Light and CO₂/cAMP Signal Cross Talk on the Promoter Elements of Chloroplastic β-Carbonic Anhydrase Genes in the Marine Diatom *Phaeodactylum tricornutum* ¹[OPEN]

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Our previous study showed that three CO₂/cAMP-responsive elements (CCRE) CCRE1, CCRE2, and CCRE3 in the promoter of the chloroplastic β-carbonic anhydrase 1 gene in the marine diatom *Phaeodactylum tricornutum* (Pptca1) were critical for the cAMP-mediated transcriptional response to ambient CO₂ concentration. Pptca1 was activated under CO₂ limitation, but the absence of light partially disabled this low-CO₂-triggered transcriptional activation. This suppression effect disappeared when CCRE2 or two of three CCREs were replaced with a NotI restriction site, strongly suggesting that light signal cross-talks with CO₂ on the cAMP-signaling transduction pathway that targets CCREs. The paralogous chloroplastic carbonic anhydrase gene, ptc2 was also CO₂/cAMP-responsive. The upstream truncation assay of the ptc2 promoter (Pptca2) revealed a short sequence of –367 to –333 relative to the transcription-start site to be a critical regulatory region for the CO₂ and light responses. This core-regulatory region comprises one CCRE1 and two CCRE2 sequences. Further detailed analysis of Pptca2 clearly indicates that two CCREs are the cis-element governing the CO₂/light response of Pptca2. The transcriptional activation of two Pptcas in CO₂ limitation was evident under illumination with a photosynthetically active light wavelength, and an artificial electron acceptor from the reduction side of PSI efficiently inhibited Pptcas activation, while neither inhibition of the linear electron transport from PSI to PSI nor inhibition of ATP synthesis showed an effect on the promoter activity, strongly suggesting a specific involvement of the redox level of the stromal side of the PSI in the CO₂/light cross talk.

Marine diatoms are one of the most successful groups of phototrophic organisms in the ocean and are responsible for up to one-fifth of annual global carbon fixation, playing also an important role in global elemental cycles (Tréguer et al., 1995; Falkowski et al., 2000). The acquisition of CO₂ in diatoms is sustained by a CO₂-concentrating mechanism (CCM), which works to supply high concentration of CO₂ to the carbon-fixation enzyme, Rubisco in diatoms. The CCM helps diatoms to overcome the problems of low CO₂ concentration in seawater and a relatively high K_m[CO₂] value of diatom Rubisco (Badger et al., 1998). The CCM in diatoms is composed of an active uptake system of both CO₂ and HCO₃⁻ from the surrounding seawater (Burkhardt et al., 2001; Rost et al., 2003; Trimborn et al., 2008; Hopkinson et al., 2011; Matsuda et al., 2011), and of the system to mobilize the accumulated dissolved-inorganic carbon (DIC) to Rubisco (Matsuda et al., 2011; Hopkinson et al., 2013). The latter process is facilitated by an enzyme, carbonic anhydrase (CA; Hopkinson et al., 2011; Matsuda et al., 2011; Samukawa et al., 2014) and may also involve, in some species of marine diatoms, a C₄-like biochemical conversion of DIC into organic acids (Reinfelder, 2011). Only a few molecular data are so far available for the diatom CCM. A mammalian-type solute-carrier protein family 4 (SLC4) functions as a Na⁺-dependent plasma membrane type HCO₃⁻ transporter in the raphid pennate diatom, *Phaeodactylum tricornutum* (Nakajima et al., 2013). Many other putative transporter genes, which apparently belong to subclasses of SLC4 and SLC26, were also found in the genomes of *P. tricornutum* and the multipolar centric diatom, *Thalassiosira pseudonana*, suggesting that these genes share the common origin with mammals and heterokonts (Nakajima et al., 2013).
Numerous CAs, a critical CCM component, were also found in the genomes of *P. tricornutum* and *T. pseudonana* and most of them have been localized (Harada et al., 2005; Tanaka et al., 2005; Tachibana et al., 2011; Samukawa et al., 2014). Interestingly, there are significant differences in the location of CAs between these two species. That is, there are pyrenoidal CAs but no cytosolic nor external CA in *P. tricornutum* (Tachibana et al., 2011), while, in contrast, there are cytosolic and external CAs but no pyrenoidal CA in *T. pseudonana* (Samukawa et al., 2014). These data suggest an occurrence of a significant diversity in the mechanism of the diatom CCMs (Samukawa et al., 2014).

Many components of the CCM are suppressed under elevated CO2 conditions but induced (or derepressed) at the transcriptional level when cells of diatoms, cyanobacteria, and green algae are exposed to a limited CO2 around or below the atmospheric level within a few hours (Kaplan et al., 1980; Miller et al., 1990; Colman and Rotatore, 1995; Matsuda and Colman, 1995; Johnston and Raven, 1996; Matsuda et al., 2001; Kucho et al., 2003; Vance and Spalding, 2005; Ma et al., 2011). This indicates that algal cells can perceive CO2 concentration as a signal and quickly acclimate to changing CO2 concentrations. In the cyanobacterium *Synechocystis* sp. FCC6803, the cytoplasmic-membrane protein (cmp ABCD operon, which encodes a Bicarbonate Transporter 1, is induced upon interaction of Cytoplasmic-membrane protein Regulator (CmpR) to the promoter under low CO2 conditions. In *P. tricornutum* (Tachibana et al., 2011), while, in contrast, there are cytosolic and external CAs but no pyrenoidal CA in *T. pseudonana* (Samukawa et al., 2014). The high photosynthetic affinity for DIC was induced concomitantly with the capacity to take up DIC upon transferring diatom cells from high CO2 to atmospheric CO2 (Johnston and Raven, 1996; Burkhardt et al., 2001; Matsuda et al., 2001), strongly suggesting that DIC uptake and internal CO2 flow toward Rubisco are strengthened under CO2 limitation in diatoms. It is also pointed out that acclimation to the extreme CO2 limitation (below 2 mL m−3) induces a distinct high-affinity state of the CCM in *P. tricornutum* (Matsuda et al., 2001). Similarly, in the green alga, *C. reinhardtii*, cells appear to take different strategies for CO2 acquisition in high CO2, atmospheric level CO2, and subatmospheric level CO2 (Spalding et al., 2002; Vance and Spalding, 2005; Yamano et al., 2010). However, the mechanism to respond differentially to a spectrum of CO2 condition is still unclear in any algal cells. The pyrenoidal β-CAs (PtCA1 and PtCA2) and plasma membrane type HCO3− transporter (PtSLC4-2) in *P. tricornutum* were induced under atmospheric CO2 at the transcriptional level (Harada and Matsuda, 2005; Nakajima et al., 2013). Three paralogous SLC4 type genes, *ptSLC4-1*, *ptSLC4-2*, and *ptSLC4-4* were also induced under atmospheric CO2 (Nakajima et al., 2013), suggesting that these CCM factors are under the control of a CO2-signaling mechanism.

The function of the CO2-responsive *ptca1* promoter (*Ptca1*) has been studied in detail (Harada et al., 2006; Ohno et al., 2012). Three cis-elements denoted CO2/cAMP-responsive elements (CCRE1, CCRE2, and CCRE3), which are tandemly aligned between −86 and −42 relative to the transcription-start site, are critical motifs for *Ptca1* to respond to changes in CO2 concentration via the second messenger, cAMP (Ohno et al., 2012). CCRE1, CCRE2, and CCRE3 are an analogous sequence (TGGCCT/C) and located in an inverted direction to each other with 12 to 15 bps of spacer sequences (Ohno et al., 2012). CCRE2 is the core element, which works with either CCRE1 or CCRE3, suggesting that two elements possessing the complementary sequence may cooperate to recruit specific transcription factors (Ohno et al., 2012). Indeed, a basic zipper (bZIP) protein PtBZIP11, which resembles the mammalian cAMP-responsive element-binding protein/activation transcription factor, is detected as a Trans-acting factor, which specifically binds to CCREs (Ohno et al., 2012). However, the upstream signal transduction mechanism of the diatom CO2-response is still unclear, and factors controlling the *Ptca1* promoter activity await further investigations.

In photosynthetic organisms, CO2 signals often cross talk with light signals that may regulate multiple stages of gene expression. It has been reported that CCMs are regulated by light and CO2 with a variety of sensitivity depending on organisms. In cyanobacteria, light is an absolute requisite for the induction of the CCM under CO2 limitation (Omata et al., 2001; Nishimura et al., 2008). As described above, 2-PG seems to be a cofactor for the inducer, CmpR, strongly suggesting the participation of the photosynthetic metabolism for the
transcriptional regulation of the CCM factors (Nishimura et al., 2008). Indeed, transcription of cyanobacterial CCM factors cmpA, ndhF3, and sbtA were found to be enhanced by strong light (Hishita et al., 2001; McGinn et al., 2003, 2004; Woodger et al., 2003). In contrast, regulation of CCMs in green algae does not necessarily depend on light. In the green alga, Chlorella ellipsoidea, the CCM was fully derepressed in low CO2 in the dark (Matsuda and Colman, 1995), while in C. reinhardtii, CCM induction was strongly light-dependent, although a trace amount of transcript of CCM factors such as that of Cah1 was detected in low CO2 in the dark (Rawat and Moroney, 1995). The photosynthetic pathway appeared to be a minor factor in the CCM regulation of C. reinhardtii (Suzuki et al., 1990; Vance and Spalding, 2005), but the light signal is more likely to cross talk with the CO2 signal independently of the balance between the C2 and C3 carbon metabolisms. Similarly to C. reinhardtii, the CCM regulation in diatoms is highly dependent of light as well as CO2; that is, the CO2-responsive regulation of Pptcas activities in P. tricornutum requires light (Harada et al., 2006). However, the mechanisms linking light signaling to the CO2 signaling pathway are yet to be elucidated.

In this study, the relationship of light to the transcriptional regulation of two pyrenoidal β-type CA genes, ptca1 and ptca2, was studied in P. tricornutum. The critical cis-elements identified to respond to light in promoters of these CA genes were identical to those required for CO2 response. The involvements of the light wavelength, photosystems (PSs) and ATP synthesis in their transcriptional regulation, were also examined and the results strongly suggest that the function of Pptcas is deeply related to photosynthetic light and to the function of the acceptor side of the PSI.

RESULTS

Requirement of Light for CO2-Responsive Regulation of the Activities of ptca1 and ptca2 Promoters

Our previous studies have demonstrated that the endogenous ptca1 and ptca2 showed, respectively, about 25 and 3.5 times increase in their transcript levels by acclimating from 5% CO2 to air (Harada and Matsuda, 2005; Harada et al., 2006) and the reporter construct of Pptca1 fused with the β-glucuronidase (GUS) gene, uidA, was a quantitative indicator for the CO2 response of Pptca1 in transformant cells expressing this fusion reporter construct (Harada et al., 2005, 2006; Sakaue et al., 2008, Ohno et al., 2012). We thus used this GUS reporter system to identify the critical element required for switching Pptcas activity in response to light/CO2 by means of sequence manipulations of heterologously introduced promoter constructs.

Both Pptca1(−1292−+61)::uidA and Pptca2(−1312−+47)::uidA constructs expressed in P. tricornutum cells showed a clear CO2 response in the light; i.e. GUS levels were about 8 and 2.5 times in air, respectively, in transformants carrying Pptca1(−1292−+61)::uidA and Pptca2(−1312−+47)::uidA compared to those of 5% CO2 (Fig. 1, black and white bars). The GUS expression in air in the light was suppressed in the presence of a permeable cAMP analog, 0.5 mM dibutyryl cAMP (dbcAMP; Fig. 1). In the absence of light, the Pptca1-driven expression of GUS in 5% CO2 was about 2.5 times that of 5% CO2 in the light (Fig. 1). The derepression of Pptca1 in air was also greatly diminished to about 50% in the dark compared to that in the light (Fig. 1). These data suggest that light is required for Pptca1 to achieve a full dynamic range of CO2 response (i.e. to be fully repressed and derepressed). In contrast, the level of expression of Pptca2-driven uidA in the dark was maintained stably at the basal 5% CO2 level both under 5% CO2 and air conditions (Fig. 1), suggesting that derepression of Pptca2 is strongly dependent on both limitation of CO2 and light, and either high CO2 or the absence of light can fully repress this promoter. This is in sharp contrast to the case of Pptca1 in which CO2 and light exhibited an additive effect on the promoter regulation. The dbcAMP treatment did not show notable difference under changing light conditions and worked repressively to both Pptca1 and Pptca2 (Fig. 1), indicating that the increase in the intracellular cAMP level constitutes the basal repression signal, and light would participate in this signaling pathway. Also these data suggest that two paralogous pyrenoidal β-CAs are under slightly different expression controls.

Determination of Critical CO2/Light-Responsive Elements in the Core-Regulatory Region of Pptca1

We tested light response of the Pptca1 core-regulatory region by a series of single nucleotide replacements of three CCREs on an assumption that the critical light responsive element was associated with the core-CO2-regulatory region and deeply related to the primary control under the CO2/cAMP signal (Fig. 2). Each point-mutation construct, which was ligated with uidA at +61 bps relative to the transcription-start site, was transformed into P. tricornutum cells by microprojectile bombardment. Obtained transformants revealed a variety of specific GUS activity levels per total protein (data not shown), presumably due to the copy number and/or positioning effects of genes integrated to the genome. However, CO2/light-response of GUS expression was stably observed in each transformant and thus switching of the Pptca1 activity in response to CO2/light was quantified with some accuracy. The results in Figure 2, B to D, showed the apparent loss of the repression of the manipulated promoters in the dark (Fig. 2B, iv–ix; Fig. 2C, iv–ix; Fig. 2D, v–x), while other reporter constructs showed a clear repression under the dark and air conditions (Fig. 2, B–D). The identified light-responsive element of Pptca1 was identical to the CO2/cAMP-responsive elements, CCRE1, CCRE2, and CCRE3. Interestingly, one-base replacements adjacent only to the CCRE1 sequence gave an equivalent repression of promoter in the absence of light to that of 5% CO2 in the light, apparently dissipating the additive
effect of CO₂ and light, and thus regulation of Pptca1 resembled that of Pptca2 (Fig. 2B, i, iii, and x–xii). As the promoter constructs in Fig. 2B also lack in the CCRE3 sequence, these data suggest that adjacent sequences of CCRE1 or the lack of CCRE3 may alter the dependency of Pptca1 to CO₂ and light.

Three CCREs in Pptca1 (−90 to +61) were manipulated in a series of combinations, and light response in P. tricornutum cells was evaluated under 5% CO₂ and air conditions (Fig. 3). The replacement of CCRE2 with the NotI restriction site completely abolished the light response of Pptca1 as well as CO₂ response (Fig. 3, iii), while the single NotI replacement of either CCRE1 or CCRE3 did not affect CO₂ or light response (Fig. 3, ii and iv). However, the NotI replacement of more than two CCREs abolished both CO₂ and light responses (Fig. 3, v–viii), in agreement with our previous CO₂-response model of the CCRE1, CCRE2, and CCRE3 that is, CCRE2 provides a core platform for the Trans-acting factor, PtbZIP11 cooperating with either CCRE1 or CCRE3 (Ohno et al., 2012). These data indicate that light response elements completely overlap with CCRE1, CCRE2, and CCRE3, strongly suggesting the occurrence of the light and CO₂ cross talk in the cAMP-signal transduction pathway at the upstream processes toward CCREs. The regulation of Pptca1 containing CCRE1 and CCRE2 with NotI replacement of CCRE3 resembled that of Pptca2 (Fig. 1, iv and Fig. 3, iv). Together with the result in Fig. 2B, the promoter regulation without CCRE3 may confer the Pptca2 type sensitivity for CO₂ and light.

**Truncation Assay of Pptca2**

PtCA2 is a paralogous CA to PtCA1, with an approximately 80% amino acid sequence identity whose transcription is also CO₂-responsive, and they colocalize at the pyrenoid of P. tricornutum (Harada and Matsuda, 2005; Tanaka et al., 2005; Tachibana et al., 2011). Both CAs are under redox regulation of the function of diatom plastidic thioredoxins (Kikutani et al., 2012) and the function of the promotors of both CA genes are responsive to CO₂ via the function of cAMP (Fig. 1; Harada et al., 2006; Ohno et al., 2012). These striking similarities raised a question why PtCA1 and PtCA2 locate together and how these enzymes differ in function. This consideration prompted us to investigate the difference in transcriptional control of the pctca2 gene from that of the pctca1 gene. The isolated promoter region of pctca2 was from +47 to −1312 bps relative to the transcription-start site. This sequence was ligated to the uidA reporter gene (Fig. 4, i) and a series of upstream truncations were engineered to this reporter construct at −642, −523, −453, −367, −333, and −257 relative to the transcription-start site (Fig. 4A, ii–vii). The reporter assay was carried out using these truncated promoter-reporter constructs after transformation into P. tricornutum cells. As a result, the promoters possessing the upstream sequence beyond −367 bps relative to the transcriptional-start site clearly maintained the responses to both CO₂ and light (Fig. 4A, i–v), while the promoters truncated at −333 completely lost the response to either CO₂ or light (Fig. 4A, vi and vii), indicating that the critical CO₂/light-responsive region of Pptca2 resides between −367 and −333. Indeed, the sequence of this region comprised a couple of CCRE2 sequences, ACCTCA (at −355 to −350, and −343 to −338), and a CCRE1 sequence, TGACGT (at −345 to −340; Fig. 4B), strongly indicative that this region is under the control of the cAMP-signal transduction pathway.

**Sequence-Replacement Assay to Determine the cis-Elements for CO₂/Light Response in the Core-Regulatory Region of Pptca2**

The CCRE1 and CCRE2 sequences in the short region of about 30 bps of Pptca2 were singly or concurrently replaced by the NotI restriction sequence and the CO₂/light response of manipulated Pptca2 was evaluated by the GUS reporter assay (Fig. 5). Introduction of the NotI restriction site to the region adjacent to the CCREs cluster gave no effect on the CO₂/light response of Pptca2 (Fig. 5, Re1 and Re6). A single insertion of the
Figure 2. CO₂ and light responses of one-base-replaced promoter. A, A schematic drawing of core-regulatory region of Pptca1. B, One base replacement assay for the CCRE1 sequence; CCRE3 sequence was replaced by Nol site to avoid CCRE3 substituting for the function of CCRE1. C, D, One base replacement assay, respectively, for CCRE2 and CCRE3; CCRE1 sequence was removed for the same reason as described in (B). Cells were grown in 5% CO₂ or air in the absence or presence of light. Vertical bar indicates the dataset for each construct. Two independent clones of transformants carrying each manipulated promoter-reporter construct were selected for a set of reporter assays. Value is mean ± SD of three replicates. Statistical significance was determined by t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
No1 restriction site to either CCRE1 or CCRE2 also showed little effect on the Pptca2 function (Fig. 5, Re2, Re3, and Re5). In the construct Re4, the overlapping part of CCRE1 and the second CCRE2 were replaced by the No1 sequence but CO2 response was retained in this manipulation (Fig. 5, Re4). Complete replacement of both CCRE1 and the second CCRE2 largely dissipated the CO2/light response; it is thus suggested that a small part of CCRE1 (the first TG sequence) may have some function [Fig. 5, Re(TG+4)]. In contrast, concurrent No1 insertions into CCREs showed a significant dissipation effect on the CO2/light responses of Pptca2. That is, a disruption of one of two CCREs with the CCRE1 disruption greatly diminished the CO2/light response of Pptca2 (Fig. 5, Re(2+3) and Re(TG+4)). A concurrent No1 insertion to two CCREs completely abolished the CO2/light response (Fig. 5, Re(2+5)). Similarly, disruption of all CCREs completely abolished the CO2/light response of Pptca2 (Fig. 5, Re(2+TG+4)).

Light Wavelength Required to Maintain the Activities of Pptca1 and Pptca2 in Air

Illumination at the photosynthetic photon flux density (PPFD) of 80 μmol m⁻² s⁻¹ of three types of monochromatic light, blue (455 nm), green (520 nm), and red (635 nm; Supplemental Fig. S1A), gave an equivalent photosynthetic O₂ evolution rate to that under the white fluorescent light of the same PPFD at a saturated DIC condition (5 mM) using a cell culture of OD₇₃₀ at 0.2, indicating that blue and green light are both photosynthetically active as well as red light. On the other hand, yellow light (595 nm) showed little activation on photosynthesis (Supplemental Fig. S1B and C). The capacities of these four monochromatic light to maintain the CO2-responsive transcriptional regulation of the endogenous Pptca1 and Pptca2 were tested by quantitative RT-PCR using ribosomal protein S1 (RPS) for a reference gene (Fig. 6A). The glyceraldehyde-3-phosphate dehydrogenase C2 gene (gapC2) was not used in this experiment, as changes in light wavelength gave a significant effect on the transcript level of gapC2. Blue, green, and red light revealed an equivalent stimulation on both Pptca1 and Pptca2 activities in air to that of white light, while yellow light showed a significantly small stimulatory effect (30–40% compared to other light) on these two promoter activities (Fig. 6A). The responses of two ptcas to different light wavelengths strongly suggest that the main switching function of these CO2-responsive promoters in response to CO2 and light is deeply related to photosynthetic light.

Changes in Transcript Levels of Endogenous ptda1 and ptda2 under Inhibitor Treatments against the PSs and ATP Synthesis

PtCA1 and PtCA2 are posttranslationally regulated by redox levels via thioredoxins in response to the energy state of the PSI and PSII (Kikutani et al., 2012), which probably functions as a part of the fine regulation systems of CO2 supply to Rubisco at the pyrenoid under CO2-limited condition where the expressions of ptcas are derepressed (Matsuda and Kroth, 2014). This prompted us to investigate the existence of a regulation system of the derepressed level of the ptcas transcripts by physiological state relating to redox conditions in the chloroplast. For this purpose, we employed a quantitative RT-PCR assay to detect differences in the derepressed levels of the endogenous ptcas transcripts. P. tricornutum cells were treated with inhibitors for the photosystems or ATP synthesis. Photosystem inhibitors used were 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a specific blocker of electron flow to plastochinone (PQ) in PSII (Izawa, 1980); 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), a blocker...
of the Q$_i$ site of the cytochrome $b$_6$f$ complex (Farineau et al., 1984; Trebst, 2007); 2,5-dichloro-1,4-benzoquinone (DCBQ), an artificial electron acceptor from Q$_i$ in PSII (Graan and Ort, 1986); and 2,6-dichlorophenolindophenol (DCPIP), a strong oxidizing reagent at the acceptor side for a full derepression of transcription of both ptca1 and ptca2 from either processes between the PSII and PQ pool or photophosphorylation. In sharp contrast to the above drug treatments, cells treated with DCPIP showed drastic decreases in transcript levels of ptca1 and ptca2, respectively, to about 10% and 28% that of the control cells (Fig. 6B). Considering the low toxicity on growth of the used concentration of DCPIP, which did not show significant inhibition on growth even at 50 $\mu$M Valinomycin and 2 $\mu$M CCCP and after 2 d of treatment, total RNAs were extracted from the inhibitor-treated cells and transcript levels of endogenous *ptca1* and *ptca2* were analyzed with quantitative RT-PCR.

The transcript levels of both *ptca1* and *ptca2* in cells treated with DCMU, DBMIB, DCBQ, CCCP or Valinomycin exhibited marginal differences compared to those of non-treated cells (Fig. 6B), suggesting the absence of influence on the transcriptional control mechanism in *Ppca1* and *Ppca2* from either processes between the PSII and PQ pool or photophosphorylation. In sharp contrast to the above drug treatments, cells treated with DCPIP showed drastic decreases in transcript levels of *ptca1* and *ptca2*, respectively, to about 10% and 28% that of the control cells (Fig. 6B). Considering the low toxicity on growth of the used concentration of DCPIP, which did not show significant inhibition on growth even at 50 $\mu$M Valinomycin and 2 $\mu$M CCCP and after 2 d of treatment, total RNAs were extracted from the inhibitor-treated cells and transcript levels of endogenous *ptca1* and *ptca2* were analyzed with quantitative RT-PCR.

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**DISCUSSION**

In this study, the light-responsive cis-elements in *Ppca1* were newly determined. Also, the structure and the function of *Ppca2* were determined and compared in detail with those of *Ppca1*. We found that *Ppca1* and *Ppca2* were essentially controlled by a similar switching mechanism of transcription, which was mediated
Figure 5. Sequence-replacement assay to determine the cis-elements for CO₂/light response in the core-regulatory region of Pptca2. CCRE1 and CCRE2 sequences and adjacent sequences within the core-regulatory region of Pptca2 were replaced by NotI restriction site (left half), and the GUS reporter assay was done with each transformant (right half). Cells were grown in 5% CO₂ or air in the absence or presence of light. Vertical bar indicates the dataset for each construct. Two independent clones of transformants carrying each manipulated promoter-reporter construct were selected for a set of reporter assays. Value is mean ± SD of three replicates. Statistical significance was determined by t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
by a second messenger cAMP, in response to CO2 and light. But in contrast, it was also strongly suggested that there were some fine-tuning systems at the transcriptional level that might differentiate the function of these two paralogous pyrenoidal CAs, PtCA1 and PtCA2, when they were derepressed under air and illuminated conditions.

The CCRE2 sequences identified in the core-regulatory regions of both Ptca1 and Ptca2 were shown to be essential to repress the promoter activity in response to CO2, and light was required for proper switching function. As indicated previously, CCRE2 was the core cis-element that worked either with CCRE1 or CCRE3 in Ptca1 (Ohno et al., 2012) and these functional combinations of cis-elements were also responsible for the light response of this promoter. Light response of Ptca2 also revealed a tight relation to CO2 response and the disruption of CCREs directly caused a disappearance of light response (Figs. 4 and 5). These results strongly suggest the existence of the CO2 and light cross talk at upstream signal transduction pathways toward CCREs both in Ptca1 and Ptca2. However, there were some differences in the alignment of CCREs between Ptca1 and Ptca2 (Figs. 2A and 4B). Our previous study demonstrated by in vitro gel shift assay that all CCREs were the specific target of a diatom basic ZIP transcription factor, PtbZIP11 (Ohno et al., 2012), strongly suggesting that CCREs in the core-regulatory region of Ptca2 are also targeted by PtbZIP11. But the difference in the alignment of CCREs probably conferred some differences in the transcriptional regulations in response to CO2 and light; that is, transcriptional activation of Ptca2 in response to the decrease in CO2 was strongly light-dependent and either CO2 or the absence of light can maximally down-regulate the promoter, while CO2 and light have additive effect on the regulation of Ptca1 (Fig. 1). One of the interesting structural features clarified in this study is that the manipulated Ptca1 only with CCRE1 and CCRE2 altered its CO2/light response to that similar to Ptca2 (Figs. 2 and 3). Interestingly, the structure of Ptca2 does not have CCRE3 sequence either (Figs. 4B and 5), although the alignment of CCREs between two promoters is not similar and the detail is yet to be made clear.

A requirement of light for the activity of CO2-responsive promoters is commonly observed in the regulation of algal CCM factors but the molecular mechanism of the CO2/light cross talk in eukaryotic algae is yet to be elucidated. In bacterial systems, in contrast, the mechanism of CO2/light cross talk is relatively clear. Physiological studies have demonstrated that cyanobacterial CCM is regulated in response to total DIC in the medium presumably corresponding to the availability of substrate inorganic carbon for photosynthesis (Mayo et al., 1986). In fact, a recent molecular study has revealed that 2-PG directly stimulated the binding of CmpR to the CO2-responsive promoter of the HCO3− transporter gene operon, cmpABCD in the cyanobacterium, Synechocystis sp. PCC 6803, strongly suggesting that CO2/light cross talk occurs at the C2/C3 photosynthetic carbon flux mediated by the accumulation of the initial photosynthetic metabolite (Nishimura et al., 2008). In contrast, regulation of CCM in eukaryotic algae is relatively complicated; that is, cells respond specifically to CO2 concentration to control CCM levels even though they possess high HCO3− uptake capacity. In the green algae, C. reinhardtii and C. ellipsoidea, a physiological determinant of the CCM level was CO2 in the bulk media but not HCO3− nor...
CO₂ was reported in the regulation of the CCM expression in the continuous illumination of photosynthetic photon flux density (F/2ASW; Guillard and Ryther, 1962; Harrison et al., 1980) under light-dependent (Dionisio-Sese et al., 1990). In the marine diatom, *P. tricornutum*, also the similar signaling role of CO₂ was reported in the regulation of the CCM expression (Matsuda et al., 2001). This evidence strongly suggests the occurrence of some direct signal reception mechanisms for CO₂ and light in eukaryotic algae, which may cross talk independently of photosynthesis. The role of the CAMP analog in CO₂/light signals to control Ptptcas in *P. tricornutum* strongly suggests the involvement of cAMP in this cross talk (Fig. 1).

Furthermore, all photosynthetically active-monochromatic-light wavelength (red, blue, and green) conferred a decrease on both Ptptcas with equivalent efficiency to the normal light, while weakly photosynthetically active-light wavelength (yellow) showed a weak derepression activity to Ptptcas (Fig. 6A; Supplemental Fig. S1). This apparent synchronization of the promoter activity with the photosynthesis strongly suggests an involvement of photosynthetic process to the CO₂/light signaling, although an involvement of a direct sensing of the light wavelength by specific photoreceptors is not ruled out.

The light-dependent chloroplastic metabolism and the redox state seemed to work to control the transcript levels of *ptca1* and *ptca2* in a mode similar to each other. Interestingly, accumulation levels of both *ptcas* transcripts showed a drastic decrease by the treatment with low dose of DCPIP, which is a specific oxidizing reagent for the acceptor side of the PSI and would enhance the proton gradient across the thylakoid membrane by accepting electron from the PSI with high affinity or by releasing proton at the thylakoid lumen (Mühlbauer and Eichacker, 1998). However, the absence of a notable effect of electron transport inhibitors from the PSI to the PSI and of the ΔPH inhibitor on the *ptcas* transcription strongly suggests that the PQ pool level or proton gradient is not involved in mediating light signal, but the redox state of the PSI acceptor side would be critical in this process. The PSI acceptor side is extremely multifunctional in sorting electron flow into a number of different metabolic pathways and the CO₂/cAMP signaling pathway might be one of the targets of this sorting system, integrating the light signal into the promoter function based upon CCREs and PtbZIP11. Although the mechanism of this process awaits further investigation, the results of this study may provide an initial clue to clarify it.

**MATERIALS AND METHODS**

**Cells and Culture Conditions**

The marine diatom *Phaeodactylum tricornutum* Behl (UTEX 642) was obtained from the University of Texas Culture Collection and was grown in artificial seawater, which was supplemented with half-strength Guillard’s “F” solution (F/2ASW; Guillard and Ryther, 1962; Harrison et al., 1980) under continuous illumination of photosynthetic photon flux density (PPFD) of 50 μmol m⁻² s⁻¹ and constant aeration with 5% CO₂ or ambient air (0.039% CO₂) at 20°C. In acclimation experiments, 5% CO₂-grown cells were harvested by centrifugation at 3,500g for 5 min at room temperature, washed twice with CO₂-free F/2ASW, and allowed to acclimate to air-aerated F/2ASW for 1–2 d. In some experiments, 0.5 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich, St. Louis, MO), 0.025 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 1 μM 2,5-dibromo-3-methyl-6-isopropylphenoxazone (DBMIB), 2 mM 2,6-dichlorophenolindophenol (DCPIP), 0.5 mM carbonylcyanide m-chlorophenylhydrazone (CCCP), and 2 μM Valinomycin were added during the acclimation to air.

**Preparation of Transformation Constructs**

The genomic DNA was extracted from air-grown *P. tricornutum* cells with a modified method as described by Sambrook and Russell (2001). The promoter region of *ptca1* (−1312 to +47 relative to the transcription-start site) was amplified in a template genomic DNA with high-fidelity PrimeSTAR HS DNA polymerase (TaKaRa Bio, Kusatsu, Shiga, Japan) and a primer pair: Fw: 5′-CGCAAATGAGCATCTCGACGGCACCAATCGCT-3′; Rv: 5′-G5TGAACAC-GATTTAATCTAAGTGGTGTTGTTACACA-3′ and was phosphorylated. To construct the plasmid fused with uidA gene, a vector pFcpApGUS (Harada et al., 2005) was double-digested with EcoRI and Ndel to remove the promoter region of fucoxanthin chlorophyll (Chl) α/b binding protein gene (*fcp*) and blunt-ended with T4 DNA polymerase. A phosphorylated PCR product was inserted into blunt-ended pFcpApGUS and sequenced (pPcA2pGUS). A series of upstream truncated constructs of Ptptca1 (−1292 to +61 and −90 to +61 relative to the transcription-start site) were used as described by Ohno et al. (2012), while Ptptca2 (−642 to +47, −523 to +47, −453 to +47, −367 to +47, −333 to +47, and −257 to +47 relative to the transcription-start site) were amplified by the inverse-PCR method using pPcA2pGUS as a template with a set of primers shown in Supplemental Table S2. These constructs were phosphorylated, self-ligated, and sequenced.

To generate one-base replacement constructs of Ptptca1, PCR reactions were carried out targeting the sequence −71 to −60 and −52 to −42 using the vector pFcpApGUS containing Ptptca1 (−90 to +61), and targeting the sequence −86 to −81 using the vector pFcpApGUS containing Ptptca1 (−90 to +61) replacing the sequence −52 to −42 with Ndel restriction site. A set of linker scan constructs with a Ndel restriction linker site was synthesized by a modified inverse-PCR method using the vector plasmid pPcA2pGUS as a template. Primer sets were designed to generate a Ndel restriction site (CGCGCCGCCG) at desired locations by self-ligation (Supplemental Table S3). In some experiments, Ndel substitutions were introduced into −358 to −351 and −350 to −344 or −344 to −337 or −338 to −331 relative to the transcription-start site using the aforementioned vector plasmids pFcpApGUS containing upstream truncated Ptptca2 (−1212 to −47) as templates.

**Transformation of *P. tricornutum***

Air-grown cells of *P. tricornutum* were harvested at the midlogarithmic growth phase (optical density at 730 nm of 0.2–0.4). Approximately 5 × 10⁷ cells were spotted as a plaque of 2.5 cm diameter on the surface of the F/2ASW agar plate. A 500 μg tungsten microcarrier (Japan New Metals, Toyonaka, Osaka, Japan; particle size: 0.79 μm) was coated with 1 μg of plasmid DNA containing 1× CaCl₂ and 16 μm spermidine. DNA PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, Hercules, CA) was used for microprojectile bombardment of the microcarrier. The bombardment was done at 10.7 MPa to the cells in the chamber under a negative pressure of 9.14 kPa with a target distance of 6 cm. Bombarded cells were maintained on the plate for 1 d in the dark and were then suspended in 300 μL of F/2ASW. This cell suspension was plated on F/2ASW agar plates containing 300 μL of 1 μg Zeocin (Invitrogen, Carlsbad, CA) and allowed to form colonies for 3–4 weeks under continuous illumination.

**Screening of GUS-Expressing Transformants**

Zeocin-resistant clones were suspended in 60 μL of GUS extraction buffer (50 mM sodium phosphate, pH 7.0; 10 mM β-mercaptoethanol, 0.1% w/v sodium lauryl sarcosine; and 0.1% v/v Triton X-100) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-GluA; Sigma-Aldrich) on 96-well plates and were incubated overnight at 37°C. Transformants expressing GUS, which displayed the dark blue-colored chromogenic reaction product of X-GluA, were inoculated and used for further experiments.

[1] Zeocin (Invitrogen, Carlsbad, CA) and allowed to form colonies for 3–4 weeks under continuous illumination.
Quantification of GUS Activity

Cells at different acclimation stages were harvested by centrifugation at 3,000g at 20°C, resuspended in 0.3–1.0 mL of GUS extraction buffer, and disrupted by sonicator (Ultrasonic disruptor model no. UD-201; Tomy Digital Biology, Tokyo, Japan), followed by five cycles of disruption for 10 s, a pause for 30 s in an ice bath, and then centrifugation at 15,400g for 5 min at 4°C. Supernatant (lysate, 18 μL) was added to 882 μL of GUS reaction buffer [10 mM p-nitrophenyl β-D-glucuronide (PNPG) in 50 mM sodium phosphate buffer, pH 7.0] and incubated at 37°C. The reaction was terminated by the addition of 80 μL of 0.5 M Na2CO3 to 200 μL reaction solutions at every 10 min after starting the reaction, and the absorbance of p-nitrophenol (PNP, Wako Chemicals USA, Richmond, VA) released from the substrate was measured at 405 nm. Protein concentrations in lysate were measured by the Bradford method (Bio-Rad Laboratories) according to the manufacturer’s protocol. The standard curve of PNP was drawn with 0.2–8.0 nmol PNP, which gave a range of ΔA405 from 0.011 to 0.454. Concentrations of lysate protein in the reaction mixture were adjusted to produce PNP within the range of the above standard curve in the reaction for 4 min. GUS activity was calculated as the rate of PNP production per min per mg lysate protein.

Determination of Photosynthetic Parameters under Illumination of Monochromatic Light

Air-grown cells at the midlogarithmic growth phase were harvested by centrifugation at 1,400g at room temperature and washed three times with CO2-free F/2ASW. Cells were then suspended in the same F/2ASW at a final Chl a concentration of 10 μg mL−1. The rate of photosynthetic O2 evolution was measured with a Clark-type oxygen electrode (Hansatech Instruments, Pentney, King’s Lynn, Norfolk, UK; and Qubit Systems, Kingston, Ontario, Canada). A quantity of 1.5 mL of cell suspension was placed in an O2-electrode chamber and illuminated with a fiber illuminator at PPFD of 40 μmol m−2 s−1 under aeration with N2 for 10 min until cells reached to the CO2-compensation point, which was determined by the gas chromatography method. For some inhibitor experiments, DCMU, DBMIB, DCBQ, or DCPIP were added to a chamber for 10 min before the measurement. PPFDs of illumination or LED bulbs were then increased to 250 μmol m−2 s−1, and a known amount of DIC was added as a form of NaHCO3 to the desired final concentrations to cell culture. The rate of O2 evolution at each DIC concentration was plotted against DIC concentration. Chlor concentration was calculated with spectrophotometer according to Jeffrey and Humphrey (1975). Based on the plot, photosynthetic parameters were determined by the least-squares method.

Quantifications of the Endogenous ptc1a and ptc2a Transcripts

Total RNAs were extracted using NucleoSpin RNA (Macherey-Nagel, Bethlehem, PA) from P. tricornutum cells grown under illumination of monochromatic light or treated with inhibitors to PS or ATP synthesis for 2 d under an air condition according to the manufacturer’s protocol. Each single-strand cDNA was synthesized from 1 μg total RNA using an oligo (dT)20 (Toyobo, Osaka, Japan) and reverse transcriptase, ReverTra Ace (Toyobo). Quantitative RT-PCR was carried out with Thermal Cycler Dice Real Time System II (Takara) and GeneAce SYBR qPCR Mix (Ro No RX, Nippon Gene, Tokyo, Japan) under PCR conditions as follows: heating at 95°C for 10 min followed by 45 cycles of denaturing at 95°C for 30 s, annealing, and elongation at 60°C for 1 min. To standardize the transcript level, ribosomal protein S1 (RPS) or endogenous glyceraldehyde-3-phosphate dehydrogenase C2 (gapC2) genes were used as the constitutive maker genes. Quantitative RT-PCR was carried out by a set of primers shown in Supplemental Table S4. The standard curves of ptc1a, ptc2a, RPS, and gapC2 for Quantitative RT-PCR were drawn with a known amount of template using plasmids containing the ptc1a, ptc2a, RPS, and gapC2 cDNAs. Each transcript level was calculated using each standard curve from the values of the threshold cycle (Ct) that were the intersection point of the threshold and the amplification curve. The levels of the ptc1a and ptc2a transcripts were normalized by the level of the RPS or gapC2 transcripts.

Sequences data from this article can be found in the JGI Genome Portal or the GenBank accession no. AF638050, ptc1a (GenBank accession no. AAL74793), Ptc2a (GenBank accession no. BAE67442).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Diatom photosynthesis under monochromatic light.

Supplemental Figure S2. Diagrams of functional mechanisms of inhibitors for the PSs and ATP synthesis.

Supplemental Figure S3. Growth and photosynthetic profiles under different doses of inhibitors for the PSs.

Supplemental Table S1. Effects of photosynthesis inhibitors and ATP synthetase inhibitors on photosynthesis parameters.

Supplemental Table S2. Primer sequences used to make Ptc2a truncated constructs.

Supplemental Table S3. Primer sequences used to make Ptc2a linker-scan constructs.

Supplemental Table S4. Primer sequences for quantitative RT-PCR.

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LITERATURE CITED


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