Light and CO_2/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 CO_2/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* (P*ptca1*) were critical for the cAMP-mediated transcriptional response to ambient CO_2 concentration. P*ptca1* was activated under CO_2 limitation, but the absence of light partially disabled this low-CO_2-triggered transcriptional activation. This suppression effect disappeared when CCRE2 or two of three CCREs were replaced with a *Not*I restriction site, strongly suggesting that light signal cross-talks with CO_2 on the cAMP-signal transduction pathway that targets CCREs. The paralogous chloroplastic carbonic anhydrase gene, *ptca2* was also CO_2/cAMP-responsive. The upstream truncation assay of the *ptca2* promoter (P*ptca2*) revealed a short sequence of −367 to −333 relative to the transcription-start site to be a critical regulatory region for the CO_2 and light responses. This core-regulatory region comprises one CCRE1 and two CCRE2 sequences. Further detailed analysis of P*ptca2* clearly indicates that two CCRE2s are the cis-element governing the CO_2/light response of P*ptca2*. The transcriptional activation of two P*ptcas* in CO_2 limitation was evident under illumination with a photosynthetically active light wavelength, and an artificial electron acceptor from the reduction side of PSI efficiently inhibited P*ptcas* activation, while neither inhibition of the linear electron transport from PSII 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 CO_2/light cross talk.

Marine diatoms are one of the most successful groups of photoautotrophic 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 CO_2 in diatoms is sustained by a CO_2-concentrating mechanism (CCM), which works to supply high concentration of CO_2 to the carbon-fixation enzyme, Rubisco in diatoms. The CCM helps diatoms to overcome the problems of low CO_2 concentration in seawater and a relatively high $K_m[CO_2]$ value of diatom Rubisco (Badger et al., 1998). The CCM in diatoms is composed of an active uptake system of both CO_2 and HCO_3^- 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 C_4-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 HCO_3^- 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).

[1] This work was supported by Grant-in-Aid for Scientific Research B (grant no. 24310015 to Y.M.), by Grant-in-Aid for Challenging Exploratory Research (grant no. 24651119 to Y.M.) from the Japan Society for the Promotion of Science (JSPS), by the MEXT-Supported Program for the Strategic Research Foundation for the Advancement of Environmental Protection Technology and for Development of Novel Intelligent Biomaterials Applying Self-Organization Capacity of Diatoms, and by Grant-in-Aid for Young Scientists B (grant no. 26870750 to K.N.).

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A.T., N.O., K.N., and Y.M. conceived research plans, designed the experiments, and analyzed the data; A.T., N.O., and K.N. performed all experiments; Y.M. supervised all experiments; Y.M. wrote the paper with contributions of all authors; and K.N. and Y.M. complemented the writing.

[OPEN] This article is available without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01738

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 CO_2 conditions but induced (or derepressed) at the transcriptional level when cells of diatoms, cyanobacteria, and green algae are exposed to a limited CO_2 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 CO_2 concentration as a signal and quickly acclimate to changing CO_2 concentrations. In the cyanobacterium *Synechocystis* sp. PCC6803, 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 CO_2 conditions (Omata et al., 2001; Nishimura et al., 2008) and this interaction on the promoter is apparently stimulated by 2-phosphoglycolate (2-PG), an initial photorespiratory metabolite (Nishimura et al., 2008). In contrast, the other CO_2-responsive operons (e.g. *sodium bicarbonate transporter* (*sbt*)*A*/*sbtB*, *NAD*(*P*)*H dehydrogenase* (*ndh*)*F3*/ndh*D3*/*CO*$_2$ *hydration protein Y*/*open reading frame 133*, and *modified NDH-1* (*mnh*)*D1*/*mnhD2*) are repressed by CO_2-concentrating mechanism Regulator/NAD(P)H dehydrogenase Regulator under high CO_2 conditions (Wang et al., 2004; Price et al., 2008). On the other hand, no common mechanism of CO_2 response is found in eukaryotic algae. The CO_2-response mechanism has been well studied in the green alga, *Chlamydomonas reinhardtii* using the transcriptional regulation system of a periplasmic CA, *Cah1* and the mitochondrial CA (*mtCA*) genes, both of which are highly responsive to the ambient CO_2 concentration (Fujiwara et al., 1990; Eriksson et al., 1998). The *Cah1* promoter is regulated positively and negatively by enhancer and silencer regions. The enhancer region is controlled by two critical elements denoted enhancer-element consensus (Kucho et al., 2003), to which a class of basic-ZIP transcription factors, enhancer-element-consensus binding proteins, interact (Yoshioka et al., 2004). A zinc finger protein, CCM1/Cia5, has been found to be involved in the up-regulation of most low-CO_2-inducible genes, strongly suggesting that CCM1/Cia5 is a master regulator for the transcriptional response of the *Chlamydomonas* CCM, but the mechanism of the initial CO_2 signal perception is still unknown (Fukuzawa et al., 2001; Xiang et al., 2001).

The high photosynthetic affinity for DIC was induced concomitantly with the capacity to take up DIC upon transferring diatom cells from high CO_2 to atmospheric CO_2 (Johnston and Raven, 1996; Burkhardt et al., 2001; Matsuda et al., 2001), strongly suggesting that DIC uptake and internal CO_2 flow toward Rubisco are strengthened under CO_2 limitation in diatoms. It is also pointed out that acclimation to the extreme CO_2 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 CO_2 acquisition in high CO_2, atmospheric level CO_2, and subatmospheric level CO_2 (Spalding et al., 2002; Vance and Spalding, 2005; Yamano et al., 2010). However, the mechanism to respond differentially to a spectrum of CO_2 condition is still unclear in any algal cells. The pyrenoidal β-CAs (PtCA1 and PtCA2) and plasma membrane type HCO_3^- transporter (PtSLC4-2) in *P. tricornutum* were induced under atmospheric CO_2 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 CO_2 (Nakajima et al., 2013), suggesting that these CCM factors are under the control of a CO_2-signaling mechanism.

The function of the CO_2-responsive *ptca1* promoter (P*ptca1*) has been studied in detail (Harada et al., 2006; Ohno et al., 2012). Three cis-elements denoted CO_2/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 P*ptca1* to respond to changes in CO_2 concentration via the second messenger, cAMP (Ohno et al., 2012). CCRE1, CCRE2, and CCRE3 are an analogous sequence (TGACGT/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 CO_2-response is still unclear, and factors controlling the P*ptca1* promoter activity await further investigations.

In photosynthetic organisms, CO_2 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 CO_2 with a variety of sensitivity depending on organisms. In cyanobacteria, light is an absolute requisite for the induction of the CCM under CO_2 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 photorespiratory metabolism for the

transcriptional regulation of the CCM factors (Nishimura et al., 2008). Indeed, transcriptions of cyanobacterial CCM factors *cmpA*, *ndhF3*, and *sbtA* were found to be enhanced by strong light (Hihara 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 CO_2 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 CO_2 in the dark (Rawat and Moroney, 1995). The photorespiratory 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 CO_2 signal independently of the balance between the C_2 and C_3 carbon metabolisms. Similarly to *C. reinhardtii*, the CCM regulation in diatoms is highly dependent of light as well as CO_2; that is, the CO_2-responsive regulation of P*ptcas* activities in *P. tricornutum* requires light (Harada et al., 2006). However, the mechanisms linking light signaling to the CO_2 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 CO_2 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 P*ptcas* is deeply related to photosynthetic light and to the function of the acceptor side of the PSI.

RESULTS

Requirement of Light for CO_2-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% CO_2 to air (Harada and Matsuda, 2005; Harada et al., 2006) and the reporter construct of P*ptca1* fused with the β-glucuronidase (GUS) gene, *uidA*, was a quantitative indicator for the CO_2 response of P*ptca1* 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 P*ptcas* activity in response to light/CO_2 by means of sequence manipulations of heterologously introduced promoter constructs.

Both P*ptca1*(−*1292*−*+61*)::*uidA* and P*ptca2*(−*1312*−*+47*)::*uidA* constructs expressed in *P. tricornutum* cells showed a clear CO_2 response in the light; i.e. GUS levels were about 8 and 2.5 times in air, respectively, in transformants carrying P*ptca1*(−*1292*−*+61*)::*uidA* and P*ptca2*(−*1312*−*+47*)::*uidA* compared to those of 5% CO_2 (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 P*ptca1*-driven expression of GUS in 5% CO_2 was about 2.5 times that of 5% CO_2 in the light (Fig. 1). The derepression of P*ptca1* 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 P*ptca1* to achieve a full dynamic range of CO_2 response (i.e. to be fully repressed and derepressed). In contrast, the level of expression of P*ptca2*-driven *uidA* in the dark was maintained stably at the basal 5% CO_2 level both under 5% CO_2 and air conditions (Fig. 1), suggesting that derepression of P*ptca2* is strongly dependent on both limitation of CO_2 and light, and either high CO_2 or the absence of light can fully repress this promoter. This is in sharp contrast to the case of P*ptca1* in which CO_2 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 P*ptca1* and P*ptca2* (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 CO_2/Light-Responsive Elements in the Core-Regulatory Region of P*ptca1*

We tested light response of the P*ptca1* 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-CO_2-regulatory region and deeply related to the primary control under the CO_2/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, CO_2/light-response of GUS expression was stably observed in each transformant and thus switching of the P*ptca1* activity in response to CO_2/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 P*ptca1* was identical to the CO_2/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% CO_2 in the light, apparently dissipating the additive

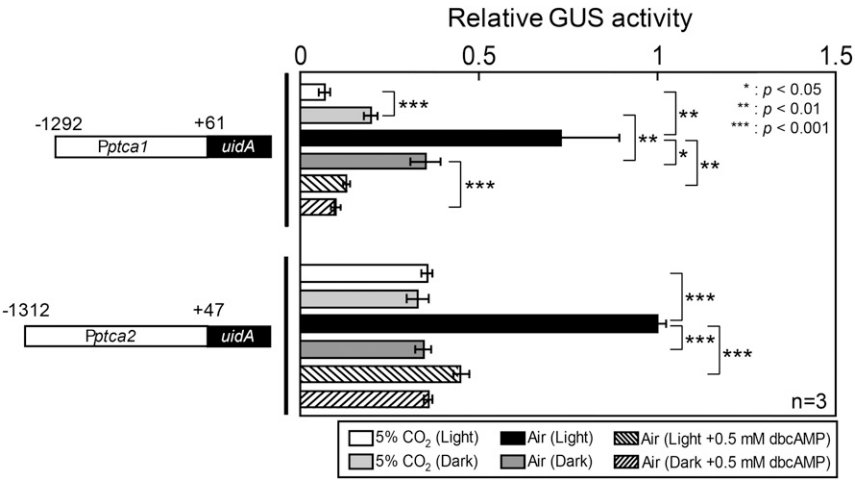

Figure 1. Promoter activity of *ptcas* reported by GUS in cells grown in changing CO_2 and light conditions in the absence or presence of dbcAMP. Isolated upstream genomic DNA sequence of *ptca1* (−1292 to +61: P*ptca1*) and *ptca2* (−1312 to +47: P*ptca2*) were fused with the GUS reporter gene, *uidA* (left half); GUS activity relative to the value in 2 d air-acclimated cells in the light (black bar in P*ptca2*) were shown for 5% CO_2 cells or 2 d air-acclimated cells (right half). Value is mean ± SD of three replicates. Statistical significance was determined by *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

effect of CO_2 and light, and thus regulation of P*ptca1* resembled that of P*ptca2* (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 P*ptca1* to CO_2 and light.

Three CCREs in P*ptca1*(−90 − +61) were manipulated in a series of combinations, and light response in *P. tricornutum* cells was evaluated under 5% CO_2 and air conditions (Fig. 3). The replacement of CCRE2 with the *Not*I restriction site completely abolished the light response of P*ptca1* as well as CO_2 response (Fig. 3, iii), while the single *Not*I replacement of either CCRE1 or CCRE3 did not affect CO_2 or light response (Fig. 3, ii and iv). However, the *Not*I replacement of more than two CCREs abolished both CO_2 and light responses (Fig. 3, v–viii), in agreement with our previous CO_2-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 CO_2 cross talk in the cAMP-signal transduction pathway at the upstream processes toward CCREs. The regulation of P*ptca1* containing CCRE1 and CCRE2 with *Not*I replacement of CCRE3 resembled that of P*ptca2* (Fig. 1, iv and Fig. 3, iv). Together with the result in Fig. 2B, the promoter regulation without CCRE3 may confer the P*ptca2* type sensitivity for CO_2 and light.

Truncation Assay of P*ptca2*

PtCA2 is a paralogous CA to PtCA1, with an approximately 80% amino acid sequence identity whose transcription is also CO_2-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 by the function of diatom plastidic thioredoxins (Kikutani et al., 2012) and the function of the promoters of both CA genes are responsive to CO_2 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 *ptca2* gene from that of the *ptca1* gene. The isolated promoter region of *ptca2* 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 CO_2 and light (Fig. 4A, i–v), while the promoters truncated at −333 completely lost the response to either CO_2 or light (Fig. 4A, vi and vii), indicating that the critical CO_2/light-responsive region of P*ptca2* resides between −367 and −333. Indeed, the sequence of this region comprised a couple of CCRE2 sequences, ACGTCA (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 CO_2/Light Response in the Core-Regulatory Region of P*ptca2*

The CCRE1 and CCRE2 sequences in the short region of about 30 bps of P*ptca2* were singly or concurrently replaced by the *Not*I restriction sequence and the CO_2/light response of manipulated P*ptca2* was evaluated by the GUS reporter assay (Fig. 5). Introduction of the *Not*I restriction site to the region adjacent to the CCREs cluster gave no effect on the CO_2/light response of P*ptca2* (Fig. 5, Re1 and Re6). A single insertion of the

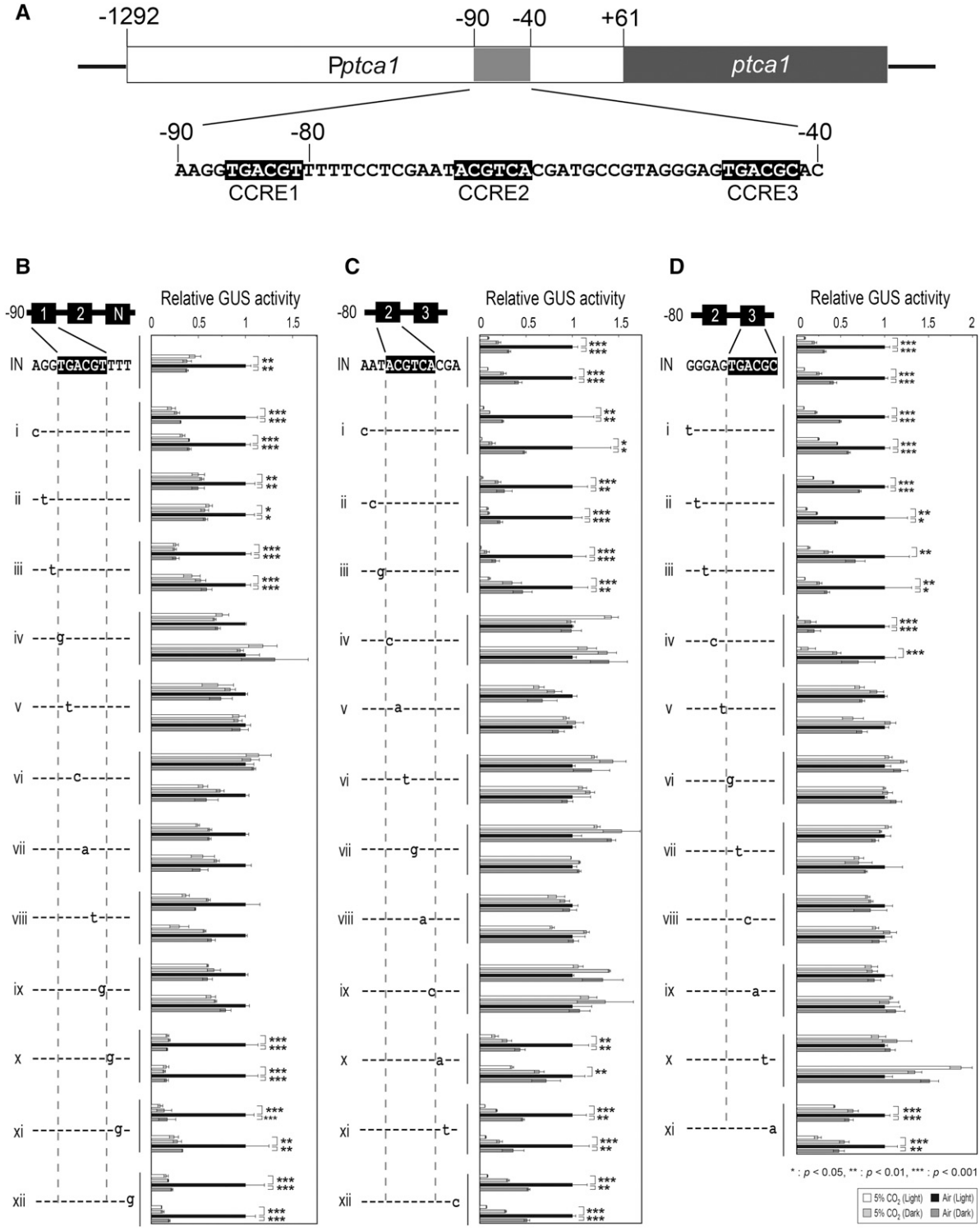

Figure 2. CO_2 and light responses of one-base-replaced promoter. A, A schematic drawing of core-regulatory region of P*ptca1*. B, One base replacement assay for the CCRE1 sequence; CCRE3 sequence was replaced by *Not*I site to avoid CCRE3 substituting for the function of CCRE1. C, D, One base replacement assay, respectively, for CCRE2 and CCRE3; CCRE1 sequence was removed for the same reason as described in (B). Cells were grown in 5% CO_2 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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

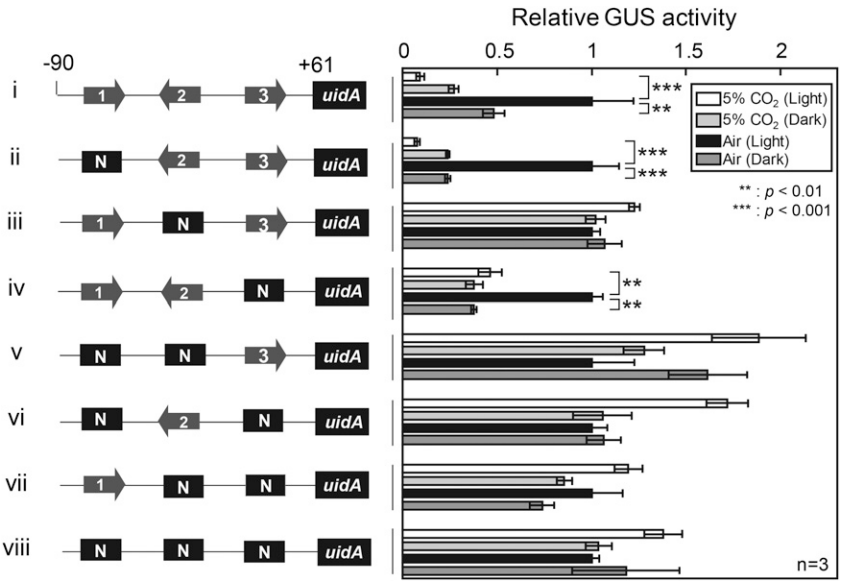

Figure 3. Determination of critical cis-element combination for light response. P*ptca1* core-regulatory region (−90 to +61) was fused with *uidA* and CCREs were replaced by *Not*I site with a set of different combinations and a GUS assay was performed. Cells were grown in 5% CO_2 or air in the absence or presence of light. Value is mean ± SD of three replicates. Statistical significance was determined by *t* test (**, $P < 0.01$; ***, $P < 0.001$).

*Not*I restriction site to either CCRE1 or CCRE2 also showed little effect on the P*ptca2* function (Fig. 5, Re2, Re3, and Re5). In the construct Re4, the overlapping part of CCRE1 and the second CCRE2 were replaced by the *Not*I sequence but CO_2 response was retained in this manipulation (Fig. 5, Re4). Complete replacement of both CCRE1 and the second CCRE2 largely dissipated the CO_2/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 *Not*I insertions into CCREs showed a significant dissipation effect on the CO_2/light responses of P*ptca2*. That is, a disruption of one of two CCRE2s with the CCRE1 disruption greatly diminished the CO_2/light response of P*ptca2* (Fig. 5, Re(2+3) and Re(TG+4)). A concurrent *Not*I insertion to two CCRE2s completely abolished the CO_2/light response (Fig. 5, Re(2+5)). Similarly, disruption of all CCREs completely abolished the CO_2/light response of P*ptca2* (Fig. 5, Re(2+TG+4)).

Light Wavelength Required to Maintain the Activities of P*ptca1* and P*ptca2* 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 O_2 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 OD_{730} 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. S1, B and C). The capacities of these four monochromatic light to maintain the CO_2-responsive transcriptional regulation of the endogenous P*ptca1* and P*ptca2* 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 P*ptca1* and P*ptca2* 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 CO_2-responsive promoters in response to CO_2 and light is deeply related to photosynthetic light.

Changes in Transcript Levels of Endogenous *ptca1* and *ptca2* 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 CO_2 supply to Rubisco at the pyrenoid under CO_2-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 plastoquinone (PQ) in PSII (Izawa, 1980); 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), a blocker

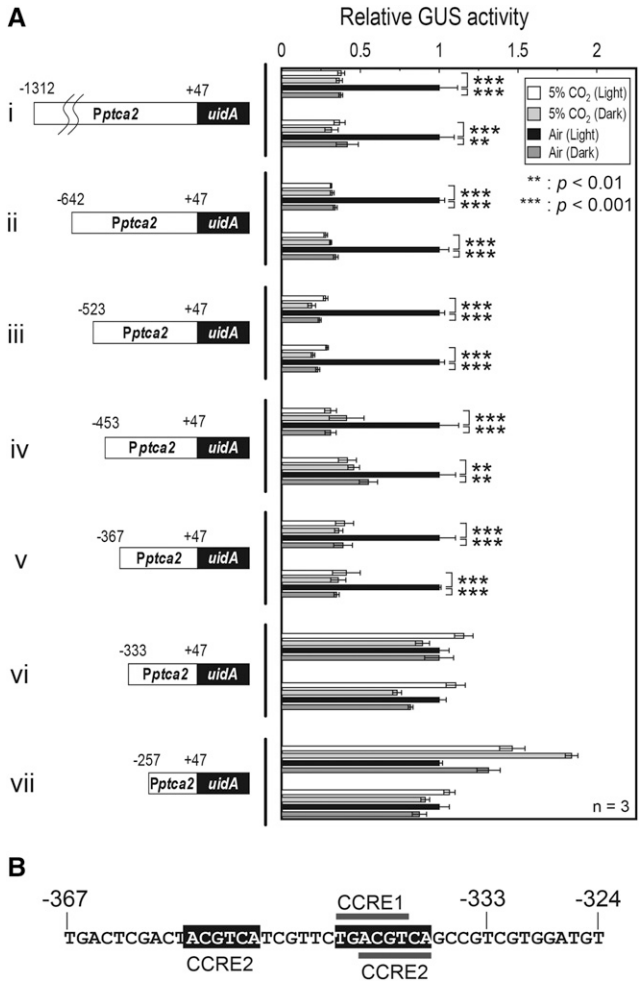

Figure 4. Truncation assay of P*ptca2* to screen the critical regulatory region for CO_2 and light responses. A, A series of upstream truncation was carried out on P*ptca2* (−1312 to +47) and each truncated sequence was fused with *uidA* (left half) and the GUS reporter assay was done with each transformant (right half). Cells were grown in 5% CO_2 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 (**, $P < 0.01$; ***, $P < 0.001$). B, The primary structure of the newly found core-regulatory region of P*ptca2*, which contains one CCRE1 and two CCRE2 sequences.

of the Q_o site of the cytochrome b_6f complex (Farineau et al., 1984; Trebst, 2007); 2,5-dichloro-1,4-benzoquinone (DCBQ), an artificial electron acceptor from Q_B in PSII (Graan and Ort, 1986); and 2,6-dichlorophenolindophenol (DCPIP), a strong oxidizing reagent at the acceptor side of PSI (Izawa, 1980; Supplemental Fig. S2A). The ATP synthesis inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), is a universal uncoupler of protonmotive force and ATP synthase (Heytler and Prichard, 1962; Terada, 1981; Supplemental Fig. S2B), and Valinomycin is a specific potassium ionophore that dissipates membrane potential, $\Delta\psi$ but not ΔpH (Harold, 1969; Gómez-Puyou and Gómez, 1977; Yildiz et al., 1994; Supplemental Fig. S2C). The growth rate of cells slowed down in 2 d treatment with 0.1 μM DCMU, 3 μM DBMIB, or 2 μM DCBQ, while DCPIP up to 50 μM did not reveal any growth inhibition over 4 d of culture (Supplemental Fig. S3A). Photosynthetic O_2 evolution under short time exposure to inhibitors showed significantly different response from that of growth response (Supplemental Fig. S3B; Supplemental Table S1). The quantities 0.1 μM and 1 μM of DCMU and DBMIB respectively were highly toxic to PSII and no O_2 evolution was observed (Supplemental Fig. S3B; Supplemental Table S1). On the other hand, DCBQ showed a clear inhibitory effect on O_2 evolution at 10 μM but below this concentration, there was no significant inhibition (Supplemental Fig. S3B; Supplemental Table S1). DCPIP showed a linear concentration dependency of inhibition to O_2 evolution from 5 μM over 100 μM (Supplemental Fig. S3B; Supplemental Table S1). In the presence of 2.5 μM CCCP, there was little O_2 evolution, while 2 μM Valinomycin inhibited the maximum photosynthesis to about 60% of the nontreated cells (data not shown). From these preliminary experiments, we set the dose of each inhibitor at the level below the acute toxic level (0.025 μM DCMU, 1 μM DBMIB, 2 μM DCBQ, 10 μM DCPIP, 0.5 μM CCCP, and 2 μM Valinomycin) 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 nontreated cells (Fig. 6B), suggesting the absence of influence on the transcriptional control mechanism in P*ptca1* and P*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 μM (Supplemental Fig. S3A), this repressive effect of DCPIP on accumulations of the *ptca1* and *ptca2* transcripts looked extremely efficient with little metabolic damage, strongly suggesting the critical requirement of a high reduced level of the PSI acceptor side for a full derepression of transcription of both P*ptca1* and P*ptca2* in air. Photosynthetic affinity for DIC was slightly inhibited in cells treated with 10 μM DCPIP (Supplemental Fig. S3B), which is presumably a result of repression of both *ptcas* by DCPIP.

DISCUSSION

In this study, the light-responsive cis-elements in P*ptca1* were newly determined. Also, the structure and the function of P*ptca2* were determined and compared in detail with those of P*ptca1*. We found that P*ptca1* and P*ptca2* were essentially controlled by a similar switching mechanism of transcription, which was mediated

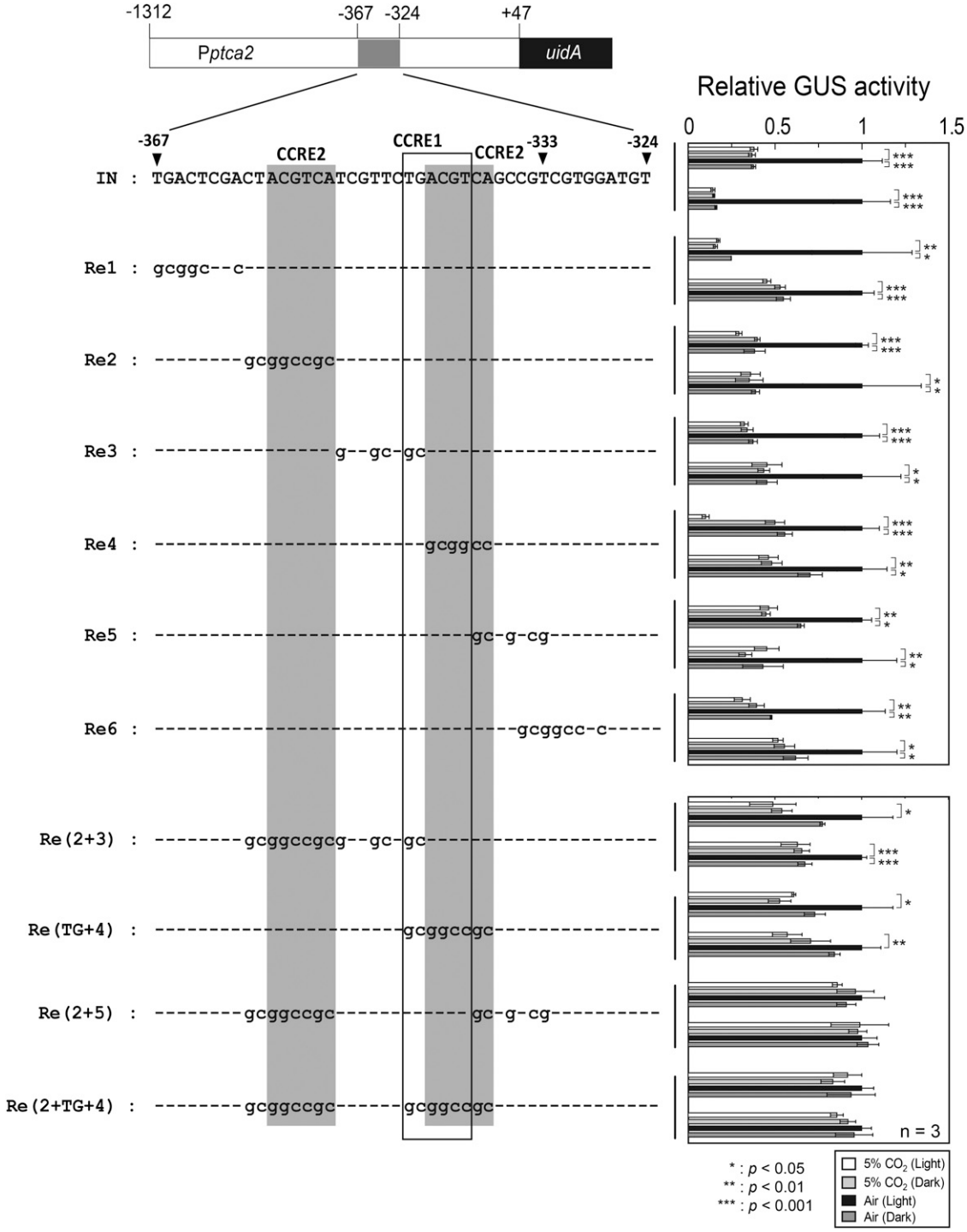

Figure 5. Sequence-replacement assay to determine the cis-elements for CO_2/light response in the core-regulatory region of P*ptca2*. CCRE1 and CCRE2 sequences and adjacent sequences within the core-regulatory region of P*ptca2* were replaced by *Not*I restriction site (left half), and the GUS reporter assay was done with each transformant (right half). Cells were grown in 5% CO_2 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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

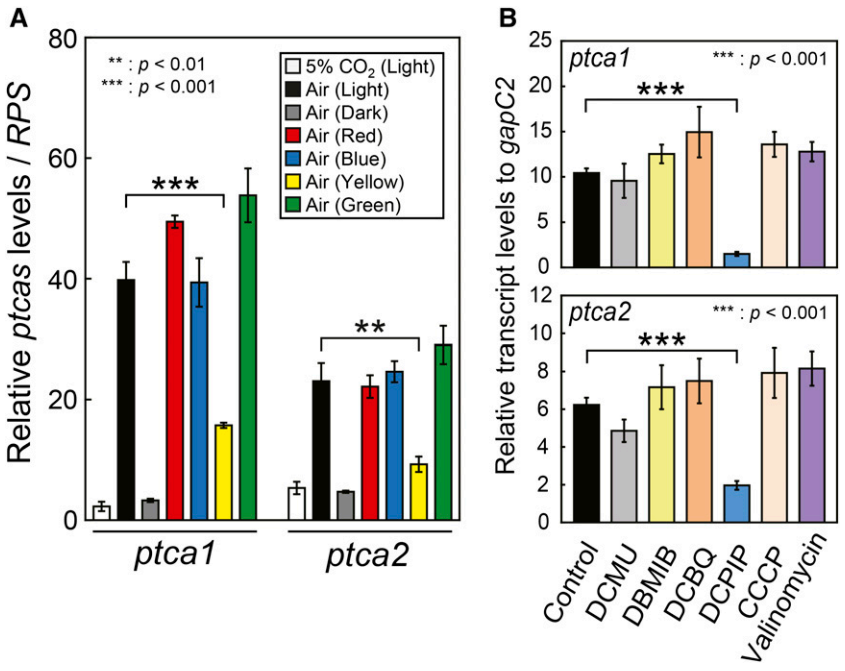

Figure 6. Changes in transcript levels of endogenous *ptca1* and *ptca2* under different light wavelength and inhibitor treatments for the PS and ATP synthesis. A, 5% CO_2-grown cells were acclimated to air under illumination with different wavelengths of light for 2 d and quantitative RT-PCR was carried out by *RPS* as an internal control. Value is mean ± SD of three replicates. Statistical significance was determined by *t* test (**, $P < 0.01$; ***, $P < 0.001$). B, Transcript levels of endogenous *ptca1* and *ptca2* in air-grown cells in the light were compared among cells treated with PS inhibitors (DCMU, DBMIB, DCBQ, and DCPIP) and ATP synthesis inhibitors (CCCP and Valinomycin). The levels of transcript were determined by quantitative RT-PCR as a relative amount to the *gapC2* transcript. Value is mean ± SD of three replicates. Statistical significance was determined by *t* test (***, $P < 0.001$).

by a second messenger cAMP, in response to CO_2 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 P*ptca1* and P*ptca2* were shown to be essential to repress the promoter activity in response to CO_2, 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 P*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 P*ptca2* also revealed a tight relation to CO_2 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 CO_2 and light cross talk at upstream signal transduction pathways toward CCREs both in P*ptca1* and P*ptca2*. However, there were some differences in the alignment of CCREs between P*ptca1* and P*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 P*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 CO_2 and light; that is, transcriptional activation of P*ptca2* in response to the decrease in CO_2 was strongly light-dependent and either CO_2 or the absence of light can maximally down-regulate the promoter, while CO_2 and light have additive effect on the regulation of P*ptca1* (Fig. 1). One of the interesting structural features clarified in this study is that the manipulated P*ptca1* only with CCRE1 and CCRE2 altered its CO_2/light response to that similar to P*ptca2* (Figs. 2 and 3). Interestingly, the structure of P*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 CO_2-responsive promoters is commonly observed in the regulation of algal CCM factors but the molecular mechanism of the CO_2/light cross talk in eukaryotic algae is yet to be elucidated. In bacterial systems, in contrast, the mechanism of CO_2/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 CO_2-responsive promoter of the HCO_3^- transporter gene operon, *cmpABCD* in the cyanobacterium, *Synechocystis* sp. PCC 6803, strongly suggesting that CO_2/light cross talk occurs at the C_2/C_3 photosynthetic carbon flux mediated by the accumulation of the initial photorespiratory metabolite (Nishimura et al., 2008). In contrast, regulation of CCM in eukaryotic algae is relatively complicated; that is, cells respond specifically to CO_2 concentration to control CCM levels even though they possess high HCO_3^- uptake capacity. In the green algae, *C. reinhardtii* and *C. ellipsoidea*, a physiological determinant of the CCM level was CO_2 in the bulk media but not HCO_3^- nor

total DIC (Matsuda and Colman, 1995; Bozzo and Colman, 2000; Vance and Spalding, 2005). In *C. ellipsoidea*, even light was not required for a regulation of the CCM expression (Matsuda and Colman, 1995) but in contrast, the CCM expression in *C. reinhardtii* was strongly blue light-dependent (Dionisio-Sese et al., 1990). In the marine diatom, *P. tricornutum*, also the similar signaling role of CO_2 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 CO_2 and light in eukaryotic algae, which may cross talk independently of photosynthesis. The role of the cAMP analog in CO_2/light signals to control P*ptcas* in *P. tricornutum* strongly suggests the involvement of cAMP in this cross talk (Fig. 1).

Furthermore, all photosynthetically active-monochromic-light wavelength (red, blue, and green) conferred a derepression on both P*ptcas* with equivalent efficiency to the normal light, while weakly photosynthetically active-light wavelength (yellow) showed a weak derepression activity to P*ptcas* (Fig. 6A; Supplemental Fig. S1). This apparent synchronization of the promoter activity with the photosynthesis strongly suggests an involvement of photosynthetic process to the CO_2/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 PSII to 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 CO_2/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* Bohlin (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% CO_2 or ambient air (0.039% CO_2) at 20°C. In acclimation experiments, 5% CO_2-grown cells were harvested by centrifugation at 3,500*g* for 5 min at room temperature, washed twice with CO_2-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-metyl-6-isopropyl-pbenzoquinone (DBMIB), 2 μM 2,6-dichlorobenzoquinone (DCBQ), 10 μM 2,6-dichlorophenolindophenol (DCPIP), 0.5 μM carbonylcyanide *m*-chlorophenyl-hydrazone (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 *ptca2* (−1312 to +47 relative to the transcription-start site) was amplified on the template genomic DNA with high-fidelity PrimeSTAR HS DNA polymerase (TaKaRa Bio, Kusatsu, Shiga, Japan) and a primer pair: Fw: 5′-CGCAATGAGCATCTCGACGGCACCAATCGCT-3′; Rv: 5′-GGTAGAA-CAGTAAACTAGGTTGGTGTACCACA-3′ and was phosphorylated. To construct the plasmid fused with *uidA* gene, a vector pFcpApGUS (Harada et al., 2005) was double-digested with *Eco*RI and *Nde*I to remove the promoter region of fucoxanthin chlorophyll (Chl) *a*/*c* binding protein gene (*fcpA*) and blunt-ended with T4 DNA polymerase. A phosphorylated PCR product was inserted into blunt-ended pFcpApGUS and sequenced (pPtCA2pGUS).

A series of upstream truncated constructs of P*ptca1* (−1292 to +61 and −90 to +61 relative to the transcription-start site) were used as described by Ohno et al. (2012), while P*ptca2* (−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 pPtCA2pGUS 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 P*ptca1*, PCR reactions were carried out targeting the sequence −71 to −60 and −52 to −42 using the vector pFcpApGUS containing P*ptca1* (−90 to +61), and targeting the sequence −86 to −81 using the vector pFcpApGUS containing P*ptca1* (−90 to +61) replacing the sequence −52 to −42 with *Not*I restriction site. A set of linker scan constructs with a *Not*I restriction linker site was synthesized by a modified inverse-PCR method using the vector plasmid pPtCA2pGUS as a template. Primer sets were designed to generate a *Not*I restriction site (GCGGCCGC) at desired locations by self-ligation (Supplemental Table S3). In some experiments, *Not*I substitutions were introduced into −358 to −351 and −350 to −343 or −344 to −337 or −338 to −331 relative to the transcription-start site using the aforementioned vector plasmids pFcpApGUS containing upstream truncated P*ptca2* (−1312 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 5×10^7 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 M $CaCl_2$ and 16 mM spermidine. The 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 91.4 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 100 μg mL^{-1} 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 lauroyl sarcosine; and 0.1% v/v Triton X-100) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcA cyclohexyl ammonium salt (X-GlucA; 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-GlucA, were inoculated and used for further experiments.

Quantification of GUS Activity

Cells at different acclimation stages were harvested by centrifugation at 3,000*g* 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 15,400*g* 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 0.5 M Na_2CO_3 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 A_{405} 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 1,400*g* at room temperature and washed three times with CO_2-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 O_2 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 O_2-electrode chamber and illuminated with a fiber illuminator at PPFD of 40 μmol m^{-2} s^{-1} under aeration with N_2 for 10 min until cells reached to the CO_2-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 $NaHCO_3$ to the desired final concentrations to cell culture. The rate of O_2 evolution at each DIC concentration was plotted against DIC concentration. Chl*a* 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 *ptca1* and *ptca2* 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 manufacture'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 Mixα No ROX (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 *ptca1*, *ptca2*, *RPS*, and *gapC2* for Quantitative RT-PCR were drawn with a known amount of template using plasmids containing the *ptca1*, *ptca2*, *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 *ptca1* and *ptca2* transcripts were normalized by the level of the *RPS* or *gapC2* transcripts.

Sequence data from this article can be found in the JGI Genome Portal or the GenBank/EMBL data libraries under accession numbers: *RPS* (protein ID: 10847), *gapC2* (GenBank accession no. AF63805), *ptca1* (GenBank accession no. AAL07493), Ptca2 (GenBank accession no. BAD67442).

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 PS.

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

Supplemental Table S2. Primer sequences used to make P*ptca2* truncated constructs.

Supplemental Table S3. Primer sequences used to make P*ptca2* linker-scan constructs.

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

ACKNOWLEDGMENTS

We thank Ms. Nobuko Higashiuchi and Keiji Goto for their technical assistance, and Ms. Miyabi Inoue for her skillful secretarial aid.

Received November 9, 2015; accepted December 7, 2015; published December 11, 2015.

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