

Heat Induction of Cyclic Electron Flow around Photosystem I in the Symbiotic Dinoflagellate *Symbiodinium*¹

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Increases in seawater temperature impair photosynthesis (photoinhibition) in the symbiotic dinoflagellate *Symbiodinium* within cnidarian hosts, such as corals and sea anemones, and may destroy their symbiotic relationship. Although the degree of photoinhibition in *Symbiodinium* under heat stress differs among strains, the differences in their responses to increased temperatures, including cyclic electron flow (CEF), which sustains photoprotective thermal energy dissipation, have not been investigated. Here, we examined CEF in cultured *Symbiodinium* cells or those in an endosymbiotic relationship within a cnidarian host. The light-dependent reduction of the primary electron donor photosystem I, i.e. P700⁺, was enhanced in any *Symbiodinium* cell by increasing temperatures, indicating CEF was induced by heat, which was accompanied by thermal energy dissipation activation. The critical temperatures for inducing CEF were different among *Symbiodinium* strains. The clade A strains with greater susceptibility to photoinhibition, OTcH-1 and Y106, exhibited higher CEF activities under moderate heat stress than a more phototolerant clade B strain Mf1.05b, suggesting that the observed CEF induction was not a preventive measure but a stress response in *Symbiodinium*.

Dinoflagellate algae of the genus *Symbiodinium* form endosymbiotic relationships with marine cnidarian animals, such as corals, sea anemones, and jellyfish (Davy et al., 2012). In their symbiotic relationships, *Symbiodinium* cells provide photosynthetically produced energy, such as Glc or glycerol, to their host, and in return, they receive CO₂ and inorganic nutrients, such as nitrogen and phosphate (Davy et al., 2012). Because the hosts rely on the symbionts for the majority of their energy, efficient photosynthesis is key to maintaining the symbiotic relationship. This symbiotic relationship can be disrupted due to elevated temperatures, which results in mortality in corals and eventually the destruction of coral reefs (Hoegh-Guldberg, 1999; Lesser, 2011). The frequency and scale of this

phenomenon, which is known as coral bleaching, are increasing due to global warming, and coral bleaching is becoming a present danger for tropical ecosystems (Hoegh-Guldberg, 1999).

The photosystem II (PSII) activity is sensitive to elevated temperature in *Symbiodinium* (Iglesias-Prieto et al., 1992; Warner et al., 1996). This down-regulation of PSII is widely observed when an imbalance between the light energy absorption and its consumption occurs, collectively called photoinhibition (Aro et al., 1993). The damaged PSII is normally repaired in *Symbiodinium*, but this process is sensitive to heat (Takahashi et al., 2004), which gives rise to the loss of PSII and its light-harvesting antenna, namely, bleaching (Takahashi et al., 2008).

To avoid photoinhibition, photosynthetic organisms have developed diverse photoprotective mechanisms, such as scavenging systems for reactive oxygen species, the rerouting of reducing equivalents to oxygen via photorespiration or the Mehler reaction, and the thermal dissipation of excess energy (qE; Takahashi and Badger, 2011). Because *Symbiodinium* cells develop high levels of qE under heat stress conditions, qE is expected to help prevent heat stress-associated photoinhibition in *Symbiodinium* (Warner et al., 1996; Roth, 2014). Thus, the capacity of qE in each *Symbiodinium* strain may determine that strain's sensitivity to photoinhibition under heat stress.

It is generally accepted that qE is activated by the generation of a proton gradient (Δ pH) across the thylakoid membrane (Müller et al., 2001). In *Symbiodinium*, qE is fully relaxed upon dissipation of Δ pH by the uncoupler NH₄Cl (Takahashi et al., 2009). Because the formation of

¹ This work was supported in part by a cooperative program between the Japan Science and Technology Agency and the Fonds de la Recherche Scientifique; the Japan Society for the Promotion of Science through Grant-in-Aid for Scientific Research (A); the Ministry of Education, Culture, Sports, Science and Technology (through the Network of Centres of Carbon Dioxide Resource Studies in Plants); and by the research project on environmental response strategies of life at the National Institute for Basic Biology.

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Y.A., S.T., and J.M. designed the study; Y.A. performed the experiments; Y.A., S.T., and J.M. analyzed the data and wrote the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01886

ΔpH is accelerated by cyclic electron flow (CEF) around PSI (Shikanai, 2014), CEF has been proposed to be crucial for establishing high qE and alleviating photoinhibition in *Symbiodinium* under heat stress conditions. *Symbiodinium* is genetically diverse (Pochon and Gates, 2010), and the members of this genus exhibit different sensitivities to photoinhibition under heat stress (Warner et al., 1996). Reynolds et al. (2008) have characterized CEF in *Symbiodinium* with chlorophyll fluorescence transients and have demonstrated that CEF capacity is higher in the heat-tolerant strain (clade A) than in the heat-sensitive strains (clades B and C). Recently, however, Roberty et al. (2014) have suggested that the fluorescence signature reported by Reynolds et al. (2008) was not necessarily due to CEF and have claimed that the CEF capacity, as estimated on the basis of the electrochromic shift of carotenoids and the rereduction kinetics of the oxidized primary electron donor in PSI ($P700^+$), is negligible in all four *Symbiodinium* strains. Therefore, the capacity and significance of CEF remain controversial in *Symbiodinium*.

CEF is induced under moderate heat stress in vascular plants, such as tobacco (Sazanov et al., 1998), spinach (Bukhov et al., 1999), maize (Egorova and Bukhov, 2002), and oat (Quiles, 2006), which presumably helps to temporally alleviate photoinhibition due to high temperature. Therefore, it is likely that CEF is also induced in *Symbiodinium* under high temperature conditions, and the extent of this induction may determine the heat sensitivities of the different strains. However, no report has examined the effects of heat stress on CEF in *Symbiodinium*. In this study, we examined the effects of increased temperatures on CEF in three cultured *Symbiodinium* strains (OTcH-1, Y106, and Mf1.05b). We demonstrated that CEF is indeed enhanced by increasing temperature in *Symbiodinium*, but the extent of this enhancement varies by strain. Possible mechanisms and the significance of CEF increases in *Symbiodinium* under moderate heat stress are discussed.

RESULTS

Acceleration of CEF around PSI in *Symbiodinium* under Moderate Heat Stress

To measure the CEF around PSI in the clade A strain of *Symbiodinium* OTcH-1, we monitored the rereduction kinetics of $P700^+$ (Iwai et al., 2010; Supplemental Fig. S1). After exposure to a saturating pulse of white light ($2,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), absorption changes at 810 nm (ΔA_{810}) were monitored in the dark (Fig. 1). The rereduction kinetics of $P700^+$ were fitted to a single-exponential-decay curve. The extent of CEF was examined in the presence of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which completely abolished the electron transfer on PSII at a concentration of $>20 \mu\text{M}$ (Supplemental Fig. S2). Normally in oxic conditions, the reduction of $P700^+$ still occurred in the presence of DCMU but at a

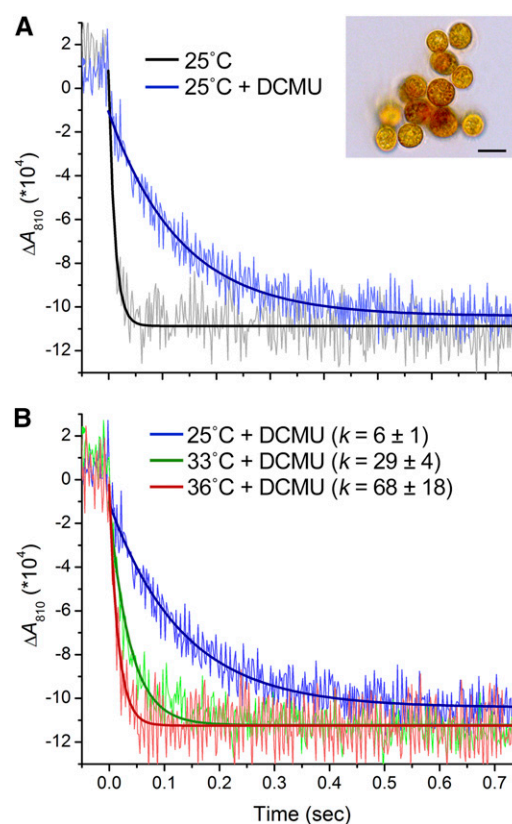


Figure 1. Dark reduction kinetics of photooxidized $P700^+$ in *Symbiodinium* OTcH-1. Five measurements taken at 10-s intervals were averaged and fit with a single-exponential decay. A, Effect of DCMU. The cells were incubated at 25°C for 15 min and subsequently treated with or without DCMU ($80 \mu\text{M}$) for 5 min in the dark before measurement. Inset, A micrograph of *Symbiodinium* cells (bar = $10 \mu\text{m}$). B, Effects of elevated temperatures. The cells were incubated at 25°C, 33°C, or 36°C for 15 min and subsequently treated with DCMU for 5 min in the dark before measurement. The reduction rate constants shown (k) are means \pm SD from three independent experiments.

considerably slower rate than in its absence (Fig. 1); however, this rate was accelerated in anoxic conditions because of the induction of CEF, much as it is in the green alga *Chlamydomonas reinhardtii* (Supplemental Fig. S3; Takahashi et al., 2013). We then examined the effects of high temperatures on CEF. Following incubation in the dark at 25°C, 33°C, or 36°C for 15 min, the $P700^+$ reduction was monitored in the presence of DCMU in *Symbiodinium* OTcH-1 cells (Fig. 1B). The rate constants for $P700^+$ reduction in the cells incubated at 33°C and 36°C were 5- and 10-fold greater, respectively, than that for the cells incubated at 25°C, which demonstrated that the levels of CEF were elevated upon incubation at higher temperatures in *Symbiodinium* OTcH-1.

To examine whether the heat induction of CEF also occurs when *Symbiodinium* cells are in an endosymbiotic relationship within a cnidarian host, we tested the sea anemone *Aiptasia* sp. H2, which harbors a clade B *Symbiodinium* (Xiang et al., 2013). The $P700^+$ reduction kinetics was monitored in the presence of DCMU in the

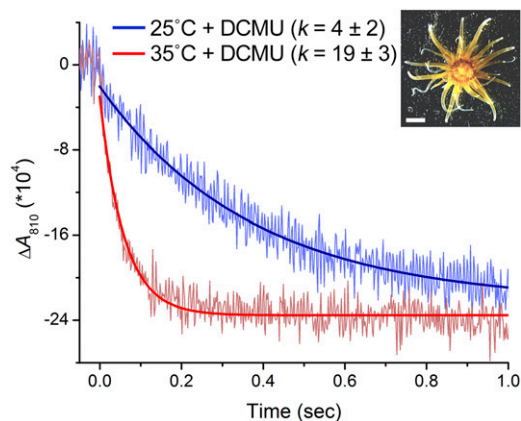


Figure 2. Dark reduction kinetics of photooxidized P700⁺ in *Aiptasia* H2. *Aiptasia* polyps were incubated at 25°C in the presence of DCMU (200 μM) for 15 min in the dark, and the reduction kinetics of P700⁺ were measured (blue line). The polyps were subsequently incubated at 35°C for 15 min in the dark, and the reduction kinetics of P700⁺ were measured (red line). The reduction rate constants shown (k) are means ± SD from three independent experiments. Inset, Micrograph of an *Aiptasia* polyp (bar = 2 mm).

Aiptasia polyps following dark incubation at 25°C or 35°C for 15 min (Fig. 2). The P700⁺ reduction in the polyps at 35°C was nearly 5-fold faster than that at 25°C. These results demonstrated that CEF was also accelerated in endosymbiotic *Symbiodinium* cells.

To further characterize the high temperature-induced CEF, we tested potential CEF inhibitors, including methyl viologen (MV) and antimycin A (AA). MV accepts electrons from PSI and limits the electron flow to CEF (Bukhov et al., 2001) in barley, whereas AA inhibits the PGR5-dependent CEF pathway in *Arabidopsis* (Munekage et al., 2002). To examine whether MV acts as an electron acceptor for PSI in *Symbiodinium*, we determined the effects of MV on the relative electron transport rate (rETR) in the presence of glycolaldehyde, which depletes the electron acceptor for PSI, i.e. NADP⁺. If MV accepted electrons from PSI, the inhibitory effect of glycolaldehyde on rETR would be abolished. However, there was no effect of MV on rETR in the presence of glycolaldehyde in *Symbiodinium* OTcH-1 (Supplemental Fig. S4A), suggesting that MV does not function as an electron acceptor for PSI in *Symbiodinium* OTcH-1. We then tested the effects of AA on CEF by measuring the nonphotochemical quenching of chlorophyll fluorescence (NPQ) to determine whether AA suppresses NPQ by impairing the generation of a ΔpH across the thylakoid membrane. NPQ development under strong light conditions was not inhibited by AA (Supplemental Fig. S4B), which suggests that AA does not function as a CEF inhibitor in *Symbiodinium*. Together, these results indicate that the characterization of electron transfer pathway of high temperature-induced CEF in *Symbiodinium* using these CEF inhibitors was unsuccessful.

In *Symbiodinium*, high temperatures have been demonstrated to inhibit the Calvin-Benson cycle (Jones

et al., 1998; Lilley et al., 2010). As described above, we determined that CEF is enhanced under similar conditions in *Symbiodinium*. To examine whether the high temperature-induced CEF was due to the inhibition of the Calvin-Benson cycle, we examined the effects of inhibitors of the Calvin-Benson cycle, including glycolaldehyde and iodoacetamide, on the P700⁺ reduction rate in *Symbiodinium* OTcH-1 (Supplemental Fig. S5). Glycolaldehyde inhibits the Calvin-Benson cycle by inactivating phosphoribulokinase without any side effects on photosynthetic electron transfer (Takahashi and Murata, 2006). Iodoacetamide inhibits the Calvin-Benson cycle though inactivating phosphoribulokinase, Fru 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and GAPDH (Ferri et al., 1981). When *Symbiodinium* cells were incubated with glycolaldehyde (120 mM) and iodoacetamide (5 mM), the net photosynthetic oxygen production rate dropped to 30% and 13% of the initial (control) levels, respectively (Supplemental Fig. S5A). Monitoring of the P700⁺ reduction revealed that both glycolaldehyde and iodoacetamide enhanced the P700⁺ reduction rate (glycolaldehyde and iodoacetamide increased the P700⁺ reduction rate to 127% and 208%, respectively, as compared with that in the absence of the inhibitor; Supplemental Fig. S5B). Thus, inhibition of the Calvin-Benson cycle enhances CEF in *Symbiodinium*. However, the effects of these inhibitors on the P700⁺ reduction rate were considerably smaller than that of high temperature. Therefore, it is unlikely that the high temperature-associated increase in CEF was primarily due to the inhibition of the Calvin-Benson cycle.

To examine whether the heat induction of CEF was reversible, we examined the time courses of P700⁺ reduction at 25°C and 33°C over 40 min following preincubation at 33°C for 15 min (Fig. 3). During the preincubation, the P700⁺ reduction rate gradually increased. The increased P700⁺ reduction rate was rapidly relaxed within 20 min of the transfer to 25°C, and it was further increased and saturated after 15 min at 33°C. Our results demonstrated that the heat-induced CEF is reversible. *Symbiodinium* might acclimatize to high temperature by up-regulating CEF.

We next examined the effects of high temperature on the induction of NPQ (Fig. 4). Three factors could contribute to NPQ: qE, state transitions (qT), and photo-inhibition (qI; Müller et al., 2001). Because qE is the only factor relevant to NPQ that is regulated by the ΔpH across the thylakoid membrane, the qE signal can be isolated by measuring the effect of the uncoupler NH₄Cl. When *Symbiodinium* cells were exposed to high light at 25°C, 33°C, and 35°C, NPQ was significantly induced to 0.7, 1.0, and 1.6, respectively (Fig. 4). However, in the presence of NH₄Cl, the induction of NPQ was nearly completely abolished at all temperatures tested (Fig. 4). These results demonstrate that the observed NPQ was due to qE and not to state transitions or photoinhibition. When DCMU was added during the light exposure, the induced NPQ was completely abolished at 25°C and 33°C (Fig. 4), which indicates that

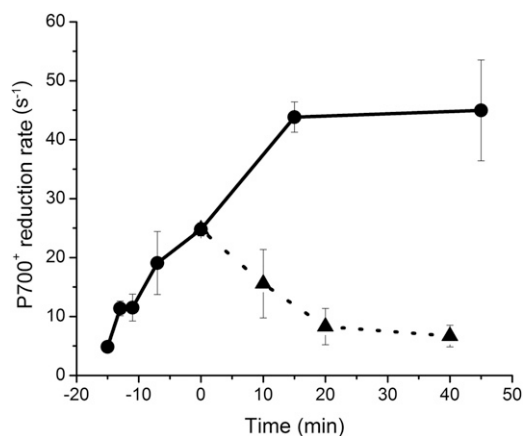


Figure 3. Time course of the P700⁺ reduction rate in *Symbiodinium* OTcH-1 after transfer from a high (33°C) temperature to the optimal growth (25°C) temperature. The P700⁺ reduction rate was monitored at 25°C (triangles) and 33°C (circles) following preincubation at 33°C for 15 min. The P700⁺ reduction rate was determined in the presence of DCMU. The values are presented as means \pm SD from three independent experiments.

qE development was solely dependent on linear electron flow. However, at 35°C, NPQ was only partially suppressed by DCMU, which suggests that CEF as well as linear electron flow contributed to the qE development at 35°C (Fig. 4).

Relationships between Photoinhibition Sensitivity and CEF Activity in Different *Symbiodinium* Strains

Symbiodinium strains are diverse, and their sensitivities to photoinhibition under heat stress vary considerably (Warner et al., 1996). We examined the

relationships between photoinhibition sensitivity and CEF activity using three different *Symbiodinium* strains, i.e. OTcH-1 (clade A), Y106 (clade A), and Mf1.05b (clade B1), at 25°C and 33°C. The extent of photoinhibition was monitored on the basis of the decrease in the maximum quantum yield of PSII (F_v/F_m) during light exposure (Fig. 5A). F_v/F_m decreased slightly faster in OTcH-1 and Y106 than in Mf1.05b at 25°C (F_v/F_m declined to 62%, 50%, and 79% of the initial values after 80 min of light exposure in OTcH-1, Y106, and Mf1.05b, respectively). At 33°C, the F_v/F_m in OTcH-1 and Y106 decreased significantly faster than at 25°C (the F_v/F_m declined to 29% and 28% of the initial values at 33°C in OTcH-1 and Y106, respectively), but there was no difference in the F_v/F_m of Mf1.05b. These results indicate that Mf1.05b is less sensitive to heat-induced photoinhibition than OTcH-1 and Y106. We further examined the effects of high temperature on the P700⁺ reduction rate (Fig. 5B). At temperatures ranging from 25°C to 30°C, P700⁺ reduction was slow in all three *Symbiodinium* strains, which is consistent with the results of a previous report (Roberty et al., 2014). However, this rate was considerably higher in all three *Symbiodinium* strains at temperatures above 30°C; the rates started to increase at 31°C in OTcH-1 and Y106 and at 34°C in Mf1.05b (Fig. 5A). The P700⁺ reduction rate was considerably higher in OTcH-1 and Y106 than in Mf1.05b at all temperatures. The P700⁺ reduction rates at 34°C in OTcH-1 and Y106 were 4.6- and 3.9-fold greater than in Mf1.05b, respectively (Fig. 5A). Our results demonstrate that *Symbiodinium* strains commonly enhance CEF under high temperature conditions and that the extent of this enhancement differs among strains. Intriguingly, the clade A *Symbiodinium* strains (OTcH-1 and Y106), which are more sensitive to photoinhibition, exhibited greater CEFs than the clade B strain (Mf1.05b), which is less sensitive to photoinhibition at

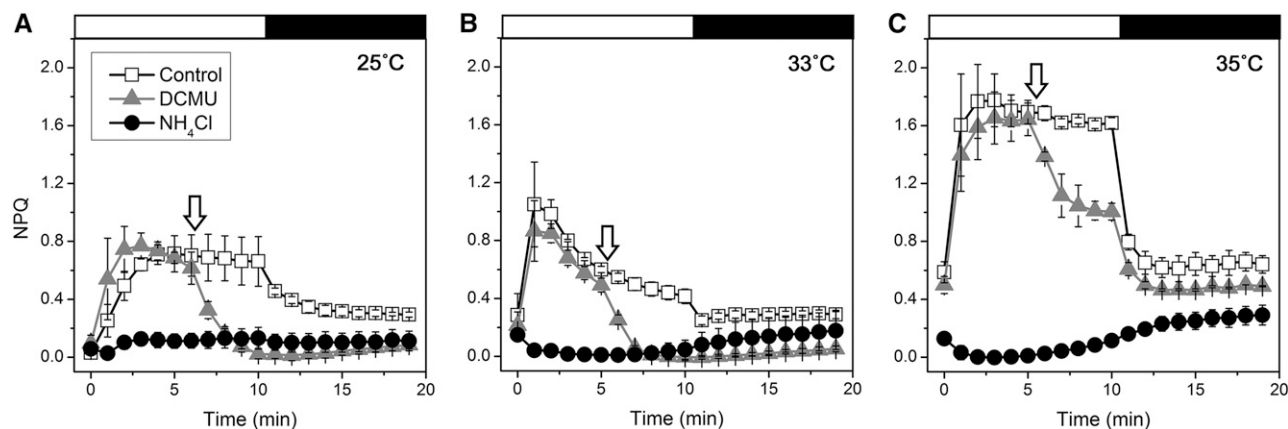


Figure 4. Effects of DCMU and NH_4Cl on NPQ at different temperatures in *Symbiodinium* OTcH-1. The cells were incubated at 25°C for 30 min in the dark with or without NH_4Cl (100 mM). Next, the cells were incubated at 25°C (A), 33°C (B), or 35°C (C) for 15 min in the dark, and the NPQ development was monitored in the light at $240 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 10 min. The relaxation of NPQ was monitored in the dark for another 10 min. DCMU was added to the cells 5 min after the onset of light exposure (arrows). The values are presented as means \pm SD of two or three independent experiments.

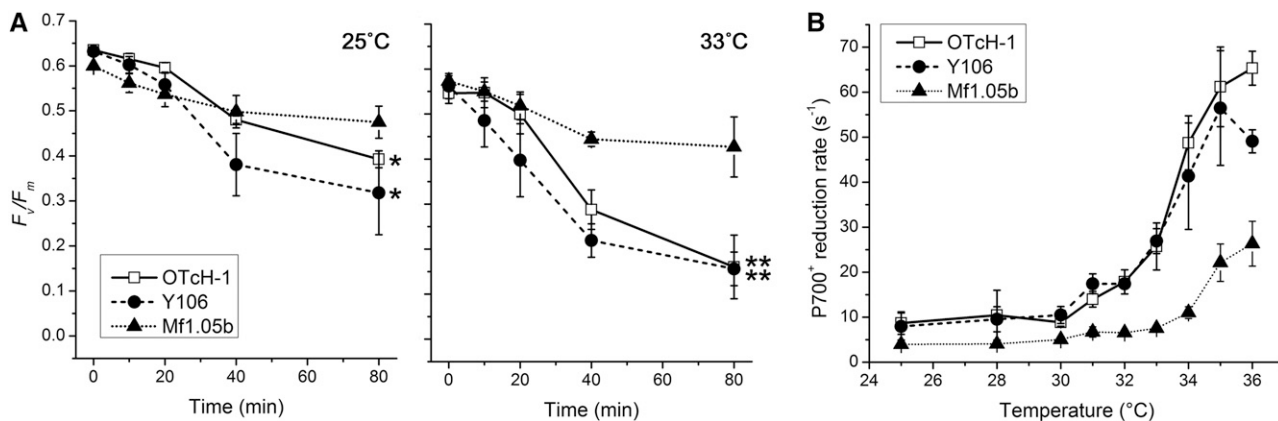


Figure 5. Relationships between the extent of CEF and the photoinhibition sensitivity in three different *Symbiodinium* strains. A, Effects of high temperature on PSII photoinhibition. The cells were preincubated at 25°C (left) and 33°C (right) for 1 h in the dark and were subsequently exposed to light at 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The maximum quantum yield of PSII, i.e. F_v/F_m , was measured at each time point after dark incubation for 10 to 15 min. The asterisks indicate statistically significant differences compared with the corresponding data from Mf1.05b (* $P < 0.05$ and ** $P < 0.01$, Student's t tests). B, Effects of elevated temperatures on the P700⁺ reduction rate. The cells were incubated at temperatures ranging from 25°C to 36°C for 15 min in the dark before the measurements. DCMU was subsequently added, and the rate constant of P700⁺ reduction was taken as demonstrated in Figure 1. The values are presented as means \pm SD from three independent experiments.

34°C, thus suggesting that the extent of CEF does not correspond to the strains' abilities to cope with photoinhibition under heat stress but rather reflects their stress responses.

DISCUSSION

High Temperature Activates CEF in *Symbiodinium*

Reynolds et al. (2008) have demonstrated that clade A *Symbiodinium* strains exhibit significant CEF activity. In contrast, Roberty et al. (2014) have reported that the CEF activities in four *Symbiodinium* strains, including clade A, are only marginal. Therefore, the significance of CEF and whether CEF is always active, always inactive, or active only under certain conditions in *Symbiodinium* have remained unclear. In our study, CEF activities were low in all three of the *Symbiodinium* strains we tested, including the clade A strains, when they were grown at the optimal growth temperatures. However, CEF activity was enhanced by increasing the temperature, and this enhancement was apparent at temperatures above 33°C (Fig. 1). Therefore, CEF can be induced by heat in *Symbiodinium*. The enhanced CEF activity after heat treatment was rapidly relaxed by the return to the optimal growth temperature (Fig. 3), which suggests that heat-induced CEF is a reversible process that responds to heat stress in *Symbiodinium*. Because similar phenomena have been observed in *Chlamydomonas* (Supplemental Fig. S6) and land plants (Sazanov et al., 1998; Bukhov et al., 1999; Egorova and Bukhov, 2002; Quiles, 2006), heat-induced CEF could be a common response of photosynthetic organisms, although its extent depends on the organism. Because such heat-induced CEF is more evident in *Symbiodinium*

than in *Chlamydomonas* (Fig. 5; Supplemental Fig. S5), such acclimation of electron flow may be important for these tropical symbiotic algae to cope with the acute heat stress that they likely encounter frequently in their niche.

CEF is necessary to activate qE because it contributes to the generation of a ΔpH across the thylakoid membrane. Accordingly, mutant land plants deficient in CEF activity exhibit significantly lower qE capacities that lead to greater susceptibility to photoinhibition compared with that of wild-type plants (Munekage et al., 2002), which explains why *Symbiodinium* becomes highly susceptible to photoinhibition after treatment with the proton uncoupler NH_4Cl (Takahashi et al., 2009). Thus, the induced CEF observed in this study could help alleviate photoinhibition under hot and/or excessive light conditions in *Symbiodinium*. Because photoinhibition causes the bleaching of the pigments in endosymbiotic *Symbiodinium* and eventually causes "coral bleaching" under high temperature conditions (Takahashi et al., 2004; Takahashi et al., 2008), the heat-induced CEF observed in this study (Fig. 2) might also help alleviate coral bleaching.

How Do Temperature Increases Enhance CEF in *Symbiodinium*?

In *Symbiodinium*, CEF was rapidly enhanced by increases in temperature (Fig. 3). In higher plants, the heat induction of CEF has been hypothesized to be caused by the accumulation of reductants, such as NADPH, in the chloroplast stroma (Bukhov and Carpentier, 2004; Livingston et al., 2010; Johnson, 2011). This hypothesis is based on findings that environmental stresses, including high temperatures, inhibit reactions in the

Calvin-Benson cycle (Weis, 1981; Jones et al., 1998; Lilley et al., 2010), which increases the NADPH/NADP⁺ ratio and in turn up-regulates CEF (Endo et al., 2005; Breyton et al., 2006; Govindachary et al., 2007). However, in this study, we demonstrated that glycolaldehyde, which inhibits the Calvin-Benson cycle, only slightly enhanced CEF in *Symbiodinium* (Supplemental Fig. S5). Therefore, the heat-induced CEF was not primarily due to the inhibition of the Calvin-Benson cycle in *Symbiodinium*.

In land plants (Joët et al., 2002) and green algae (Takahashi et al., 2013), CEF has been demonstrated to be accelerated under anaerobic conditions. Because inhibitors of mitorespiration and chlororespiration accelerate CEF in tobacco leaves (Joët et al., 2002), the anaerobic acceleration of CEF is likely due to the accumulation of reductants in the stroma (Bulté et al., 1990; Joët et al., 2002; Takahashi et al., 2013). Our results demonstrated that CEF was accelerated under anaerobic conditions in *Symbiodinium* (Supplemental Fig. S3), which suggests that the accumulation of reductants could also accelerate CEF in this alga. It is also possible that the heat-induced CEF was caused by the inhibition of mitorespiration or chlororespiration in heat-treated *Symbiodinium*. However, mitorespiration is generally less sensitive to increased temperature than photosynthesis in photosynthetic organisms, including *Symbiodinium* (Iglesias-Prieto et al., 1992). Furthermore, we cannot exclude other mechanisms, for example, the conformational changes in the cytochrome *b₆f* complex that have previously been demonstrated in isolated chloroplasts from peas (Thomas et al., 1986).

Two possible CEF pathways have been proposed for CEF: an NAD(P)H dehydrogenase (NDH)-dependent pathway and an Fd-dependent pathway (Shikanai, 2007). In the former pathway, NDH mediates NADPH oxidation and the plastoquinone pool reduction in much the same manner as complex I does in the mitochondria (Ogawa, 1991). Some algae, such as *Chlamydomonas*, do not have type I NDH but have only a single subunit of NDH (Nda2) that is localized in the chloroplast (Jans et al., 2008). The genome of *Symbiodinium* Mf1.05b (Shoguchi et al., 2013) contains only type II NDH (Supplemental Table S1). In the latter pathway, two molecular models have been proposed: the CEF supercomplex comprising PSI and its own antenna system (the PSI-LHCI supercomplex), LHCIIs, the cytochrome *b₆f* complex, FNR, and PGRL1 with equimolar amounts of the cytochrome *b₆f* complex and PSI (Iwai et al., 2010), and the PGR5-PGRL1 heterocomplex (Hertle et al., 2013) in which the 25-kD single membrane-spanning protein PGRL1 is reduced by Fd when it forms a heterocomplex with PGR5, which is an 8-kD membrane peripheral polypeptide (Munekage et al., 2002). The reduced and monomerized PGRL1 in turn reduces a plastoquinone (Hertle et al., 2013). The *Symbiodinium* genome contains copies of type II NDH (Nda2), PGR5, and PGRL1 in addition to the authentic components for CEF, including the cytochrome *b₆f* complex, PSI, and FNR (Supplemental Table S1;

Shoguchi et al., 2013). Thus, *Symbiodinium* has at least one type of NDH and possible machinery for an Fd-dependent pathway. Because the inhibitor of the PGR5-PGRL1 heterocomplex, i.e. AA, did not inhibit CEF in *Symbiodinium* (Supplemental Fig. S4), the PGR5-PGRL1 heterocomplex may not operate in this organism, and the NDA2-dependent and/or CEF supercomplexes may be responsible for heat-induced CEF. However, AA may simply not be taken into *Symbiodinium* cells as has been commonly observed for various chemical compounds in marine algae. This CEF pathway issue must be further clarified in future studies.

Differences in the Extent of CEF among *Symbiodinium* Strains under High Temperature Conditions

Symbiodinium is genetically diverse and is grouped into nine clades from A to I (Pochon and Gates, 2010). Each clade contains multiple phylotypes (Correa and Baker, 2009). The physiological characteristics, such as photosynthetic activity (Hennige et al., 2009) and thermal sensitivity (Tchernov et al., 2004; Takahashi et al., 2008; Takahashi et al., 2009), have been demonstrated to differ among *Symbiodinium* strains. In this study, we used three different *Symbiodinium* strains and found that the extent of CEF at high temperatures differed among the strains (Fig. 5A). Because CEF contributes to the activation of qE and the synthesis of ATP, CEF efficiency might differ among *Symbiodinium* strains under heat stress. We initially expected that the *Symbiodinium* strains that exhibit greater CEF would be more tolerant to heat stress, as proposed previously (Reynolds et al., 2008). However, the *Symbiodinium* strains that exhibited greater CEF (Fig. 5B) were actually more susceptible to photoinhibition at both moderate and high temperatures (Fig. 5A). Thus, the thermal tolerances of *Symbiodinium* strains do not readily correlate with their CEF activities but are rather inversely correlated. Thus, CEF is activated as an acute response to heat stress, which explains why the “heat-sensitive” strains that sensed heat stress at a lower temperature (33°C) induced greater CEF at that temperature.

MATERIALS AND METHODS

Strains and Growth Conditions

Cultures of *Symbiodinium* sp. OTCH-1 (clade A) were obtained from the National Institute of Technology and Evaluation (Chiba, Japan). The Y106 strain (clade A) was originally maintained in Dr. Michio Hidaka's laboratory (University of the Ryukyus, Okinawa, Japan) and was a gift from Dr. Eiichi Shoguchi (Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan). The Mf1.05b strain (clade B) was a gift from Dr. Mary Alice Coffroth (State University of New York at Buffalo, Buffalo, NY). The clades of these *Symbiodinium* cultures were determined as previously described (Toller et al., 2001; Santos et al., 2003). The *Symbiodinium* cells were grown in artificial seawater (sea salts no. S-9883; Sigma-Aldrich) containing Daigo's IMK medium for marine microalgae at 25°C under white fluorescent bulbs at 60 μmol photons m⁻² s⁻¹ with a light/dark cycle of 12 h/12 h. *Chlamydomonas reinhardtii* 137c cells were grown in a high-salt minimal medium (Sueoka, 1960) at 25°C under continuous irradiation from white fluorescent lights at 40 μmol photons m⁻² s⁻¹. The sea anemone *Aiptasia* sp. (H2 strain) was a gift from Dr. John

Pringle (Stanford University, Stanford, CA) and was grown in artificial seawater (sea salts REI-SEA; IWAKI) under white fluorescent bulbs at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a light/dark cycle of 12 h/12 h at 25°C. *Artemia* was fed to *Aiptasia* once per week.

Sample Preparation

AA, DCMU, MV, glycolaldehyde, Glc oxidase, and catalase were obtained from Sigma-Aldrich. Iodoacetamide was obtained from Tokyo Chemical Industry. DCMU, AA, and iodoacetamide were made up as stock solutions in ethanol at concentrations of 40, 50, and 500 mM, respectively. Glycolaldehyde, MV, and NH_4Cl were made up as stock solutions in water.

The *Symbiodinium* cells were collected by mild centrifugation (715g for 1 min) during their late-logarithmic growth phase and suspended in fresh growth medium. The total concentrations of chlorophylls *a* and *c*₂ were measured as described previously (Jeffrey and Humphrey, 1975; Takahashi et al., 2008). The harvested cells were diluted to 3 to 5, 20, and 80 $\mu\text{g Chl/mL}$ for the measurements of chlorophyll fluorescence, oxygen evolution, and P700 absorption changes, respectively. Anoxic conditions were induced by the addition of Glc oxidase (1 mg/mL), Glc (20 mM), and catalase (200 units/mL) and subsequent incubation for 30 min in the dark. AA, NH_4Cl , MV, glycolaldehyde, or iodoacetamide was added to the sample 30 min before the measurements or temperature treatments. For the measurements of P700 absorption changes and NPQ, 20% (w/v) Ficoll PM 400 (Sigma-Aldrich) was added to prevent cell sedimentation.

Aiptasia polyps were paralyzed by the addition of 0.37 M MgCl_2 in a 1-to-1 ratio to artificial seawater and then used for experiments.

Temperature Treatment

The *Symbiodinium* or *Aiptasia* polyps were placed in a glass vial or in a plastic cuvette and incubated at different temperatures using an aluminum gradient heat bar as previously described (Takahashi et al., 2008) or in a MiniT-H2C aluminum heat block (BMS). For the induction of photoinhibition, the *Symbiodinium* cells were exposed to halogen lamps from the top during the heat incubation. During the NPQ measurement, the cells were placed in a plastic cuvette, and the temperature was controlled with a cuvette holder connected to a water bath (UA-100; EYELA).

Spectrophotometry

Measurements of P700 absorption changes and chlorophyll fluorescence were performed with a custom-made spectrophotometer as described previously (Takizawa et al., 2009; Iwai et al., 2010). For the analysis of the photoinhibition of PSII, the F_v/F_m was measured after dark incubation for 10 min. The fluorescence transient parameters were calculated as follows: $F_v/F_m = (F_m - F_0)/F_m$; NPQ = $(F_m - F_m')/F_m'$; and $\text{rETR} = \text{PAR} \times (F_m' - F)/F_m'$, where PAR is the photosynthetically active radiation (Ralph et al., 2002).

Photosynthetic Oxygen Production Rate Measurements

Light-dependent oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech Instruments) in a closed cuvette in the light at 2,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 25°C. The cells (20 $\mu\text{g Chl/mL}$, 1.5 mL) were preincubated in the dark for 3 min and then exposed to saturating light for 3 min. The light-dependent oxygen evolution rate was calculated as the sum of the oxygen consumption rate in the dark and the oxygen production rate in the light.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Kinetics of P700 oxidation/reduction in *Symbiodinium* OTcH-1 during light exposure.

Supplemental Figure S2. Effect of DCMU on electron transfer in PSII in *Symbiodinium* OTcH-1.

Supplemental Figure S3. Effects of anoxia on the rate of P700⁺ reduction in *Symbiodinium* OTcH-1 in the presence of DCMU at 25°C.

Supplemental Figure S4. MV and AA were not effective in *Symbiodinium* OTcH-1.

Supplemental Figure S5. Effects of glycolaldehyde and iodoacetamide on the reduction of P700⁺ in the presence of DCMU in *Symbiodinium* OTcH-1.

Supplemental Figure S6. Effects of elevated temperatures on the P700⁺ reduction rates in *C. reinhardtii*.

Supplemental Table S1. Possible CEF components in *Symbiodinium*.

ACKNOWLEDGMENTS

We thank Dr. John Pringle for *Aiptasia* H2 strain and Drs. Eiichi Shoguchi and Mary Alice Coffroth for *Symbiodinium* strains Y105 and Mf1.05b, respectively. We also thank the Model Plant Research Facility, National Institute for Basic Biology BioResource Center, for their technical support.

Received December 2, 2015; accepted March 7, 2016; published March 7, 2016.

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