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Isolation, cloning, and gene expression analysis of phosphoglycolate phosphatase from green alga *Chlamydomonas reinhardtii*

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Abstract

Phosphoglycolate phosphatase (PGPase), a key enzyme in photosynthetic organisms, catalyzes the dephosphorylation of phosphoglycolate, which is largely produced by the oxygenase activity of Rubisco, and is a potent inhibitor of several Calvin cycle enzymes. PGPase (CrPGPase 1) was previously cloned, purified, and characterized from unicellular green *Chlamydomonas reinhardtii*. *In silico* analysis revealed two more candidates encoding PGPase enzymes in the *C. reinhardtii* genome. In this study, we isolated, cloned, and overexpressed three PGPase genes (*pgp1*, *pgp2*, *pgp3*) from *C. reinhardtii* and performed gene expression analysis at high and low ammonium [NH_4^+] concentrations. We demonstrate that all three *pgp* genes encode functionally active PGPases in *C. reinhardtii*. In addition, we show that *pgp1* and *pgp2* genes are N-responsive genes and are upregulated under low ammonium concentrations. *In silico* analysis revealed that PGPase exists mainly in three isoforms in higher plants and algae.

Keywords: *Chlamydomonas reinhardtii*; gene expression; N-deficiency; phosphoglycolate; phosphoglycolate phosphatase; photorespiration.

Introduction

Phosphoglycolate phosphatase, (PGPase, EC 3.1.3.18) is a key enzyme in the photorespiration pathway of photosynthetic organisms, especially algae, plants, and cyanobacteria. PGPase plays a critical role in the photorespiration pathway by catalyzing the hydrolysis of phosphoglycolate (PG), which is produced through the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and is important for the growth of photosynthetic organisms in light. This enzymatic reaction is essential for recycling and salvaging carbon compounds in the process of photorespiration, which helps prevent the loss of fixed carbon from the Calvin cycle and maintains the overall efficiency of photosynthesis. In higher plants, algae, and cyanobacteria, multiple PGPase isoforms are found, each probably adapted to different environmental conditions or cellular roles. The regulatory mechanism of PGPase is still unclear in many organisms (Mamedov *et al.* 2001).

Although in earlier studies, PGPases were partially purified from several plant and algal species, including pea (Kerr and Gear 1974), corn (Hardy and Baldy 1986), spinach (Christeller and Tolbert 1978, Husic and Tolbert 1984), tobacco (Christeller and Tolbert 1978, Belanger and Ogren 1987), *Halimeda cylindracea* (Randall 1976), *Coccochromis peniocystis* (Norman and Colman 1991), and *C. reinhardtii* (Husic and Tolbert 1985), however,

Highlights

- Three phosphoglycolate phosphatase genes encode functionally active enzymes in *Chlamydomonas reinhardtii*
- The *pgp1* and *pgp2* genes are activated by low concentrations of ammonium
- The results also support a nonphotosynthetic role of PGPase in *C. reinhardtii*

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Abbreviations: [NH_4^+] – ammonium concentration; ORF – open reading frame; PG – phosphoglycolate; PGPase – phosphoglycolate phosphatase; TAP – Tris-acetate phosphate.
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no sequence information for PGPase from eukaryotic or prokaryotic organisms was reported. The first molecular report about complete nucleotide and amino acid sequences for PGPase was published in 2001 (Mamedov *et al*. 2001) as the first PGPase sequence from eukaryotic organisms. In the study of Mamedov *et al.* (2001), PGPase protein (CrPGP1) was purified from *C. reinhardtii* to a high enough purity which allowed determination of the N-terminal amino acid sequence of PGPase protein, and enabled determination of the complete nucleotide sequence of eukaryotic PGPase for the first time (Mamedov *et al*. 2001). This work was pioneering in the field that revealed the sequences of PGPases in plants, human, and animal genomes. Later, two candidate PGPase genes from the *Arabidopsis thaliana* genome were selected for overexpression and knockout studies (Schwarte and Bauwe 2007) based on the highest similarity to the amino acid sequence of the *C. reinhardtii* PGPase (Mamedov *et al.* 2001). Although thirteen potential PGPase genes are annotated in the *Arabidopsis* genome, based on their highest similarity to CrPGP1 (Mamedov *et al*. 2001), two forms of *Arabidopsis* PGPase were selected for overexpression in *E. coli* as well as for knockout studies (Schwarte and Bauwe 2007) in *A. thaliana.* These two forms, AtPGP1 and AtPGP2, were overexpressed in *E. coli*, and both PGPase genes were confirmed to encode functionally active enzymes (Schwarte and Bauwe 2007). AtPGP1 has been demonstrated to play an important role in photorespiratory metabolism, but AtPGP2, encoded by the *At5g47760* gene, was shown to be not involved in photorespiratory metabolism (Schwarte and Bauwe 2007). Since then, on molecular level, a few works have been devoted to the study of PGPase in plants, algae, and humans. More recent studies have shown that PGPase also plays a role in starch accumulation in *Arabidopsis* (Flügel *et al.* 2017). Altered AtPGP1 activity has also been shown to cause changes in amino acid metabolism, including the γ-aminobutyric acid (GABA) shunt in *A. thaliana* (Flügel *et al*. 2017).

PG has been shown to be an important metabolite in many organisms including mammals (Badwey 1977, Barker and Hopkinson 1978, Rose and Liebowitz 1970, Rose 1981, Rose *et al.* 1986). However, PGPase has been only partially purified from human red blood cells (Zecher *et al*. 1982) and surprisingly, PGPase has been unexplored to date in molecular terms and no information is still available on the molecular structure (*e.g.*, amino acid sequences) of human PGPase. The regulatory mechanism of PGPase activity in humans and animals is also not clear.

PGPase was also found in *Escherichia coli* and was purified and characterized (Pellicer *et al*. 2003). In *E. coli*, PGPase was shown to be involved in DNA-repair processes, particularly the repair of DNA lesions caused by oxidative damage (Pellicer *et al.* 2003). Based on sequence similarity, structural and functional features of bacterial PGPase with other PGPases it was suggested that bacterial PGPase uses of the same catalytic mechanism (Pellicer *et al*. 2003).

As mentioned above, nucleotide and deduced amino acid sequence of *C. reinhardtii* PGPase (CrPGP1) was reported and *pgp1* gene and CrPGP1 protein well characterized (Mamedov *et al*. 2001). In this work, CrPGP1 enzyme was purified to homogeneity and characterized (Mamedov *et al.* 2001). CrPGP1 enzyme is homodimer with molecular mass of ~65 kDa composed of ~32-kDa subunits (Mamedov *et al*. 2001).

BLAST search revealed two more candidate genes in *C. reinhardtii* (*pgp2* and *pgp3*). Transcriptional analysis of these three *pgp* genes in *C. reinhardtii* under high and low CO_2 conditions were reported (Ma *et al*. 2013). A *pgp1* mutant was shown to naturally revert and regain its ability to survive under low CO_2 conditions (Ma *et al*. 2013). Based on quantitative RT-PCR analysis, it was shown that upregulation of PGP2 in the *pgp1* revertants might contribute to this reversion in the growth phenotype (Ma *et al*. 2013).

Here, we reported the isolation and cloning, overexpression in *E. coli* and gene expression analysis of three *pgp* genes from *C. reinhardtii*. We demonstrated for the first time that *pgp1*, *pgp2*, and *pgp3* genes encode functionally active PGPase enzymes in *C. reinhardtii*. We also showed that *pgp1* and *pgp2* genes are N-sensitive genes and upregulated under low ammonium concentrations. Since *C. reinhardtii* can accumulate a relatively high lipid content under stress conditions, particularly when it is subjected to nutrient deprivation, such as nitrogen starvation, these studies could be helpful for development of strategies to increase oil production in *C. reinhardtii.*

Materials and methods

Strains and growth conditions: *C. reinhardtii* wild type (wt) strain CC-125 was purchased from the Chlamydomonas Resource Center at the University of Minnesota, Twin Cities, MN (http://www.chlamycollection.org/). *Chlamydomonas* cells were grown under continuous illumination at 25°C, ~100 μE m^{-2} s^{-1}, and 120 rpm in Tris-acetate phosphate (Gorman and Levine 1965) and high salt (HS) medium as described previously (Harris 1989, Mamedov *et al*. 2001, 2005). Expression analyses were performed in exponential growth phase (10^6 cells ml^{-1}). For gene expression analyses, HS medium was used by changing the ammonium concentration in the Beijerinck solution (100 g of NH_4Cl, 4 g of $MgSO_4 \cdot 7H_2O$, 2 g of $CaCl_2 \cdot 2H_2O$ for 1 L). Different HS mediums were prepared by calculating the concentrations to contain 1 and 0.5 mM NH_4.

E. coli DH5α was used as host strain for genetic application and *E. coli* BL21 (DE3) (*Invitrogen*, Carlsbad, CA, USA) was employed as host strain for protein expression. *E. coli* strains were grown in Luria–Bertani (LB) broth with or without kanamycin (30 mg l^{-1}) and incubated at 37°C, under continuous shaking at 200 rpm.

RNA isolation, quantification, and cDNA synthesis: To isolate three PGPase genes and perform expression analysis, total RNA and then cDNA was first prepared from *C. reinhardtii*. About 20 mL of cells were centrifuged at room temperature, and after the media was completely removed, TRIzol reagent (*Ambion*, USA) was immediately added to the cell pellet (1 mL of TRIzol per 1 × 10^6 tissue culture cells) and then mixed by vortex for 10 s. Samples were kept at room temperature for 5 min and 0.2 mL of chloroform was added per mL of TRIzol and then followed mixing by vortex for 15 s and incubation at room temperature for 5 min. The sample was then centrifuged in a microcentrifuge at maximum speed (16,000 × *g*) for 10 min at room temperature. The upper clear phase was carefully transferred to a new tube and 0.5 mL of isopropanol was added to each milliliter of the clear phase. After incubation at room temperature for 10 min, the sample was vortexed vigorously. RNA was precipitated by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was carefully decanted using Pasteur pipette. The solution was re-extracted with phenol and precipitated with ethanol as recommended by the manufacturer (Rio *et al.* 2010). RNA samples treated with DNase I (*Thermo Scientific*) and were stored at –80°C until needed for the cDNA synthesis. Quality of RNA was determined using ethidium bromide (EB)-stained agarose gel electrophoresis. RNA concentration was determined by measuring absorbance at 260 nm using *BioDrop* (*Indolab*, Utama, Indonesia). Using primers specifically designed for the three *pgp* genes of *C. reinhardtii*, three genes were isolated, cloned, and overexpressed in *E. coli*. First strand cDNA synthesis was conducted using AMV-Reverse Transcriptase (*New England Biolabs*, Cat no.: MO277S) according to manufacturer's instructions. A 20-µl reaction mixture [total RNA up to 1 µg, 2 µl of $d(T)_{23}N$ (50 µM), 2 µl of 10X AMV buffer, 0.2 µl of AMV RT (10 U/µl), 1 µl of 10 mM dNTP Mix, 0.2 µl of RNase inhibitor (40 U/µl) and nuclease-free water to a total volume of 20 µl] was incubated at 42°C for 1 h.

Cloning of ORF of *pgp1*, *pgp2*, and *pgp3* genes for overexpression in *E. coli*: First, we isolated ORF of three *pgp* genes from cDNA prepared as described above. For amplification of the ORF of *pgp1* primers 5-ATGCTGAGCCTGAAGCAGCTG-3 (PGP1F1, Tm: 60.3°C) and 5-AGCTCACGCCGCCACCATG-3 (PGP1R1, Tm: 63.7°C) primers were used. For amplification of the ORF of *pgp2*, primers 5-ATGAAG-AAAGCTACGGACCG-3 (PGP2F1, Tm: 64°C) and 5-CACATGATGGCGCAGTTG-3 (PGP2R1, Tm: 65°C) were used. For amplification of the ORF of *pgp3*, 5-ATGGCGCTCGGTGCAACTC-3 (PGP3F1, Tm: 72°C) and 5-TCTCACGCCGGCAGTCCC-3 (PGP3R1, Tm: 74°C) primers were used. The PCR fragments were cloned into pGEM-T (*Promega*) and sequenced.

pET28a(+) bacterial protein expression system (*Novagen*) was used for expression of three *pgp* genes in *E. coli*. First, three *pgp* genes were isolated and cloned from cDNA of strain CC-125, as described above. pGEM-T plasmids with ORF of *pgp1*, *pgp2*, and *pgp3* genes were used as a template for amplification of these genes for overexpression in *E. coli*. For cloning of open reading frames of three *pgp* genes specific primers pairs with restriction sites were designed on the basis of *pgp1*, *pgp2*, and *pgp3* sequences. For cloning and overexpression of *pgp1* gene, tctagcatATGCTGAGCCTGAAGCAGCTG (*Nde I* at N-terminal) and tagtcctcgagTTACGCACG-AAGCCGCCCGTGC (Xho I at C-terminal) primer pairs were designed. For cloning and overexpression of *pgp2* gene, tctagcatATGAGCAATTTGGCGCTAC (*Nde I* at N-terminal) and tagtcgaattcCTAGTACGATATCCCCCAC (*EcoRI* at C-terminal) primer pairs were designed. Similarly, for cloning and overexpression of *pgp3* gene, tctagcatATGGCGCTCGGTGCAACTC (*Nde I* at N-terminal) and tagtctgtacaTCTCACGCCGGCAGTCCC (*BrsG1* at C-terminal) primer pairs were used.

Before ligation of the open reading frames of three *pgp* genes into pET28a(+), PCR fragments were first cloned into pGEM-T (*Promega*) and then after cutting with restriction enzymes, the fragments were ligated into pET28a(+) and the resulting plasmid was transformed into competent *E. coli* cells, strain BL21 DE3 (*Invitrogen*, Carlsbad, CA, USA) using the heat shock method (by placing the bottom of the tube into a 42°C water bath for 45 s). The sequence identity was confirmed after cloning into pET28a(+) vector.

PGPase activity assay: PGPase activity was determined as described previously by measuring the PG-dependent release of inorganic phosphate (Ames 1966) as described previously (Suzuki *et al*. 1999, Mamedov *et al*. 2001). The enzymatic assay mixture contained 20 mM MES-bistrispropane, 4 mM $MgCl_2$, 10 mM phosphoglycolate, pH 8.0. Protein concentration was measured by *BioDrop* (*Indolab*, Utama, Indonesia).

Sequence-alignment, phylogenetic tree construction, and gene-structure analyses: Amino acid sequence alignments of *C. reinhardtii* CrPGP1, CrPGP2, and CrPGP3 were performed using the *MAFFT* (*Multiple Alignment using Fast Fourier Transform*, *version 7*, https://mafft.cbrc.jp/alignment/server/index.html) program with representative plant, algal, cyanobacterial, and prokaryotic PGPases. *MEGA X* (*Molecular Evolutionary Genetics Analysis*) software was used to align amino acids sequences from PGPase proteins. Multiple sequence alignments were constructed using *ClustalW* and *MUSCLE* algorithms implemented, using default settings. A phylogenetic tree constructed using the maximum likelihood method [Jones–Taylor–Thornton (JTT) model] based on comparison of nucleotide sequences of the gene.

Gene expression analysis by quantitative real-time PCR (qPCR): Primers designed according to *pgp1*, *pgp2*, and *pgp3* sequences were used in real-time PCR for expression analysis of three *pgp* genes in high and low ammonium. The ammonium transporter (*Amt*) gene was used as an N-sensitive reference gene (Mamedov *et al.* 2005). This gene has been shown to encode a putative gas channel for the uncharged NH_3 species (Soupene *et al*. 2004).

In qPCR analyses, *Maxima SYBR Green/ROX qPCR Master Mix* (2X) was used as described by the manufacturer (*Thermo Scientific*). The samples generated by first-strand cDNA synthesis were diluted 1:50. The experiment was carried out on a 96-well plate and each reaction contained 12.5 µL *SYBR Green Master Mix*

(*Thermo Scientific*), 5 μL of cDNA, 0.3 μM each of the forward and reverse primers, and water to a final volume of 25 μL. The primers sequences used for quantification are given in the following table.

Primers	Primer sequence (5'-3')
qCBLP-F2	CCGCTGTACAGGGTGGAG
qCBLP-R2	CAAGATCTGGGACCTGGAGA
qPGPase1F	TGGAGCATGACCACGACGTG
qPGPase1R	ATGGAGCCGTTGCCCGCC
qPGPase2F	TGGCATGGGCAAGAAGGTG
qPGPase2R	TCCTCCGCCTTGACGTTGAG
qPGPase3F	ATGCTGAGCCTGAAGCAGCTG
qPGPase3R	AGCTCACGCCGCCACCATG
qAMT4-F1	GCTGGCCAAGAAGGAGTACA
qAMT4-R1	AGGCGAAGATGGAGATGATG

Relative expression levels were normalized using the *C. reinhardtii* reference gene *CBLP*. Reaction conditions were 10 min at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 30 s, and a 1-s hold at 95°C to eliminate background fluorescence generated by the formation of primer dimers, followed by detection of the fluorescence. $2^{-\Delta\Delta Ct}$ statistical approach was performed for gene expression stability. Melt-curve analyses were performed to determine whether the products represent a single amplified species.

Statistical analysis: *GraphPad Prism 9* program was used. *Student*'s *t*-test was used to evaluate the findings statistically. The results were evaluated within the 95% confidence interval and the significance level was $p<0.05$.

Results

Cloning of ORF of three *pgp* genes: We first set out to identify possible genes encoding the PGPase enzyme in *C. reinhardtii*. Based on similarity to the CrPGP1 amino acid sequence (Mamedov *et al*. 2001) and previous studies (Ma *et al*. 2013), three *pgp* genes (*pgp1*, *pgp2*, and *pgp3*) are available in the *Chlamydomonas* genome database. CrPGP1 gene and protein was well characterized (Mamedov *et al*. 2001). For expression in *E. coli*, we first amplified ORF of three *pgp* genes from *C. reinhardtii* cDNA as described in 'Materials and methods'. Amplified PCR fragments are shown in Fig. 1. As can be seen from Fig. 1, expected DNA fragments (990 bp for *pgp1*, 915 bp for *pgp2*, and 1,044 bp for *pgp3* genes) were amplified. Amplified *pgp1*, *pgp2*, and *pgp3* genes were cloned into pGEM-T vector and confirmed by sequencing. pGEM-T plasmid harboring three *pgp* genes was used as DNA templates for amplification of *pgp* genes with restriction enzyme sites, for cloning into pET28a(+) vector, as described in 'Materials and methods'.

Sequence analyses of *pgp* genes in *C. reinhardtii*: We performed sequence analysis of *pgp* genes and corresponding CrPGP1, CrPGP2, and CrPGP3 proteins in *C. reinhardtii*. Amino acid sequences used for alignment are listed in Fig. 2.

In silico analysis revealed that phosphoglycolate phosphatase mainly exists in three isoforms in higher plants and algae, this suggests that there are different variants of this enzyme in these organisms, each with distinct characteristics or functions. The sequence identity and similarity of CrPGP1, CrPGP2, and CrPGP3 with PGPases from various organisms have been presented in Table 1. The identity/similarity of CrRPGP1 to CrPGP2 and CrPGP3 proteins are 48/68% and 41/58%, respectively. Three *C. reinhardtii* PGPase proteins share the highest identity with PGPase from *Volvox carteri*, 84.1 and 74.6% for CrPGP1-VcPGP1 and CrPGP2-VcPGP2, respectively. CrPGp1 shares 55 and 66% identities to the PGPase proteins from *Oryza sativa* (accession number: BAD38247.1) and *Arabidopsis thaliana* (accession number: NP_198495.1). CrPGP1, CrPGP2, and CrPGP3

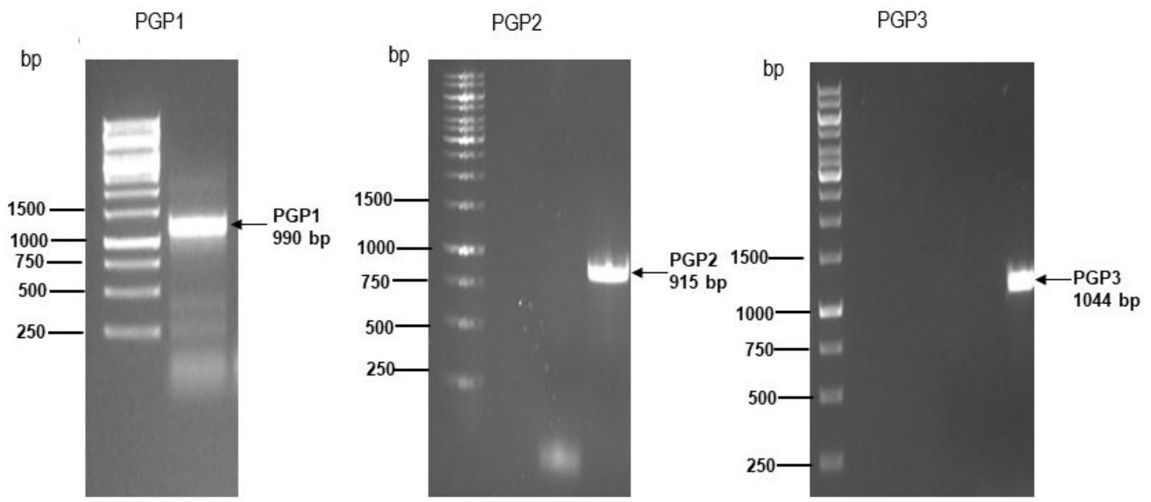
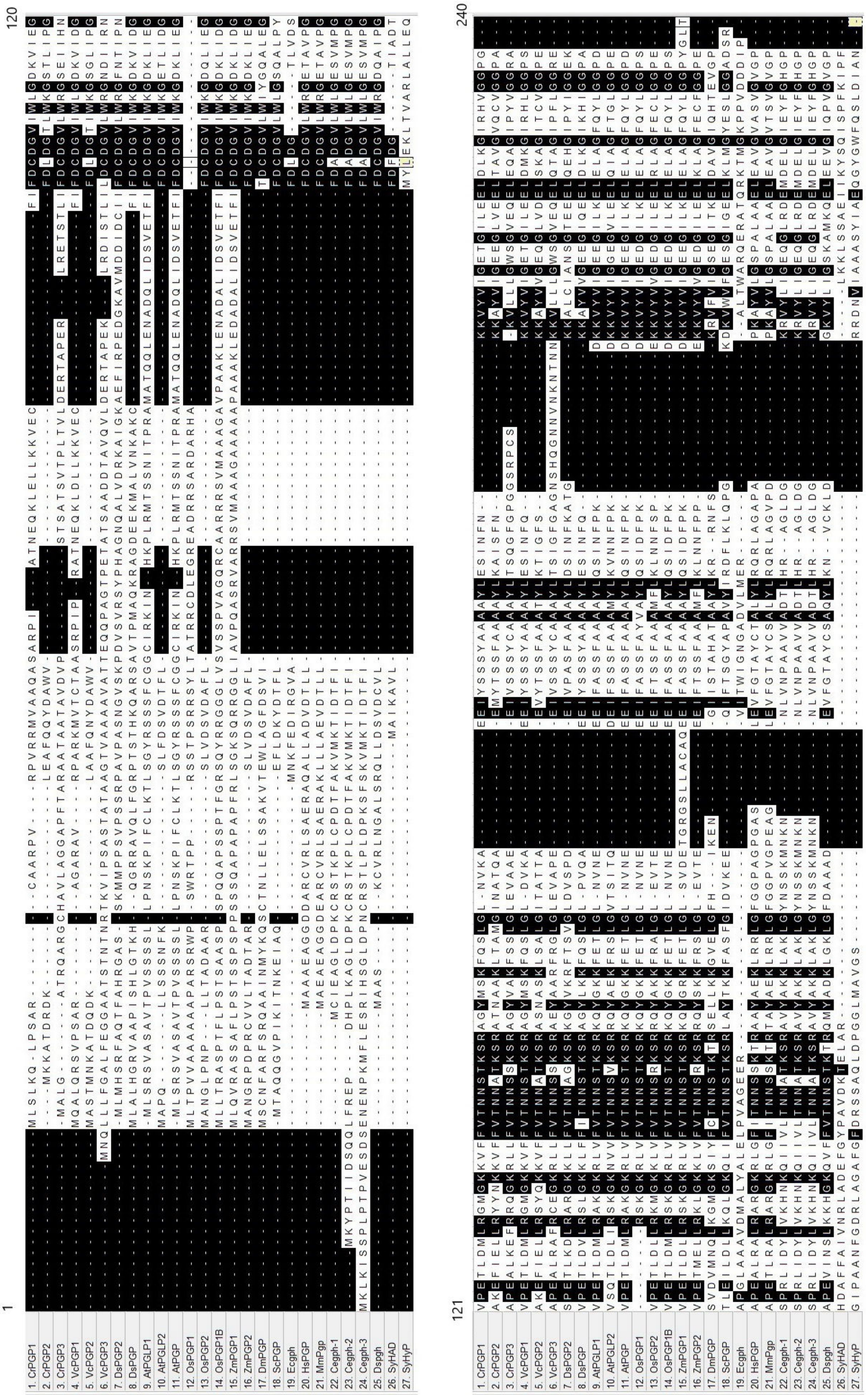


Fig. 1. Agarose gel electrophoresis of amplification of *pgp1*, *pgp2*, and *pgp3* genes from *Chlamydomonas reinhardtii*, PCR amplification was performed as described in 'Materials and methods'.


1
120
121
240
1. CrPGP1
2. CrPGP2
3. CrPGP3
4. VcPGP1
5. VcPGP2
6. VcPGP3
7. DsPGP2
8. DsPGP
9. AtPGLP1
10. AtPGLP2
11. AtPGP
12. OsPGP1
13. OsPGP2
14. OsPGP1B
15. ZmPGP1
16. ZmPGP2
17. DmPGP
18. ScPGP
19. Ecgph
20. HsPGP
21. MmPgp
22. Cegph-1
23. Cegph-2
24. Cegph-3
25. Dspgh
26. SyHAD
27. SyHyP

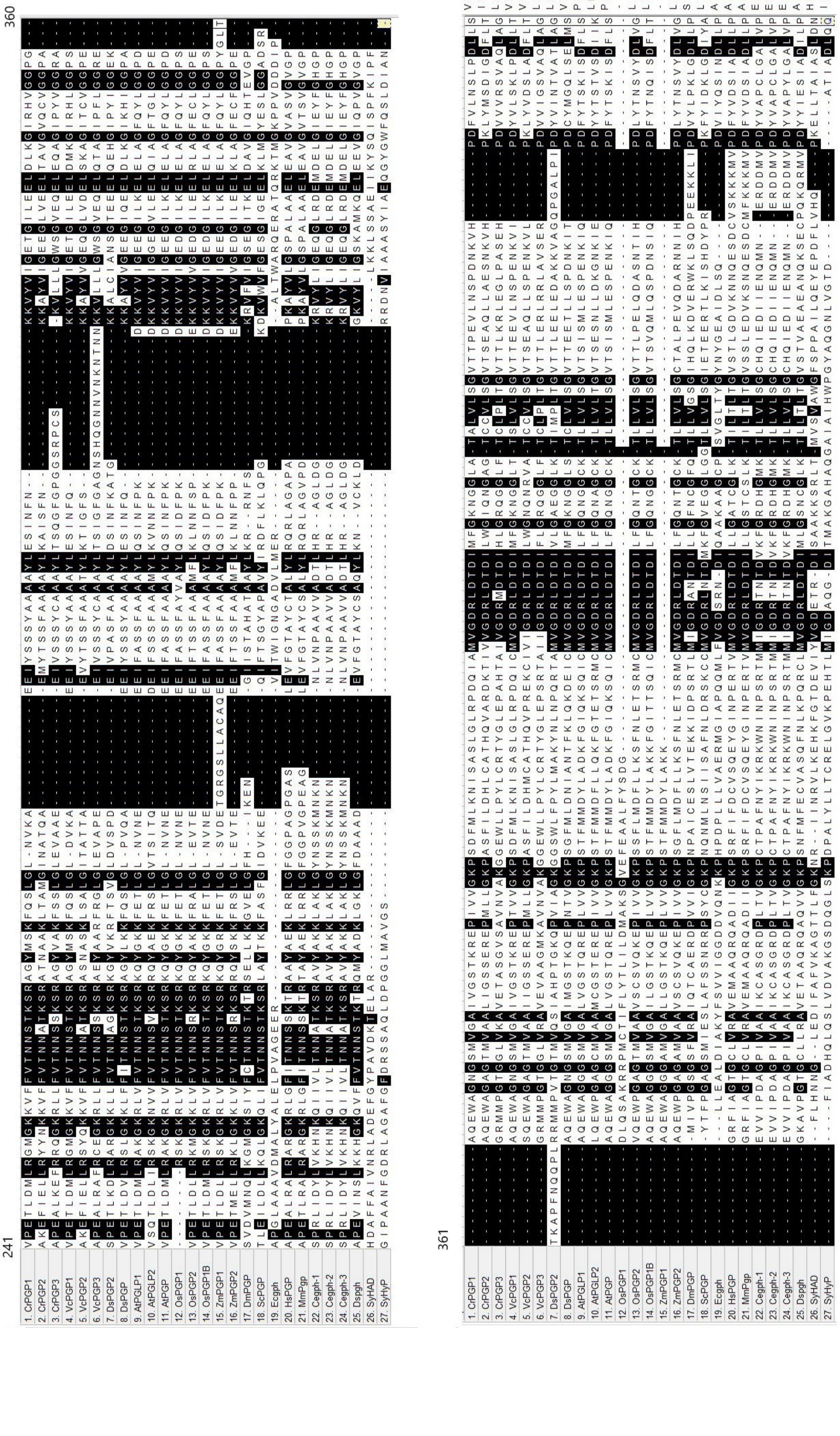


Fig. 2. Selected amino acid sequence alignments of three PGPase amino acid sequences from *Chlamydomonas reinhardtii* and representative vascular plants, human, algae, *Cyanobacteriota*, and prokaryotic species were performed using *MAFFT* (*Multiple Alignment using Fast Fourier Transform*) alignment program. *MAFFT version 7* (https://mafft.cbrc.jp/alignment/server/index.html) using amino acid sequences listed in Table 1S, *supplement*.

from *C. reinhardtii* share low identity with human PGPase (accession number: NP_001035830.1), 35.4, 31, and 37.1%, respectively. Notably, CrPGP1, CrPGP2, and CrPGP3 from *C. reinhardtii* also share low identity, 28%, with PGPase from *E. coli* (accession number: p32662).

Table 1. Identity and similarity of CrPGP1, CrPGP2, and CrPGP3 with PGPases from various organisms.

PGPases	Identity [%]	Similarity [%]
CrPGP1-CrPGP2	45.9	65.9
CrPGP1-CrPGP3	35.9	50.1
CrPGP2-CrPGP3	28.4	44.1
CrPGP1-VcPGP1	84.1	88.9
CrPGP2-VcPGP2	77.6	87.8
CrPGP2-VcPGP3	25.0	40.2
CrPGP2-VcPGP1	42.1	61.1
CrPGP1-AtPGLP1	55.9	67.3
CrPGP1-AtPGLP2	51.8	66.9
CrPGP1-AtPGP	55.9	67.3
CrPGP2-AtPGP	37.3	53.1
CrPGP2-AtPGLP2	44.4	60.8
CrPGP1-ZmPGP1	32.9	41.1
CrPGP2-ZmPGP1	23.4	33.7
CrPGP3-ZmPGP1	23.1	32.8
CrPGP1-ZmPGP2	52.8	67.7
CrPGP2-ZmPGP2	40.3	60.0
CrPGP3-ZmPGP2	33.6	47.2
CrPGP1-Ecgph	15.8	27.7
CrPGP2-Ecgph	18.6	29.6
CrPGP3-Ecgph	18.0	29.2
CrPGP1-HsPGP	35.4	52.5
CrPGP2-HsPGP	31.0	47.4
CrPGP3-HsPGP	37.1	48.2

CrPGP1 has been shown to be inhibited by Ca^{2+} and has properties similar to those of calcium-binding proteins such as CaM from *C. reinhardtii* (accession number: P04352) and is predicted to be a calcium-binding protein (Mamedov *et al*. 2001). When we analyzed the amino acid sequences of CrPGP2 and CrPGP3, we found that CrPGP2 and CrPGP3 have also the FDXDG motif, like CrPGP1 (Fig. 2). In addition, CrPGP2 has been predicted to have regions similar to EF-hand motifs of Ca^{2+}-binding proteins such as *C. reinhardtii* calmodulin (Fig. 3).

Phylogenetic analysis of three PGPases from *C. reinhardtii* and several other PGPases from vascular plants (*Z. mays*, *A. thaliana*, *O. sativa*), algae (*V. carteri*, *D. salina*), human, and yeast was presented in Fig. 4. Phylogenetic analysis revealed that CrPGPase 1 from *C. reinhardtii* is a close member of PGP1 from vascular plants and algae. Similarly, CrPGP2 from *C. reinhardtii* is the closer member to PGP2 from vascular plants and algae. As can be seen, *C. reinhardtii* PGP3 is a distant member of CrPGP1 from *C. reinhardtii* but is closer to PGP3 in *V. carteri*.

We performed gene structure analysis of three *pgp* genes using *Phytozome* (https://phytozome-next.jgi.doe.gov/blast-search). The lengths of the genomic sequences of the *pgp1*, *pgp2*, and *pgp3* genes were found to be 5.23 kb, 3.12 kb, and 3.9 kb, respectively. *pgp1*, *pgp2*, and *pgp3* genes were predicted to have 9, 10, and 11 exons, respectively. The gene structure of three *pgp* genes (indicated as CrPGP1, CrPGP2, and CrPGP3) and respective *pgp* genes from algae and vascular plant were presented in Fig. 5. As can be seen in Fig. 5. *C. reinhardtii pgp1* (accession no: BAB69477.1), *pgp2* (accession no: XP_001690570.1), and *pgp3* (accession no: XP_001696217.1) genes are predicted to have 9, 10, and 11 exons, respectively. Notably, one gene in *Volvox carteri* (accession no: XP_002955952.1) and two genes, *DsPGP* (accession no: KAF5829162.1) and *DsPGP2* (accession no: KAF5842629.1) in *Dunaliella salina* are predicted to have 11 exons. Two *pgp* genes, *AtPGLP1* (accession no: NP_001119316.1) and *AtPGLP2* (accession no: BAB11323.1) in *A. thaliana* and two *pgp* genes in rice, *OsPGP1* (accession no: XP_015620633.1) and *OsPGP2*

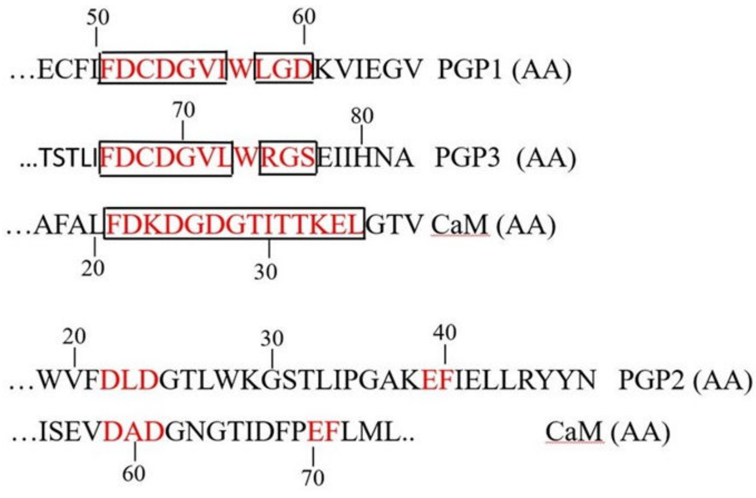


Fig. 3. Comparison of amino acid sequence regions of PGP1, PGP2, PGP3, and calmodulin (*CaM*) from *Chlamydomonas reinhardtii* for prediction of FDXDG motif and EF-hand motifs of Ca^{2+}-binding proteins. Residues in red, *boxed,* match with the FDXDG motif; residues in red, not boxed, match EF-hand region of Ca^{2+}-binding proteins.

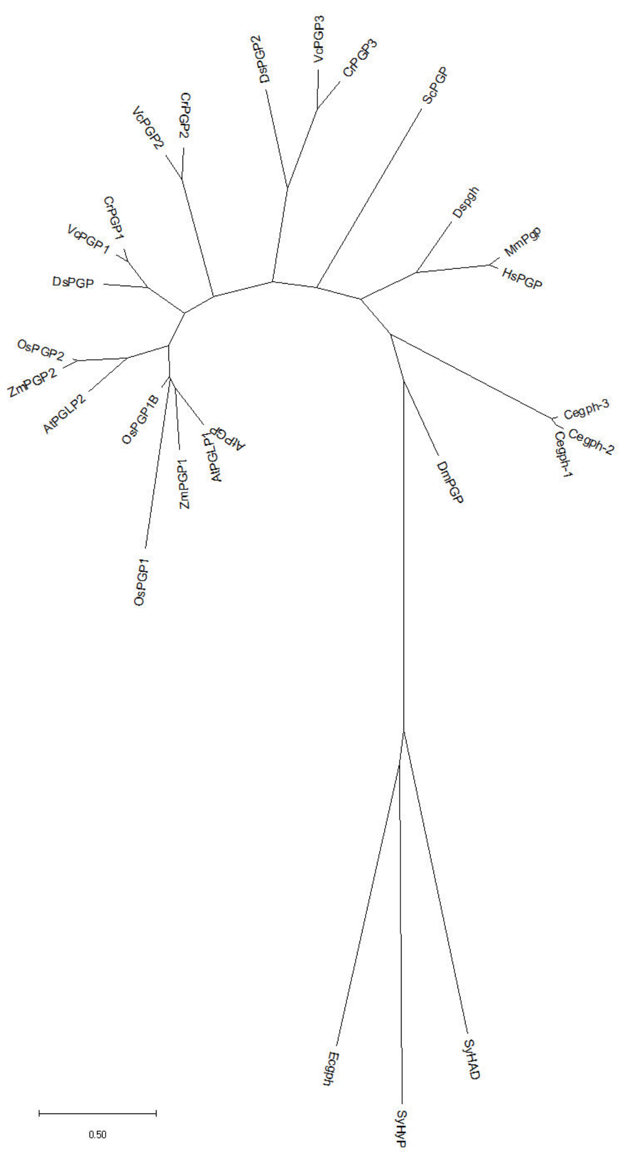


Fig. 4. Phylogenetic relationships of CrPGP1, CrPGP2, and CrPGP3 from *Chlamydomonas reinhardtii* and representative human, vascular plants, algae, cyanobacteria, yeast, and prokaryotes PGPases. Phylogenetic tree was constructed using *MEGA X* program.

(accession no: XP_015612535.1) and one *pgp* gene in *Zea mays* (*ZmPGP1*, accession no: AQK44459.1) are predicted to have 11 exons.

Overexpression of three *C. reinhardtii pgp* genes in *E. coli*: To confirm that the *pgp2* and *pgp3* genes encode functionally active enzymes in *C. reinhardtii*, we overexpressed the *pgp2* and *pgp3* genes, as well as the *pgp1* gene in *E. coli*. For expression of three *pgp* genes of *C. reinhardtii* in *E. coli*, the genes were amplified and cloned into pET28a(+) vector as described in 'Materials and methods'. The expression of CrPGP1, CrPGP2, and CrPGP3 proteins was induced using 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). PGPase activities were analyzed in total cell extracts. Very low activity of PGP2 and PGP3 compared to that of PGP1 was observed (Fig. 6).

Expression of *pgp* genes in high and low ammonium: Expression analysis of three *pgp* genes and the ammonium transporter (*Amt*), as inorganic-N responsive reference gene, were performed under high (10 mM) and low ammonium (0.5 mM) conditions. As can be seen from Fig. 7, as with the *Amt4* gene, the *pgp1* and *pgp2* genes were also upregulated at low ammonium concentration (0.5 mM). Unlike *pgp1* and *pgp2*, the *pgp3* gene is suppressed at low ammonium concentration (0.5 mM).

Discussion

PGPase enzyme that affects the PG content is essential for all photosynthetic organisms and is also important for the function of human red blood cells (Rose and Liebowitz 1970, Rose 1981, Rose *et al.* 1986). However, the regulatory mechanism of PGPase activity in plants and animals is not clear. In previous studies, *pgp1* (Suzuki *et al*. 1999) and CrPGP1 protein (Mamedov *et al.* 2001) were characterized from *C. reinhardtii*. Collective findings from a query of this publicly available *C. reinhardtii* genomic database using two different *pgp* genes revealed the presence of two more *pgp* genes (*pgp2* and *pgp3*) in this green microalga, which have high similarity to the CrPGP1 protein.

Three *C. reinhardtii* PGPase proteins (CrPGP1, CrPGP2, and CrPGP3) share highest identity with PGPase from *Volvox carteri*, *Oryza sativa*, and *Arabidopsis thaliana*, and share low identity with PGPase from human and *E. coli*. Based on amino acid analysis and phylogenetic tree, PGPase genes are more conserved in vascular plant and algae. These PGPase isoforms may have evolved to perform specific roles in different cellular and environmental conditions. Variations in isoforms could be related to factors such as substrate specificity, regulatory mechanisms, or tissue-specific expression. Further experimental studies would be needed to understand the functional differences and significance of these isoforms in plants and algae.

Regarding overall gene structure, *C. reinhardtii*, *D. salina*, and most of vascular plant *pgp* genes are highly conserved and composed of approximately 11 exons interrupted by introns. The *pgp1* gene in *C. reinhardtii* is predicted to have 9 exons.

As reported previously, 330 amino acids long PGP1 protein is ~65-kDa homodimer, migrating as a ~32-kDa protein on SDS-PAGE and not connected by any S–S bridges (Mamedov *et al.* 2001). CrPGP2 consists of 303 amino acids and has a predicted molecular mass of 32.97 kDa. CrPGP3 is composed of 347 amino acids with predicted molecular mass of 36.43 kDa and larger than CrPGP1 and CrPGP2.

Here we present the first report about isolation, cloning, and expression of three *pgp* genes from unicellular green alga *C. reinhardtii*. In this study, we overexpressed three *C. reinhardtii pgp* genes in *E. coli* and confirmed that

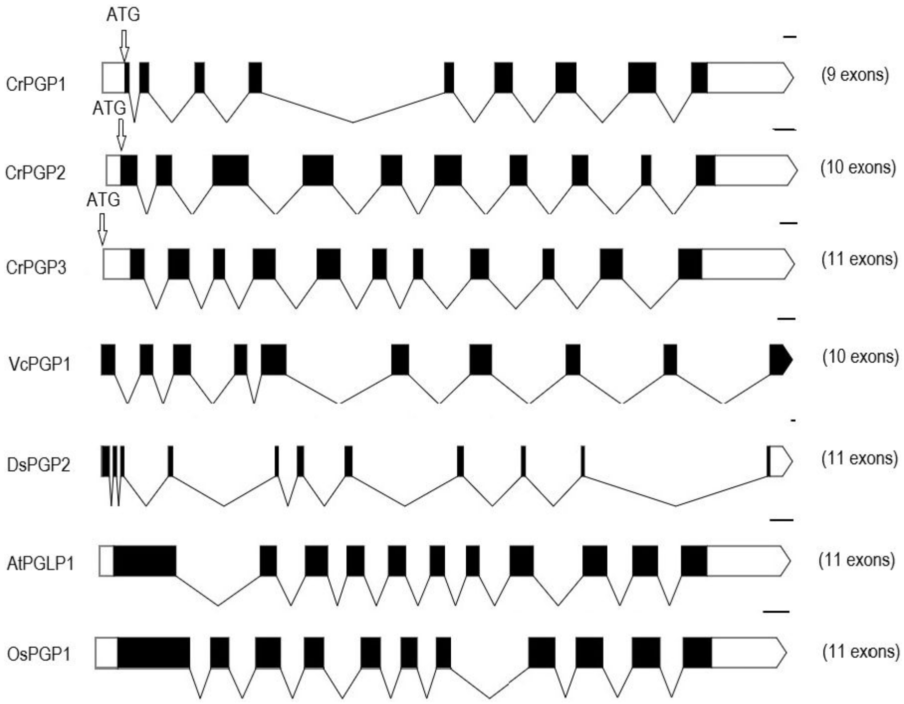


Fig. 5. Gene structures of various eukaryotic *pgp* genes predicted from the *Chlamydomonas reihartii*, *Arabidopsis*, rice, *Volvox carteri*, and *Dunaliella salina* nuclear genomes. The intron and exon positions were deduced using *Exon-Intron Graphic Maker* (http://wormweb.org/exonintron).

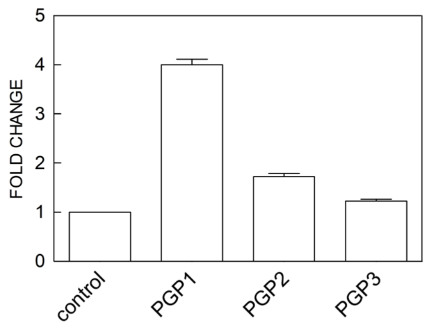


Fig. 6. Phosphoglycolate phosphatase (PGPase) activities of overexpressed PGP1, PGP2, and PGP3 recombinant proteins. The recombinant PGPases from *Chlamydomonas reihnardtii* were expressed in *E. coli* (BL21 cells). Soluble cell extracts were used for PGPase enzyme activity assays. The cell extracts from cells transformed with the empty pET28a(+) used as control. The empty vector control showed PGPase activities of about 5.5 nmol(Pi) min^{-1} mg^{-1}(protein), which were taken as 1 and relative values given are means ± SD.

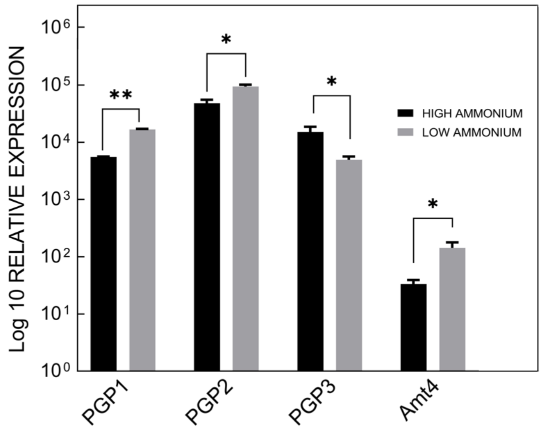


Fig. 7. Expression levels of *pgp1*, *pgp2*, *pgp3*, and *Amt4* genes (n = 2) in low (0.5 mM) and high (10 mM) ammonium.

these genes encode functional active PGPase enzymes. Very low activity of CrPGP2 and CrPGP3 compared to that of CrPGP1 was observed. Notably, *in silico* analysis of cyanobacterium *Synechocystis* sp. PCC 6803 revealed four genes, which could possibly encode PGPase proteins (Rai *et al*. 2018). Low specific activity was observed when expressed recombinantly in *E. coli* (Rai *et al*. 2018).

As described above, the *pgp1* gene expression and PGP1 (CrPGP1) protein have been characterized in previous studies (Suzuki *et al.* 1999, Mamedov *et al.* 2001). *Pgp1* mutation has been shown to result in a photorespiration-deficient phenotype, which can grow under high CO_2 conditions but not under ambient air, under photorespiratory conditions (Suzuki *et al.* 1999, 2005). It should be noted that CrPGP2 and CrPGP3 may have some capacity to compensate for the loss of CrPGP1, but if their activity is significantly reduced or not functioning optimally, they might not be able to fully take over CrPGP1's role. This can result in an accumulation of PG which can inhibit normal photosynthesis and plant growth. In other words, if PGP2 and PGP3 have low activity or are not functioning optimally, it could be a significant reason why a PGP1 mutant plant cannot grow under ambient air conditions (Suzuki *et al*. 1999). Recombinant CrPGP2, produced in *E. coli*, had relatively higher activity compared to that of CrPGP3. As we mentioned above, based on quantitative RT-PCR analysis, it was shown that upregulation of *pgp2* gene in the *pgp1* revertants might contribute to this reversion in the growth phenotype (Ma *et al*. 2013). At this point, PGP2 may have comparably high capacity to compensate for the loss of PGP1 compared with PGP3 enzyme. Notably, the photorespiratory pathway is a complex process that relies on multiple enzymes, including PGPase enzymes, to efficiently detoxify glycolate and allow normal photosynthesis to occur.

Based on previous studies, it was demonstrated that Ca^{2+} may have an important role in the regulation of PGPase activity in *C. reinhardtii* (Mamedov *et al.* 2001). CrPGPase1 was shown to be a Ca^{2+}-binding protein and was potently but reversibly inhibited by Ca^{2+}. As previously reported, Ca^{2+}-binding or phosphate-related proteins found in the *Swiss-Prot* database at *Expasy* site (https://www.expasy.org/), contain the motif FDXDG (Mamedov *et al.* 2001). It was shown that PGPase from *C. reinhardtii* (CrPGP1) and maize was strongly inhibited by Ca^{2+} and this inhibition was reversed by Mg^{2+} (Hardy and Baldy 1986, Mamedov *et al*. 2001). In addition, it was predicted that *C. reinhardtii* CrPGPase1 has characteristics found in calcium-binding proteins, such as calmodulin (*CaM*) from *C. reinhardtii* (accession number: P04352). When we analyzed the amino acid sequences of CrPGP2 and CrPGP3, we found that although CrPGP2 does not have the FDXDG motif, CrPGP3 has the FDXDG motif, like CrPGP1 (Fig. 2). Instead CrPGP2 has been predicted to have regions similar to EF-hand motifs of Ca^{2+}-binding proteins such as *C. reinhardtii* calmodulin in *C. reinhardtii* (Fig. 2). In particular, plant, algal, and human PGPase sequences are also predicted to have the FDXDG motif. Thus, the present and previous collective findings suggest that Ca^{2+} plays an important role in regulating the activity of the three PGPase isoforms *in vivo*.

Since the VAAQA sequence upstream of the N-terminus of the purified CrPGP1 enzymes (Mamedov *et al.* 2001) was similar to the VXA motif (Franzén *et al.* 1990), therefore, CrPGP1 from *C. reinhardtii* (Mamedov *et al.* 2001) was shown to have characteristics consistent with typical characteristics of a stromal transit peptide (Franzén *et al.* 1990, Krimm *et al.* 1999). Several VXA motifs (VLA, VAA, VLA, VKA, VGA) have been found in the CrPGP3 sequence, including one that is located upstream of the N-terminus and one VAAL (aa 201–204) motif was found in CrPGP2 sequence. Taken together, these and previous results on PGP1 from *C. reinhardtii* (Mamedov *et al.* 2001) indicate that, like CrPGP1, CrPGP3 most probably is a stromal protein (Husic and Tolbert 1985), as reported in higher plants (Hardy and Baldy 1986).

CrPGP1 from *C. reinhardtii* has been shown to possess a single CXXXXC motif (Mamedov *et al*. 2001) as a regulatory site, which has been observed in stromal thioredoxin-targeting enzymes (Schürmann and Jacquot 2000). This motif was found in CrPGP2 but not in CrPGP3. Notably, two stroma phosphatases, and sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase, are called thioredoxin-targeted enzymes with the CXXXXC motif (Schürmann and Jacquot 2000) was also shown to be inhibited by Ca^{2+} (Charles and Halliwell 1980, Hertig and Wolosiuk 1980, Wolosiuk *et al*. 1982).

Our results showed that the *C. reinhardtii pgp1* and *pgp2* genes are upregulated at low ammonium concentrations (0.5 mM). N-responsive *Amt4* gene was shown to be upregulated at N-sufficient or low (0.5 mM) N concentrations, which is consistent with our previously published results where the *Amt4* gene was upregulated at low ammonium concentrations (Mamedov *et al*. 2005). The *Amt* gene (AY542491) used in this study is one of four *Amt* genes in *C. reinhardtii*, which was shown to encode a putative gas channel for the uncharged NH_3 species (Soupene *et al*. 2004). Notably, ammonium depletion has also been shown to induce upregulation of *GLB1* gene in *C. reinhardtii* (Ermilova *et al*. 2013). Upregulation at low ammonium was also observed for two phosphoenol-pyruvate carboxylase (PEPC) genes (*Ppc1* and *Ppc2*) in *C. reinhardtii* (Mamedov *et al*. 2005), where the expression of two PEPC genes mirrored the response of cytoplasmic glutamine synthetase transcript abundance to changes in inorganic [N] (Mamedov *et al.* 2005). These results also support the nonphotosynthetic role for PGPase enzymes in *C. reinhardtii* under certain conditions, as was the case for the PEPC enzyme (Mamedov *et al.* 2005). Notably, AtPGPase2 was shown not to be involved in photorespiratory metabolism (Schwarte and Bauwe 2007). Notably, understanding the role of PGPase in *C. reinhardtii* and other photosynthetic organisms is important for optimizing photosynthetic efficiency, especially, under conditions of high oxygen and low carbon dioxide, and for understanding how these organisms adapt to different environmental conditions. Rubisco is a key enzyme in photosynthesis, and catalyze two competing reactions in the Calvin–Benson cycle, which is the primary carbon fixation pathway in plants, algae, and cyanobacteria. Rubisco can also catalyze the oxygenation of ribulose 1,5-bisphosphate, resulting in the formation of PG, a compound that inhibits triose-phosphate isomerase (Husic *et al.* 1987) and sedoheptulose 1,7-bisphosphate phosphatase (Flügel *et al.* 2017) enzymes, causing a decrease in the rate of photosynthesis. PG is then converted back into 3-phosphoglycerate (3-PGA) (Tolbert 1997, Bauwe *et al*. 2010). This process involves several enzymes and requires the expenditure of energy in the form of ATP and reducing power in the form of NADH. The recovery of 3-PGA from PG is important because 3-PGA is a key intermediate in the Calvin cycle, which is responsible for carbon fixation in photosynthesis. During the conversion of PG to 3-PGA in the photorespiratory pathway, one carbon atom is lost as photorespiratory CO_2. This loss of carbon is a significant drawback of the photorespiratory pathway, as it results in a net loss of fixed carbon and reduced photosynthetic efficiency. This is one of the reasons why C_3 plants, which are more prone to photorespiration, are less efficient in terms of carbon fixation compared to C_4 and CAM plants, which have evolved mechanisms to minimize photorespiration.

Conclusions: PGPase, a key enzyme in photosynthetic organisms, catalyzes the dephosphorylation of PG, which is largely produced by the oxygenase activity of Rubisco, a potent inhibitor of several Calvin cycle enzymes. Our findings suggest that Ca^{2+} may have an important role in regulating the activity of PGPases *in vivo*. In addition, we demonstrate that *pgp1* and *pgp2* genes are upregulated under N-limiting conditions. Multiple studies demonstrate that green alga can accumulate significant quantities of lipids, particularly under stress conditions of nutrient deprivation, *i.e.*, under N-limiting conditions. These studies contribute significantly to the understanding of the regulatory mechanisms of PGPase enzymes in the green alga *C. reinhardtii* and in plants in general and are essential for high rates of carbon dioxide fixation in plants, which are important for high biomass accumulation of algae. Biomass enhancement would be essential for optimizing the yield of lipids (oils) that can be converted into biofuels. Thus, understanding the role of PGPase and the molecular mechanism underlying the broader photorespiratory pathway in *C. reinhardtii* and other photosynthetic organisms would be useful for optimizing photosynthetic efficiency and increasing the productivity of these organisms, which is important for applications such as biofuel production. *C. reinhardtii* and related microalgae have a great potential for bioenergy and biofuel production due to their unique characteristics and versatility. However, at the moment, little information is available on molecular mechanisms that control oil accumulation in microalgae (Siaut *et al.* 2011). Thus, these studies are important for optimizing photosynthetic efficiency and genetically engineering *C. reinhardtii* to enhance its oil production capabilities, and possible to optimize its performance for biofuel production.

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