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ORIGINAL ARTICLE

Characterization of high temperature-tolerant strains of *Pyropia yezoensis*

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Abstract

High-temperature stress related to global warming reduces the growth and productivity of seaweeds. Thus, the development of new strains is urgently required for maintaining or even enhancing the productivity of useful seaweeds such as red alga *Pyropia yezoenesis* in an increasingly warmer sea environment. To develop competitive high-temperature-tolerant strains of *P. yezoensis* (Sugwawon no. 104), we screened libraries of gamma-irradiated strains and identified high-temperature-resistant (HTR) mutants. Our results showed that HTR-1 and HTR-2 grew well at higher temperatures that inhibited the growth of the wild-type strain. The efficiency of conchosporangium maturation and conchospore release of HTR-1 was similar to or higher than the wild-type strain. Moreover, thallus growth, pigment content, photosynthetic efficiency, and monospore release from the growing thallus in HTR-1 could be maintained even at high temperature. Taken together, our data demonstrate that HTR-1 may be suitable for industrial cultivation at sea, even at elevated temperatures.

Keywords *Pyropia yezoensis* · Conchocelis · Gametophyte · Gamma radiation mutants · High temperature

Introduction

Elevated temperature due to global warming affects the growth and productivity of seaweed. High temperature stress causes morphological, physiological, and biochemical changes that reduce photosynthesis and thus limit plant growth and productivity (Ashraf and Harris 2013; Wang et al. 2000a, b). To increase seaweed productivity at elevated temperature, many methods have been attempted to develop high temperature-tolerant strains, including mutagenesis and genetic transformation.

Genetic studies have been shown to be critical for farming *Pyropia* species, an economically important marine red alga used for food, fertilizer, medicine, and chemicals. Since the identification of spontaneous and gamma rays induced pigmentation mutants in *P. yezoensis* (Miura and Shin 1989; Wang et al. 2000a, b), much research has focused on improving the productivity of this cultivar by genetic mutagenesis. However, elevated seawater temperature reduces the farming productivity of *Pyropia*. A recent report demonstrated that persistent high temperature severely inhibits the growth of the *Pyropia* gametophyte, and the thallus can become deconstructed and detached from nets during cultivation (Ding et al. 2016).

Several high temperature-tolerant strains have been reported for *P. yezoensis* (Ding et al. 2016; Fu et al. 2011; Wang et al. 2012; Zhang et al. 2011), *P. haitanensis* (Yan et al. 2010), and *P. chauhanii* (Chen et al. 2016). To utilize these newly developed strains for sea cultivation, characteristics such as conchosporangium maturation, conchospore release, growth under high temperature conditions, and

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monospore release are critical and thus should be analyzed to determine the optimal cultivar. For *P. haitanensis*, which grows in temperatures above 25 °C and is mainly cultivated in China, mutant strains capable of growing at 30 °C were developed and cultivated for industrial purposes (Yan et al. 2010). *Pyropia yezoensis*, an economically important species cultivated mainly in Korea, Japan and China, grows at temperatures below 18 °C. High temperature-tolerant mutant strains of *P. yezoensis* capable of growing under conditions higher than 20 °C have also been developed (Ding et al. 2016; Fu et al. 2011; Wang et al. 2012; Zhang et al. 2011). These studies clearly demonstrate the improved performance in monospore division, conchospore division and releasing, thallus growth, and pigment content in these mutant strains relative to wild type at temperatures above 18 °C. However, additional analysis of these important characteristics is still required.

In this study, we isolated and characterized two high temperature-tolerant strains of *P. yezoensis*. The thallus of these mutants showed high temperature tolerance in growth and photosynthetic efficiency. At normal temperature, one mutant exhibited similar growth to wild type, whereas the other grew poorly. Moreover, gametophyte and conchocelis development differed between the two mutants.

Materials and methods

Isolation of high temperature-tolerant thallus

Gametophyte thallus of *P. yezoensis* strain Sugwawon no. 104, which was kindly provided by Seaweed Research Center, National Fisheries Research and Development Institute in Korea, was maintained in ESL medium (Nikaido et al. 2000) under 80 µmol photons m^{-2} s^{-1} with a photoperiod of 10 h in light and 14 h in darkness (10L:14D) at 12 °C. The culture medium was replaced weekly with fresh medium. Growth performance of the thallus was measured weekly for 42 days at 12 °C and 20 °C under 80 µmol photons m^{-2} s^{-1} with a photoperiod of 10 h in light and 14 h in darkness (10L:14D).

The thallus was irradiated with 100, 250, 500, 1000, and 2000 Gy in a ^{60}Co gamma irradiator (150 TBq capacity; ACEL, Nordion, Ottawa, ON, Canada) at the Korean Atomic Energy Research Institute. LD50 was determined by visualization of cells stained with 0.01% erythrosine (Wako Pure Chemical Industries, Japan) after 1 day incubation with the irradiated thallus. Thallus treated with 1000 Gy was cultured in ESL medium with aeration under 80 µmol photons m^{-2} s^{-1} with 10L:14D at 20 °C for 3 weeks to release monospores, which were subsequently incubated at 20 °C to isolate growing thallus for 2 months.

Isolated thallus of 1 cm in length was cut and cultured in ESL medium at 20 °C to release monospores. Each monospore was cultured to develop the thallus for 2 weeks at 20 °C. This process from monospore to clonal gametophytic thalli was repeated three times to isolate each pure line through the asexual cycle by the methods (Kuwano et al. 1996; Park et al. 2007).

Conchocelis from pure lines was developed as described previously (Park 2006) with some modifications. Carpospores formed from the thallus were developed into conchocelis, which were cultured in ESL medium under 40 µmol photons m^{-2} s^{-1} illumination (14L:10D) at 20 °C. The culture medium was replaced weekly with fresh medium.

RT-PCR and genomic PCR analyses

To compare the genetic difference between wild type and mutants, PCR analyses were performed. For semi-quantitative PCR analysis, reverse transcription (RT) was carried out using 2 µg of total RNA with oligo-dT, 200 U of murine Moloney leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA), 500 µM of each dNTP, and 20 U of ribonuclease inhibitor. RT-PCR analysis was performed using each gene-specific primer (Table S1) and 27–40 cycles at 95 °C for 30 s, 50–58 °C for 30 s, and 72 °C for 30 s. The expression of the small subunit 3 of the actin gene was used as a loading control. Genomic PCR was performed using specific primers (Table S1) and *Pfu* DNA Polymerase (Promega, Madison, WI, USA) with 35 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s.

Conchosporangium maturation and conchospores release

To mature the conchosporangium, the conchocelis was incubated with aeration at different temperatures and a photoperiod of 40 µmol photons m^{-2} s^{-1} (15 °C with 10L:14D, 20 °C with 10L:14D, 20 °C with 14L:10D, and 25 °C with 10L:14D). Maturation of conchosporangium was observed by microscopy weekly for 8 weeks. Next, conchospore release was observed at various temperatures (15, 19, 21, 23, and 25 °C) under 100 µmol photons m^{-2} s^{-1} 10L:14D for 7 days by the filament method that is used to observe monospore release (Kim and Kim 2012). Conchosporangia (100 mg) were incubated with ten filaments (3 mm in length) for 7 days. Conchospores or germinated conchospores on the filaments were counted under UV light by fluorescence microscopy (Axioskop, ZEISS, Germany) as published previously (Kim and Kim 2012).

Measurement of photosynthetic efficiency and pigment content

Gametophyte thallus cultured at 12 °C or 20 °C under 80 µmol photons m^{-2} s^{-1} with a photoperiod of 10 h in light and 14 h in darkness (10L:14D) was used to measure the photosynthetic efficiency. Maximal photochemical efficiency as estimated by the Chl fluorescence parameter *Fv*/*Fm* was measured using a Plant Efficiency Analyzer (PEA meter, Hansatech). Thallus exhibiting a length of 1 cm was used to measure chlorophyll (Chl *a*) (Porra et al. 1989), phycoerythrin (PE) (Hongfeng 1993), and phycocyanin (PC) (Hongfeng 1993) content with a spectrophotometer (UV-2450, Shimadzu, Japan).

Comparison of monospore release

Monospore release from the thallus was measured as described previously (Kim and Kim 2012). Ten 4-mm segments sliced from two to three thalli (2–3 cm in length) were incubated with filaments at 12, 15, and 21 °C under 80 µmol photons m^{-2} s^{-1} (10L:14D) with aeration. Monospores attached onto the filaments were counted weekly under UV light by fluorescence microscopy (Axioskop, ZEISS, Germany) as reported previously (Kim and Kim 2012).

Results

Growth of *P. yezoensis* (wild-type strain) at high temperature

Growth of gametophyte thalli from wild-type strain was severely inhibited when cultured at 20 °C for 3 weeks; however, the gametophyte thalli grew well at 12 °C (Fig. 1a, c). The thalli cultured at 20 °C were discolored, deconstructed, and produced monospores and/or carpospores (Fig. 1b, d). The maximal photochemical efficiency (*Fv*/*Fm*) of the thallus at 20 °C gradually decreased from 0.63 at day 0 to 0.42 at day 7, 0.29 at day 15, 0.22 at day 17, and finally 0 at day 21 (Fig. 1e).

Radiation breeding of *P. yezoensis* with high temperature tolerance

To develop high temperature-tolerant strain of *P. yezoensis* (Sugwawon no. 104), gamma ray mutagenesis was performed. To accomplish this, we first determined the dosages of gamma irradiation needed to achieve LD50, or the median lethal dosage that causes 50% mortality of cells within the thallus. The erythromycin assay demonstrated that gamma irradiation (0–2000 Gy) affected the thallus survival rate (Fig. 2) and that 1000 Gy was the LD50.

Next, thalli treated with 1000 Gy were cultured at 20 °C to release survival spores and isolate the high temperature-tolerant mutant strain. After 1–2 months, 20 rapidly growing thalli strains were found on the 20 °C culture (Fig. 3),

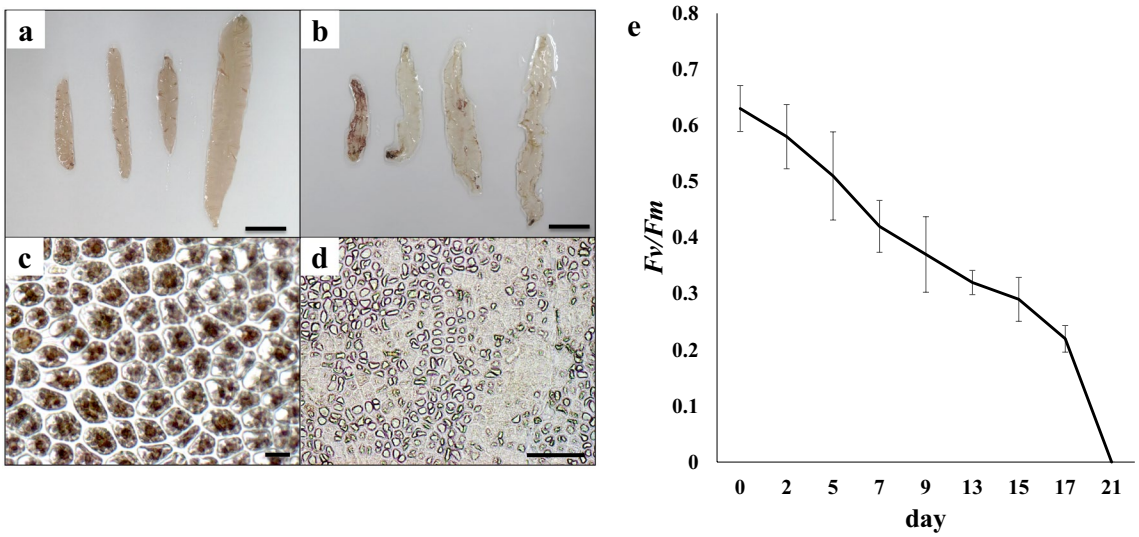

Fig. 1 Growth of *Pyropia yezoensis* (Sugwawon no. 104) at high temperature. **a, b** Gametophyte thalli of *P. yezoensis* cultured at 12 °C (**a**) and 20 °C (**b**) for 3 weeks were photographed. **c, d** Microscopic visualization of *P. yezoensis* gametophyte thallus cultured at 12 °C (**c**) and 20 °C (**d**) for 3 weeks. **e** Photochemical efficiency of photosystem II (*Fv*/*Fm*) of gametophyte thallus exposed to 20 °C for 3 weeks. Error bars represent the standard deviation of three replicates. Scale bars represent: **a, b** 1 cm; **c** 20 µm; **d** 50 µm

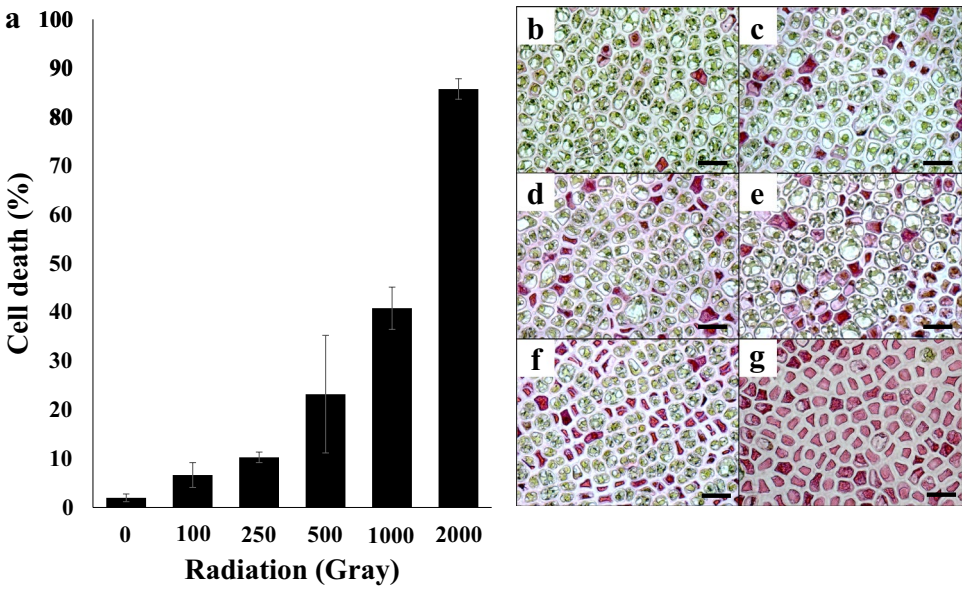

Fig. 2 Effects of gamma irradiation on the survival of *P. yezoensis* gametophytes. **a** Mortality of cells in thallus treated with increasing intensity of gamma radiation. Error bars represent the standard deviation of three replicates. **b**–**g** Visualization of erythrosine-stained thallus treated with varying intensities of gamma radiation by microscopy. Control cells that were left untreated (**b**). Thallus irradiated with 100 Gy (**c**), 250 Gy (**d**), 500 Gy (**e**), 1000 Gy (**f**), and 2000 Gy (**g**). Scale bars represent 20 μm

whereas no thallus developed from the non-irradiated wild-type control. Interestingly, only 7 of the 20 strains showed comparable growth at 12 °C (data not shown). Growth of the mutants was retarded or stopped above 23 °C; therefore, high temperature experiments for thallus growing were performed at 20 °C. Two of the lines (HTR-1 and HTR-2) were selected for further investigation. Finally, each conchocelis line from the two mutants was established after three rounds of the asexual cycle from single monospore to thallus.

Genetic difference between wild type and mutants was analyzed by PCR and sequencing. RT-PCR analysis detected increased accumulation of transcripts of genes associated with the heat shock protein family (HSF, HSP70, and HSP90) in mutants cultured at 12 °C or 20 °C compared with wild type (Fig. 3e; Table S1). Only HSF gene among three heat shock-related genes was clearly upregulated at 12 °C and 20 °C. As part of our HTR-1 transcript sequencing project (funded by Golden Seed Project), genetic mutations between wild-type and the mutant HTR-1 were analyzed (data not shown). A transcript read (TRINITY_DN48859_c0_g2_i1) selected from transcriptomes comparison showed 6 bases difference between those in HTR-1 and wild type; the difference was confirmed again by the sequencing of the genomic PCR fragments (Fig. 3f; Fig. S1).

Efficiency of conchosporangium maturation and conchospore release

Vegetative growth of conchocelis was indiscernible between the mutant and wild-type strains (data not shown). Different temperatures and photoperiods were examined to compare the efficiency of conchosporangium maturation and release of conchospores from conchocelis cultured for 8 weeks. Our data demonstrate that the maturation efficiency of conchosporangium in the mutants was comparable to wild type in all conditions analyzed (Fig. S2). However, conchosporangium maturation at 25 °C was hardly detected.

Investigation of the efficiency of conchospore release from the matured conchosporangium revealed differences between the wild-type and mutant strains (Fig. 4). Bright fluorescent conchospores or germinating conchospores attached onto filaments were observed and counted for each condition (Fig. 4b). The efficiency of conchospore release was slightly lower in the mutant strains relative to wild type at 15 °C and 19 °C (Fig. 4a). However, we observed the highest conchospore release and the efficiency of the mutant strains (104% ± 10.7 for HTR-1 and 103% ± 13.0 for HTR-2) was similar to wild type (100%) at 21 °C, which is the temperature normally applied to induce conchospore release for industrial cultivation in Korea. At temperatures higher than 21 °C, slightly higher efficiencies (110% ± 8.0 at 23 °C and 120–135% ± 14.9 at 25 °C) were observed in both mutant strains relative to wild type (100%).

Growth performance of the mutants

Growth performance of the conchospore germlings to the thallus in response to elevated temperature was compared between the wild-type and mutant strains (Fig. 5). At 12 °C, which is nearly the optimal temperature for *P. yezoensis* growth, HTR-1 showed similar growth compared with wild type, while HTR-2 exhibited slower growth. After 28 days of culture, the HTR-1 thallus was 35.9 cm in length and that of HTR-2 was 14.8 cm, while wild type was 37.5 cm. After culture for 42 days, the HTR-1 and

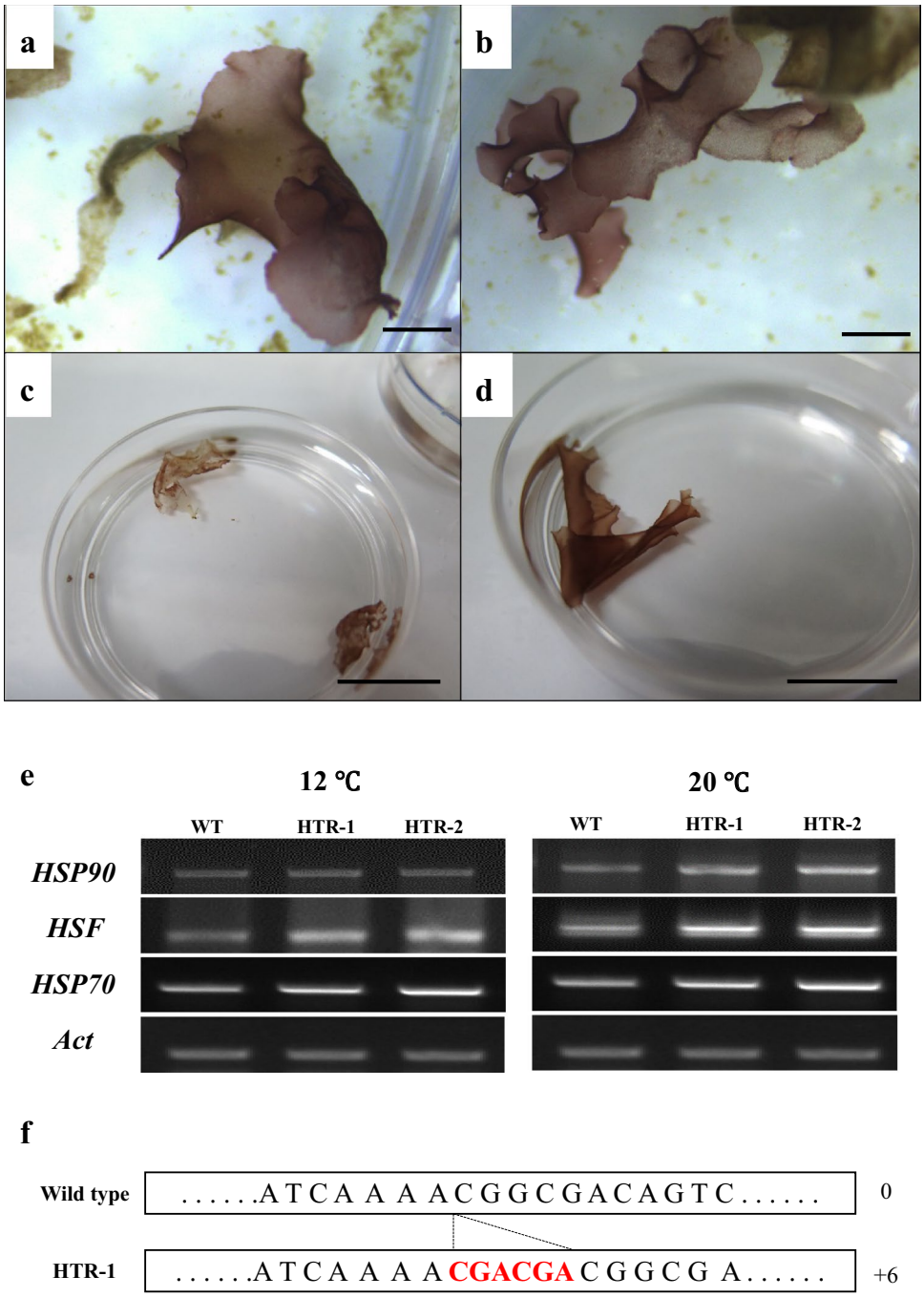

Fig. 3 Isolation of four high temperature-tolerant strains. Each panel displays the gametophyte isolated at 20 °C after gamma irradiation. Scale bars represent: **a, b** 1 cm; **c, d** 3 cm. **e** RT-PCR analysis of heat shock-related gene expression in the HTR-1 and HTR-2 mutant lines. Thalli were incubated at 12 °C and 20 °C for 3 h. **f** Illustration of 6 nt insertion in HTR-1. Insertion of HTR-1 was confirmed by sequencing of PCR fragment using genomic DNA for a TRINITY_DN48859_c0_g2_i1 (Fig. S1) which was identified from transcriptomes-based comparing and variant calling between wild type and mutants

HTR-2 thalli were 44.1 cm and 17.7 cm, respectively, while the wild-type thallus was 47.3 cm.

The wild-type strain is incapable of growth at 20 °C and the thallus displayed a deconstructed morphology. However, HTR-1 exhibited a fast growth rate under this condition that was similar to that observed following culture at 12 °C. In contrast, HTR-2 grew slowly compared to HTR-1. After 28 days of culture at 20 °C, the lengths of HTR-1 and HTR-2 thalli were 32.3 cm and 20 cm, respectively. At day 42, the HTR-1 and HTR-2 thalli were measured at 45.5 cm and 28.1 cm, respectively.

Pigment content and photosynthesis efficiency of the mutants

To characterize the photosynthetic efficiency of the mutants, we measured pigment content and maximal photochemical efficiency of photosystem II (*Fv*/*Fm*) in thallus

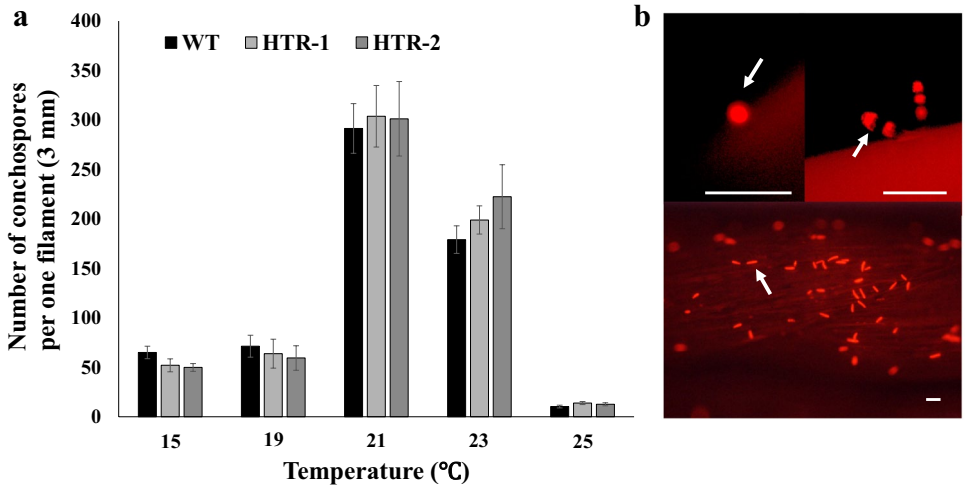

Fig. 4 Efficiency of conchospore release. **a** The efficiency of conchospore release in the wild-type (WT) and mutant (HTR-1 and HTR-2) strains after incubation for 7 days at the temperatures indicated was determined by counting. Error bars represent the standard deviation of ten replicates. **b** Fluorescence micrographs of germinated conchosopores on filament. Scale bars represent 100 μm

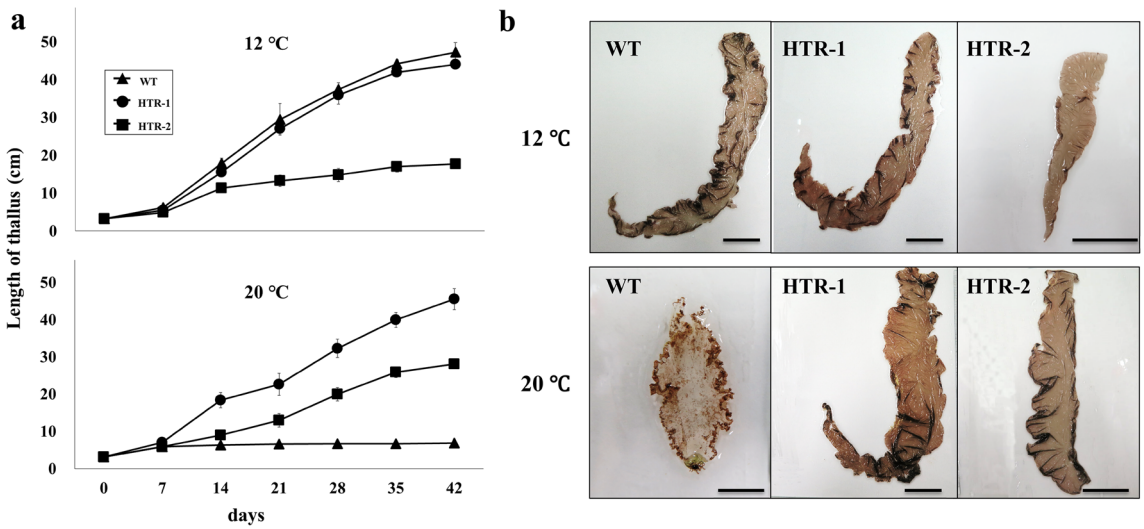

Fig. 5 Growth performance of the high temperature-tolerant mutants. **a** conchospore germlings (3 cm) from the wild-type (WT) and mutant (HTR-1 and HTR-2) strains were cultured at 12 °C and 20 °C. The length of the thallus was measured weekly for 42 days. Error bars represent the standard deviation of the three replicates. **b** Photographed image of thallus from wild-type and mutant strains grown for 42 days at 12 °C and 20 °C. Scale bars represent 5 cm except for WT (20 °C). Scale bar in WT (20 °C) represents 1 cm

incubated at 20 °C for 14 days. Initially, the level of chlorophyll *a* (Chl *a*), phycoerythrin (PE), and phycocyanin (PC) in the mutants was comparable to those in the wild-type strain (Fig. 6). However, these levels decreased over time in the wild-type strain, but were maintained in the mutants. Chl *a* content in wild type decreased drastically to 78%, 59%, and 54% of the initial level on days 3, 7, and 14, respectively. However, the Chl *a* content in both mutants was maintained at 87–107% relative to wild type (100%). The PE content in wild type was reduced to 70%, 56%, and 59% on days 3, 7, and 14, respectively, from (100%) value of the initial culture. However, 87–91% of the initial day content was maintained in HTR-1, while PE in HTR-2 was slightly reduced to 81% after 14 days. The PC content in wild type decreased dramatically to 82%, 40%, and 40% at 3, 7, and 14 days, respectively (Fig. 6). However, the level of PC content in both mutants was maintained throughout the time period assessed.

Next, to compare the photosynthetic efficiency between the mutants and wild type, the maximal photochemical efficiency (*Fv*/*Fm*) was analyzed. The wild-type and both mutant strains maintained an *Fv*/*Fm* above 0.6 when cultured at 12 °C (Fig. 7). However, at 20 °C, the *Fv*/*Fm* in wild type decreased gradually to 0.50, 0.40, and 0.30 at days 3, 7, and 14, respectively. In contrast, the *Fv*/*Fm* of both mutants was maintained around 0.6, similar to what was observed following culture at 12 °C.

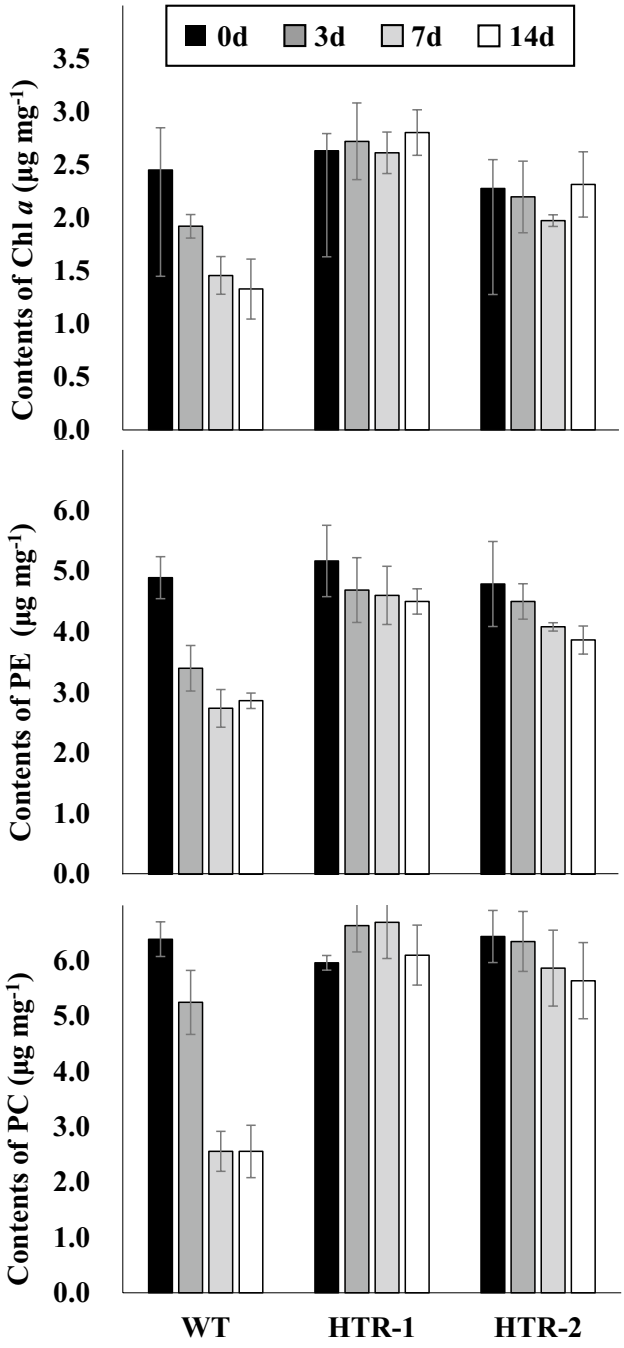

Fig. 6 Analysis of pigment content. Chlorophyll *a* (Chl *a*), phycoerythrin (PE), and phycocyanin (PC). The contents were assessed in conchospore germlings (1 cm) grown at 20 °C under 80 μmol photons m^{-2} s^{-1} (10L:14D) for 14 days. Pigment contents were measured in each thallus at days 3, 7, 10, and 14. Error bars represent the standard deviation of three replicates

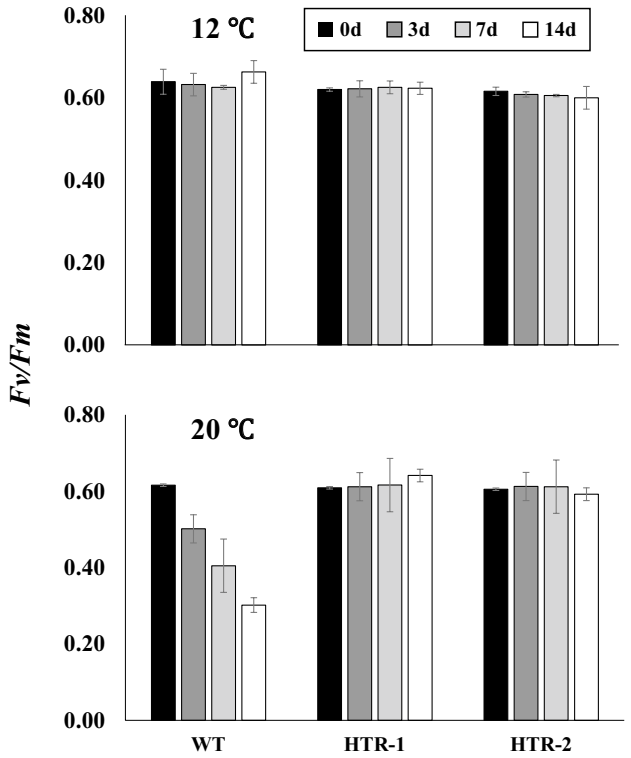

Fig. 7 Photosynthesis efficiency of the HTR mutants. The maximal photochemical efficiency (*Fv*/*Fm*) of thalli grown at 12 °C and at 20 °C was compared. Wild-type and mutant conchospore germlings (3 cm) were cultured under 80 μmol photons m^{-2} s^{-1} (10L:14D) at 12 °C and 20 °C for 14 days. *Fv*/*Fm* was measured at days 3, 7, 10, and 14. Error bars represent the standard deviation of three replicates

Monospore release

We compared the number of monospores released from the wild-type and mutant lines following growth at different temperatures for 4 weeks. Bright fluorescent monospores or germinating monospores attached onto filaments were observed and counted for each condition (Fig. 8; Fig. S3; Table S2). Monospore release was hardly detected in 2 weeks. After culture at 12–20 °C for 4 weeks, the number of monospores released by the mutants was lower than that of wild type (Fig. 8), with HTR-2 exhibiting a lower count than HTR-1. The greatest monospore release was found during culture at 20 °C, with the mutants exhibiting a lower quantity of release than wild type. More specifically, at 12–15 °C, the number of monospores released by HTR-1 and HTR-2 was 75–88% and 48–83% relative to wild type, respectively. Culture at 20 °C reduced monospore release of HTR-1 and HTR-2 to 89% and 78% that of wild type, respectively. However, monospore release was three times higher in the mutants than wild type following culture at 25 °C (Fig. 8).

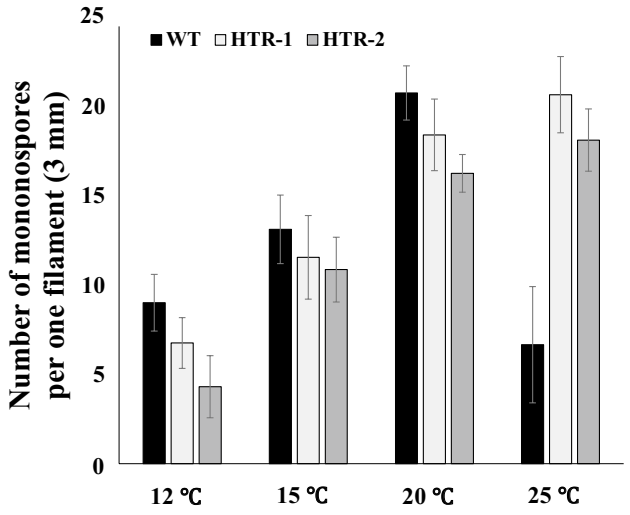

Fig. 8 Efficiency of monospore release. The efficiency of monospore release was determined under microscopic observations (Fig. S3) in wild-type and mutant strains cultured at the temperatures indicated. Error bars represent the standard deviation of ten replicates

Discussion

Global warming threatens the production of *Pyropia* on sea cultivation. To develop the competitive high temperature-tolerant strain of *P. yezoenesis* (Sugwawon no. 104), we isolated mutant strains that were capable of growing at high temperatures and selected those with characteristics important for sea cultivation. Genetic difference between wild type and mutants was confirmed by RT-PCR and genomic PCR analyses. Interestingly, only heat shock factor gene (HSF) was clearly upregulated at 12 °C and 20 °C where change of HSP70 and HSP90 expressions was not clear (Fig. 3e). These results suggest that other unidentified genes which are regulated by HSF are involved in conferring the high temperature tolerance in the mutants. Detection of 6 nt insertion in a selected transcript by genomic PCR sequencing (and many mutated transcripts from transcript sequencing) in HTR-1 mutant revealed genetic mutations induced by gamma irradiation. Wild-type *P. yezoenesis* cannot grow at 20 °C and displayed many defects, such as discoloration and a deconstructed thallus morphology (Fig. 1), indicating that the wild-type strain was sensitive to temperatures above 20 °C. However, the newly isolated mutant strains, HTR-1 and HTR-2, grew at 20 °C without any morphological and physiological defects (Figs. 5, 6, 7). The phenotypes of the mutants were maintained for 4 years, suggesting that the mutation conferring high temperature tolerance was stable. In addition, the mutants showed greater expression of gene transcripts related to the heat shock factor gene among the heat shock protein family (Choi et al. 2013) under elevated temperature compared to wild type (Fig. 3e). The photosynthetic efficiency (*Fv*/*Fm*) and level of pigments such as Chl *a*, PE, and PC were maintained during culture at 20 °C, whereas the contents in wild type decreased under these conditions (Figs. 6, 7). Analysis of *Fv*/*Fm* and pigment content indicates that the mutants can grow with normal photosynthetic efficiency at high temperatures (20 °C), unlike wild type, which cannot grow because photosynthesis is severely inhibited. Taken together, these results indicate that the mutants clearly show a tolerance for high temperature at the molecular and physiological level, which leads to improved growth performance. Efficiency of monospore release from growing thallus in the mutants was slightly lower than that of wild type at temperatures between 12 and 20 °C, but three times higher at 25 °C (Fig. 8), providing additional evidence of the high temperature tolerance of the mutants.

High temperature-tolerant-strains of *P. yezoensis* have been reported previously (Ding et al. 2016; Fu et al. 2011; Wang et al. 2012; Zhang et al. 2011). However, little to no analysis has been performed on the characteristics that are critical for industrial application, except for a study (Fu et al. 2011) reporting similar conchospores release between wild type and mutant. Our data demonstrate that vegetative growth, conchosporangium maturation, and conchospore release in conchocelis stage were similar or higher in the HTR-1 and HTR-2 mutants compared to wild type at temperature conditions which have been optimized for industrial and sea cultivation (Figs. 4, 5; Fig. S2). In *Pyropia* farming, mass release of monospores from young thallus cause slow growth in this tissue. Therefore, it is necessary to understand the characteristics of monospore release during temperature changes for the development of new strains. Monospore release from the mutant thalli during the gametophyte stage was low compared to wild type grown at temperatures above 15 °C (Fig. 8). These characteristics indicate that HTR-1 and HTR-2 are competitive strains that can be utilized for sea cultivation during periods of persistent high temperature.

Even though the growth characteristics of HTR-1 and HTR-2 were highly promising at high temperature, high growth performance at a normal temperature (around 12 °C) is also necessary for cultivation at sea. Our data demonstrate that HTR-1 growth was comparable to wild type at 12 °C, while HTR-2 exhibited slower growth (Fig. 5). We initially isolated 20 strains showing high temperature tolerance. However, only seven of them, including HTR-1, displayed a growth rate that was faster or comparable to wild type at 12 °C. The remaining 13 strains, including HTR-2, showed poor growth performance at 12 °C. Therefore, isolated mutants showing high temperature tolerance need to be analyzed to determine their optimal growth conditions prior to use in industrial farming.

Taken together, our results demonstrate that HTR-1 and HTR-2 are tolerant to high temperature (20 °C), with the

former strain exhibiting greater tolerance. These mutant strains are capable of growing under conditions that are severely inhibiting for the wild-type strain. HTR-1 and HTR-2 also exhibit high performance in the characteristics that are important for industrial cultivation at sea. When cultured at 12 °C, the growth and physiological performance of the HTR-1 gametophyte was similar to wild type; however, HTR-2 grew slower than the wild-type strain. Therefore, HTR-1 is a more promising strain than HTR-2 for sea cultivation under elevated temperatures. Further analysis for identifying the mutated genes in HTR-1 will be useful to understand a mechanism for high temperature tolerance in *Pyropia* species. Our results and approach will contribute to develop and isolate new strains capable of being cultivated in an increasingly warmer sea environment.

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References

Ashraf M, Harris PJC (2013) Photosynthesis under stressful environments: an overview. Photosynthetica 51(2):163–190

Chen SS, Ding HC, Yan XH (2016) Isolation and characterization of an improved strain of *Porphyra chauhanii* (Bangiales, Rhodophyta) with high-temperature resistance. J Appl Phycol 28(5):3031–3041

Choi S, Hwang MS, Im S, Kim N, Jeong WJ, Park EJ, Gong YG, Choi DW (2013) Transcriptome sequencing and comparative analysis of the gametophyte thalli of *Pyropia tenera* under normal and high temperature conditions. J Appl Phycol 25(4):1237–1246

Ding H, Zhang B, Yan X (2016) Isolation and characterization of a heat-resistant strain with high yield of *Pyropia yezoensis* Ueda (Bangiales, Rhodophyta). Aquacult Fish 1:24–33

Fu CH, Yan XH, Huang LB, Li L (2011) The releasing and high temperature resistance of conchospores from an improved strain in *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta). Oceanol Limnol Sinica 42:460–466

Hongfeng G (1993) The variation in the contents of phycobiliproteins from *Porphyra haitanensis* collected in different growing stages. Oceanol Limnol Sinica 24:645–648

Kim SO, Kim NG (2012) Effects of deep sea water on the liberation of monospore and growth in three species Porphyra (Rhodophyta, Bangiales). The Sea 17(1):1–8

Kuwano K, Aruga Y, Saga N (1996) Cryopreservation of clonal gametophytic thalli of Porphyra (Rhodophyta). Plant Sci 116:117–124

Miura A, Shin JA (1989) Cross breeding in cultivars of *Porphyra yezoensis* Ueda(Bangiales, Rhodophyta). A preliminary report. J Phycol Korean 4:207–211

Nikaido I, Asamizu E, Nakajima M, Nakamura Y, Saga N, Tabata S (2000) Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga, *Porphyra yezoensis*. DNA Res 7(3):223–227

Park EJ (2006) Genetic polymorphism within *Porphyra yezoensis*-related species (Bangiales, Rhodophyta) from Japan and Korea detected by CAPS analysis CAPS. Ph.D. Thesis Nat'1. Univ. Hokkaido, Japan

Park EJ, Fukuda S, Endo H, Kitade Y, Saga N (2007) Genetic polymorphism within Porphyrayezoensis (Bangiales, Rhodophyta) and related species from Japan and Korea detected by cleaved amplified polymorphic sequence analysis. Eur J Phycol 42(1):29–40

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975(3):384–394

Wang SJ, Zheng YZ, Ma LB, Xu P, Zhu JY (2000a) Gamma-rays induction of mutation in conchocelis of *Porphyra yezoensis*. Chin J Oceanol Limno 18(1):47–53

Wang WX, Vinocur B, Shoseyov O, Altman A (2000b) Biotechnology of plant osmotic stress tolerance physiological and molecular considerations. Acta Hortic 560:285–292

Wang HZ, Yan XH, Li L (2012) Selection and characterization of a high-temperature resistant strain of *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta). Oceanol Limnol Sinica 2:363–369

Yan XH, Lv F, Liu CJ, Zheng YF (2010) Selection and characterization of a high-temperature tolerant strain of *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta). J Appl Phycol 22(4):511–516

Zhang BL, Yan XH, Huang LB (2011) Evaluation of an improved strain of *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta) with high-temperature tolerance. J Appl Phycol 23(5):841–847