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The phosphoglycolate phosphatase gene and the mutation in the phosphoglycolate phosphatase-deficient mutant (*pgp1-1*) of *Chlamydomonas reinhardtii*

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The phosphoglycolate phosphatase gene and the mutation in the phosphoglycolate phosphatase-deficient mutant (*pgp1-1*) of *Chlamydomonas reinhardtii*[1]

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Abstract: The sequences of the phosphoglycolate phosphatase (PGPase) gene *Pgp1* and the 5′-upstream region from *Chlamydomonas reinhardtii* wildtype 2137 and the *pgp1-1* mutant N142 that lacks the activity of PGPase (PGP1) were determined. The comparison revealed the alteration of a G to A at position 98 relative to the start codon. This destroyed the "GT" splice donor site at the beginning of the first intron of this gene, resulting in an extension of the first exon to 49 translatable codons followed by a stop codon, containing the codons corresponding to whole transit peptide for the chloroplast stroma and the first four N-terminal amino-acid residues of the PGP1 subunit. The comparison of the upstream nucleotide sequence of *Pgp1* with those of 37 other genes including those involved in the CO_2-concentrating mechanism and (or) photorespiration showed the high similarity of *Pgp1* upstream to a periplasmic carbonic anhydrase gene *Cah1*; the motifs RAGGTCAGN$_{8–9}$CCR and TTGGCAG were found only within the low-CO_2 responsive genes, including *Pgp1* and *Cah1*. GAN$_7$CGNTTGGAAN$_2$AG, TTGGAAGGAG, and CAGAGGTCAGN$_8$CCG were found only with *Pgp1* and *Cah1*, and ACGCTTGGCAGT and CATTACCAT were found only with *Pgp1* and alanine aminotransferase gene *Aat1*. The possibility of functional PGPase isozyme(s) in *C. reinhardtii* is also discussed.

Key words: Chlamydomonas reinhardtii, CO_2-concentrating mechanism, low-CO_2 responsive gene, *pgp1-1* mutation, phosphoglycolate phosphatase.

Résumé : Les auteurs ont déterminé les séquences du gène de la phosphatase du phosphoglycolate (PGPase) et de la région 5′ en amont, chez la souche sauvage 2137 du *Chlamydomonas reinhardtii,* ainsi que du gène *pgpl-1* du mutant N142 qui est dépourvu de l'activité de la PGPase (PGP1). Une comparaison révèle l'altération d'un G vers un A à la position 98, par rapport au codon de départ, ce qui détruit le site du fournisseur de jonction « GT », au début du premier intron de ce gène. Ceci conduit à une extension du premier exon à 49 codons transposables, suivi d'un codon d'arrêt, et contenant les codons correspondants au peptide de transition entier du stroma chloroplastique ainsi qu'à la première sous unité N terminale à quatre résidus d'acides aminés du PGP1. La comparaison de la séquence des nucléotides en amont du gène *Pgp1* avec ceux de 37 autres gènes, incluant ceux impliqués dans le mécanisme de concentration du CO_2 et (ou) de la photorespiration, montre une forte similarité du *Pgp1* en amont, jusqu'au gène de l'anhydrase carbonique périplasmique *Cah1*; on ne retrouve les motifs RAGGTCAGN$_{8–9}$CCR et TTGGCAG uniquement avec les gènes de réaction au faible CO_2, incluant *Pgp1* et *Cah1*. Les séquences GAN$_7$CGNTTGGAAN$_2$AG, TTGGAAGGAG et CAGAGGTCAGN$_8$CCG ne se retrouvent qu'avec les gènes *Pgp1* et *Cah1*, et les séquences ACGCTTGGCAGT et CATTACCAT qu'avec le gène *Pgp1* ainsi que le gène *Aat1* de l'aminotransférase de l'alanine. Les auteurs discutent la possibilité de l'existence d'une ou plusieurs isoenzyme(s) fonctionnelle(s) PGPase, chez le *C. reinhardtii*.

Mots clés : Chlamydomonas reinhardtii, mécanisme de concentration du CO_2, gène de réaction au faible CO_2, mutation *pgp1-1*, phosphatase du phosphoglycolate.

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Introduction

Phosphoglycolate phosphatase (PGPase), a photorespiratory enzyme that catalyzes the hydrolysis of phosphoglycolate produced by the ribulose-1,5-bisphosphate oxygenase activity of ribulose-1,5-bisphosphate carboxylase–oxygenase, is essential for the growth of photosynthetic organisms in the light. The PGPase-deficient mutant of *Chlamydomonas reinhardtii pgp1-1* requires elevated levels of CO_2 and cannot grow under air (Suzuki et al. 1990). This high-CO_2 requirement likely results because phosphoglycolate accumulates in the *pgp1-1* cells under air during photosynthesis (Suzuki et al. 1999), as phosphoglycolate is a strong inhibitor of triosephosphate isomerase (Wolfenden 1970; Anderson 1971; Norman and Colman 1991). However, it is also possible that the mutation blocks the trigger for the induction of the CO_2-concentrating mechanism (CCM). In the *pgp1-1* mutant, the function of the CCM was considerably lower than that in the wild type, in terms of photosynthetic affinity for inorganic carbon and total carbonic anhydrase activity (Suzuki et al. 1990), and intracellular inorganic carbon accumulation (Suzuki and Spalding 1987). On the other hand, it has been reported that PGPase is quickly and transiently upregulated by transfer of high-CO_2-grown cells to air (Marek and Spalding 1991), although the mechanism is not clear. Thus, the regulation of PGPase activity seems to be closely related to that of the CCM when the cells adapt to different CO_2 conditions, and use of the PGPase mutants should provide valuable information to investigate both regulatory mechanisms. In this report, we compared DNA sequences of the *Pgp1* gene and upstream regions of *C. reinhardtii* wild-type 2137 and *pgp1-1* mutant N142 to determine the *pgp1-1* mutation. Possible regulatory sites in the upstream region of *Pgp1* and the possibility of functional PGPase isozyme(s) are also discussed.

Materials and methods

Strains and growth conditions

Chlamydomonas reinhardtii mutant strain N142 mt– (*pgp1-1*, *cw15*) was isolated from the progenies of RPR-*pgp1* mutant 7FR2N (Suzuki 1995; Suzuki et al. 1999) after three crossings with strains containing the CC125-background, such as 124^8 mt– (wild type) and TW3 mt+ (*thi10*, *cw15*), which were obtained from K. Shimogawara (Teikyo University, Japan). The *pgp1-1* mutant N142 and wild-type 2137 (Suzuki et al. 1990) were grown photoautotrophycally as described previously (Suzuki 1995).

Determination of DNA sequences

DNA sequences of the PCR products and (or) the subclones ligated into pGEM-T (Promega, Madison, Wisconsin) were determined with automated DNA-sequencing systems 310 and 373 (Applied Biosystems, Foster City, California) using both the Dye Terminator Cycle Sequencing Kit and the Dye Primer Cycle Sequencing Kit (Applied Biosystems). The PCR reactions were performed using TAKARA LA *Taq* with GC Buffer (TAKARA BIO INC., Otsu, Japan) with purified whole genomic DNA from 2137 and N142 cells, and combinations of the following primers. The primers for the forward direction were PGP1S1 (GCACGAGGGATTATCTGACG), PGP1iSi12 (GCGCAGTTGCCACCAACGAG), PGP1iS51 (CTCAAGAGCGGCGAGTTCAT), PGP1iS65 (TGTTCATCGCCACCAACCG), PGP1tS2 (AAGGTCCACCCCGACTTCGT), PGP1p41 (TGTGAACTCTCGTTTGGAAG), and PGP1p61 (TTGCTGATGGCCCGTCAGAG). The primers for the reverse direction were PGP1iA21 (GAAGATGAAGCACTCCACCT), PGP1iA24 (TCCACCTTCTTCAGCAGCTC), PGP1iA62 (ACGTAGCGGTCAAAACCAAC), PGP1iA81 (CATGAAGTCCGAGGGCTTG), PGP1A1 (ACCAGGCTCCCTTAGC), and PGP1A162 (AGCAACTACGTTTACTGAGC). Most primers were designed based on the cDNA sequence of PGPase (AB052169). PGP1iSi12 was designed based on the sequence containing the first intron determined in the present study, and PGP1p41 and PGP1p61 were designed based on the 5′-upstream sequence found in the scaffold 1006 obtained from the JGI *Chlamydomonas reinhardtii* version 1.0 database, using the BLAST search (http://genome.jgi-psf.org/cgi-bin/runBlast?db = chlre1). The sequence determination was done at least twice for both forward and reverse directions. Sequences were analyzed with DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Sequence interpretation

To help find candidates for regulatory DNA elements, the sequences of a 1000-bp 5′-upstream region of *Pgp1* were also analyzed with PLACE, the motif database for plant *cis*-acting regulatory DNA elements (Higo et al. 1999; Prestridge 1991) via Internet (http://www.dna.affrc.go.jp/PLACE/), and TESS, transcription element search software on the Internet (Schug and Overton 1997; http://www.cbil.upenn.edu/tess/). Because the DNA sequence for the *Pgp1* gene we determined was from –634 to 5090 relative to the start codon, the complete 1000-bp 5′-upstream sequence of *Pgp1* was obtained from JGI *Chlamydomonas reinhardtii* version 2.0 database (JGI Chlamy 2.0), which was found in scaffold 34 using the BLAST search (http://genome.jgi-psf.org/cgi-bin/runBlast?db = chlre2), where there was no mismatch within the 634-bp upstream sequences, although the complete sequence of *Pgp1* gene was not available in the database. The results obtained were compared with those of the following genes: *Aat1* (alanine aminotransferase, GenBank accession No. U31975,); *Ald1* (fructose-1,6-bisphosphate aldolase, GenBank accession No. S72951, upstream from JGI Chlamy 2.0); *AOX1* (alternative oxidase, GenBank accession No. AF047832, upstream from JGI Chlamy 2.0); *AOX2* (alternative oxidase, GenBank accession No. AF285187, upstream from JGI Chlamy 2.0); *Cah1* (periplasmic carbonic anhydrase, GenBank accession No. AB026126); *Cah2* (periplasmic carbonic anhydrase, GenBank accession No. X54488, upstream from JGI Chlamy 2.0); *Cah3* (chloroplast carbonic anhydrase, GenBank accession No. U73856, upstream from JGI Chlamy 2.0); *Cah6* (chloroplast carbonic anhydrase, GenBank accession No. AY463238, upstream from JGI Chlamy 2.0); *Ccp1* (chloroplast envelop protein LIP36G1, GenBank accession No.U75345, upstream from JGI Chlamy 2.0); *Ccp2* (chloroplast envelop protein LIP36G2, U75346, upstream from JGI Chlamy 2.0); *Ccm1* (a regulatory gene for CCM, Miura et al. 2002, GenBank accession No.

Fig. 1. The position of the *pgp1-1* mutation in the sequence of the *Pgp1* gene from the mutant N142. (A) PCR fragments that were used for nucleotide sequencing, directly or after the subcloning. Each PCR product was obtained from *Chlamydomonas reinhardtii* wild-type 2137 and *pgp1-1* mutant N142, and was sequenced at least twice for both directions. (B) Outline of the *Pgp1* gene, with the exons indicated by thick lines. (C) Comparison of the nucleotide and deduced amino acid (bold letters) sequences from wild-type 2137 and the mutant N142. The sequence of the whole mutant gene with the 634-bp 5′-upstream region is available in the DDBJ, EMBL, and GenBank databases (accession No. AB191485).

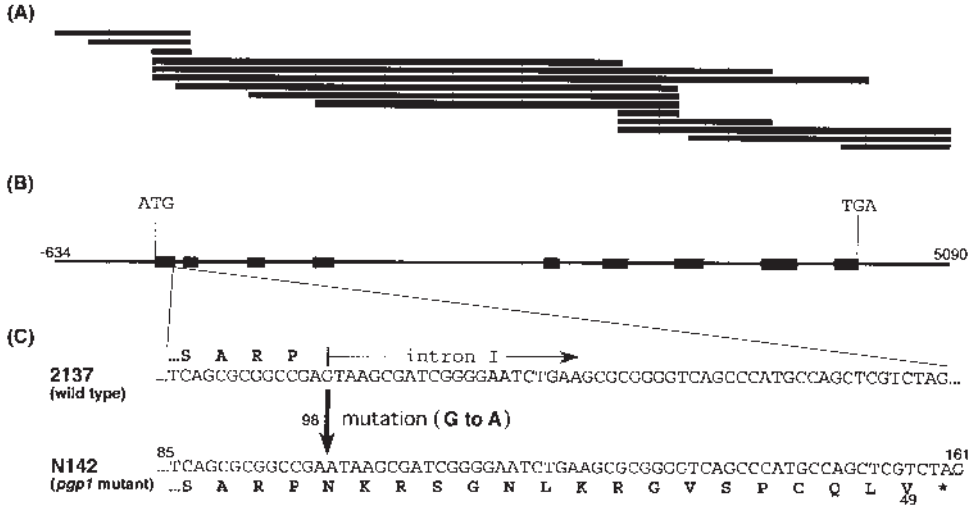

Fig. 2. Nucleotide sequence of the *Pgp1* gene and the 5′-upstream region that is expected to contain the promoter region, as compiled from the gene sequence with the 634-bp 5′-upstream region (GenBank accession No. AB091101, version AB091101.2) from *Chlamydomonas reinhardtii* wild-type 2137, and that obtained using the BLAST search from the JGI *Chlamydomonas reinhardtii* version 2.0 database (http://genome.jgi-psf.org/cgi-bin/runBlast?db = chlre2). Deduced amino acid sequence of the PGPase precursor protein (accession No. AB052169) is shown by one-letter codes under the nucleotide sequence. The nucleotide sequences found within the enhancer element EE-1 (Kucho et al. 2003) of *Cah1* upstream are boxed by regular lines. The sequences indicated by bold letters were found only in the low-CO_2 responsive genes, and those underlined by thick lines were found only with *Aat1* and *Pgp1* (Fig. 3). The CAAT and GATA motifs (putative promoter elements) are boxed by thick lines.

```
.....GCATCAACAT   -991
TTAGAACCACATCGAAATCCGTGTCAGCGCACGCCCGCGCTCGTGAGGTTTGTAGTGCGTGCCACGGTCACCCGCAACCCAGCTGCACAC   -901
CCGCCATCCGCGTACACGCTGCAGCCCCTGCGCCACCTCCGCCCTCCCAAAACCCCCAACACACACACACACCCATCCACACAGGTATGC   -811
ATCCCACCTGCATCCTCAGCAGGTCCTCCTCCGAATCCAAGTGAAGGTCGTCGTCTCCCCGGACACTCTCCTGAGGCCCGCCAAGCGCCG   -721
CGCGCGCAGCACGCAGGCTGCTGCCGGCGCCGCGCCGCGAGCTGCCGGCAGTAGATGCTGGAGCGCTTGCTGATGGCCCGTCAGAGGTCA   -631
GGCCGGTGGCCGGGGAAGCTGCGGCCGCAGGCGGCGCCGCGACGGCGGGTGCCGCTGTGGCGCCTGCGCGCGCTGCCGCCGTGCGACGGA   -541
CCACGGTCGCCGCTGCCGACCCCACCTGCCCAGCCATAAACGCTTCCTGCGGCCGCGAGGCAAAGCGACACCGTCAGCTCACCAAGCAATT   -451
CATGTGTGAACTCTCGTTTGGAAGGAGTCGGCGCCGGCGGGATCTCCATAATACCTGCTGCCGGAGCAGGTCATCCTCGGAGCCAAAGTC   -361
      |   | | ||  ||||||||||
   agattttcac-cggttggaaggaggt (EE-1 in Cah1 upstream)

CAGGTCGCGGCGCCTGGGAAGCGCTTCCGGCAGTTCCTCTGATGCCTCCATCTTGAACCGGTTCGGCTCGGCTACCTAAGTGCCAGACTC   -271
GCGACGATTGGAACAAGGGTCAGCTATTTCCTTCCAAACCTAGTGACCATAGTATACTGTAATGCATACATTACGCTTCAACTTATCCTG   -181
GATGTCAGTTTGGCAGTTCCTTAGTGACCGCTTGCAATTGCAGCCCATCCCGATGCTGCTCTGTGCAGCTCGTTTGCTTGTTTGCCCCGC    -91
CCATGTACGCTTGGCAGTCGCATTACCATCTTGCACAGATCGTCGGACCAGGACGCTGCAGGCACTTGCATATTGTAGGATTATCTGACG     -1
ATGCTGAGCCTGAAGCAGCTGCCCAGCGCGCGCTGCGCCGCTCGTCCCGTCCGTCCGGTTCGCAGGATGGTTGCTGCCCAGGCTTCAGCG     90
 M  L  S  L  K  Q  L  P  S  A  R  C  A  A  R  P  V  R  P  V  R  R  M  V  A  A  Q  A  S  A
CGGCCGAGTAAGCGATCGGGGAATCTGAAGCGCGGGGTCAGCCCATGCCAGCTCGTCTAGATAACGCTCCACCCCGCGCTGCGCGCAGTT    180
 R  P                                                                                    I
GCCACCAACGAGCAGAAGCTGGAGCTGCTGAAGAAGGTGGAGTGCTTCATCTTCGACTGCGATGGTGCGTTTGCCCGCCCGGTGGGGGTT    270
 A  T  N  E  Q  K  L  E  L  L  K  K  V  E  C  F  I  F  D  C  D
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AB052694); *Csbp* (sedoheptulose-1,7-bisphosphatase, GenBank accession No. Y14608); *GapA* (chloroplastic glyceraldehydes 3-phosphate dehydrogenase, GenBank accession No. L27668, upstream from JGI Chlamy 2.0); *Gdh1* (putative glycolate dehydrogenase, Miura et al. 2004, upstream from JGI Chlamy 2.0); *GscP* (putative glycine cleavage system P-protein, Miura et al. 2004, upstream from JGI Chlamy 2.0); *H43* (high-CO_2-inducible periplasmic protein, GenBank accession No. AB042098, upstream from JGI Chlamy 2.0); *LciA* (putative inorganic carbon transporter, GenBank accession No. AB168092, upstream from JGI Chlamy 2.0); *LciB* (putative membrane protein, GenBank accession No. AB168093, upstream from JGI Chlamy 2.0); *LciC* (putative chloroplast protein, GenBank accession No. AB168094, upstream from JGI Chlamy 2.0); *Lci1* (putative membrane protein, GenBank accession No. U31976, upstream from JGI Chlamy 2.0); *Lcr1* (a low-CO_2-inducible protein, AB168089); *Mca1* (mitochondrial carbonic anhydrase, GenBank accession No. U80804); *Mca2* (mitochondrial carbonic anhydrase, GenBank accession No.

U80805); *Mdh1* (NADP-malate dehydrogenase, GenBank accession No. AJ277281, upstream from JGI Chlamy 2.0); *Nar1* (nitrate transporter, GenBank accession No. AF149737, upstream from JGI Chlamy 2.0); *nad-MDH* (NAD-malate dehydrogenase, GenBank accession No.AJ250844, upstream from JGI Chlamy 2.0); *Pgk* (phosphoglycerate kinase, GenBank accession No. U14912,); *Pgp2* (C_15019, a putative PGPase gene proposed in JGI Chlamy 2.0); *Pgp3* (C_270015, a putative PGPase gene proposed in JGI Chlamy 2.0); *PrkA* (phosphoribulokinase, GenBank accession No. AF228914, upstream from JGI Chlamy 2.0); *RbcS1* (ribulose-1,5-bisphosphate carboxylase–oxygenase small subunit, GenBank accession No. X04471, upstream from JGI Chlamy 2.0); *RbcS2* (ribulose-1,5-bisphosphate carboxylase–oxygenase small subunit, GenBank accession No. X04472); *Rh1* (a CO_2-channel protein, GenBank accession No. AY013258, upstream from JGI Chlamy 2.0); *Sgat* (putative serine glyoxylate aminotransferase, upstream from JGI Chlamy 2.0); *Shmt* (serine hydroxymethyltransferase, GenBank accession No. AF442558, upstream from JGI Chlamy 2.0); *Sta2* (granule-bound starch synthase, GenBank accession No. AF026420, upstream from JGI Chlamy 2.0); and *Sta3* (soluble starch synthase, GenBank accession No. AF026422, upstream from JGI Chlamy 2.0). To determine whether the genes were low-CO_2 responsive or not, we referred to Miura et al. (2004) and the supplemental data of Yoshioka et al. (2004), which is available on the Internet (http://www.plantcell.org/cgi/content/full/tpc.021162/DC1).

Results and discussion

pgp1-1 mutation

The cDNA sequence for the *Chlamydomonas* PGPase, which we have reported previously (Mamedov et al. 2001), allowed us to determine the *pgp1-1* mutation. The sequences of the *Pgp1* gene and the upstream region were determined for *Chlamydomonas reinhardtii* wild type 2137 and PGPase-deficient mutant *pgp1-1*-N142, from the position –634 to 5112 relative to the start codon (Figs. 1 and 2, entire sequences not shown). The comparison of these two sequences revealed the alteration of a G to A at position 98 (Fig. 1), a result consistent with the recent quantitative finding that more than 99% of ethylmethane sulfonate-induced mutations were G/C-to-A/T transitions (Greene et al. 2003). This change destroyed the "GT" splice donor site at the beginning of the first intron of this gene and resulted in an extension of the first exon to 49 translatable codons, followed by a stop codon (Fig 1). Because the expected peptide contained the complete signal peptide for stroma and four N-terminal-amino-acid residues of the PGPase subunit, it is quite likely that the gene product is digested soon after being translated, even if the mRNA is fully functional and is not digested soon. As we have not been able to detect both the small peptide (Mamedov et al. 2001) and the responsible mRNA (data not shown) in either high-CO_2-grown or air-adapted cells of the *pgp1* mutant, it is necessary to determine if it was because of a quick digestion of the mutant peptide or mRNA, or some other reason. Because of such characteristics of the mutation, the suppressor double mutants of *pgp1-1* mutant in which PGPase activity is recovered fully or partially to allow the growth under air (Suzuki 1995) should help define the regulatory mechanism of PGPase.

Thus, the deficiency of PGPase activity in the *pgp1-1* mutant was caused by the lack of the functional enzyme, and further investigation is required to make clear why the mutant shows quite low CCM activity even in air-adapted cells.

The upstream sequence of *Pgp1* gene

The regulation of PGPase activity seems to be closely related to that of the CCM when the cells adapt to different CO_2 conditions. PGPase is quickly and transiently upregulated after transfer of high-CO_2-grown cells to air (Marek and Spalding 1991). Although the mechanism is not clear, such a response is expected to be a result of a different gene regulation from that proposed for the periplasmic carbonic anhydrase gene *Cah1* (Kucho et al. 2003). In addition, phosphoglycolate, the substrate of PGPase, has been thought to be a candidate for the trigger of low-CO_2 adaptation (e.g., Suzuki et al. 1990; Kaplan and Reinhold 1999). To help define the regulatory mechanism of the *Pgp1* gene, the DNA sequence of the 1000-bp upstream region was compared with that of 37 other genes, including the genes and putative genes involved in the CCM and photorespiration, most of which are low-CO_2 responsive (Fig. 3).

The upstream region of *Pgp1* gene seems to have the highest similarity to that of the *Cah1* gene in Fig. 3. Although the CAAT motif was found with all genes, except *Aat1* and *Aox2* (data not shown), which are listed in Fig. 3, two motifs containing CAAT were observed at similar positions in *Pgp1* and *Cah1*. The first one, CAATCA, was found at –454 in *Pgp1* and –473 in *Cah1*, and the second one, CAATTG, was found at –146 in *Pgp1* and at –150 in *Cah1*. Such similarity was not observed with any other genes that we compared. The region between the two CAAT motifs of *Pgp1* upstream has a sequence similar to the EE-1 motif (Kucho et al. 2003) of *Cah1* upstream (Fig. 2). Although the EEC motif (GANTTNC, Kucho et al. 2003) was not found, two regions have the consensus sequence GAN_7CGN-$TTGGAAN_2AG$ (–436 to –424 and –266 to –254), which was also found in EE-1 of *Cah1* upstream (–283 to –271). The sequence TTGGAAGGAG, which contained the TGGAAGG motif (R02828 in the TRANSFAC database), was found in *Pgp1* upstream and the EE-1 motif in *Cah1* upstream (Fig. 3). Such motifs are not found in any other genes, and likely involved in the common regulatory mechanism for *Pgp1* and *Cah1*.

It has been proposed that the EE-1 and EE-2 motifs, which contain the EEC motif as the consensus sequence, are essential for binding the transcriptional regulator CCM1 (CIA1) (Fukuzawa et al. 2001; Xiang et al. 2001). Recently it has been proposed that the CCM1 is a master regulatory factor of low-CO_2 inducible genes (Fukuzawa et al. 2001; Xiang et al. 2001; Miura et al. 2004). Although *Pgp1* gene may be one of the CCM1-regulated genes (Miura et al. 2004), it is not likely that this gene is regulated directly by CCM1 because of the lack of the EEC motif, but it is likely that this gene has another regulatory mechanism that the *Cah1* gene also has. It is also possible that CCM1 is able to bind to another site in the upstream region or that it requires other factor(s) for proper operation because the EEC motif was not found in the 1000-bp 5′-upstream region of 11 low-

Fig. 3. Comparison of the 5′-upstream 1000-base-nucleotide sequence of the *Pgp1* gene with those of 37 other genes mostly involved in carbon and nitrogen metabolism. The upstream sequences were obtained from the JGI *Chlamydomonas reinhardtii* version 2.0 database (JIG Chlamy 2.0, http://genome.jgi-psf.org/chlre2/chlre2.home.html), after searching corresponding genes with BLAST or a key word search with the gene or cDNA sequences available in the DDBJ, EMBL, GenBank, and Kazusa *Chlamydomonas reinhardtii* EST (Kazusa Chlamy EST, Asamizu et al. 2000, http://www.kazusa.or.jp/en/plant/chlamy/EST/index.html) databases or published papers. Numbers starting with "C_" are the gene model names in JGI Chlamy 2.0, and others are DDBJ, EMBL, or GenBank accession numbers. The accession numbers in parentheses indicate sequences that were used only for the BLAST searches. CGCGCC, GGGTTGAANTCCC, and AACCCCNGNTGCA are the sequences reported to be conserved in some CO_2-responsive genes, including *Cah1* (Kucho et al. 1999). AACCAA is the sequence annotated as "REalpha" in the *Lemna gibba Lhcb21* gene promoter (Degenhardt and Tobin 1996). TTGGAAGGAG is the sequence observed within the EE-1 region (Kucho et al. 2003) of the *Cah1* gene upstream. AGGTCA is the sequence annotated as "Q (quantitative)-element" in the maize *Zm13* gene promoter in the PLACE database (http://www.dna.affrc.go.jp/PLACE/). ACGCTTGGCAGT and CATTACCAT are sequences observed only in the *Aat1* and *Pgp1* upstreams in this report. *Pgp2* and *Pgp3* are putative genes, based on sequence similarity with the *Pgp1* gene, as annotated in JGI Chlamy 2.0. +, positive low-CO_2 response; (+), probable positive low-CO_2 response; –, negative low-CO_2 response (or positive high-CO_2 response); (blank), no apparent CO_2 response. The EST fragments corresponding to *Pgp2* and *Pgp3*, identified by using the BLAST search in Kazusa Chlamy EST, did not have low- or high-CO_2 responsive characteristics in the web-only supplemental table (Yoshioka et al. 2004, http://www.plantcell.org/cgi/content/full/tpc.021162/DC1). The position from the start codon is indicated for each motif found in the upstream region. The motifs found only in low-CO_2 responsive genes are indicated by bold letters, and the motifs found only in *Pgp1* and other genes involved in CCM and photorespiration are boxed by thick lines.

Fig. 4. Comparison of the amino acid sequences of *Pgp2* and *Pgp3* gene products (CrPGP2 and CrPGP3, respectively) with PGPase precursor (CrPGP1). The alignment was calculated using ClustalW version 1.8 (http://clustalw.genome.jp/), and the similarity is indicated below the amino acid sequences with the following characters: *, positions that have a single, fully conserved residue; :, one of the following "strong" groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, or FYW; ., one of the following "weaker" groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, or HFY. These are all the positively scoring groups that occur in the Gonnet Pam250 matrix. Strong and weak groups had scores >0.5 and 0.5, respectively. Core motifs for the families of *p*-nitrophenyl phosphatases and bacterial PGPases within the haloacid dehalogenase superfamily (Selengut 2001) are indicated below the similarity indicators, using the following groupings: a, aromatic (FHWY); c, charged (DEHKR); f, small polar (CDNST); h, hydrophobic (ACFGHIKLMPRTVWY); l, aliphatic (ILV); o, hydroxyllic (ST); p, polar (CDEHKNQRST); X, any; and Z, E or Q.

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                                                                                ▼
CrPGP1    1 --MLSLKQLPSARCAARPVR----------------------PVRRMVAAQASARPIAT
CrPGP2    1 ------------------------------------------------------MKKAT
CrPGP3    1 MSNLALQARRMALGATRQARGCHAVLAGGAPFTARAATAATAVDVPSTSATSVTPLTVLD

CrPGP1   36 NEQKLELLKKVECFIFDCDGVIWLGDKVIEGVPETLDMLRGMGKKVFFVTNNSTKSRAGY
CrPGP2    6 DRDKLEAFQQYDAWVFDLDGTLWKGSTLIPGAKEFIELLRYYNKKVFFVTNNATKSRATN
CrPGP3   61 ERTAPERLRETSTLIFDCDGVLWRGSEIIHNAPEALKEFRRQGKRLLFVTNNSSKSRAGY
           :.   * ::: .  :** **.:* *. :* .. * :. :*  .*:::*****::****
(NPPase)              hhlhDhDGzlh                    hXhhhloNN
(bact.PGP)            hlhFDhDGTLh                    hhhzlhoNN
                       (Motif I)                      (Motif II)

CrPGP1   96 MSKFQSLGLNVKAEEIYSSSYAAAAYLESINFN-------KKVYVIGETGILEELDLKGI
CrPGP2   66 AAKLTAMGINATQAEMYTSSFAAAAYLKAISFN-------KKAYVIGEEGLVEELTAVGV
CrPGP3  121 VAKFSSLGLEVAAEEIVSSSYCAAAYLTSQGFGPGGSRPCSKVLLLGWSGVEQELEQAGI
             :*: ::*::.   *: :**:.***** : .*.       .*. ::*  *: :**   *:

CrPGP1  149 RHVGGPGDADKKVTLKSGE-FMEHDHDVGAVVVGFDRYVNYYKIQYATLCIRENPGCMFI
CrPGP2  119 QCVGGPAHRGVEVDWSQAEPHVEVDPEVGAVVVGLDRYISYYKLQYATLCLANNDSCMFL
CrPGP3  181 PYVGGRALKVPPMDDLDAMKALKVDPDVGAVVVGWDPNFSYSRLVYASIHLRELPGCLLV
              *** .     :   ..   :: * :******* *  ..* :: **:: : :  .*:::

CrPGP1  208 ATNRDAVTHLTDAQEWAGNGSMVGAIVGSTKREPIVVGKPSDFMLKNISASLGLRPDQIA
CrPGP2  179 ACNTDARGHFSQAQEWAGAGTMVAALIGSSEREPMLLGKPASFILDHLCATHQVARDKTI
CrPGP3  241 ATNMDCADHIGGGRMMPGTGGLVKAVETASGVSAVNVAKGGEWLLPYLCRTYGLEPAHTA
           * * *.  *:  .:  .* * :* *:  ::  ..: :.* ..::*  :. :  :   :
(NPPase)                                          KPphh              hh
(bact.PGP)                                        KPpzX              zl
                                                (Motif IIIa)

CrPGP1  268 MVGDRLDTDIMFGKNGGLATALVLSGVTTPEVLNSPDNKVHPDFVLNSLPDLLSVKEKAM
CrPGP2  239 VVGDRLDTDILWGIQNGAGTCCVLSGVTSEAQLLAESNKVHPKLYMSDIGDFLTIKNQIP
CrPGP3  301 IVGDRMDTDIHLGRQGGLFTCLPLTGVTTLKRLEGLPASEHPDVVVRSVAQLAGLPA---
           :****:****  * :.*  *.  *:***:   * .   . **.. : .: ::  :
(NPPase)   hlGDphXoDlXhz
(bact.PGP) hlGD-fXXDlZzz
           (Motif IIb)

CrPGP1  328 VAA---
CrPGP2  299 SNCAIM
CrPGP3      ------
```

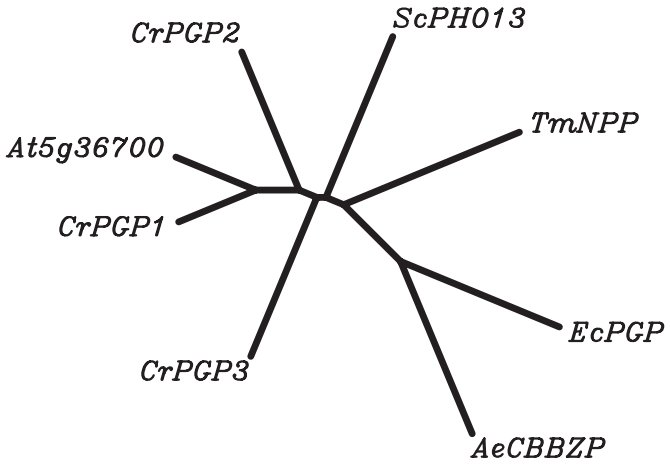
	Gene	Response to low CO_2	Motif: CGCGCC	GANTTNC	GGGTTGAANTCCC	AACCCCNGNTGCA	AACCAA	CAATCA	CAATTG	TTGGAA	TTGGAAGGAG	AGGTCA	RAGGTCAGN$_{8-9}$CCR	TTGGCAG	ACGCTTGGCAGT	TACCAT	CATTACCAT	Corresponding gene model in JGI Chlamy v2.0 and/or DDBJ/EMBL/GenBank accesion number
Photrespiration	*Aat1*	+	-914 -791 -97	-	-	-	-	-	-	-	-	-	-	**-46**	**-50**	**-129**	**-132**	C_320082, (U31975)
	GcsP	(+)	-547	-64	-	-	-752	-	-	-	-	-	-	-	-	-	-	C_910041, putative gene
	Gdh1	+	-	-	-	-	-	-	-210	-	-	-	-	-	-	-	-	C_290029, putative gene
	Pgp1	+	-690	-	-	-	-	-454	-146	-433 -263	**-433**	-636 -167	**-637**	**-171** **-80**	**-84**	**-67**	**-70**	C_340088, AB091101.2
	Sgat	+	-562 -475	-15	-	-	-	-	-540	-	-	-	-	-	-	-	-	C_900004, putative gene
	Shmt	+	-	-604	-	-	-938	-	-	-	-	-	-	-	-	-	-	C_970018, (AF442558)
CCM	*Cah1*	+	-890 -360	-656 -591 -334 -282	**-596**	**-200**	-218	-473	-150	-322	**-322**	-696	**-697**	**-824** **-348**	-	-	-	AB026126, C_40150
	Cah3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C_710031, U73856
	Cah6	+	-68	-	-	-	-	-954	-	-975	-	-	-	-	-	-	-	C_1150014, AY463238
	Ccm1		-563	-135	-	-	-	-	-	-	-	-873	-	-	-	-	-	AB052694, C_2060016
	Ccp1	+	-367	-288	-	-	-494	-	-	-	-	-	-	-	-	-	-	C_40052, (U75345)
	Ccp2	+	-	-	-	-	-	-	-	-	-	-323	**-324**	-	-	-	-	C_40147, (U75346)
	Lci1	+	-	-639	-	-	-	-228	-	-	-	-	-	-	-	-	-	C_1350038?, (U31976)
	Lci5	+	-	-	-	-	-	-	-	-	-	-	-	**-725**	-	-	-	C_150094, AF394230
	LciA	+	-	-	-	-	-	-	-	-	-	-360	**-361**	-	-	-	-	C_90197, (AB168092)
	LciB	+	-329	-953 -373	-	-	-554	-	-484	-628	-	-	-	-	-	-	-	C_8740001, (AB168093)
	LciC	+	-822	-	-	-	-365	-	-143	-	-	-	-	-	-	-	-	C_90183, (AB168094)
	Lcr1	+	-733	-523	-	-	-173	-385	-	-	-	-	-	**-820** **-446**	-	-	-	AB168089, C_1530002
	Mca1	+	-261	-165	**-299**	**-284**	-696	-	-	-	-	-	-	-	-	**-949**	-	U80804
	Mca2	+	-261	-165	**-299**	**-284**	-696	-	-	-	-	-725	**-726**	-	-	-	-	U80805, C_2030011
Others	*Ald1*		-101	-653	-	-	-	-	-	-	-	-	-	-	-	-	-	C_4220001, (S72951)
	Aox1		-565	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C_330029, (AF047832)
	Aox2		-947	-996	-	-	-	-	-	-	-	-	-	-	-	-	-	C_340013, AF314255.2
	Cah2	-	-	-693	-	-	-		-20 -12	-	-	-	-	-	-	-	-	C_40149, X54488
	Mdh1	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	**-805**	-	C_520009, (AJ277281)
	Csbp		-	-	-	-	-		-141	-	-	-	-	-	-	-	-	Y14608, C_30202
	GapA		-529	-173	-	-	-	-	-	-	-	-737	-	-	-	-	-	C_280107, L27668
	H43	-	-742	-297	-	-	-	-	-989	-	-	-	-	-	-	-	-	C_70187, AB042099
	nad-MDH		-230	-	-	-	-671	-707	-	-	-	-	-	-	-	-	-	C_550067, (AJ250844)
	Nar1		-19	-740	-	-	-	-	-	-	-	-	-	-	-	-	-	AF149738, C_520040
	Pgk	-	-164 -138	-	-	-	-	-5	-	-	-	-	-	-	-	-	-	C_140184, (U14912)
	PrkA		-379	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C_950008, (AF228914)
	RbcS1		-	-422	-	-	-	-	-	-	-	-	-	-	-	-	-	C_660013, X04471
	RbcS2		-378	-	-	-	-	-	-	-	-	-	-	-	-	-	-	X04472, C_660012
	Rh1	-	-	-	-	-	-	-	-842	-297	-	-	-	-	-	-	-	C_1460033, (AY013258)
	Sta2	+	-539 -25	-	-	-	-	-	-	-379	-	-315	-	-	-	-	-	C_10205, (AF026420)
	Sta3	+	-	-157	-	-	-	-	-284	-	-	-	-	-	-	-	-	C_970042, (AF026422)
Pgp1-like	*Pgp2**		-576	-667	-	-	-163	-	-	-	-	-	-	-	-	-	-	C_150191, putative gene
	*Pgp3**		-658 -273	-303	-	-	-	-5	-	-967	-	-723	-	-	-	-	-	C_270015, putative gene

CO_2 responsive genes such as *Aat1*, *LciA*, and *Sta2*, and was also found with eight genes that were not low-CO_2 responsive, such as *GapA*, *H43*, and *Cah2* (Fig. 3).

The motif CAGAGGTCAGN$_8$CCG, which contains the AGGTCA motif annotated as the "Q (quantitative)-element" in the maize *Zm13* gene promoter (Hamilton et al. 1998) in the PLACE database, is a good candidate for the region involved in the common regulatory mechanism of *Pgp1* and *Cah1*. This motif was found only with these two genes at the similar positions (–639 to –619 and –699 to –679, respectively).

Although CAGAGGTCAGN$_8$CCG was not found with any other genes in Fig. 3 (data not shown), a more leaky motif, RAGGTCAGN$_{8-9}$CCR, was also found with *Ccm1*, *Ccp2*, *LciA*, and *Mca2* (Fig. 3). TTGGCAG, which contains the TTGGCA motif (Paonessa et al. 1988), was also found with *Pgp1* and *Cah1*, as well as with *Aat1*, *Lci5*, and *Lcr1* (Fig. 3). Thus, although there was no motif that was com-

Fig. 5. A phylogenetic tree calculated from an alignment of the PGPase subunit (CrPGP1), *Pgp2* and *Pgp3* gene products (CrPGP2 and CrPGP3, respectively), and NPPases and bacterial PGPases. The output tree is displayed as an unrooted N-J tree after the alignment using ClustalW version 1.8 (http://clustalw.genome.jp/). CrPGP1, *Chlamydomonas reinhardtii* PGPase (PGP1) subunit; CrPGP2, *C. reinhardtii Pgp2* gene product (C_150191 in JGI Chlamy 2.0); CrPGP3, *C. reinhardtii Pgp3* gene product (C_270015 in JGI Chlamy 2.0); At5g36700, *Arabidopsis thaliana* putative PGPase gene product (AT5G36700.1 in the *Arabidopsis* Information Resource, http://www.arabidopsis.org/index.jsp); AeCBBZP, *Alcaligenes eutrophus* PGPase (AAA20195.1); EcPGP, *Escherichia coli* PGPase (AAC76410.1); ScPHO13, *Saccharomyces cerevisiae p*-nitrophenylphosphatase (CAB56540.1); TmNPP, *Thermotoga maritima* PGPase (AAD36807.1).



mon among the low-CO_2 responsive genes shown in Fig. 3, the two motifs, RAGGTCAGN$_{8-9}$CCR and TTGGCAG, were found only within the low-CO_2 responsive genes involved in the CCM and photorespiration, suggesting that these two motifs are involved in the signal transduction for low-CO_2 adaptation in *C. reinhardtii*.

The *Pgp1* upstream region also has some motifs quite similar to those in *Aat1* upstream (Figs. 2 and 3). The following sequences were found only in the similar regions of *Pgp1* and *Aat1* upstreams (Fig. 3): ACGCTTGGCAGT was found at –84 to –73 in *Pgp1* and 50 to –39 *Aat1*, and CATTACCAT was found at –50 to –39 in *Pgp1* and –132 to –124 in *Aat1*. Thus, *Pgp1* and *Aat1* genes are quite likely to have common regulatory mechanism(s) in which these two sequences are involved together with the above-mentioned sequence TTGGCAG, although these motifs were not found in the upstreams of the other photorespiratory genes *GcsP*, *Gdh1*, *Shmt*, and *Sgat*.

Further investigations are necessary to clarify the regulatory mechanism of the *Pgp1* gene to help understand the mechanism that optimizes photosynthesis when organisms adapt to different concentrations of inorganic carbon.

Possible PGPase isozymes

The deduced sequence of mutant PGP1 contains the complete stroma-targeting signal peptide, followed by 21 amino acid residues, but contains only four amino acid residues of the wild-type PGPase mature subunit (Fig. 1). It is, therefore, quite unlikely that the mutant PGP1 is functional and responsible for the residual PGPase activity observed in the *pgp1-1* mutants (Suzuki 1995; Suzuki et al. 1990, 1999). The observed residual activity in the mutants could be caused by nonspecific phosphatase(s) and (or) isozyme(s) of PGPase.

Two putative PGPase genes, *Pgp2* and *Pgp3*, have been proposed in JGI Chlamy 2.0 based on similarity with the *Pgp1* cDNA sequence. Comparison of the deduced amino acid sequences of putative gene products with that of PGP1 showed that all three gene products had all consensus motifs of *p*-nitrophenyl phosphatases (Selengut 2001), except motif IIIa (Fig. 4). PGP2 was very likely to lack any transit peptide, but PGP3 seems to have a transit peptide region quite similar to those of thylakoid lumen-targeted peptides (Fig. 4). On the other hand, a phylogenetic tree produced after the ClustalW alignment with the related gene products showed that the sequences of putative PGP2 and PGP3 subunits were rather similar to that of *p*-nitrophenyl phosphatases rather than that of PGP1 subunit, and were less similar to those of bacterial PGPases (Fig. 5). Based on a comparison of these sequence characteristics, PGP2 and PGP3 are likely to have PGPase activity, although they are less similar to PGP1 than the *Arabidopsis* putative PGPase, and it is not clear if *Pgp2* and *Pgp3* are really functional or not. As the expression of *Pgp2* and *Pgp3* did not change after transfer from high- to low-CO_2 levels (Fig. 3), based on the supplemental data of Yoshioka et al. (2004), the physiological role(s) is likely not in photorespiration if they are functional PGPases but rather in nonphotosynthetic reactions such as DNA repair (Pellicer et al. 2003; Kim et al. 2004). However, some of the suppressor double mutants of *pgp1-1*-18-7F with a partly recovered PGPase activity (Suzuki 1995) may have enhanced *Pgp2* and (or) *Pgp3* expression. It is, on the other hand, still possible that the low rate of glycolate production observed in *pgp1* mutants (Suzuki et al. 1999) are catalyzed by other phosphatase(s) such as galactose-1-phosphatase that has been reported in *Arabidopsis thaliana* to have some PGPase activity (Laing et al. 2004).

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