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HEAT-STRESS MEMORY IS RESPONSIBLE FOR ACQUIRED THERMOTOLERANCE IN *BANGIA FUSCOPURPUREA* [1]

Ikuya Kishimoto, Inori Ariga*

Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan

Yutaka Itabashi[#] and Koji Mikami[2]

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan

Running title: Heat-stress memory in *Bangia*

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[2]Corresponding author: Koji Mikami, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan. TEL/FAX: +81-138-40-899. E-mail: komikami@fish.hokudai.ac.jp

*Present address: Rokkatei Confectory Co. Ltd., 3-19 Kita 1, Nisi 24, Obihiro 080-2496, Japan

Present address: Department of Chemistry, Faculty of Science, Rikkyo University, 3-34-1 Nishi-Ikebukuro, Toshima, Tokyo 171-8501, Japan

The environmental stresses that sessile organisms experience usually fluctuate dramatically and are often recurrent. Terrestrial plants can acquire memory of exposure to sub-lethal heat stress to acquire thermotolerance and survive subsequent lethal high-temperature stress; however, little is known concerning whether seaweeds acquire thermotolerance via heat-stress memory. We have demonstrated that the red seaweed *Bangia fuscopurpurea* can indeed acquire memory of sub-lethal high-temperature stress, resulting in the acquisition of thermotolerance that protects against subsequent lethal high-temperature stress. Moreover, the maintenance of heat-stress memory was associated with a slight increase in the saturation level of membrane fatty acids. This suggests that the modification of membrane fluidity via changes in membrane fatty acid composition is involved in the establishment and maintenance of heat-stress memory in *B. fuscopurpurea*. These findings provide insights into the physiological survival and growth strategies of sessile red seaweeds to cope with recurrent changes in environmental conditions.

***Key index words*: *Bangia fuscopurpurea*, Fatty acid, Heat stress, Rhodophyta, Stress memory, Thermotolerance**

Temperature fluctuation represents one of the most prominent environmental stresses for sessile plants (Cramer et al. 2011, Kumar et al. 2012, Pandey et al. 2017). Increases in ambient temperature can cause heat stress and affect the survival, growth, development and reproduction of plants (Barnabás et al. 2008, Zinn et al. 2010; Hartfield and Prueger 2015). In the natural environment, heat stress is often chronic or

recurring. Although the molecular mechanisms that regulate the response to a single acute heat stress have been analyzed in detail, the response to recurring stress is only beginning to be elucidated. In plants, priming by exposure to a sub-lethal heat stress promotes the acquisition of thermotolerance, which allows plants to survive a subsequent and temporally distinct second potentially lethal heat stress (Bruce et al., 2007, Bäurle 2016, Sanyal et al., 2018). These findings indicate that plants can maintain the primed state to respond strongly to a recurring stress, a phenomenon that is termed heat-stress memory (Bruce et al. 2007, Bäurle 2016, Crisp et al. 2016, Sanyal et al. 2018).

Bangiales is an industrially and economically important marine resource and a large-scale mariculture system has been established for *Pyropia yezoensis* in Japan and *P. haitanensis* and *Bangia fuscopurpurea* in China (Mumford and Miura 1988, Wang et al. 2008, Blouin et al. 2011). Because an increase in temperature affects the growth and productivity of maricultured seaweeds, much effort has been invested to establish and analyze heat stress-tolerant strains of Bangiales (Yan et al. 2010, Ding et al. 2016, Lee et al 2017, Wang et al 2018a). In addition, transcriptome analyses have generated information concerning heat-inducible and -repressible genes (Choi et al. 2013, Im et al. 2015, Sun et al. 2015, Cao et al. 2017, Wang et al. 2018a, 2018b), which has indicated that Bangiales can respond to heat stress. However, knowledge concerning the regulatory mechanisms underlying the heat-stress response, tolerance and memory in Bangiales and other seaweeds is lacking.

The effects of heat stress on survival of the red seaweed *B. fuscopurpurea* have been investigated using strains harvested from various locations (Sommerfeild and Nichols 1973, Notoya and Iijima 2003, Wang et al. 2008, Mikami and Kishimoto 2018).

Sub-lethal heat stress promotes the asexual life cycle, which releases large numbers of asexual spores to increase the number of clones (Notoya and Iijima 2003, Wang et al. 2008, Mikami and Kishimoto 2018). Because these findings suggest that *B. fuscopurpurea* responds to heat stress like other Bangiales, we addressed whether *B. fuscopurpurea* can acquire heat-stress tolerance via acquiring heat-stress memory.

We first examined whether *B. fuscopurpurea* can acquire heat-stress tolerance (see Appendix S1 for details of experimental procedure). After growth at 15°C, *B. fuscopurpurea* was primed at the sub-lethal high temperature 28°C for 1 week and was further incubated at 30, 32 or 34°C for 1 week (Fig. 1A). As reported previously (Mikami and Kishimoto 2018), the direct transfer of unprimed control samples from 15°C to 32 or 34°C for 1 week strongly reduced their viability (Figs. 1B, S1). By contrast, *B. fuscopurpurea* samples that were primed at 28°C for 1 week could survive exposure to lethal conditions for 1 week (Fig. 1B). In this case, priming for 3 days was enough to acquire the heat-stress tolerance (Fig. S2). These findings indicate that the priming of *B. fuscopurpurea* by sub-lethal high temperature promotes the acquisition of heat-stress tolerance, enabling survival of subsequent exposure to lethal high temperature as a short-term response.

To assess whether this priming in *B. fuscopurpurea* leads to heat-stress memory, we designed experiments that included the insertion of a recovery phase by returning the samples to non-stress 15°C conditions between the priming phase and triggering phase as a secondary heat-stress exposure to a lethal temperature (Fig. 1C). Importantly, the viability of samples exposed to a 1-day recovery period was equal to that of primed samples without a recovery phase (Fig 1D). Thus, we concluded that *B. fuscopurpurea* can acquire heat-stress memory and maintain it after being returned to

non-stress conditions. However, increasing the duration of the recovery phase by 1 to 5 days gradually decreased viability and 6- and 7-day recovery periods abolished heat-stress memory (Fig. 1D), indicating that increasing the length of the recovery phase reduces heat-stress memory. Thus, we conclude that heat-stress memory is detectable for only 5 days after transfer to normal growth conditions.

There is a close relationship between membrane rigidification via a reduction in fluidity and heat-stress tolerance in Poikilothermic organisms, where membrane fatty acid composition influences the fluidity of membranes (Carratù et al. 1996, Horváth et al. 1998, Murakami et al. 2000, Sangwan et al. 2002, Königshofer et al. 2008, Leach and Cowen 2014). We therefore addressed whether the acquisition of the heat-stress memory in *B. fuscopurpurea* is related to changes in the composition of membrane fatty acids (Appendix S1). Firstly, we examined whether the membrane fatty acid composition was modulated by heat stress. When *B. fuscopurpurea* was cultured at 15°C followed by 32°C for a week, the relative amounts of saturated fatty acids and monoenes increased compared to those in the 15°C controls, whereas the relative amounts of polyenes decreased (compare the white and black bars in Fig. 2A–C). Thus, heat stress induces an increased saturation level of membrane fatty acids in *B. fuscopurpurea*, which likely results in increased membrane rigidification. Secondly, we investigated the effects of priming by exposure to sub-lethal high temperatures on the composition of membrane fatty acids. Cells from samples induced to acquire heat-stress memory exhibited intermediate levels of fatty acid saturation and unsaturation compared to those in the control and non-primed 32°C-shifted samples (Fig. 2A–C; gray bars at 0 days). Thus, the modification of fatty acid composition by priming appears to be important for the acquisition and maintenance of heat-stress tolerance.

Lastly, we investigated the relationship between the loss of heat-stress memory (Fig. 1D) and changes in fatty acid composition. The relative levels of monounsaturated fatty acids (monoenes) and polyunsaturated fatty acids (polyenes) in cells induced to acquire heat-stress memory gradually became more similar to those observed in non-primed controls (black bars), depending on the length of the recovery phase (Fig. 2B and C). Thus, the loss of heat-stress memory, which resulted in more dead cells at 32°C was closely associated with changes in membrane fatty acid composition. These findings indicate that priming of *B. fuscopurpurea* can lead to the acquisition of heat-stress memory and recovery treatments to its loss, and that both states are associated with a specific membrane fatty acid composition. We therefore conclude that the maintenance of heat-stress memory requires a specific membrane physical state and an appropriate level of membrane fluidity.

Our data demonstrate that *B. fuscopurpurea* can acquire heat-stress memory as a heat-stress tolerance strategy (Figs. 1 and 2), confirming that this phenomenon is widely conserved among seaweeds and terrestrial plants. This represents an important strategy that enables plants to adapt and survive under recurring or chronic heat stress in natural environments. Although heat-stress memory involves the maintenance of a heat-stress tolerance-specific composition of membrane fatty acids (Fig. 2), the regulatory mechanisms that alter membrane fatty acid composition by priming are largely unknown. The establishment and maintenance of heat-stress memory, which plays a central role in the maintenance of thermotolerance in response to recurring heat stress in terrestrial plants, is regulated epigenetically (Crisp et al. 2016, Lämke and Bäurle 2017, Friedrich et al. 2019). For example, a relationship exists between the maintenance of heat-stress memory and the accumulation of histone methylation markers H3K4me3

and H3K4me2 within chromatin at the promoters of genes that are highly expressed for several days during heat-stress memory (Kusch et al. 2014, Lämke et al. 2016; Liu et al. 2018). Thus, heat-stress memory is maintained by structural chromatin changes caused by H3 methylation during the memory phase. Because little is known concerning epigenetic regulation in response to abiotic stress in any Bangiales species, it is necessary to investigate whether genes associated with memory and their epigenetic control potentially regulate membrane fatty acid composition and maintain heat-stress memory in *B. fuscopurpurea*.

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Supporting Information

Fig. S1. Priming-induced thermotolerance in *Bangia fuscopurpurea*. Viability of *B. fuscopurpurea* under lethal high temperatures (32 and 34ºC) following prior priming at 28ºC were examined. For primed experiments (right half), samples grown at 15ºC were primed at 28ºC for a week, then triggering was performed at 30, 32 or 34ºC for a further week. For non-primed control experiments (left half), samples grown at 15ºC were directly transferred to 30, 32 or 34ºC and incubated for a further week. Mean values ± SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

Fig. S2. Time course analysis for acquisition of heat-stress tolerance during the priming phase in *Bangia fuscopurpurea*. Changes in viability associated with the duration of the priming phase were examined. Algal samples grown at 15ºC were separately primed at 28ºC for one to seven days, then triggering was performed for all of samples at 32ºC for a further week. Mean values ± SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

Appendix S1. Details of experimental procedure.

Legends to Figures

Fig. 1. Thermotolerance is dependent on heat-stress memory in *Bangia fuscipurpurea*. (A) Schematic representation of the experimental design to assess the ability of *B. fuscopurpurea* to acquire thermotolerance. After growth at 15°C and priming at 28°C for a week, triggering was performed at 30, 32 or 34°C for a further week. The upper panel indicates the control treatment without priming. (B) Priming-induced thermotolerance; *B. fuscopurpurea* survived lethal high temperatures (32 or 34°C) following prior priming under sub-lethal conditions. (C) Schematic representation of the experimental design to assess the ability of *B. fuscopurpurea* to acquire heat-stress memory. Varying durations of the recovery phase at 15°C were inserted between priming and triggering phases. (D) Changes in viability associated with the duration of the recovery phase. Mean values ± SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

Fig. 2. Changes in membrane fatty acid composition are associated with the acquisition and loss of heat-stress memory in *B. fuscopurpurea*. Changes in the relative amounts of saturated fatty acids (Saturates, A), monounsaturated fatty acids (Monoenes, B) and polyunsaturated fatty acids (Polyenes, C) were analyzed in control samples (white bars) or those triggered either with a lethal high temperature (32°C) without priming (black bars) or with priming and recovery treatments (gray bars). Mean values ± SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters

denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

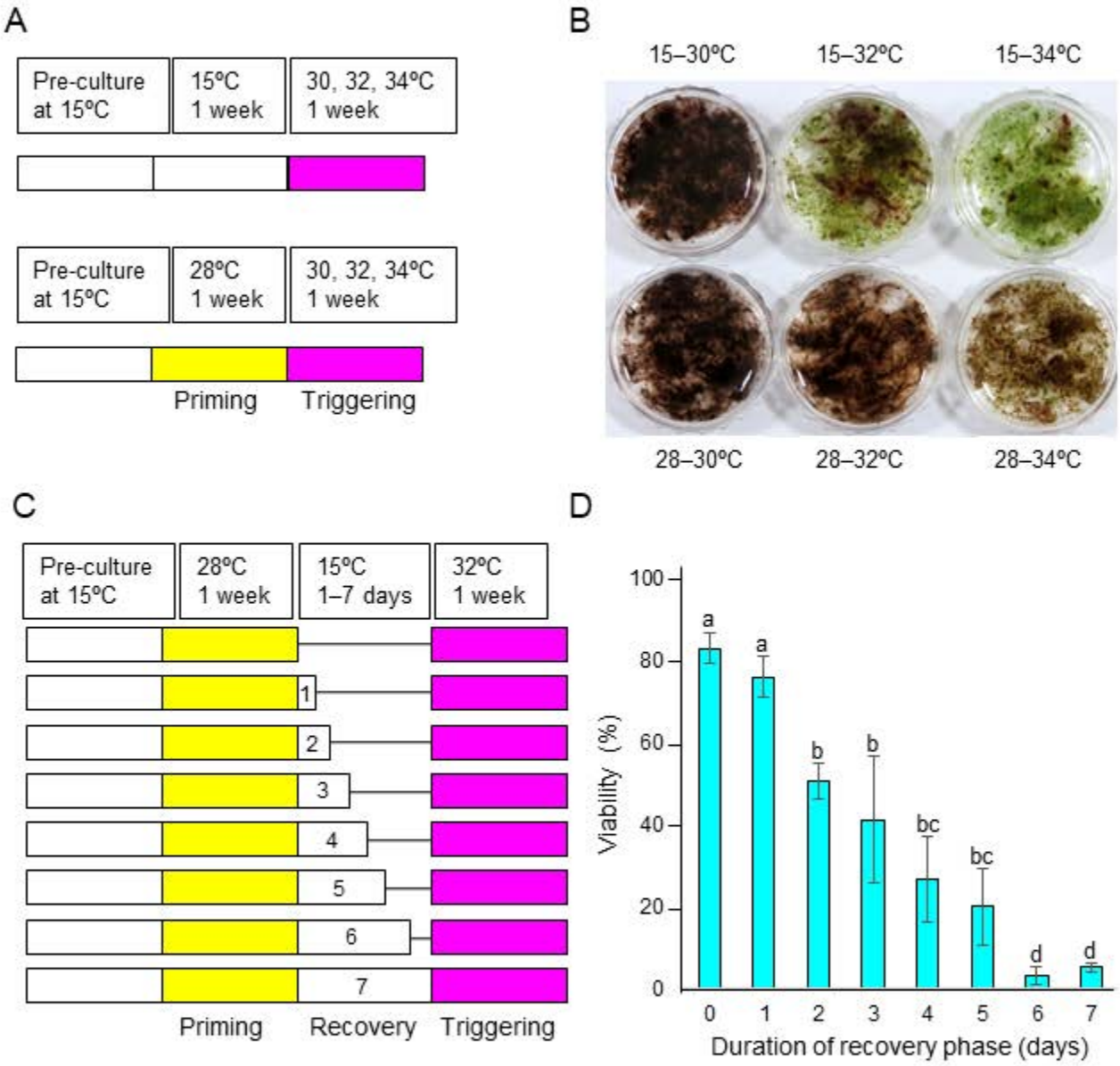
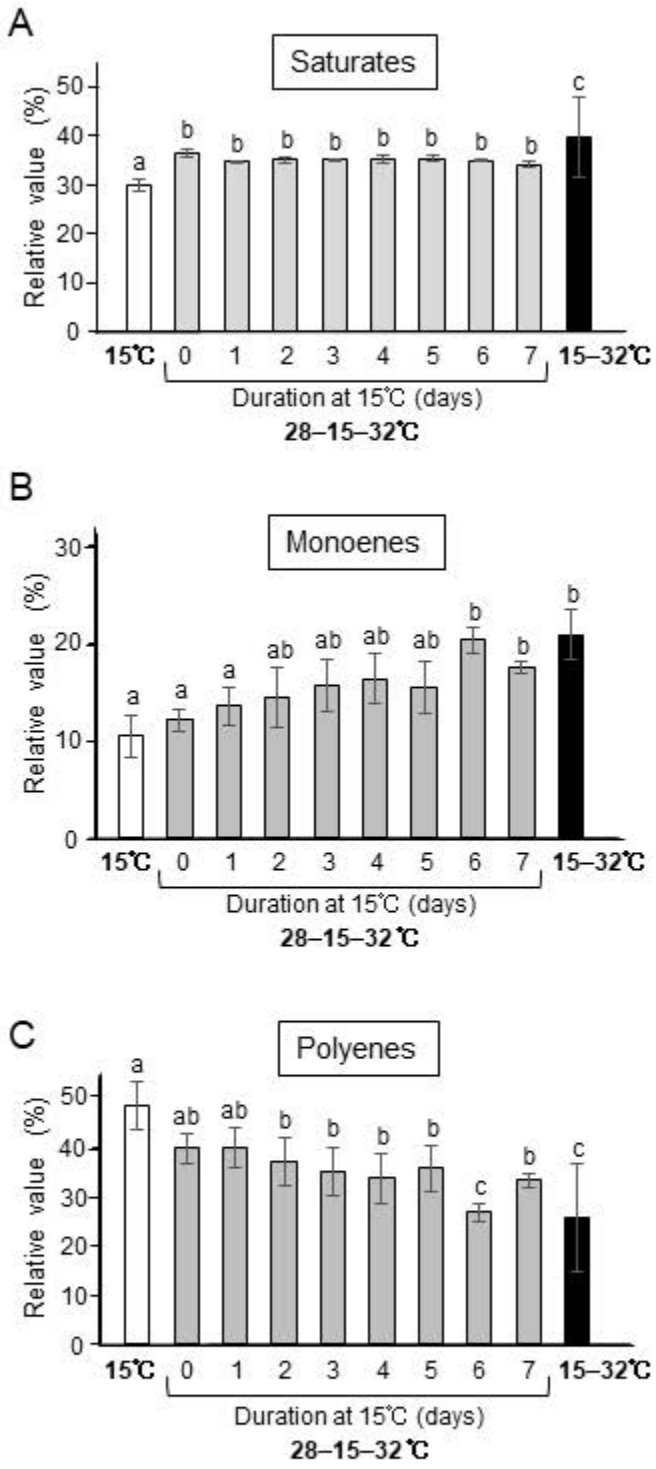

A
Pre-culture at 15ºC
15ºC 1 week
30, 32, 34ºC 1 week
Pre-culture at 15ºC
28ºC 1 week
30, 32, 34ºC 1 week
Priming
Triggering
B
15–30ºC
15–32ºC
15–34ºC
28–30ºC
28–32ºC
28–34ºC
C
Pre-culture at 15ºC
28ºC 1 week
15ºC 1–7 days
32ºC 1 week
1
2
3
4
5
6
7
Priming
Recovery
Triggering
D
Viability (%)
100
80
60
40
20
0
a
a
b
b
bc
bc
d
d
0
1
2
3
4
5
6
7
Duration of recovery phase (days)

Fig. 1


A
Saturates
Relative value (%)
50
40
30
20
10
0
a
b
b
b
b
b
b
b
b
c
15℃
0
1
2
3
4
5
6
7
15–32℃
Duration at 15℃ (days)
28–15–32℃
B
Monoenes
Relative value (%)
30
20
10
0
a
a
a
ab
ab
ab
ab
b
b
b
15℃
0
1
2
3
4
5
6
7
15–32℃
Duration at 15℃ (days)
28–15–32℃
C
Polyenes
Relative value (%)
50
40
30
20
10
0
a
ab
ab
b
b
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c
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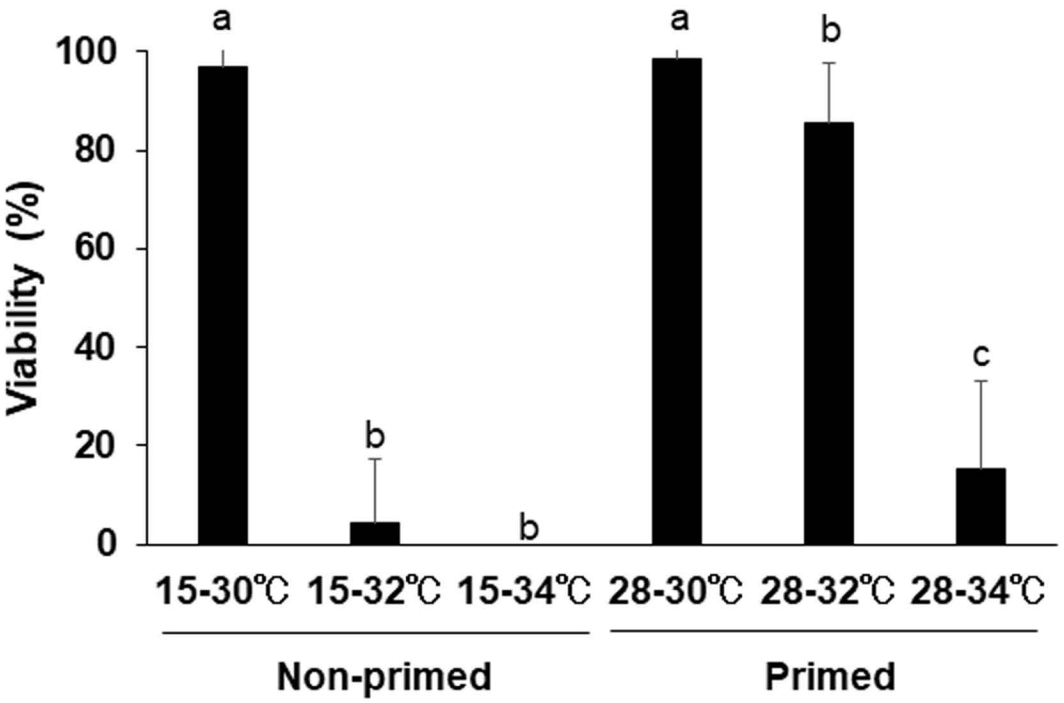
Duration at 15℃ (days)
28–15–32℃

Fig. 2



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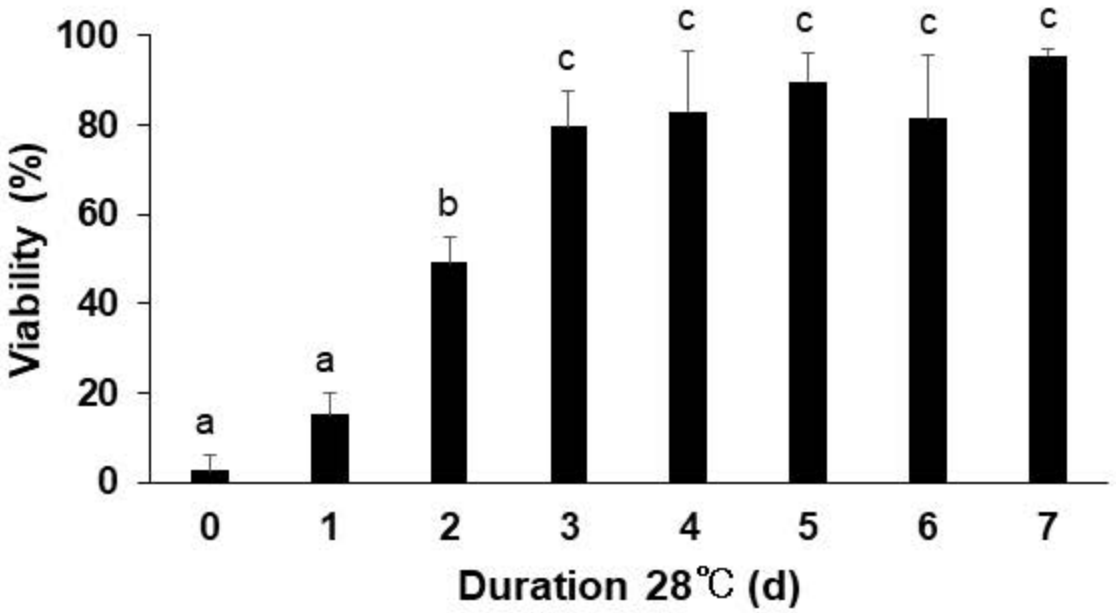

Fig. S2 Time course analysis for acquisition of heat-stress tolerance during the priming phase in *Bangia fuscopurpurea*.

Changes in viability associated with the duration of the priming phase were examined. Algal samples grown at 15°C were separately primed at 28°C for one to seven days, then triggering was performed for all of samples at 32°C for a further week. Mean values ± SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

Appendix S1

Detailed Materials and Methods

Algal material and heat-stress treatment

Gametophytic thalli of the marine red seaweed *Bangia fuscopurpurea* were collected at Esashi, Hokkaido, Japan on 14 May 2010, and a clean single thallus of unknown sex was maintained in the laboratory via the asexual propagation producing clones as an experimental line. To prepare control and starting samples, each 0.1 g (fresh weight) of *B. fuscopurpurea* thalli was cultured in dishes (Azunoru dish φ90 × 20 mm height, As One) containing 50 mL esterized artificial seawater at 15°C under 60 μmol m^{-2} s^{-1} irradiation (see Mikami and Kishimoto 2018, for details). Priming of *B. fuscopurpurea* thalli was performed by cultivating 15°C-grown samples at 28°C as a sub-lethal high temperature for 1 week. To analyze thermotolerance acquisition, primed samples were exposed to 32 or 34°C as lethal triggering conditions for 1 week. To assess the acquisition of heat-stress memory, recovery phases consisting of cultivation at 15°C for one to seven days were inserted between phases of priming at 28°C and triggering at 32 or 34°C. To determine the duration required for establishment of heat-stress tolerance, samples were separately primed at 28°C for one to seven days and then all samples were transferred to 32°C and incubated for seven days. Every treatment was repeated three times. The viability of all samples was tested, and the membrane fatty acid composition was analyzed as follows.

Viability test

As described previously (Mikami and Kishimoto, 2018), 0.1 g (fresh weight) of *B.*

fuscopurpurea thalli exposed to priming, recovery and triggering conditions as described above, were visualized daily by staining with ESL medium containing 0.01% erythrosine (Wako Pure Chemical Industries, Japan). After staining for 5 min at room temperature, thalli were gently rinsed with ESL medium to remove excess erythrosine and were mounted on a slide with ESL medium. Thalli were observed and photographed using an Olympus IX73 light microscope equipped with an Olympus DP22 camera. Cells stained by the dye were defined as dead cells. Viability was calculated from the number of living and dead cells obtained using micrographs. Analysis of every treatment was repeated three times and statistical analysis was performed as described in Mikami et al. (2018).

Extraction of lipids and preparation of fatty acid methyl esters

Fresh samples of *Bangia fuscopurpurea* were immersed in boiling water for 3 min to deactivate lipid hydrolytic enzymes and were then freeze-dried and homogenized using a grinder. Lipids were extracted via the Bligh-Dyer method (Bligh and Dyer, 1959) with some modifications. In brief, 0.1 g powdered algal sample was homogenized in a mixture of 2 mL MeOH and 1 mL $CHCl_3$, 1 mL $CHCl_3$ was added and the sample was vigorously mixed. After filtration, 2 mL $CHCl_3$ was added to the algal residue, which was then homogenized and filtered. All the obtained solvent fractions were combined, 1 mL water was added, and the sample was mixed. The organic solvent layer was recovered by centrifugation and evaporated to dryness under reduced pressure. The resultant lipids were resuspended to a known concentration with $CHCl_3$/MeOH (2:1, v/v) and stored at –30°C until use.

Lipids were converted to fatty acid methyl esters by heating at 90°C for 1 h in a

solution of 1–2% H_2SO_4/MeOH (Christie and Han, 2010) and were then purified in a Pasteur pipette filled with silica gel using hexane-Et_2O (95:5, v/v) as the mobile phase. The purified fatty acid methyl esters were confirmed on a silica gel 60 F_{254} aluminum TLC sheet (Merck, Darmstadt, Germany) using hexane-Et_2O (90:10, v/v) as the developing solvent (Christie and Han, 2010), and were then dissolved in 5 mg/mL hexane for gas chromatography.

GC analysis of fatty acid methyl esters

Fatty acid methyl esters were analyzed using a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an Omegawax 320 column (30 m × 0.32 mm i.d., Supelco, PA, USA). Helium was used as the carrier gas at a constant flow-rate of 2.2 mL/min. The split ratio was 1:25. The column temperature was maintained at 200°C isothermally. The injector and flame-ionization detector (FID) temperatures were set at 230°C and 240°C, respectively. Peaks were monitored on a Chromatopac C-R6A and identified by comparison with the retention data of some authentic standards (GLC-462, Nu-Chek Prep., Elysian, MN), and those of known fatty acids from marine algae (Takagi et al. 1985). Analysis of samples for every treatment was repeated three times and statistical analysis was performed as described in Mikami et al. (2018).

References

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