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A Mutant of *Chlamydomonas reinhardtii* with Reduced Rate of Photorespiration

Kensaku Suzuki [1, 5], Tarlan G. Mamedov [1, 3] and Tomoyoshi Ikawa [2, 4]

[1] *Plant Eco-Physiology Laboratory, Tohoku National Agricultural Experiment Station, Shimo-kuriyagawa, Morioka, 020-0198 Japan*
[2] *Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305-8572 Japan*

Photorespiration rates under air-equilibrated conditions (0.04% CO_2 and 21% O_2) were measured in *Chlamydomonas reinhardtii* wild-type 2137, a phosphoglycolate-phosphatase-deficient (*pgp1*) mutant and a suppressor double mutant (7FR2N) derived from the *pgp1* mutant. In both cells grown under 5% CO_2 and adapted air for 24 h in the suppressor double mutant, the maximal rate of photorespiration (phosphoglycolate synthesis) was only about half of that in either the wild type or the *pgp1* mutant (18-7F) cells. In the progeny, the reduced rate of photorespiration was accompanied by increased photosynthetic affinity for inorganic carbon and the capacity for growth under air whether accompanied by the *pgp1* background or not. Tetrad analyses suggested that these three characteristics all resulted from a nuclear single-gene mutation at a site unlinked to the *pgp1* mutation. The decrease in photorespiration was, however, not due to an increase in the CO_2/O_2 relative specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase of 7FR2N or of any other suppressor double mutants tested. The relationship between the decrease in the rate of photorespiration and the CO_2-concentrating mechanism is discussed.

Key words: *Chlamydomonas reinhardtii* — CO_2-concentrating mechanism (CCM) — Glycolate excretion — Phosphoglycolate phosphatase — Photorespiration — Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

Photorespiration inhibits photosynthetic carbon assimilation by 30% or more in C_3 plants (Ogren 1984). Therefore, many efforts have been made to obtain a mutant with reduced activity of photorespiration for the possible improvement of plant productivity, and the use of phosphoglycolate-phosphatase (PGPase)-deficient mutants has been suggested to provide one of the easiest strategies (Spreitzer 1993).

PGPase-deficient mutants have been isolated in *Arabidopsis thaliana* (Somerville and Ogren 1979), *Hordeum vulgare* (Hall et al. 1987) and *Chlamydomonas reinhardtii* (Suzuki et al. 1990). They cannot grow under air and require elevated levels of CO_2, most likely because phosphoglycolate accumulates in the chloroplasts during photosynthesis. When they are exposed to air-equilibrated conditions in the light, they accumulate phosphoglycolate in the cells, the first direct product of photorespiration produced by the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Phosphoglycolate is a potent inhibitor of triose phosphate isomerase (Wolfenden 1970, Anderson 1971, Norman and Colman 1991), and significantly lowers the rate of photosynthesis (Somerville and Ogren 1979, Suzuki et al. 1990). Therefore, in second site suppressor mutants capable of growth under air without restoration of the missing PGPase, photorespiration may be reduced by a decreased ratio of oxygenase to carboxylase activities of Rubisco.

Although an attempt to exploit such a strategy has been made in *Arabidopsis thaliana* (Somerville and Ogren 1982, Spreitzer 1993), no second site suppressors have been identified. More recently, however, such suppressors have been selected from the PGPase-deficient mutant of the green alga *Chlamydomonas reinhardtii, pgp1-1* (=*pgp1*-18-7F, Suzuki 1995, Spalding 1998). Both the suppressor double mutants and 18-7F still have very low PGPase activities; about 1 to 2 μmol (mg Chl)$^{-1}$ h^{-1} detected only in the membrane fraction, whereas the PGPase activity in the wild type, about 50 μmol (mg Chl)$^{-1}$ h^{-1} in cells adapted to air for 5 h, is mostly catalyzed by the soluble enzyme (Suzuki 1995). Some of the suppressor double mutants have higher photosynthetic affinity for CO_2 than the wild type especially in cells grown under high CO_2 concentrations, suggesting reduced photorespiration activity resulting either from the improved relative CO_2/O_2 specificity of Rubisco or from an enhanced CO_2-concentrating mechanism (CCM) in both cells grown under 5% CO_2 and adapted to air for 24 h. In this report, we compare the rates of photorespiration among the mutants and the wild type,

Abbreviations: AOA, aminooxyacetate; CCM, CO_2-concentrating mechanism; EZA, ethoxyzolamide; $K_{0.5}$, substrate concentration at half-maximum rate of photosynthesis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PGA, 3-phosphoglycerate; PGPase, phosphoglycolate phosphatase; Rubisco, RuBP carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

[3] Current address: Institute of Botany, Academy of Sciences, Patamdar Shosse, 40, Baku, 370073, Azerbaijan.
[4] Current address: Department of Management and Information Science, Jobu University, 270-1, Shinmachi, Tano, Gunma, 370-1393 Japan.
[5] Corresponding author. Fax, 019-641-7794; E-mail, suzuki@affrc.go.jp.

using a newly developed method applicable even when phosphoglycolate accumulates, and show that one of the mutants, 7FR2N, has a reduced rate of photorespiration. This is also the first report on the measurement of intracellular levels of phosphoglycolate.

Materials and Methods

Strains and culture conditions—Chlamydomonas reinhardtii wild-type strains 2137 mt^+ (Spreitzer and Mets 1981), CC124 mt^- and CC125 mt^+, and PGPase-deficient mutants (*pgp1-1*) 18-7F mt^+ (Suzuki et al. 1990), 96H74 mt^+ (a *pgp1-1* progeny from crosses of 18-7F with CC124 mt^- and CC125 mt^+) and 7FR2N mt^+ (Suzuki 1995) were grown photoautotrophically as described previously (Suzuki 1995). The minimal growth medium designated min-70 (Suzuki 1995) contained 0.82 mM K_2HPO_4, 0.54 mM KH_2PO_4, 5.0 mM NH_4NO_3, 0.41 mM $MgSO_4 \cdot 7H_2O$, 0.34 mM $CaCl_2 \cdot 2H_2O$, 1 ml liter^{-1} Hutner's trace elements (Surzycki 1971) and 20 mM MOPS-Tris (pH 7.2). The pH of the min-70 was 7.0 after equilibration with air at 25°C. The pH was adjusted to pH 7.4 with solid Tris base (0.85 g per liter) for growth under 5% CO_2 (designated min-74). Min-74 was used after equilibration with 5% CO_2 for 1 h at 25°C, and the pH was approximately 7.0 after equilibration. The cultures were aerated with 5% CO_2 and, when necessary, with air or about 0.04% CO_2 after transfer of cells from min-74 to min-70 or to the medium described below.

Measurements for phosphoglycolate accumulation and glycolate excretion—Cells grown under 5% CO_2 in min-74 or adapted to air for 24 h in min-70 were transferred in the dark to the medium designated 70CX, a modified min-70 where NH_4NO_3 was replaced with 5.2 mM NH_4Cl and the trace elements were omitted. Cells were aerated with 0.03 to 0.04% CO_2 on a shaker under irradiation of about 100 μmol photons m^{-2} s^{-1} at 25°C. For phosphoglycolate measurements, 1 ml aliquots of cells were taken 0, 5, 10 and 15 min after transfer to air, and mixed quickly with 17 μl perchloric acid in microtubes and kept on ice. The cells, when grown under air, were left in the dark for about 30 min to minimize the intracellular phosphoglycolate level before samples were taken for phosphoglycolate determinations. For glycolate measurements, 1 ml aliquots of cell suspension were taken 0, 30, 45, 75, 105 and 135 min after transfer to air, the cells were removed quickly by centrifugation in the light, and the supernatants were filtered using a syringe filter unit (4 mm Millex LG, Nihon Millipore Ltd., Japan). The filtrates were kept in an ice bath until use, or frozen when measured on another day. To determine the excretion rate of glycolate, aminooxyacetate (AOA) was added to the cell culture 45 min after transfer to air to give a final concentration of 2 mM (Tolbert et al. 1983, Yokota and Kitaoka 1987).

Separation and determination of phosphoglycolate—The cell extracts were neutralized with an appropriate amount (about 27 μl) of 2 M KOH and frozen or left on ice at least for 1 h until use. Samples were applied to solid phase extraction columns (LiChrolut SAX, Merck, Germany) and washed with 3 ml of water and 2 ml of 50 mM HCl, and then eluted with 3 ml of 50 mM HCl. The eluates were dried in vacuo to remove HCl and then re-dissolved in 3 ml of water. Hundred microliter aliquots were subjected to HPLC anion exchange chromatography with a Dionex ion chromatograph system (Dionex, Sunnyvale, CA, U.S.A.) and detected by conductivity (Uemura et al. 1996). Phosphoglycolate was separated by Dionex IonPac AG11 guard and AS11 analytical columns with a NaOH gradient from 0.5 to 100 mM at the flow rate of 1 ml min^{-1}. Phosphoglycolate was eluted at 20 to 25 mM of NaOH, and to be separated from other anions the slope was kept to be about 0.67 mM NaOH-increase min^{-1} between these concentrations. The eluate was passed through an anion self-regenerating suppressor (Model ASRS-I, Dionex) regenerated with pure water (higher than 18 MΩ cm) at about 10 ml min^{-1}, and phosphoglycolate was detected by conductivity (Fig. 1).

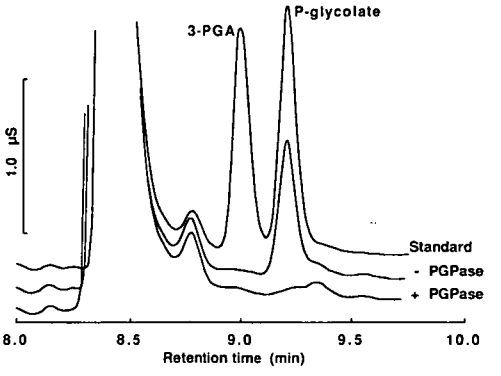

Fig. 1 Separation and detection of phosphoglycolate by HPLC anion exchange chromatography. (A) Authentic 3-PGA (0.26 nmol) and phosphoglycolate (0.26 nmol) dissolved in 70CX. (B and C) The extract from a PGPase-deficient mutant cells (*pgp1*-96H74) 15 min after transfer from 5% CO_2 to air. The extract was incubated in 20 mM Tris-maleate, pH 6.3, at 25°C for 6 h with (C) or without (B) PGPase as described in Materials and Methods.

Determination of glycolate—The glycolate concentration in the growth medium was measured by Calkins' method (Calkins 1943, Goyal and Tolbert 1996). After being dried in vacuo, the above mentioned filtrates were mixed with 2 ml of Calkins' reagent (0.01% (w/v) 2,7-napthalenediol in sulfuric acid) and boiled for 20 min. The boiled mixture was cooled on ice and mixed with 4 ml of 1 M sulfuric acid. Then glycolate was determined by measurement of its absorbance at 530 nm. Glycolate in the growth medium was also determined using HPLC ion-exclusion chromatography after being filtered as mentioned above. Glycolate was separated from lactate and formate using Shodex Ionpac KC-810P guard and KC-811 analytical columns (Showa Denko, Tokyo, Japan) with 1 mM *p*-toluenesulfonic acid at the flow rate of 0.8 ml min^{-1} at 60°C. The organic acids were detected by conductivity after mixture with 20 mM BisTris containing 1 mM *p*-toluenesulfonic acid and 100 μM EDTA at the same flow rate.

Enzyme purification— All purification steps were carried out at 4°C unless otherwise specified. PGPase was partially purified from the wild-type strain 2137. The crude extract prepared as described previously (Suzuki 1995) was incubated at 50°C for 5 min and the precipitate was removed by centrifugation at 45,000 × *g* for 20 min. A fine powder of $(NH_4)_2SO_4$ (Wako Chemicals, Osaka, Japan, enzyme-purification grade) was added to the supernatant to 50% saturation. Thirty min later, solid $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation. Thirty min later, the mixture was centrifuged at 45,000 × *g* for 20 min, and the precipitate was dissolved in 2.5 ml 20 mM MOPS-KOH (pH 6.3) containing 5 mM $MgCl_2$, passed through a small column of Sephadex G25 (PD-10, Amersham Pharmacia, Uppsala, Sweden) equilibrated with the same buffer and applied to a 1 × 7 cm DEAE-cellulose column (DEAE-Sephacel, Amersham Pharmacia) equilibrated with the same buffer. The enzyme was eluted by changing the buffer to 20 mM Tris-maleate (pH 6.3) containing 5 mM

$MgCl_2$, and the fractions with PGPase activity were collected. The eluate had no detectable *p*-nitrophenyl phosphate phosphatase activity and was used to identify phosphoglycolate in the analyses by HPLC anion-exchange chromatography (Fig. 1). Phosphatase activities were determined by measurement of the substrate-dependent release of P_i at pH 8.0 or 6.3 (Suzuki 1995) with Ames reagent (Ames 1966).

Rubisco was purified from cells grown under 5% CO_2. The cells were washed twice with water and suspended in 150 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$ and 1 mM phenylmethanesulfonyl fluoride, and the crude extract was prepared in the same manner as for PGPase (Suzuki 1995). A fine powder of $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation. Thirty min later, the mixture was centrifuged at 150,000× *g* for 20 min and solid $(NH_4)_2SO_4$ was added to the supernatant to 65% saturation. Thirty min later, the mixture was centrifuged again and the precipitate was dissolved in 150 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$ and 10 mM $NaHCO_3$, and applied to a HiLoad 16/60 Superdex 200 pg column (Amersham Pharmacia) equilibrated with 150 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$. The fractions with Rubisco activity were collected and precipitated by the addition of solid $(NH_4)_2SO_4$ to 65% saturation, and the supernatant was discarded after centrifugation. The precipitate was dissolved in 80 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$ and 10 mM $NaHCO_3$, and passed through PD-10 equilibrated with the same buffer at room temperature before use. This preparation had no detectable phosphatase activity for 3-phosphoglycerate (PGA) or phosphoglycolate.

CO_2/O_2 relative specificity factor of Rubisco—The CO_2/O_2 relative specificity factor ($S_{c/o}$) of Rubisco was determined principally based on the method described by Uemura et al. (1996). The enzyme reaction was allowed to proceed in a rubber-capped test tube with a side-arm. The contents of tube were equilibrated with 0.05% CO_2 and 99.95% O_2 for 60 min prior to initiation of the reaction by mixing ribulose-1,5-bisphosphate (RuBP) in the side-arm with the other components in the main tube (Kane et al. 1994, Uemura et al. 1996). The reaction mixture (1 ml) contained 80 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM RuBP and the partially purified enzyme. The reaction was terminated 30 min later by the addition of 17 μl perchloric acid. PGA and phosphoglycolate were measured by the same method as that for the intracellular phosphoglycolate measurement described above. The $S_{c/o}$ was calculated as follows (Kane et al. 1994, Uemura et al. 1996):

$$S_{C/O}=0.0375\times\frac{[PGA]-[PGO]}{2\times[PGO]}\times\frac{[O_2]}{[CO_2]}$$

where 0.0375 is the ratio between the solubilities of CO_2 and O_2 in the aqueous phase (Uemura et al. 1996), [PGA] and [PGO] are the final concentrations of PGA and phosphoglycolate after the reaction, and $[O_2]$ and $[CO_2]$ are the concentrations of O_2 and CO_2 in the gas phase, respectively.

Genetic analyses—The suppressor double mutant 7FR2N mt^+ and wild type CC124 mt^- were grown on min-70 plates solidified with 1.5% (w/v) agar, for a few days in the light. Each strain was incubated separately for 2–3 h or overnight in the light after being suspended in a nitrogen-free liquid medium, a modified min-70 in which NH_4NO_3 was omitted, for gametogenesis. Mating, zygote maturation, and germination were performed as described by Sears et al. (1980). Mating type was determined by mating with CC124 mt^- and CC125 mt^+, and phenotypes of tetrads were determined using spot tests on agar plates (Winder and Spalding 1988).

Photosynthetic oxygen exchange—The photosynthetic exchange of O_2 was measured using an O_2 electrode (Rank Brothers, Cambridge, England) as described previously (Suzuki 1995).

Chl content—Chl content was determined after extraction with 96% (v/v) ethanol (Wintermans and DeMots 1965).

Results

Phosphoglycolate accumulation—Phosphoglycolate accumulated after transfer of cells grown under 5% CO_2 to air in both mutants 7FR2N and 18-7F, but not in the wild-type 2137 (Fig. 2). The maximal rate of accumulation in 7FR2N was one-third of that in 18-7F; about 1.2 μmol (mg Chl)$^{-1}$ h^{-1} in 7FR2N and about 3.9 μmol (mg Chl)$^{-1}$ h^{-1} in 18-7F. The rate in 7FR2N decreased with increasing intracellular level after reaching about 0.4 μmol (mg Chl)$^{-1}$, to attain the highest level of about 0.7 μmol (mg Chl)$^{-1}$ after 120 min (Fig. 2). In 18-7F, it took only about 20 min to reach the highest level of about 0.9 μmol (mg Chl)$^{-1}$ (Fig. 2). After adaptation to air for 24 h, the levels decreased to 0.4 μmol (mg Chl)$^{-1}$ in 7FR2N and 0.6 in 18-7F (data not shown). The accumulated phosphoglycolate was metabolized in the dark at almost the same rates in both 7FR2N and *pgp1*-18-7F (Fig. 2); about 3 to 4 μmol (mg Chl)$^{-1}$ h^{-1} at a phosphoglycolate concentration of about 0.4 μmol (mg Chl)$^{-1}$. In 24-h air-adapted cells, the maximal accumulation rate was only 0.2 μmol (mg Chl)$^{-1}$ h^{-1} in 7FR2N whereas 1.2 μmol (mg Chl)$^{-1}$ h^{-1} in 18-7F (Table 1).

Glycolate excretion in the presence of aminooxyacetate (AOA)—The rate of glycolate production was assayed as the rate of glycolate excretion in the presence of AOA (Tolbert et al. 1983, Yokota and Kitaoka 1987). For the determination of glycolate in the growth media by Calkins' method, it was necessary to modify the growth media, because nitrate and EDTA interfere with the glycolate assay, and other organic acids excreted from the cells also may react with the reagent. Formate and lactate were detected by HPLC in the growth medium when the cells were grown for about 2 d. Formate was detected in high

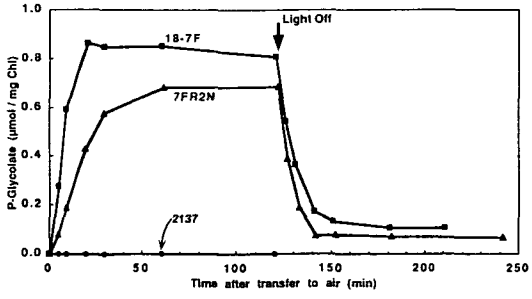

Fig. 2 The accumulation of phosphoglycolate in 5% CO_2-grown cells of *Chlamydomonas reinhardtii* mutants 18-7F and 7FR2N, and wild type 2137 after transfer to air, and its consumption during the subsequent dark period.

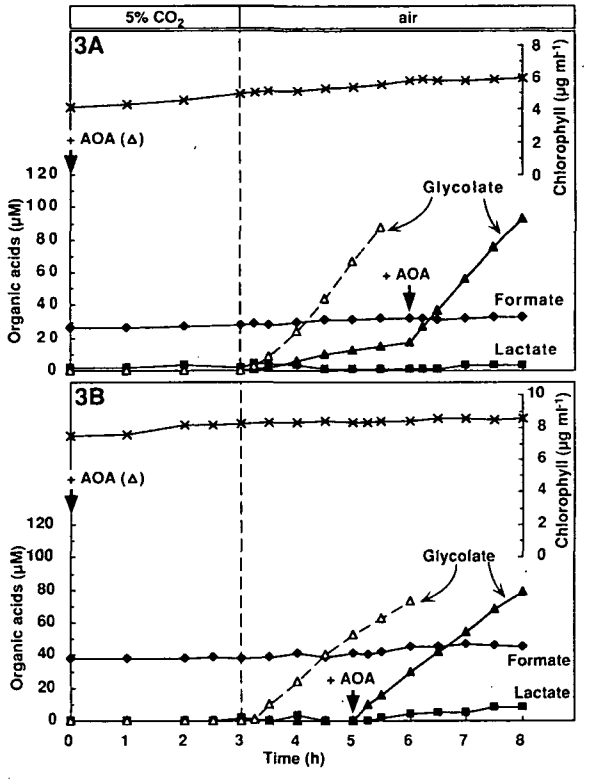

Fig. 3 The excretion of organic acids from 5% CO_2-grown cells of *Chlamydomonas reinhardtii* 2137 (A) and 18-7F (B) after transfer to air. AOA was added at 3 h before transfer to air (0 time, open triangles), or 3 h (A) or 2 h (B) after transfer to air (closed symbols), as indicated by arrow.

concentrations, although it increased quite slowly and was not affected by either a change in CO_2 concentration or

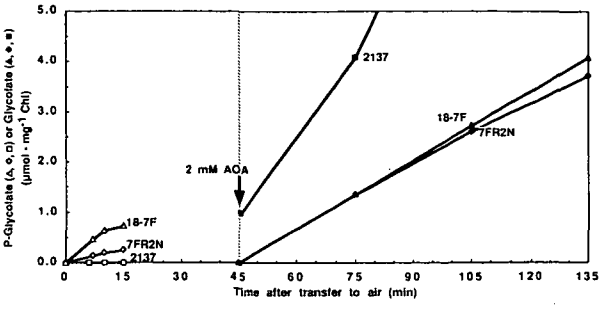

Fig. 4 Phosphoglycolate accumulation after transfer from 5% CO_2 to air and glycolate excretion after addition of 2 mM AOA in *Chlamydomonas reinhardtii* mutants 18-7F and 7FR2N, and a wild type 2137.

addition of AOA (Fig. 3). Lactate was also detected, but the excretion was quite small and transient and seems to respond to a variety of stimuli (Fig. 3). Formate and lactate were not detected by Calkins' method at least 3 h after transfer of the cells from growth medium to 70CX (data not shown). As AOA also reacts with Calkins' reagent, controls were taken with or without AOA at 45 min after transfer.

Unlike in wild type cells as shown here (Fig. 3, 4) and by others (Tolbert et al. 1983), glycolate excretion was not detected after transfer to air in the absence of AOA from both 7FR2N and 18-7F cells (Fig. 3, 4). In the presence of AOA, the rate of glycolate excretion from 7FR2N cells was almost the same as that from 18-7F, was much lower than that from wild type cells (Fig. 4), and was comparable with the activity of membrane-bound PGPase in these mutants (Suzuki 1995). The rate of AOA-dependent glycolate excretion was almost the same for at least 3 h after transfer

Table 1 Comparison of photorespiratory and photosynthetic characteristics of 7FR2N with those of wild type 2137 and *pgp1* mutant 18-7F under air-equilibrating conditions

Strain and cell type	Maximal rate of P-glycolate accumulation under air (PG)	Rate of glycolate excretion under air in the presence of 2 mM AOA (G)	Estimated rate of photorespiration under air (PG+G)	Rate of photosynthesis under air-equilibrating condition	Relative rate of photorespiration under air
	(μmol (mg Chl)$^{-1}$ h^{-1})				
2137					
5% CO_2	0.0±0.0	6.5±0.2	6.5	32±2	20%
24 h air	0.0±0.0	2.8±0.3	2.8	77±9	4%
18-7F					
5% CO_2	3.9±0.0	2.6±0.1	6.5	8±2	78%
24 h air	1.2±0.0	2.7±0.1	3.9	65±7	6%
7FR2N					
5% CO_2	1.2±0.1	2.4±0.2	3.6	44±5	8%
24 h air	0.2±0.0	1.2±0.2	1.4	43±2	3%

Table 2 Comparison of photorespiratory and photosynthetic characteristics in tetrad progeny from crosses between 7FR2N and wild type CC124

Tetrad progeny	Mating type	Photoautotrophic growth under		PGPase activity in 5% CO_2 grown cells permeabilized with 2% toluene	Photosynthetic O_2 exchange at $NaHCO_3$ concentration of		Relative affinity for inorganic carbon (P_{80}/P_{5000})	Photorespiration		
		Air	5% CO_2		80 μM (P_{80})	5,000 μM (P_{5000})		P-glycolate accumulation under air (PG)	AOA-dependent glycolate excretion (G)	Estimated PR rate under air (PG+G)
				(μmol (mg Chl)$^{-1}$ h^{-1})				(μmol (mg Chl)$^{-1}$ h^{-1})		
4-1	−	+	+	25	26	99	0.26	0.0	6.7	6.7
4-2	+	−	+	1	17	70	0.25	3.3	2.1	5.4
4-3	+	+	+	38	34	64	0.52	0.0	2.6	2.6
4-4	−	+	+	1	40	72	0.55	0.9	2.3	3.3
8-1	+	+	+	2	34	72	0.48	1.0	2.3	3.3
8-2	−	+	+	27	46	79	0.59	0.0	3.2	3.2
8-3	+	−	+	1	26	137	0.19	3.8	2.8	6.5
8-4	−	+	+	25	22	127	0.18	0.0	6.7	6.7

to air in wild-type and mutant cells when measured by Calkins' method (data not shown), whereas phosphoglycolate accumulation was saturated within 1 h in mutant cells (Fig. 2). These results are consistent with the results from HPLC analysis for both 2137 and *pgp1*-18-7F in the absence and presence of AOA (Fig. 3), whereas it took about 30 min to reach air-equilibration after transfer to air in the experiments which were performed only by changing the gas from 5% CO_2 to air without changing the growth medium (Fig. 3).

The rate of photorespiration—We added the maximal rate of phosphoglycolate accumulation in the absence of AOA to the rate of glycolate excretion in the presence of AOA to estimate the maximal rate of photorespiration (Fig. 4, Table 1). The estimated rate in 18-7F cells was almost the same as that in wild type 2137 (Table 1). On the other hand, the rate of photorespiration in 7FR2N was about half of those in 2137 and 18-7F, in both cells grown under 5% CO_2 and adapted to air for 24 h. The relative rate of photorespiration against photosynthesis was also much lower in 7FR2N than in other strains (Table 1).

Characteristics of tetrad progeny—It was very difficult to obtain complete tetrad sets from zygotes after crossing 7FR2N with any strains tested. So far, only 8 complete tetrads have been obtained from a large number of zygotes after crossing 7FR2N with CC124 or *pgp1*-96H32. In a cross between 7FR2N and a wild-type CC124, the reduced rate of photorespiration (RPR) phenotype from 7FR2N was accompanied by increased photosynthetic affinity for inorganic carbon and the capability for growth under air whether accompanied by the *pgp1* background or not (Table 2). Thus two strains with the RPR phenotype segregated from the *pgp1* background were obtained (progeny 4-3 and 8-2). The 2 : 2 segregation of the RPR and non-RPR phenotypes was observed in all 8 tetrads tested (data not shown), suggesting that all the RPR phenotypes resulted from a single-gene, nuclear mutation at a site unlinked to *pgp1*.

CO_2/O_2 relative specificity factor of Rubisco—The CO_2/O_2 relative specificity factor ($S_{c/o}$) of Rubisco was determined using a partially purified enzyme with no detectable phosphatase activities to degrade PGA and phosphoglycolate (data not shown). There was no significant difference in the $S_{c/o}$ among 7FR2N, 18-7F and 2137, and was calculated to be about 67 (Table 3), consistent with the value of 70 for the wild type reported by Uemura et al. (1996).

Effect of ethoxyzolamide (EZA)—The effect of EZA, a carbonic anhydrase (CA) inhibitor, on photosynthetic O_2 exchange in cells of 7FR2N grown under 5% CO_2 was compared with that of the wild type 2137 (Fig. 5). In the absence of EZA, the photosynthetic affinity for inorganic carbon was much higher in 7FR2N than that in 2137.

Table 3 The CO_2/O_2 relative specificity factor ($S_{c/o}$) of Rubisco from wild-type and mutant strains of *Chlamydomonas reinhardtii*

Strain	$S_{c/o}$	±SE
2137	67.4	±1.3
7FR2B	68.4	±0.8
7FR2F	66.9	±1.5
7FR2H	66.7	±3.9
$7FR2K_1$	66.9	±0.8
7FR2N	67.0	±1.8

The values are averages of three measurements ± standard errors.

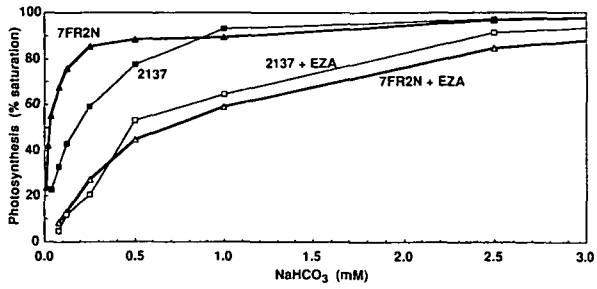

Fig. 5 Effect of 50 μM EZA, a CA inhibitor, on photosynthetic affinity for CO_2 in the wild type 2137 and the suppressor double mutant 7FR2N cells grown under 5% CO_2. The rates of photosynthesis at 5 mM $NaHCO_3$ in the absence and presence of 50 μM EZA were 92 and 71 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, respectively, in 2137, and 76 and 67 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, respectively, in 7FR2N.

As reported previously (Suzuki 1995), $NaHCO_3$ concentrations at half-maximum rate of photosynthesis, $K_{0.5}$ ($NaHCO_3$), were about 30 and 170 μM in 7FR2N and 2137, respectively (Fig. 5). In the presence of 50 μM EZA, on the other hand, the difference in the affinity disappeared and the value of $K_{0.5}$ increased to about 500 μM in both strains (Fig. 5).

Discussion

Eight mutant strains were selected on the basis of higher photosynthetic affinity for inorganic carbon in cells grown under 5% CO_2 (Suzuki 1995). They were expected to have reduced rates of photorespiration, because they were recovered as suppressors of *pgp1* from the PGPase-deficient high-CO_2-requiring mutant *pgp1*-18-7F (Suzuki et al. 1990) after a second mutagenesis. These suppressor double mutants were capable of growth under air without any restoration of the missing soluble PGPase activity (Suzuki 1995). In the present study 7FR2N, one of the selected suppressor double mutants, was confirmed to have a reduced rate of photorespiration; about half of that in the wild-type 2137 or the original *pgp1* mutant 18-7F (Table 1). However, it is quite unlikely that the suppressor mutation affected the CO_2/O_2 relative specificity of Rubisco, since we have not found any significant increase in the $S_{c/o}$ of Rubisco from 7FR2N and any other suppressor double mutants, so far. The $S_{c/o}$ should have been about twice that in the wild type if its increase were responsible for the decreased rate of photorespiration in 7FR2N. Based on the $S_{c/o}$ of Rubisco of about 70 in *C. reinhardtii*, the relative rate of photorespiration should be about 30% of the photosynthesis under air-equilibrating condition if CCM does not function at all. It was, however, about 20% in cells of the wild type grown under 5% CO_2 (Table 1), suggesting that CCM functions partially even in these cells, as if the $S_{c/o}$ were about 100 just as in higher plants.

The photosynthetic affinity for inorganic carbon of cells of 7FR2N grown under 5% CO_2 was much higher than that of the wild-type 2137 in the absence of the CA inhibitor, EZA, but in the presence of EZA, the $K_{0.5}$ for inorganic carbon increased to about 500 μM in both 7FR2N and 2137. This value is actually comparable with that in the *ca1* mutants that cannot utilize the over-accumulated inorganic carbon in the cells, as well as with that in the *pmp1* mutant that cannot accumulate inorganic carbon at all (Suzuki and Spalding 1989). This suggests the partial operation of CCM even in cells of the wild type grown under 5% CO_2, and its enhanced operation in the suppressor double mutant 7FR2N, as well.

The enhanced operation of CCM is also suggested by the photosynthetic characteristics of 7FR2N and its progeny. As the operation of CCM requires energy produced by photosynthesis (Sültemeyer et al. 1986, Spalding 1990), it is possible that the over-expression of CCM could limit the capacity of photosynthetic carbon assimilation. This may explain why the rates of photosynthetic oxygen exchange were always lower than those in the wild type at saturating $NaHCO_3$ concentrations in 7FR2N and RPR progeny, even though they were higher in these strains at $NaHCO_3$ concentrations below air level (Table 2).

Thus, the suppressor mutation responsible for reducing photorespiration does not seem to be the result of an increase in the CO_2/O_2 relative specificity of Rubisco in the suppressor double mutant 7FR2N, but of an enhanced operation of CCM in both cells adapted to air and grown under 5% CO_2, probably by reducing the sensitivity of the cells to CO_2.

It has been assumed that the rate of glycolate excretion in the presence of 1 or 2 mM AOA represents the rate of photorespiration in the wild type strains of *C. reinhardtii* (Tolbert et al. 1983, Yokota and Kitaoka 1987), as the accumulation of glycolate inside the cells is very small relative to the excretion (Tolbert et al. 1985). This method was, however, not applicable to the mutants in which phosphoglycolate accumulates. To compare the rates of photorespiraton in mutant strains with the wild type, it is necessary to estimate the maximal rate of photorespiration before the accumulated phosphoglycolate inhibits photosynthesis in the mutants. Under such conditions, it is necessary to add the rate of phosphoglycolate accumulation to the rate of glycolate production, to estimate the maximal rate of photorespiration in *pgp1* mutants. In order to make the experiments easier, however, we did not measure the rate of glycolate excretion and phosphoglycolate accumulation simultaneously because almost the same rate was obtained for glycolate excretion for about 5 h after transfer to air whereas phosphoglycolate accumulation was saturated within 20 to 120 min. Thus, glycolate measurement was made 45 min after the phosphoglycolate

measurement under the optimal conditions, using the same cell suspension. In order to obtain reliable values for cells adapted to air for 24 h, it was, however, necessary to incubate in the dark for about 30 min or more before the measurements to deplete the accumulated phosphoglycolate. The estimated rates of photorespiration in 2137 and 18-7F using our method were consistent with the rate in wild type reported by Yokota and Kitaoka (1987).

Phosphoglycolate seems to be an important metabolite in both plant and animal cells. In plant cells, it is the first product of photorespiration known as a potent inhibitor of triosephosphate isomerase (Wolfenden 1970, Anderson 1971, Norman and Colman 1991), and it may also act as a trigger for the induction of CCM in *C. reinhardtii* (Suzuki et al. 1990). It is also known, at least in animal cells, as a strong activator for both diphosphoglycerate mutase and diphosphoglycerate phosphatase reactions (Black et al. 1985). Although it has been believed that phosphoglycolate does not accumulate in plant cells, its actual amount has not been measured because a sensitive assay method has not been available. The method presented in this paper may help elucidate the physiological roles of phosphoglycolate in the adaptation to environmental stresses in plant cells or even in animal cells.

Although intracellular levels of phosphoglycolate have not been determined in any organism, the methods for separation and determination of phosphoglycolate have recently been developed to evaluate the RuBP oxygenase activity in purified Rubisco (Uemura et al. 1996, Zhu et al. 1998). In the present study, we successfully measured the phosphoglycolate in the cells by HPLC anion exchange chromatography (Uemura et al. 1996, Zhu et al. 1998) after SAX-fractionation of the $HClO_4$ extract (Zhu et al. 1992) with some modifications.

Although a similarly low, but a detectable rate of glycolate production was observed in both 7FR2N and 18-7F, the rate of phosphoglycolate accumulation in 7FR2N was much lower than that in 18-7F (Table 1). The rate of glycolate excretion, detectable only in the presence of AOA, should be limited by the in vivo activity of a phosphatase that produces glycolate in *pgp1* mutants. In 18-7F, phosphoglycolate-dependent phosphatase activity was about 1 to 2 μmol (mg Chl)$^{-1}$ h^{-1} and was found only in the membrane fraction, whereas in the wild type 2137 cells adapted to air for 5 h, it was about 50 μmol (mg Chl)$^{-1}$ h^{-1} and mostly found in the soluble fraction (Suzuki 1995). The rates of glycolate excretion appear, however, somewhat higher than the in vitro PGPase activities in these mutants, probably because the lack of solubilization of the membrane fraction resulted in a slight underestimate of the in vitro activity (Suzuki 1995). The rate of AOA-dependent glycolate excretion in these mutants was also comparable with the rate of phosphoglycolate consumption in the dark after irradiation (Fig. 2).

The level of phosphoglycolate accumulated in 7FR2N cells appears to be too high, and too similar to that in 18-7F cells at the steady state, to explain the growth differences between these two strains, which is significant because photosynthesis was inhibited strongly in 18-7F only 5 to 10 min after transfer to air (Suzuki et al. 1990), whereas no detectable inhibition was observed in 7FR2N over this time period (Suzuki 1995). Although the concentration of phosphoglycolate in the chloroplast could not be determined, triose-phosphate isomerase activities in 2137, 18-7F and the suppressor double mutants seem to be higher than the rate of photosynthesis even in the presence of 1 mM phosphoglycolate; 80 μmol (mg Chl)$^{-1}$ h^{-1} or higher when 0.2 mM glyceraldehyde 3-phosphate was used as the substrate (Suzuki and Ikawa, unpublished data). When adapted to air, 7FR2N cells may be able to keep the phosphoglycolate level in the chloroplast low enough not to inhibit triose-phosphate isomerase activity below the level required to maintain the rate of photosynthesis high enough to grow under air.

Now we have obtained progeny of 7FR2N which have a RPR without the *pgp1* background (Table 2). These progeny, together with other RPR progeny with the *pgp1* background, as in 7FR2N, should be useful for further investigations to define the second-site suppressor mutation in 7FR2N to help clarify the CO_2-sensing mechanism in *C. reinhardtii*, whether it is a direct "CO_2 sensor" (Matsuda et al. 1998) or not, and to investigate the most effective way to reduce photorespiration, although it is still necessary to obtain more suppressor double mutants with different suppressor mutations. Selecting the mutants with reduced CO_2-sensitivity might be one of the easiest strategies to reduce photorespiration to improve plant productivity.

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