Title: Thermal acclimation of the symbiotic alga *Symbiodinium* alleviates photobleaching under heat stress

Shunichi Takahashi*, Miho Yoshioka-Nishimura, Daisuke Nanba, and Murray R. Badger

ARC Center of Excellence in Plant Energy Biology, Research School of Biology, The Australian National University, Canberra, Australian Capital Territory, 2601 Australia (S.T., M.R.B.); and Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan (M.Y.-N., D.N).

*Corresponding author;
Dr. Shunichi Takahashi; Address: ARC Center of Excellence in Plant Energy Biology, Research School of Biology, The Australian National University, Canberra, Australian Capital Territory, 2601 Australia; Tel: +61-2-6125-0299; Fax: +61-2-6125-5075; Email: shunichi.takahashi@anu.edu.au

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Abstract

Moderate increase in seawater temperature causes coral bleaching at least partially through photobleaching of the symbiotic algae *Symbiodinium*. Photobleaching of *Symbiodinium* is primarily associated with the loss of light-harvesting proteins of photosystem II (PSII) and follows the inactivation of PSII under heat stress. Here we examined the effect of increased growth temperature on the change in sensitivity of *Symbiodinium* PSII inactivation and photobleaching under heat stress. When *Symbiodinium* cells were grown at 25°C and 30°C, the thermal tolerance of PSII, measured by the thermal stability of the maximum quantum yield of PSII ($F_v/F_m$) in darkness, was commonly enhanced in all six *Symbiodinium* species tested. In *Symbiodinium* CCMP827, it took 6 h to acquire the maximum PSII thermal tolerance after transfer from 25°C to 30°C. The effect of increased growth temperature on the thermal tolerance of PSII was completely abolished by chloramphenicol, indicating that the acclimation mechanism of PSII is associated with the *de novo* synthesis of proteins. When CCMP827 cells were exposed to light at temperature ranging from 25°C to 35°C, the sensitivity of cells to high temperature induced photoinhibition and photobleaching were both ameliorated by increased growth temperatures. These results demonstrate that thermal acclimation of *Symbiodinium* helps to improve the thermal tolerance of PSII, resulting in reduced inactivation of PSII and algal photobleaching. These results suggest that whole organism coral bleaching associated with algal photobleaching can be at least partially suppressed by the thermal acclimation of *Symbiodinium* at higher growth temperatures.
Introduction

Reef-building corals harbor symbiotic dinoflagellate algae of the genus *Symbiodinium*. Corals generally show a brownish coloration due to algal photosynthetic pigments, such as peridinin, chlorophyll *a* and *c*₂ present in *in situ Symbiodinium*. However, under increased seawater temperatures, corals become pale through the loss of *Symbiodinium* cells and/or loss of photosynthetic pigments of *in situ Symbiodinium* (Glynn, 1993, 1996; Hoegghguldberg, 1999; Fitt et al., 2001; Coles and Brown, 2003). This phenomenon is so called coral bleaching. Since a healthy algae-coral symbiotic relationship is important for coral survival (Yellowlees et al., 2008), severe coral bleaching leads to the mortality of corals and even destruction of entire coral reef ecosystems. The frequency and intensity of coral bleaching has been increasing since the early 1980s and it is predicted to become more severe in the future due to ongoing global climate change and warming (Hughes et al., 2003). Coral reef ecosystems are in serious decline; with an estimated 30% already severely damaged, and it is predicted that globally as much as 60% of the world’s coral reef ecosystems may be lost by 2030 (Hughes et al., 2003).

Coral bleaching caused by heat stress is at least partially attributed to the photobleaching of photosynthetic pigments in *Symbiodinium* algae within corals (Kleppel et al., 1989; Porter et al., 1989; Fitt et al., 2001; Takahashi et al., 2004; Venn et al., 2006). The photobleaching commonly occurs in photosynthetic organisms under conditions where the absorbed light energy for photosynthesis is in excess of the capacity to use it particularly under environmental stress conditions in high light (Niyogi, 1999). In cultured *Symbiodinium* cells, heat stress-associated algal photobleaching is attributed to loss of major light harvesting proteins such as the peridinin-chlorophyll *a*-binding proteins and the chlorophyll *a*-chlorophyll *c*₂-peridin-protein complexes (Takahashi et al., 2008). A recent study has also demonstrated that the heat stress-associated loss of light harvesting proteins in *Symbiodinium* is attributed to the suppression of the *de novo* synthesis of light harvesting proteins but not acceleration of the photodamage and subsequent degradation of light harvesting proteins (Takahashi et al., 2008). High temperature sensitivity of *Symbiodinium*
cells to photobleaching differs among Symbiodinium species and this is at least partially attributed to the thermal sensitivity of the de novo synthesis of light harvesting proteins (Takahashi et al., 2008).

Heat stress-associated photobleaching in Symbiodinium follows severe photoinhibition of photosystem II (PSII) (Takahashi et al., 2008). The extent of photoinhibition is a result of the dynamic balance between the rate of photodamage to PSII and the rate of its repair. In plants and green algae, the PSII repair process is primarily composed of the degradation and the de novo synthesis of the D1 proteins in photodamaged PSII protein complexes (Aro et al., 1993; Takahashi and Murata, 2008; Takahashi and Badger, 2011). However, in Symbiodinium this differs, in that the photodamaged PSII can be repaired without the de novo synthesis of D1 proteins (Takahashi et al., 2009). Furthermore, a part of photodamaged PSII is repaired without protein synthesis (Takahashi et al., 2009), indicating that Symbiodinium algae have a unique PSII repair mechanism. In Symbiodinium found within corals and also in culture, heat stress accelerates photoinhibition at least partially through suppression of the PSII repair (Warner et al., 1999; Takahashi et al., 2004; Takahashi et al., 2009). However, the sensitivity of PSII repair to heat stress differs among Symbiodinium species and is strongly related to the sensitivity of PSII to photoinhibition under heat stress (Takahashi et al., 2009).

The high temperature sensitivity of corals to bleaching is changed by their growth temperature and this is suggested to be due to changing in situ Symbiodinium populations from heat sensitive to heat resistant ecotypes (Baker, 2001, 2003; Baker et al., 2004; Berkelmans and van Oppen, 2006; Jones et al., 2008; Jones and Berkelmans, 2010). However, thermal tolerance of the population might be also enhanced by thermal acclimation mechanism(s) associated with both the corals and Symbiodinium, although experimental data that directly supports this hypothesis is lacking. In this study, we examine the effect of increased growth temperature (thermal acclimation treatment) on the extent of heat stress-associated algal photobleaching using cultured Symbiodinium species. Our results demonstrate that Symbiodinium species commonly have thermal acclimation
mechanisms that enhance the high temperature tolerance of PSII and alleviate heat stress-associated photobleaching. Our results strongly suggested that thermal acclimation of *Symbiodinium* plays a role in alleviating algal photobleaching-associated coral bleaching under heat stress.
Results

Effect of growth temperature on thermal tolerance of PSII. To determine if growth of *Symbiodinium* at moderately elevated temperatures results in an acclimatory shift in the thermal tolerance of PSII, the maximum quantum yield of PSII ($F_v/F_m$) was measured in six different *Symbiodinium* species grown at 25ºC or 30ºC after exposure treatments at temperatures ranging from 25ºC to 38ºC for 1h in darkness (Fig. 1). In *Symbiodinium* cells grown at 25ºC, the measured $F_v/F_m$ remained unchanged up to 33ºC in all species but declined to zero at 34ºC in CCMP827, at 35ºC in CCMP831, CCMP830 and CCMP421, and at 36ºC in Mf1.05b and OAH-1. When *Symbiodinium* cells were grown at 30ºC for 8 days, the thermal tolerance of PSII (the minimum temperature that leads to a reduction of $F_v/F_m$ to zero) increased 1ºC in CCMP827, CCMP831 and Mf1.05b, and 2ºC in OAH-1, CCMP830 and CCMP421. These results demonstrate that all *Symbiodinium* species tested in our study commonly had a thermal acclimation mechanism that was able to improve the high temperature tolerance of PSII. Furthermore, our results indicate that the thermal acclimation ability shows slight differences among the *Symbiodinium* species.

To further understand the thermal acclimation mechanism in *Symbiodinium*, we examined the effect of different growth temperatures ranging from 25ºC to 32ºC on the thermal tolerance of PSII using *Symbiodinium* CCMP827 (Fig. 2A). When growth temperature increased from 25ºC to 28ºC, the thermal tolerance of PSII apparently increased 1ºC. There was no difference in the thermal tolerance of PSII between cells grown at 28ºC and 30ºC. In cells grown at 32ºC, the thermal tolerance of PSII was improved as much as cells grown at 28ºC and 30ºC, but the $F_v/F_m$ value after incubation at each growth temperature was much lower in cells grown at 32ºC, indicating that PSII was severely impaired in cells grown at 32ºC. These results demonstrated that a small increase (<3ºC) in growth temperature is enough to fully activate the thermal acclimation mechanism in CCMP827. To test whether the minimum temperature that fully activates the thermal acclimation mechanism is common in *Symbiodinium* species, we performed the same experiments in OAH-1 and CCMP830. In OAH-1, the thermal tolerance showed
improvement in cells grown at 30ºC compared with those at 28ºC (Fig. S1A). Furthermore, in CCMP830, the thermal tolerance was increased further in cells grown at 32ºC (Fig. S1B). These results demonstrate that the minimum temperature that fully activates the thermal acclimation mechanism shows differences among *Symbiodinium* species.

We examined how long it takes to acquire the maximum thermal tolerance of PSII in CCMP827 (Fig. 2B). Cells grown at 25ºC were transferred to 30ºC and incubated for 3 h, 6 h, 1 day or 8 days. The thermal tolerance of PSII was slightly enhanced after 3 h incubation at 30ºC and was significantly enhanced after 6 h. Further incubation had no effect on the thermal tolerance of PSII up to 8 days. The results demonstrate that the process of thermal acclimation was completed after 6 h in CCMP827.

We examined how long it takes to lose the acquired thermal tolerance of PSII in CCMP827 (Fig. 2C). Cells grown at 30ºC for 3 days were transferred to 25ºC and incubated for 2, 4, or 6 days. The thermal tolerance of PSII was slightly reduced after 2 days and significantly after 4 days. After 6 days incubation at 25ºC, there was no significant difference in the thermal tolerance of PSII with cells continuously grown at 25 ºC. The results demonstrate that the acquired thermal tolerance of PSII is completely lost in 6 days in CCMP827.

Thermal acclimation may be associated with the *de novo* synthesis of proteins. To examine this CCMP827 cells were transferred from 25ºC to 30ºC and incubated in the presence or absence of chloramphenicol in the darkness for 12 h. In general chloramphenicol inhibits the *de novo* synthesis of chloroplast-encoded proteins. However, in *Symbiodinium*, chloramphenicol has been demonstrated to inhibit the *de novo* synthesis of both chloroplast- and nuclear-encoded proteins (Takahashi et al., 2009). Since chloramphenicol inhibits protein synthesis-dependent repair of photodamaged PSII and also causes photoinhibition in light, experiments were carried out under darkness. In the absence of chloramphenicol, the thermal tolerance of PSII was enhanced by moderately increased temperature in the darkness (Fig. 3A) to an extent similar to that in the light (Fig. 1A & 2B), indicating that thermal acclimation of PSII in *Symbiodinium* is not light
dependent. The effect of moderately increased growth temperature on the thermal
tolerance of PSII was completely abolished by chloramphenicol (Fig. 3B). These results
demonstrate that the thermal acclimation mechanism in CCMP827 is associated with the de
novo synthesis of proteins.

The thermal acclimation helps maintain higher photosynthetic performance under
heat stress in light. An increase in seawater temperature causes acceleration of the
photoinhibition of PSII in the light in *Symbiodinium* (Warner et al., 1999; Takahashi et al.,
2004; Takahashi et al., 2009). To examine the effects of the thermal acclimation on
sensitivity of PSII to photoinhibition under heat stress, the extent of photoinhibition was
monitored in CCMP827 cells grown at 25°C or 30°C at temperature ranging from 25°C to
34°C under exposure to light (Fig. 4A). In cells grown at 25°C, the extent of
photoinhibition (decrease in the value of $F_v/F_m$ during light exposure) was drastically
enhanced at temperature above 31°C. However, in cells grown at 30°C, the temperature
that caused a rapid onset of photoinhibition was 1°C higher than that in cells grown at 25°C.
The results demonstrate that thermal acclimation not only improves the stability of PSII in
the dark but also alleviates photoinhibition of PSII in the light under heat stress in
CCMP827. Thus, thermal acclimation improves the photosynthetic performance under heat
stress conditions through enhancing the thermal tolerance of PSII (Fig. 1A & 2A) and
suppressing the thermal sensitivity of PSII to photoinhibition in the light in CCMP827 (Fig.
4A). However, this was different in other *Symbiodinium* species (Fig. S2). In OAH-1 and
CCMP830 cells, an increase in growth temperature did not help alleviate photoinhibition
under heat stress (Fig. S2), although it enhanced the thermal tolerance of PSII (see Figs 1D
and 1E). These results demonstrate that thermal acclimation improves photosynthetic
performance in OAH-1 and CCMP830 primarily through enhancing the thermal tolerance
of PSII but not through suppressing the thermal sensitivity of PSII to photoinhibition in the
light.
To further understand how thermal acclimation alleviates photoinhibition under heat stress, the thermal sensitivity of photoinhibition in the presence of chloramphenicol was examined in CCMP827 cells grown at 25°C or 30°C (Fig 4B). When cells grown at 25°C and 30°C were exposed to light at 30°C, there was no difference in the extent of photoinhibition in the presence of chloramphenicol between them. However, when cells were exposed to light at 33°C, the extent of photoinhibition was much higher in cells grown at 25°C than 30°C. These results show that thermal acclimation reduces photoinhibition in the presence of chloramphenicol under heat stress in CCMP827. As chloramphenicol inhibits the protein synthesis-dependent repair of photodamaged PSII, it is possible that thermal acclimation may suppress either or both acceleration of photodamage or inhibition of the protein synthesis-independent repair under heat stress.

To examine the effect of growth temperatures (25°C and 30°C) on the sensitivity of the PSII repair process to heat stress, we monitored the recovery of $F_v/F_m$ after photoinhibition treatment by strong light in *Symbiodinium* CCMP827 (Fig. 4C). Cells grown at 25°C and 30°C were exposed to strong light (2,000 μmol m$^{-2}$ s$^{-1}$) at each growth temperature for 1h. Cells were then pre-incubated in darkness at 30°C or 33°C for 1 h and subsequently exposed to low light (20 μmol m$^{-2}$ s$^{-1}$) to allow repair. The value of $F_v/F_m$ before the low light exposure (after photoinhibition and pre-incubation treatments) was 40% of the initial level in all conditions except at 33°C in cells grown at 25°C (in this case $F_v/F_m$ declined to 20% of initial values). This is presumably because PSII is thermally inactivated under 33°C in cells grown at 25°C as shown in Fig. 1A. In cells grown at both 25°C and 30°C, the recovery of $F_v/F_m$ was significantly lower at 33°C than 30°C, indicating that heat stress reduces the repair of photodamaged PSII in CCMP827 cells (Fig. 4C). Importantly, there was no effect of growth temperature on the recovery of $F_v/F_m$ at either 30°C or 33°C (Fig. 4C). This demonstrates that thermal acclimation had no influence on the sensitivity of the PSII repair to heat stress. Thus, in the presence of chloramphenicol, suppression of heat stress-associated photoinhibition by increased growth temperature (Fig. 4B) could be attributed to reducing photodamage to PSII caused by heat stress. This demonstrates that heat stress accelerates the photoinhibition process through both
increasing photodamage to PSII and reducing the PSII repair process and that consequence thermal acclimation alleviates photoinhibition through avoiding acceleration of photodamage to PSII under heat stress.

**Thermal acclimation alleviates photobleaching under heat stress.** Moderate increase in seawater temperature causes acceleration of photobleaching in *Symbiodinium* (Takahashi et al., 2004; Takahashi et al., 2008). To examine the effect of thermal acclimation processes on the extent of photobleaching under heat stress, total chlorophyll (chlorophyll *a* and chlorophyll *c*₂) content was measured before and after light exposure for 12 h at temperature ranging from 25ºC to 35ºC in *Symbiodinium CCMP827* cells grown at 25ºC or 30ºC (Fig. 5). In cells grown at 25ºC, loss of chlorophyll content was drastically enhanced at temperature above 33ºC and 50% of initial chlorophyll content was lost at 34ºC. However, in cells grown at 30ºC, the temperature that initiated the drastic loss of chlorophyll was 1ºC higher than that in cells grown at 25ºC and only 20% of initial chlorophyll content was lost at 34ºC. These results demonstrated that thermal acclimation increased the thermal threshold for initiating photobleaching in *Symbiodinium CCMP827*. Similar results were obtained in other *Symbiodinium* species of OAH-1 and CCMP830 (Fig. S3); where the thermal threshold for initiating drastic photobleaching increased 1ºC after growing cells at moderately increased temperatures.

**Photobleaching of *Symbiodinium* is not associated with inactivation of PSII.** Photobleaching of *Symbiodinium* commonly follows severe inactivation of PSII under heat stress. To determine whether the heat stress-associated photobleaching is due to inactivation of PSII, the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the extent of photobleaching was examined. When *Symbiodinium CCMP827* cells were incubated with 5 µM DCMU, the photosynthetic O₂ production rate decreased to 6% of that in the absence of DCMU (Fig. 6A). After incubation of light at 200 µmol m⁻² s⁻¹ for 12h,
DCMU slightly enhanced the extent of photobleaching (Fig. 6B). However, the effect of DCMU on photobleaching was much lower than the effect of heat stress on photobleaching in Figure 5. These results demonstrate that heat stress-associated photobleaching in *Symbiodinium* CCMP827 is not due to inactivation of PSII.
Discussion

Thermal acclimation alleviates photobleaching in *Symbiodinium* under heat stress.

Heat stress causes coral bleaching at least partially through acceleration of photobleaching in *Symbiodinium* living within corals (Kleppel et al., 1989; Porter et al., 1989; Fitt et al., 2001; Takahashi et al., 2004; Venn et al., 2006). In the present study, we demonstrate that the thermal tolerance threshold of *Symbiodinium* for initiating photobleaching increases after growing at moderately increased temperature (Fig. 5). Extrapolation of our results strongly suggests that photobleaching-associated coral bleaching can be alleviated by thermal acclimation of *Symbiodinium* growing within corals.

Consistent with previous studies (Takahashi et al., 2008), sensitivity of *Symbiodinium* to bleaching under heat stress differs among *Symbiodinium* species; e.g., CCMP827 (Fig. 5) showed more severe photobleaching under heat stress conditions than OAH-1 (Fig. S3A) and CCMP830 (Fig. S3B) in cells grown at 25ºC. In all three *Symbiodinium* species tested in the current study, thermal acclimation commonly showed a 1ºC increase in the thermal threshold for initiating photobleaching (Figs. 5 and S3). However, the results demonstrate that different thermal sensitivity of *Symbiodinium* among species is not due to different thermal acclimation ability.

The thermal sensitivity of *Symbiodinium* to photobleaching corresponds with the sensitivity of PSII to inactivation under heat stress (Chen et al., 2004; Takahashi et al., 2008). Since the process of photobleaching occurs after severe inactivation of PSII under heat stress, inactivation of PSII is expected to cause photobleaching in *Symbiodinium*. However, in the present study, inhibition of photosynthetic O₂ production at PSII by DCMU had little effect on the extent of photobleaching under our experimental conditions (Fig. 6B). In a previous study using *Symbiodinium*, heat stress-associated photobleaching has been demonstrated to be primarily due to suppression of the *de novo* synthesis of light harvesting proteins in thylakoid membranes (Takahashi et al., 2008). However, there was no effect of DCMU on the *de novo* synthesis of any membrane proteins (Fig. S4). It is therefore likely that heat stress-associated photobleaching of *Symbiodinium* is not due to
lack of PSII activity. Thus, the alleviation of photobleaching in thermally acclimated *Symbiodinium* cells (Fig. 5) is not due to remaining PSII activity. Our results suggest that heat stress causes both inactivation of PSII and photobleaching with a time delay. This might be because photobleaching is a slow process through proteolytic degradation of light harvesting proteins, while inactivation of PSII is quick process though non-enzymatic photochemical reactions.

In OAH-1 and CCMP830, the thermal threshold for initiating apparent photobleaching increased by 1°C after thermal acclimation (Fig. S3), although the thermal tolerance of PSII in the dark increased 2-3°C (Fig. S1). These results demonstrate that thermal tolerance of PSII that was examined in the dark does not always correspond with photobleaching sensitivity under heat stress. This lack of correlation might be because thermal sensitivity of PSII to inactivation is determined not only by the thermal tolerance of PSII but also the thermal sensitivity of PSII to photoinhibition. In OAH-1 and CCMP830, PSII performance was much higher in cells grown at 30°C or 32°C than cells grown at 25°C under heat stress (>34°C) before light exposure (Fig. S2). However, the difference was gradually minimized during light exposure due to the acceleration of photoinhibition in cells grown at higher temperature (Fig. S2). Thus, the thermal sensitivity of PSII in *Symbiodinium* differs between before and after light exposure treatments. The thermal sensitivity of *Symbiodinium* to photobleaching seems to correspond more closely with the thermal tolerance of PSII in the light rather than its stability in the dark.

**How does thermal acclimation enhance the thermal tolerance of PSII in *Symbiodinium***? Moderately increased growth temperature has been demonstrated to enhance the thermal tolerance of PSII in photosynthetic organisms (Armond et al., 1978; Nishiyama et al., 1999; Tanaka et al., 2000; Kimura et al., 2002; Nanjo et al., 2010) including *Symbiodinium* (Díaz-Almeyda et al., 2011). In the present study, small increases in temperature (<3°C) were found to be sufficient to activate the thermal acclimation mechanism of PSII in *Symbiodinium* (Fig. 2A). Furthermore, we found that it takes only 6
h to acquire the maximum thermal tolerance of PSII in *Symbiodinium* CCMP827 (Fig. 2B). The thermal acclimation mechanism of *Symbiodinium* was associated with the *de novo* synthesis of proteins (Fig. 3) as has been shown in other photosynthetic organisms (Tanaka et al., 2000; Nanjo et al., 2010). Furthermore, the thermal tolerance of PSII was enhanced in darkness (Fig. 3A) to a similar extent as in the light (Fig. 1A & 2). Thus, the synthesis of proteins which are responsible for the thermal acclimation of PSII in *Symbiodinium* is regulated solely by temperature as has been shown in green algae *Chlamydomonas reinhardtii* (Tanaka et al., 2000). Our results suggest that *Symbiodinium* cells acquire the thermal tolerance of PSII at any time of the day under increased seawater temperature.

A variety of factors have been shown to enhance the thermal tolerance of PSII in studies using plants, algae, and cyanobacteria, e.g., psbU protein in PSII (Nishiyama et al., 1997; Kimura et al., 2002), lipid compounds (Sato et al., 2003; Sakurai et al., 2007; Sakurai et al., 2007; Mizusawa et al., 2009), xanthophyll zeaxanthin (Havaux et al., 1996; Havaux, 1998), heat-shock proteins (Stapel et al., 1993; Eriksson and Clarke, 1996; Heckathorn et al., 1998; Tsvetkova et al., 2002), sigma factors (Singh et al., 2006; Tuominen et al., 2006), and isoprene (Sharkey and Singsaas, 1995). In *Symbiodinium*, membrane lipid composition has been suggested to determine the thermal tolerance of PSII (Tchernov et al., 2004). However, it is still controversial whether changes in lipid compositions are responsible for the thermal acclimation of PSII in *Symbiodinium* (Díaz-Almeyda et al., 2011). In cyanobacteria, the synthesis of fatty acids is involved, but stability increases of PSII are not associated with either the total amount of fatty acids nor the levels of unsaturated fatty acids in thylakoid membrane (Nanjo et al., 2010). Models suggest that the synthesis of fatty acids is expected to be associated with binding the PSII stabilizing proteins, such as lipoprotein. Inhibition of thermal acclimation of PSII by an inhibitor of protein synthesis might therefore be due to inhibition of the synthesis of proteins associated with the synthesis of fatty acids or stabilizing PSII under heat stress (Nishiyama et al., 1999; Kimura et al., 2002; Nanjo et al., 2010).
Thermal acclimation decreases the sensitivity of PSII to photoinhibition under heat stress. Heat stress causes acceleration of photoinhibition of PSII in cultured *Symbiodinium* and *in situ* *Symbiodinium* within corals (Warner et al., 1999; Takahashi et al., 2004; Takahashi et al., 2009). Heat stress-associated photoinhibition in *Symbiodinium* CCMP827 cells grown at 25°C was due to acceleration of photodamage to PSII (Fig. 4B) and also through inhibition of the PSII repair (Fig. 4C). We found that increased growth temperature alleviates the heat-stress-associated photoinhibition (Fig. 4A) through suppressing the photodamage to PSII (Fig. 4B) but not suppressing the inhibition of PSII repair (Fig. 4C). In *Symbiodinium*, heat stress has been demonstrated to damage thylakoid membranes (Tchernov et al., 2004), which impairs the generation of the proton gradient across thylakoid membrane. Since impairment of the proton gradation causes acceleration of photodamage to PSII (Takahashi et al., 2009; Takahashi et al., 2009), alleviation of thermal-stress-associated photodamage to PSII in *Symbiodinium* after growing at moderately increased temperature might therefore be due to suppressing damage to thylakoid membranes under heat stress.

Thermal acclimation of *Symbiodinium* may alleviate coral bleaching under heat stress. When corals are grown under moderately increased temperature, heat stress-associated coral bleaching is suppressed (Coles and Jokiel, 1978; Coles and Brown, 2003). Since the sensitivity of corals to bleaching under heat stress is changed by the thermal sensitivity of *Symbiodinium* living within corals and this differs among *Symbiodinium* species, the sensitivity of corals to bleaching under heat stress can be decreased by changing the dominant *in situ* *Symbiodinium* species population from a heat sensitive to tolerant one (Baker, 2001, 2003; Baker et al., 2004; Berkelmans and van Oppen, 2006; Jones et al., 2008; Jones and Berkelmans, 2010). In the present study, we found that the sensitivity of *Symbiodinium* to photobleaching under heat stress is suppressed by acclimation to moderately increased growth temperatures. In three cultured *Symbiodinium* species tested in this study, the thermal threshold for initiating apparent photobleaching commonly
increased by 1°C after thermal acclimation (Fig. 5 & S3). By extrapolation, these findings suggest that the threshold for initiating photobleaching-associated coral bleaching might increase by at least 1°C through a thermal acclimation process of *Symbiodinium* within corals without involving a change in the overall *Symbiodinium* population. It is still uncertain whether there are *Symbiodinium* species which show a greater acclimation ability and which increase their thermal threshold for initiating photobleaching by more than 1°C after thermal acclimation.

In the present study, thermal acclimation was demonstrated to alleviate inactivation of PSII through both enhancing the thermal tolerance of PSII (Fig. 1) and suppressing photoinhibition of PSII (Fig. 4A) under heat stress. Since inactivation of PSII has been hypothesized to be a trigger of expulsion of *Symbiodinium* from host cells (Brown, 1997; Hoeghguldberg, 1999; Warner et al., 1999), thermal acclimation of *Symbiodinium* might also reduce coral bleaching that is associated with the loss of *Symbiodinium* under heat stress. However, further study is needed to elucidate this hypothesis.

The thermal acclimation of *Symbiodinium* occurs in a period of hours (Fig. 2B), while dominant *Symbiodinium* species within corals are changed in the order of days (Baker, 2003). It is therefore conceivable that thermal acclimation of *Symbiodinium* and changes of dominant *Symbiodinium* species are associated with short- and long-term thermal acclimation, respectively, and both help to alleviate coral bleaching under heat stress.
Materials and Methods

Cultures and Growth Conditions. Cultures of *Symbiodinium* spp., CCMP827 (Clade A), CCMP831 (Clade A), CCMP830 (Clade B), CCMP421 (Clade E) were obtained from National Center for Marine Algae and Microbiota (Maine, USA) (Tchernov et al., 2004). OAH-1 (Clade B) (Ishikura et al., 2004) and Mf1.05b (Clade B) (Voolstra et al., 2009) were a gift from Dr. Tadashi Maruyama (JAMSTEC, Kanagawa, Japan) and Dr. Mary Alice Coffroth (University at Buffalo, NY, USA), respectively. Clades of each *Symbiodinium* cultures are according to previous studies (Ishikura et al., 2004; Tchernov et al., 2004; Voolstra et al., 2009). *Symbiodinium* cells (200–400 ml) were grown in artificial seawater (sea salts, SIGMA, St. Louis, USA) containing Daigo’s IMK medium for marine microalgae (Wako, Osaka, Japan) in 2L shaker flask with filter cap under fluorescent lights at 80 µmol photons m⁻² s⁻¹ with a light/dark cycle of 12/12 h. The flask was mixed once a day for aeration. Growth temperatures were controlled with electronic aquarium heater (A-761, Hagen, Germany) in 40 L aquarium tank. The cells were collected by filtration (0.22 µm, Stericup, Millipore Corporation, Massachusetts, USA) during their mid-logarithmic growth phase (< 0.5 µg of Chl ml⁻¹) and suspended in fresh growth medium for experiments.

Temperature Treatments. Freshly harvested cells were diluted to 5 µg Chl per ml and equal volumes incubated at different temperatures in darkness for 1h before either being illuminated at 200 µmol photons m⁻² s⁻¹ with halogen lamps or maintained in darkness. All temperature treatments represented in each experiment were performed simultaneously using an aluminium gradient heat bar with glass vials containing cells positions at appropriate temperatures along the bar in wells and illuminated with halogen lamps from the top where necessary. Light intensity (400-700 nm) was measured with a LI-250 light meter (LI-COR).

Photoinhibition, Chlorophyll and Photosynthetic O₂ Production Rate Measurements. Maximum quantum yield of PSII ($F_v/F_m$) was measured with a PAM-2000 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany) after the cells had been incubated for 10 min in darkness. The concentration of Chl $a$ and $c_2$ was measured by
treating cells collected by centrifugation (16,000 x g, 1 min) with 80% (v/v) methanol at
70°C for 10 min (Takahashi et al., 2008). Cell debris was removed by centrifugation
(16,000 x g, 1 min) and the absorption spectrum of the supernatant was measured using a
diode-array spectrophotometer (Cary 50 Bio, Varian) and the total Chl \(a\) and \(c_2\)
concentration calculated according to (Jeffrey and Humphrey, 1975). To measure the
photosynthetic \(O_2\) production rate, light-dependent \(O_2\) production was measured in
*Symbiodinium* cells (10 \(\mu\)g total Chl \(a\) and \(c_2\) in 1 ml) with a Clark-type oxygen electrode
(Hansatech Instruments) in the closed cuvette with stirring in the light at 1,000 \(\mu\)mol
photons m\(^{-2}\) s\(^{-1}\) at 25°C.

**Pulse Labeling of proteins and Separation of Membrane Proteins.**
*Symbiodinium* CCMP827 cells (5 \(\mu\)g Chl per 1 ml) were incubated with or without 5 \(\mu\)M
DCMU in the dark at 25°C for 1h. Then, cells were exposed to light at 200 \(\mu\)mol photons
m\(^{-2}\) s\(^{-1}\) in the presence of \([^{35}\text{S}]\text{Met/Cys}\) (10 mCi mL\(^{-1}\)) at 25°C for 15 min. Cells were
released by centrifuged and membrane proteins (corresponding to 1.5 \(\mu\)g of Chl) were
separated by NuPAGE Novex 4-12% Bis-Tris gel electrophoresis (Invitrogen) (Takahashi
et al., 2008). Gel was stained with Coomassie G-250 (GelCode Blue Stain Reagent,
Thermo Scientific) and dried on a paper with the gel dryer (Model 583, BioRad) at 80°C for
1h. The dried gel was exposed to an imaging Screen-K (Kodak) and visualized with a
Molecular Imager PharosFX Plus System (BioRad).

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Figure Legends

Fig. 1. Effect of growth temperature on the thermal tolerance of PSII in six different Symbiodinium species. Cells were grown at 25°C or 30°C for 8 days. The maximum quantum yield of PSII (Fv/Fm) was measured after incubation of cells for 1 h in darkness at temperatures ranging from 25°C to 38°C. The values are means ±SD (bars) from three independent experiments.

Fig. 2. Thermal acclimation of PSII stability in Symbiodinium CCMP827. A, Symbiodinium CCMP827 cells were grown at different temperatures ranging from 25°C to 32°C for 8 days. B, Symbiodinium CCMP827 cells grown at 25°C were transferred to 30°C and incubated for different periods (3 h, 6 h, 1 day, or 8 days). C, Symbiodinium CCMP827 cells grown at 30°C for 3 days were transferred to 25°C and incubated for different periods (2, 4, or 6 days). Control is a result of experiments of cells continuously grown at 25°C. The maximum quantum yield of PSII (Fv/Fm) was measured after incubation for 1 h in darkness at temperatures ranging from 25°C to 35°C. The values are means ±SD (bars) from three independent experiments.

Fig. 3. Effect of moderately increased growth temperature on the thermal tolerance of PSII in the presence and absence of chloramphenicol in Symbiodinium CCMP827. The maximum quantum yield of PSII (Fv/Fm) was measured in CCMP827 cells incubated at 25°C or 30°C for 12 h in darkness in the absence (A) or presence of 1 mM chloramphenicol (B). The values are means ±SD (bars) from three independent experiments. Cm, chloramphenicol.

Fig. 4. Effect of moderately increased growth temperature on the thermal sensitivity of PSII to photoinhibition and subsequent repair in Symbiodinium CCMP827. CCMP827
cells grown at 25°C or 30°C for more than 3 days were used for experiments.  A, Cells were incubated at temperatures ranging from 25°C to 34°C in darkness for 1 h and then exposed to light at 200 µmol m^{-2} s^{-1} for 12 h.  B, Cells were incubated at 30°C or 33°C in darkness for 1 h and then exposed to light at 200 µmol m^{-2} s^{-1} for 3 h in the presence of 1 mM chloramphenicol.  C, Cells were pre-exposed to light at 2,000 µmol m^{-2} s^{-1} at their respective growth temperatures for 1 h.  Cells were then incubated at 30°C or 33°C in darkness for 1 h before monitoring the recovery of $F_v/F_m$ for 3 h at 20 µmol m^{-2} s^{-1}.  In all experiments the maximum quantum yield of PSII ($F_v/F_m$) was measured after 10 min incubation in darkness.  The values are means ±SD (bars) from three independent experiments.  g.t., growth temperature, t.t., treated temperature.

**Fig. 5.** Effect of moderately increased growth temperature on the thermal sensitivity of *Symbiodinium* to photobleaching.  *Symbiodinium* CCMP827 cells grown at 25°C or 30°C were incubated at different temperatures ranging from 25°C to 35°C for 1 h in darkness.  Subsequently, cells (5 µg Chl per 1 ml) were exposed to light at 200 µmol m^{-2} s^{-1} for 12 h at the same temperature.  Total Chl content (Chl $a$ and Chl $c_2$) was measured before and after light exposure and the loss of Chl content (% of initial) was calculated.  The values are means ±SD (bars) from three independent experiments.

**Fig. 6.** Effect of DCMU on the sensitivity of *Symbiodinium* to photobleaching.  *Symbiodinium* CCMP827 cells grown at 25°C were incubated for 1 h in darkness with (+) or without (-) 5 µM DCMU and used for experiments.  All experiments were carried out at 25°C.  A, Effect of DCMU on photosynthesis.  Photosynthetic O$_2$ production rate was measured under the light at 1,000 µmol photons m^{-2} s^{-1}.  The photosynthetic O$_2$ production rate was 115±7.6 µmol O$_2$ mg Chl$^{-1}$ h$^{-1}$ in the absence of DCMU (control).  B, Effect of DCMU on photobleaching.  Cells (5 µg Chl per 1 ml) were exposed to light at 200 µmol m^{-2} s^{-1} for 12 h.  Total Chl content (Chl $a$ and Chl $c_2$) was measured before and after light
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