiosa* were observed according to Funano (1983).

Gametophyte selection has been used as an effective tool for the genetic improvement of higher plants, especially angiosperms. Pollen grains compete in stress environments for fertilization. For *Saccharina*, the gametophyte cloning method has been developed, making the selection of haploid gametophytes possible and convenient. Direct selection with high temperature during gametophyte development is time and labor saving. In the present study it was found that temperature significantly affected the development of gametophytes and that spores were more sensitive to high temperature than young gametophytes. Once the sporophyte-yielding ability of survival gametophytes from nonlethal high temperature damage is determined, they may be used to breed high temperature-resistant varieties and hybrids immediately.

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