Adaptation of a *Chlamydomonas* mutant with reduced rate of photorespiration to different concentrations of CO_2[1]

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Abstract: It has been widely accepted that *Chlamydomonas reinhardtii* cells utilize inorganic carbon very efficiently for photosynthesis by operating a CO_2-concentrating mechanism (CCM) under conditions of limited CO_2. To help define the mechanism, 7FR2N, one of the suppressor double mutants of phosphoglycolate phosphatase-deficient (*pgp1*) mutants that have a reduced photorespiration rate (RPR) was crossed with wild-type strains to generate the strain N21 as a single RPR mutant. The comparison of photosynthetic characteristics with wild-type strains after the cells adapted to different concentrations of CO_2 revealed that photosynthetic affinity for inorganic carbon was higher than that in wild-type strains after adaptation to concentrations between 50 $\mu L \cdot L^{-1}$ CO_2 and 5% CO_2. Chlorophyll fluorescence parameters were also compared, and the biggest difference between N21 and the wild-type strains was observed in the photochemical quenching and effective quantum yield of photosystem II ($\Delta F/F_m'$) at the CO_2 compensation point. These values in N21 increased in a similar manner to the photosynthetic affinity for CO_2, and increased significantly when the cells adapted to low-CO_2 levels, whereas the values in the wild-type strains were apparently lower without any significant changes, regardless of the CO_2 concentrations to which they were adapted. Although it was not clear if a nonphotochemical quenching parameter (NPQ) in N21 was higher than that in wild-type strains, NPQ increased coincidentally with the increase in photosynthetic affinity for inorganic carbon when the CO_2 concentrations to which the strains were adapted decreased, in both the mutant and wild-type strain, suggesting that this form of NPQ reflects the operation of CCM in certain conditions. Possible candidates for the RPR mutation and the relationship between CCM and photosynthetic electron flow are discussed.

Key words: Chlamydomonas reinhardtii, chlorophyll fluorescence, CO_2-concentrating mechanism, low-CO_2 responsive gene, phosphoglycolate phosphatase, photorespiration.

Résumé : On accepte généralement que les cellules du *Chlamydomonas reinhardtii* utilisent le carbone inorganique très efficacement pour la photosynthèse, grâce à un mécanisme de concentration du CO_2 (CCM), lorsqu'elles s'adaptent à des conditions de CO_2 limitantes. Afin de contribuer à la définition de ce mécanisme, les auteurs ont croisé le 7FR2N, un des doubles mutants suppresseurs déficient en phosphatase du phosphoglycolate (*pgp1*), lesquels mutants ont des taux réduits de photorespiration (RPR), avec des souches sauvages, pour finalement générer la souche N2l, cun mutant simple RPR. Une comparaison des caractéristiques photosynthétiques avec des souches sauvages, après une adaptation à différentes concentrations en CO_2, révèle que l'affinité photosynthétique pour le carbone inorganique y est plus élevée que chez les souches sauvages, après une adaptation à des concentrations allant de 50 $\mu L \cdot L^{-1}$ à 5 % en CO_2. Ils ont également comparé les paramètres de fluorescence de la chlorophylle; la plus forte différence entre les souches N2l et sauvages apparaît dans le quenching photochimique et le rendement quantique réel du photosystème II ($\Delta F/F_m'$), au point de compensation en CO_2. Chez le N2l, ces valeurs augmentent de la même façon que l'affinité photostyntétique pour le CO_2, et augmentent significativement lorsque les cellules s'adaptent au CO_2 faible, alors que ces valeurs chez les souches sauvages, sont apparemment plus faibles sans changement significatif, indépendamment des concentrations d'adaptation au CO_2. Bien qu'il ne soit pas certain qu'un quenching non photochimique (NPQ) soit plus élevé chez le N2l que chez les souches sauvages, le NPQ augmente concurremment avec l'augmentation de l'affinité pour le carbone inorganique, lorsque la concentration d'adaptation au CO_2 diminue, chez le mutant aussi bien que chez une souche sauvage; ceci suggère que cette forme du NPQ reflète l'activité du CCM. Les auteurs discutent les candidatures possibles pour la mutation RPR et la relation qui existe entre le CCM et le flux photosynthétique des électrons.

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Mots clés : Chlamydomonas reinhardtii, fluorescence de la chlorophylle, mécanisme de concentration du CO_2, gène réagissant au CO_2 faible, phosphatase du phosphoglycolate, photorespiration.

[Traduit par la Rédaction]

Introduction

It has been widely accepted that *Chlamydomonas reinhardtii* cells utilize inorganic carbon very efficiently for photosynthesis by the operation of a CO_2-concentrating mechanism (CCM) when adapted to limited CO_2 conditions (Kaplan and Reinhold 1999; Spalding et al. 2002). However, the detailed mechanism and its regulation are still not clear. Mutant analysis is suitable to study mechanisms such as CCM, but so far, only a limited number of mutants are available for analysis of the CCM. Most mutants, including insertional mutants, have been selected as high CO_2-requiring mutants (Spalding et al. 2002; Thyssen et al. 2003). For this reason, it may be helpful if the new mutants can be isolated using new selection strategies.

We have isolated suppressor double mutants of phosphoglycolate phosphatase (PGPase)-deficient (*pgp1*) mutants, capable of growth under air without any recovery from the deficiency of PGPase activity. Several strains were found to have higher photosynthetic affinity for CO_2 in cells grown in 5% CO_2 (Suzuki 1995). The rate of photorespiration suppressed in the mutants was about half or less than half of that of wild-type 2137 or PGPase-deficient mutant *pgp1-1*-18-7F, in both air-adapted and 5% CO_2-grown cells of a suppressor double mutant 7FR2N and the progeny (Suzuki et al. 1999). However, it was not clear, at the time of initial characterization, whether the mutants also had the higher affinity when air-adapted. We called these suppressor mutants RPR (reduced rate of photorespiration) mutants, although the mutation was suggested to be involved in the CCM (Suzuki et al. 1999).

To help define the RPR mutation of the suppressor double mutant 7FR2N, in the present report, we selected the strain N21, the "single" RPR mutant, after crossings with CC125-background strains, and compared the photosynthetic characteristics carefully with those of wild-type strains, especially in terms of adaptation to different concentrations of CO_2. Chlorophyll fluorescence analyses were employed not only to provide photosynthetic characteristics to help define the mutation, but also to provide an easy way for quick and simple selection or detection of this kind of mutant.

Materials and methods

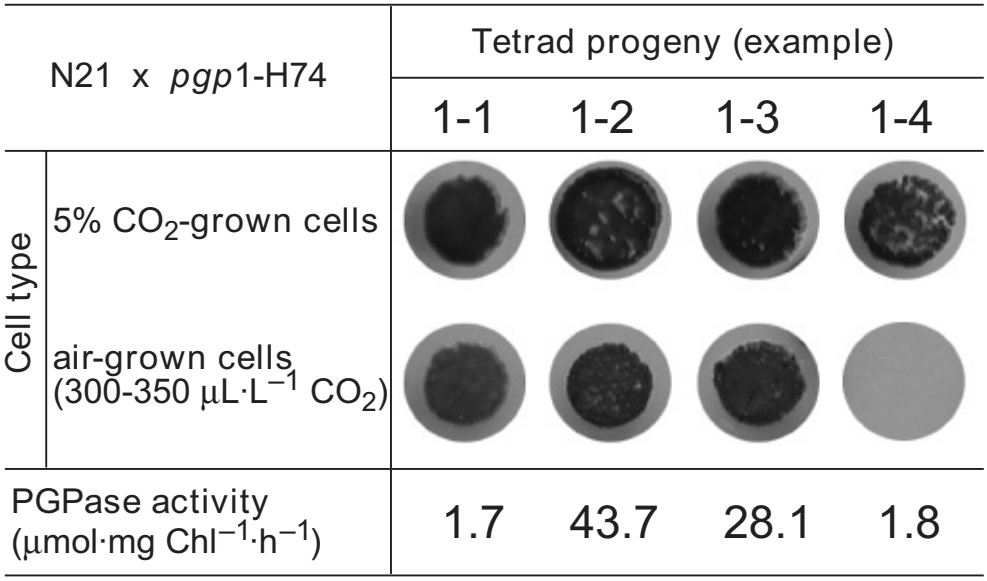
Wild-type strains of *Chlamydomonas reinhardtii* 2137 mt^+ and CC125 mt^+ (CC125-A) were obtained from Dr. M.H. Spalding, Department of Botany, Iowa State University, USA, and another clone of CC125 mt^+ (CC125-B) and CC124 mt^- was obtained from Dr. Y. Matsuda, Department of Biology, Kobe University, Japan. *pgp1-1*-H74 is a progeny of *pgp1-1*-18-7F (Suzuki et al. 1990) obtained after crossing with CC124. 7FR2N, a suppressor double mutant obtained after ethylmethane sulfonate / 5-fluorodeozyuridine (EMS/FdU) treatment of *pgp1-1*-18-7F (Suzuki et al. 1990), which has a RPR phenotype (reduced rate of photorespiration; Suzuki 1995; Suzuki et al. 1999), was crossed three times with the strains with CC125-background. The RPR-*pgp1-1* double mutant was selected after the first and second crossing to facilitate the selection, and then the RPR mutant without *pgp1* mutation, N21, was selected after the third crossing. The cells were grown under 5% CO_2, as described previously (Suzuki et al. 1999), and then adapted to various concentrations of CO_2 by transferring to new growth medium equilibrated with bubbling gas at a specific CO_2 concentration. CO_2 concentration in the bubbling gas was monitored using CO_2 transmitters GMT221 and (or) GMT222 (Vaisala, Helsinki, Finland). The pH of growth medium was 7.0 after equilibration with the bubbling gas at 25 °C.

Oxygen exchange and chlorophyll fluorescence were measured simultaneously in a DW2/2 O_2-electrode chamber (Hansatech, King's Lynn, Norfolk, UK) connected with a multibranched fiberoptics (101-F5, Walz, Effeltrich, Germany) by which all optical links were made. Both oxygen and fluorescence signals were monitored with a two-pen recorder. O_2 measurements were controlled using the CBD1 control box (Hansatech), which was made as described previously (Suzuki 1995). Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM 101, 102, and 103, Walz). Two of the branched ends of the fiberoptics were connected with 100ED (Walz) controlled with PAM 101 to supply a measuring beam and to detect the modulated fluorescence; the third branch was connected with a light source KL1500LCD (Schott, Mainz, Germany) to supply actinic light; the fourth branch was connected with another light source (KL1500LCD) controlled with PAM 103 to supply a saturation pulse; and the fifth branch was connected with 101FR (Walz) controlled with PAM 102 to supply far-red light. The fluorescence nomenclature follows Van Kooten and Snel (1990). The maximum quantum yield of photosystem II (PSII) (F_v/F_m) was determined in dark-adapted (15–20 min) cells. Other parameters in the CO_2-dependent oxygen exchange experiments (Suzuki 1995) such as the effective quantum yield of PSII ($\Delta F/F_m'$), photochemical quenching (qP; $\Delta F/F_v'$), and nonphotochemical quenching (NPQ; $F_m/F_m' - 1$) were determined after the oxygen evolution ceased and fluorescence level (F and F_m') reached a steady state, as a consequence of the cells consuming all the usable inorganic carbon that was carried over with the cells.

The determination of inorganic ^{14}C accumulated after 10 s of photosynthesis was determined as described previously (Suzuki and Spalding 1989) with two exceptions: the cell volume was not determined using the $^3H_2O/^{14}C$-sorbitol double label technique, and the silicone–oil mixture was changed from AR20 and AR200 (5:1; Wacker-Chemie, München, Germany) to SH556 and SH550 (2:1; Dow Corning Toray Silicone, Tokyo, Japan).

For enzyme assays, cells were disrupted by sonication in the extraction buffer described in the following assay systems. The supernatant was kept on ice and used as the crude extract after centrifugation (20 000*g*, 20 min, 4 °C). PGPase

Fig. 1. Spot test for growth response to 5% CO_2 and air (300–350 $\mu L \cdot L^{-1}$ CO_2) for the strains in a typical set of tetrad progeny obtained when N21 cells were crossed with *pgp1* mutant H74 cells. The plates containing minimal medium (min 70; Suzuki 1995) and 1.5% (*m*/*v*) agar were kept either at 5% CO_2 for 7 d or in air (300–350 $\mu L \cdot L^{-1}$ CO_2) for 10 d. PGPase activity was assayed in the cells adapted to air for 24 h after being grown under 5% CO_2 in the minimal liquid growth medium.



activity was assayed as described previously (Mamedov et al. 2001). Phosphoenolpyruvate (PEP) carboxylase activity was assayed in a reaction mixture containing 50 $mmol \cdot L^{-1}$ 3-(*N*-morpholino)propoanesulfonic acid – Tris (pH 7.3), 5 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ $NaHCO_3$, 0.2 $mmol \cdot L^{-1}$ NADH, 10 units malate dehydrogenase (410-13, Sigma, St. Louis, Missouri, USA), and crude extract. The reaction was initiated by the addition of 2.5 $mmol \cdot L^{-1}$ PEP, and the PEP-dependent decrease in A_{340} was monitored using a spectrophotometer (Akagawa et al. 1972). The extraction buffer contained 100 $mmol \cdot L^{-1}$ 3-(*N*-morpholino) propoanesulfonic acid – Tris (pH 7.3), 20% (*v*/*v*) glycerol, 10 $mmol \cdot L^{-1}$ $MgCl_2$, 2 $mmol \cdot L^{-1}$ K_2HPO_4, 1 $mmol \cdot L^{-1}$ EDTA, 10 $mmol \cdot L^{-1}$ KF, 5 $mmol \cdot L^{-1}$ dithiothreitol (DTT), and 1 $mmol \cdot L^{-1}$ phenylmethylsulfonyl fluoride. PEP carboxykinase activity was assayed in a reaction mixture containing 50 $mmol \cdot L^{-1}$ 3-(*N*-morpholino)propoanesulfonic acid – Tris (pH 7.3), 6 $mmol \cdot L^{-1}$ $MnCl_2$, 1 $mmol \cdot L^{-1}$ EDTA, 10 $mmol \cdot L^{-1}$ $NaHCO_3$, 2.5 $mmol \cdot L^{-1}$ ADP, and crude extract. The reaction was initiated by the addition of 2.5 $mmol \cdot L^{-1}$ PEP, and the PEP- and ADP-dependent decrease in A_{340} was monitored. The extraction buffer was the same as that for PEP carboxylase. Phosphoglycerokinase (PGK) activity was assayed in a reaction mixture containing 100 $mmol \cdot L^{-1}$ Tris – HCl (pH 8.0), 10 $mmol \cdot L^{-1}$ $MgCl_2$, 1 $mmol \cdot L^{-1}$ DTT, 0.2 $mmol \cdot L^{-1}$ NADH, 2 $mmol \cdot L^{-1}$ ATP, 10 $mmol \cdot L^{-1}$ phosphocreatine (P7936, Sigma), 10 units creatine phosphokinase (C3755, Sigma), NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH; G2267, Sigma), and crude extract. The reaction was initiated by the addition of 5 $mmol \cdot L^{-1}$ 3-phosphoglycerate (PGA), and the PGA-dependent decrease in A_{340} was monitored (Macioszek et al. 1990). The extraction buffer contained 100 $mmol \cdot L^{-1}$ Tris–HCl, pH 8.0, 20% (*v*/*v*) glycerol, 10 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ EDTA, 20 $mmol \cdot L^{-1}$ $NaHCO_3$, 5 $mmol \cdot L^{-1}$ DTT, and 1 $mmol \cdot L^{-1}$ phenylmethylsulfonyl fluoride. NADPH-dependent GAPDH activity was assayed in a reaction mixture containing 100 $mmol \cdot L^{-1}$ Tris–HCl, pH 8.0, 10 $mmol \cdot L^{-1}$ $MgCl_2$, 20% (*v*/*v*) glycerol, 1 $mmol \cdot L^{-1}$ DTT, 0.2 $mmol \cdot L^{-1}$ NADPH, 2 $mmol \cdot L^{-1}$ ATP, 10 $mmol \cdot L^{-1}$ phosphocreatine, 10 units creatine phosphokinase, PGK (P7634, Sigma), and crude extract. The reaction was initiated by the addition of 5 $mmol \cdot L^{-1}$ PGA, and the PGA-dependent decrease in A_{340} was monitored (Li et al. 1997). The extraction buffer contained 100 $mmol \cdot L^{-1}$ Tris–HCl, pH 8.0, 20% (*v*/*v*) glycerol, 10 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ EDTA, 20 $mmol \cdot L^{-1}$ $NaHCO_3$, 5 $mmol \cdot L^{-1}$ DTT, and 1 $mmol \cdot L^{-1}$ phenylmethylsulfonyl fluoride was the same as that for PGK. Ribulose-1,5-bisphosphate (RuBP) carboxylase activity was assayed in a reaction mixture containing 100 $mmol \cdot L^{-1}$ Tris–HCl, pH 8.0, 10 $mmol \cdot L^{-1}$ $MgCl_2$, 20 $mmol \cdot L^{-1}$ $NaHCO_3$, 1 $mmol \cdot L^{-1}$ DTT, 0.2 $mmol \cdot L^{-1}$ NADH, 2 $mmol \cdot L^{-1}$ ATP, 10 $mmol \cdot L^{-1}$ phosphocreatine, and crude extract. The reaction mixture was incubated at 25 °C for 10 min, and 2.5 units PGK, 2.5 units GAPDH, 10 units creatine phosphokinase, and 50 units carbonic anhydrase (C3934, Sigma) were added. The reaction was initiated by the addition of 4 $mmol \cdot L^{-1}$ RuBP (R0878, Sigma), and the RuBP-dependent decrease in A_{340} was monitored (Larson et al. 1997). The extraction buffer was the same as that for PGK.

Mating experiments and determination of chlorophyll concentration were performed as described previously (Suzuki et al. 1999).

Results

Genetic analysis of a RPR (reduced rate of photorespiration) mutant N21

A RPR mutant strain, N21, was selected after crossings of 7FR2N (Suzuki 1995) with the CC125-background strains, to minimize the effect of EMS/FdU mutagenesis (Suzuki

Fig. 2. $NaHCO_3$-response curves of photosynthetic O_2 exchange in 5% CO_2-grown cells (closed symbols) and 24-h air (300–350 $\mu L \cdot L^{-1}$ CO_2) adapted cells (open symbols) of *Chlamydomonas reinhardtii* strains N21 (△, ▲), CC125-A (□, ■), CC125-B (◇, ◆), and 2137 (○, ●). The chlorophyll concentration was approximately 10 $\mu g \cdot mL^{-1}$, and the light intensity was 500 μmol photons$\cdot m^{-1} \cdot s^{-1}$.

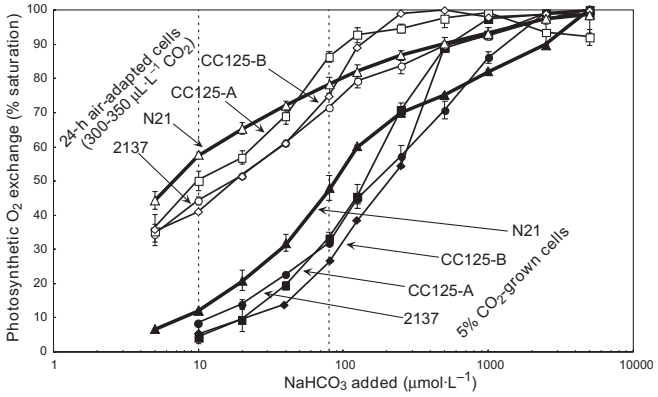
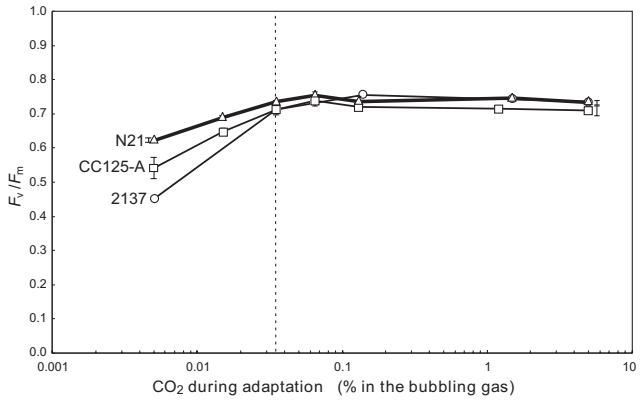
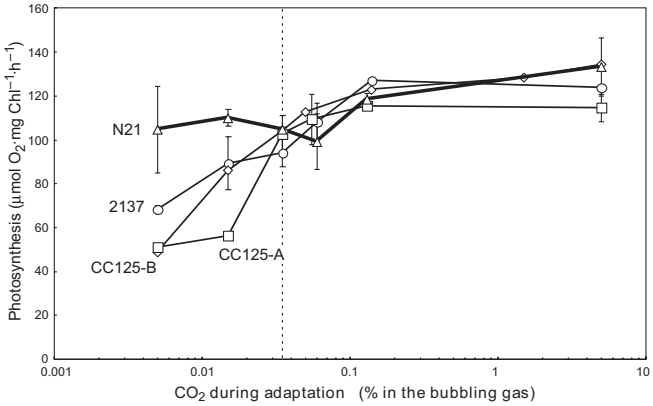


Fig. 3. Effect of adaptation to different concentrations of CO_2 on F_v/F_m in *Chlamydomonas reinhardtii* strains N21 (△), CC125-A (□), and 2137 (○). The chlorophyll concentration was approximately 10 $\mu g \cdot mL^{-1}$.



1995) and to remove *pgp1* mutation. In these crossings, the tetrads obtained always showed a 2:2 segregation in terms of the RPR phenotype assayed by measuring the relative photosynthesis rate at 80 $\mu mol \cdot L^{-1}$ $NaHCO_3$ in 5% CO_2-grown cells. The RPR phenotype was not linked with *pgp1* phenotype (data not shown). Figure 1 shows the phenotypes of a typical tetrad set when N21 was crossed with *pgp1-1*-H74. A 2:2 segregation of wild type and mutant was observed for PGPase activity in 18 tetrad sets tested. In regard to the high CO_2 requirement of the growth, on the other hand, three patterns of segregation of wild type and mutant were observed: 4:0, 3:1, and 2:2. Such segregation was related to the number of the suppressor double mutants, PRP1-*pgp1*, capable of growth under air. The numbers of parental ditype (PD), nonparental ditype (NPD), and tetratype (T) were 4, 3, and 11, respectively, suggesting both mutations were not linked and located at a distance from the centromeres on the different chromosomes involved.

Fig. 4. Effect of adaptation to different concentrations of CO_2 on the rate of photosynthetic O_2 exchange at 5 $mmol \cdot L^{-1}$ $NaHCO_3$ in *Chlamydomonas reinhardtii* strains N21 (△), CC125-A (□), CC125-B (◇), and 2137 (○). The chlorophyll concentration was approximately 10 $\mu g \cdot mL^{-1}$, and the light intensity was 500 μmol photons$\cdot m^{-1} \cdot s^{-1}$.



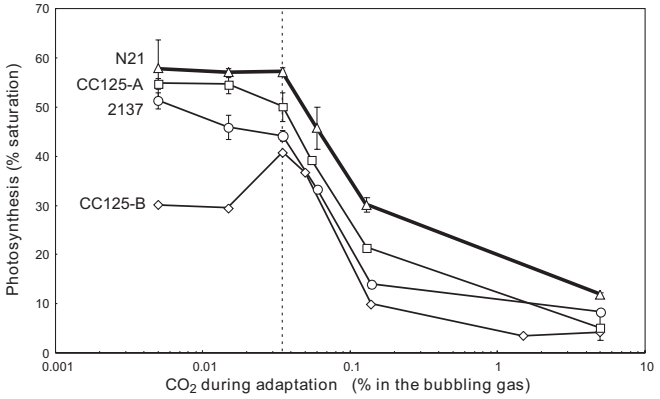
Photosynthetic affinity for inorganic carbon in 24-h air-adapted and 5% CO_2-adapted cells of N21 and wild-type strains

The relative rate (or saturation rate) of photosynthesis at 80 $\mu mol \cdot L^{-1}$ $NaHCO_3$ in 24-h air-adapted cells of N21 was not higher than that of wild-type strains but rather lower than that of CC125-A, a result consistent with that reported previously for the RPR1-*pgp1* double mutant 7FR2N (Suzuki 1995). However, the saturated rate of photosynthesis in 5% CO_2-grown cells was apparently higher than that of wild-type strains (Fig. 2). The relative rate in N21 was higher than that of CC125-B and 2137 in both air-adapted (350 $\mu L \cdot L^{-1}$ CO_2) and 5% CO_2-grown cells at $NaHCO_3$ concentrations lower than 80 $\mu mol \cdot L^{-1}$. The affinity for inorganic carbon was apparently higher in CC125-A than in the other two wild-type strains of air-adapted cells, but was still lower than that of N21 at $NaHCO_3$ concentrations lower than 40 $\mu mol \cdot L^{-1}$. Thus, N21 cells showed higher photosynthetic affinity for CO_2, even in air-adapted cells, especially at very low CO_2 conditions. Moreover, the relative rate of photosynthesis at 10 or 20 $\mu mol \cdot L^{-1}$ $NaHCO_3$ seems useful to distinguish this mutant from others when the cells are adapted to air, and that at 80 $\mu mol \cdot L^{-1}$ seems useful for 5% CO_2-grown cells.

Effect of adaptation to different concentrations of CO_2 on F_v/F_m and maximal rate of photosynthesis

When cells adapted to 300 $\mu L \cdot L^{-1}$ CO_2 or higher, F_v/F_m was almost the same in all strains tested, but decreased when the cells adapted to CO_2 below 300 $\mu L \cdot L^{-1}$. The decrease in F_v/F_m was bigger in wild-type strains than that in N21 (Fig. 3). A similar but clearer difference was observed between N21 and wild-type strains in the maximal rate of photosynthetic O_2 evolution (Fig. 4). The rates were almost the same in N21 when the cells adapted to any CO_2 concentrations from 50 $\mu L \cdot L^{-1}$ to 5%. The rates were almost the same also in CC125-A, CC125-B, and 2137 when the cells were adapted to 300 $\mu L \cdot L^{-1}$ CO_2 or higher, but decreased significantly when the cells adapted to below 300 $\mu L \cdot L^{-1}$

Fig. 5. Effect of adaptation to different concentrations of CO_2 on the relative rate of photosynthetic O_2 exchange at 10 μmol·L^{-1} $NaHCO_3$ in *Chlamydomonas reinhardtii* strains N21 (△), CC125-A (□), CC125-B (◇), and 2137 (○). The relative rate was expressed as % saturation of the rate at 5 mmol·L^{-1} $NaHCO_3$. The chlorophyll concentration was approximately 10 μg·mL^{-1}, and the light intensity was 500 μmol photons·m^{-1}·s^{-1}.



(Fig. 4). Thus, N21 cells do not seem to suffer as much stress as CC125-A or 2137 does when the cells adapted to very low CO_2 such as 50 or 150 μL·L^{-1}.

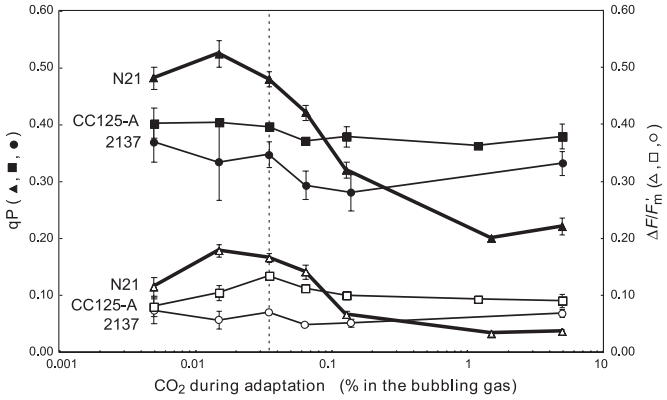
Effect of adaptation to different concentrations of CO_2 on photosynthetic affinity for inorganic carbon

Photosynthetic affinity for CO_2 in N21 was compared using the relative rate of photosynthesis (% saturation) at 10 μmol·L^{-1} $NaHCO_3$ (Fig. 5). The rate in N21 was higher than those in all wild-type strains tested. The rate in CC125-A was close to that in N21 when the cells adapted to 50–150 μL·L^{-1} CO_2, and was higher than those in 2137 and CC125-B. Such high rates in CC125-A, however, likely are an overestimate, as the maximal rate of photosynthesis in CC125-A was only about half of that in N21 in these conditions (Fig. 4).

Effect of adaptation to different concentrations of CO_2 on chlorophyll fluorescence parameters in cells maintained at CO_2 compensation point

Because the difference in photosynthetic affinity for inorganic carbon between N21 and other strains was easier to distinguish at very low CO_2 levels, chlorophyll fluorescence parameters were determined at CO_2 compensation point. Both photochemical quenching (qP) and effective quantum yield of PSII ($\Delta F/F_m'$) changed significantly only in N21 when the cells adapted to different concentrations of CO_2 (Fig. 6). Both were maximal when N21 cells adapted to 300 μL·L^{-1} CO_2, and decreased with increasing or decreasing CO_2 concentrations. Both qP and $\Delta F/F_m'$ in wild-type CC125-A and 2137 were lower than those in N21 when the cells adapted to 50–600 μL·L^{-1} CO_2, and did not change by increasing the CO_2 concentrations to adapt (Fig. 5). On the other hand, any significant differences were not detected among the strains in either qP or $\Delta F/F_m'$ in the presence of 5 mmol·L^{-1} $NaHCO_3$, in both 24-h air (300–350 μL·L^{-1} CO_2)-adapted cells and 5% CO_2-grown cells (data not shown).

Fig. 6. Effect of adaptation to different concentrations of CO_2 on photochemical quenching (qP) and effective quantum yield of PSII ($\Delta F/F_m'$) at the CO_2 compensation point in *Chlamydomonas reinhardtii* strains N21 (△, ▲), CC125-A (□, ■), and 2137 (○, ●). The chlorophyll concentration was approximately 10 μg·mL^{-1}, and the actinic light intensity was 500 μmol photons·m^{-1}·s^{-1}.



NPQ increased in both N21 and CC125-A when the cells adapted to 600 μL·L^{-1} or lower CO_2 concentrations (Fig. 7). Because of the large standard error, the difference between N21 and CC125-A appears not significant, although NPQ appears higher in N21 when the cells adapted to 300 μL·L^{-1} CO_2. Because NPQ increased coincidentally with the increase in photosynthetic affinity for inorganic carbon, measurements of NPQ may provide a good tool to monitor operation of the CCM.

Inorganic carbon accumulation

To evaluate the inorganic carbon accumulation in 24-h air (300–350 μL·L^{-1} CO_2)-adapted cells of N21, the silicon oil-filtration method was employed to measure ^{14}C-inorganic carbon incorporated into cells after a 60-s period of photosynthesis in the presence of $NaH^{14}CO_3$. The ratio of the inorganic and total ^{14}C incorporated in N21 cells was about 50% higher than that in CC125-A, 60 s after the addition of 80 μmol·L^{-1} $NaH^{14}CO_3$ (Table 1). This result is consistent with our results, in which CC125-A showed higher relative rates of photosynthesis at 80 μmol·L^{-1} $NaHCO_3$ than that of N21 (Fig. 2), as the actual concentration of inorganic carbon after 60 s photosynthesis for inorganic carbon accumulation should be lower than 60 μmol·L^{-1}, because of the dense cell concentration (30 μg Chl·mL^{-1}), where the relative rate of photosynthesis in N21 was rather higher than in CC125-A. Thus the results suggest that N21 has the ability to accumulate inorganic carbon much more efficiently than wild type at very low CO_2 conditions.

Enzyme activities

The activities of six major enzymes related to photosynthetic carbon metabolism were compared to determine if these enzymes were directly or indirectly involved in the RPR mutation (Table 2). No significant difference between N21 and wild type was observed, although PEP carboxykinase activity was significantly low in both the 5% CO_2-adapted and 24-h air-adapted cells of CC125-A. Since the activity was almost the same as that in CC125-B when

Table 1. Inorganic carbon accumulation measured using the silicon oil-layer filtration method.

	^{14}C in the cells 60 s after the addition of $NaH^{14}CO_3$		
Strain	Inorganic ^{14}C (nmol)	Total ^{14}C (nmol)	Inorganic/total
N21	2.8±0.5	4.8±0.2	0.57
CC125-A	1.5±0.9	4.1±0.4	0.38
N21/CC125-A	1.9	1.2	1.5

Note: Chlorophyll in the cell suspension was 30 μg·mL^{-1}, $NaHCO_3$ concentration after the addition of $NaH^{14}CO_3$ was 80 μmol·L^{-1}, the duration of $^{14}CO_2$ fixation was 60 s, and the light intensity was 500 μmol photons·m^{-2}·s^{-1}. The values followed by ± standard errors are averages from two separate experiments.

Table 2. Comparison of the activities of six major enzymes related to photosynthesis in *Chlamydomonas reinhardtii* strains N21, CC125-A, CC125-B, and 2137.

		Strain (μmol·mg Chl^{-1}·h^{-1})			
Enzyme	Cell type	CC125-A	CC125-B	2137	N21
RuBPcarboxylase	5% CO_2	123.0±8.2	157.6±3.1	—	133.9±1.8
PEP carboxykinase	5% CO_2	2.8±0.2	17.3±0.6	14.0±2.7	13.4±0.7
	24 h air	2.0±0.4	14.9±0.6	—	7.0±0.7
	AC–dark	28.5	21.0	—	—
PEP carboxylase	5% CO_2	10.1	22.1	—	17.0±0.5
	24 h air	16.8±0.3	16.7±0.9	—	25.3
	AC–dark	19.1	—	—	—
PGK	5% CO_2	2802.5±310.4	3061.9±35.7	—	3268.0±139.0
GAPDH (NADP)	5% CO_2	1476.7±129.6	1476.7±129.6	—	971.5±61.5
PGPase	5% CO_2	31.5	21.5	34.8±0.7	31.5±0.2
	24 h air	—	43.7	49.8±1.0	49.6±2.4

Note: The growth conditions used were: 5% CO_2-grown cells (designated "5% CO_2" in this table), 24-h air-adapted cells after being grown under 5% CO_2 (designated "24 h air"), dark-grown cells in AC medium containing 0.1% (*m*/*v*) sodium acetate (designated "AC–dark"). The values followed by ± standard errors are averages from two or three separate experiments. Other values are averages of two measurements in one experiment. PEP, phosphoenolpyruvate; PGK, phosphoglycerokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the cells were grown in the dark in the presence of 0.1% (*m*/*v*) sodium acetate, the low activity may be caused by a spontaneous mutation in CC125-A that suppress PEP carboxykinase activity under photoautotrophic conditions. It is not clear, however, whether the low PEP carboxykinase activity affects CCM function in CC125-A cells or not.

Discussion

The rate of photorespiration in RPR mutants was about half of that displayed by wild-type strains in both air-adapted and 5% CO_2-grown cells. This was suggested to be a consequence of higher CCM activity (Suzuki et al. 1999) in the RPR mutants. This appears to be the major reason that RPR-*pgp1* suppressor double mutants can grow in air. There was, however, no significant difference between the mutant and wild-type cells in the photosynthetic affinity for inorganic carbon in the air-adapted cells when comparing the relative rates of photosynthesis at 80 μmol·L^{-1} $NaHCO_3$ (P_{80}/P_{5000}; Suzuki et al. 1999). However, the affinity was apparently higher in the mutant than in wild-type cells grown in 5% CO_2-grown cells (Suzuki 1995; Suzuki et al. 1999).

In the present report, we carefully compared the photosynthetic response to inorganic carbon especially at very low concentrations of inorganic carbon, and found that, even when the cells were adapted to limited CO_2, the photosynthetic affinity for inorganic carbon in the RPR mutant N21 was higher than that of wild-type cells at lower than air levels of $NaHCO_3$ (Figs. 2 and 5). Inorganic carbon accumulation also seems higher in N21 in such conditions

Fig. 7. Effect of adaptation to different concentrations of CO_2 on nonphotochemical quenching (NPQ) at the CO_2 compensation point in *Chlamydomonas reinhardtii* strains N21 (△), CC125-A (□), and 2137 (○). The chlorophyll concentration was approximately 10 μg·mL^{-1}, and the actinic light intensity was 500 μmol photons·m^{-1}·s^{-1}.

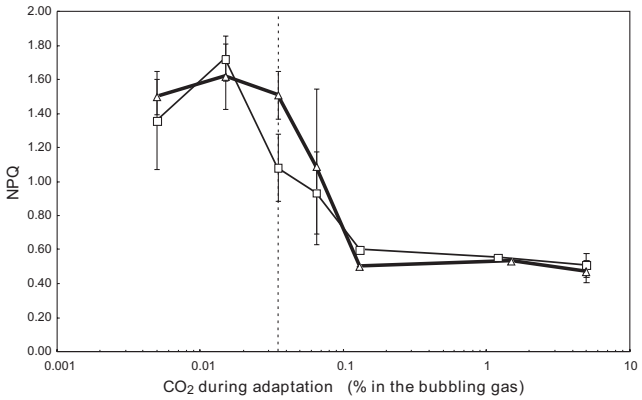
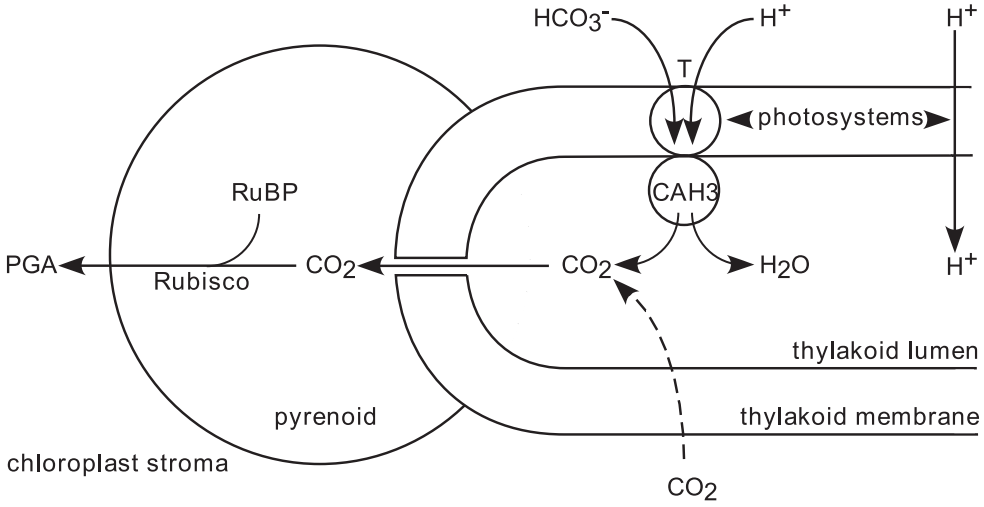


Fig. 8. A scheme for the possible flow of inorganic carbon during photosynthesis at the CO_2 compensation point in the chloroplast in air-adapted cells of *Chlamydomonas reinhardtii*. CAH3, a chloroplast carbonic anhydrase; T, a putative inorganic carbon transporter.



(Table 1). Thus, our results suggest that the reduced rate of photorespiration in N21 is caused by an enhancement of the CCM in the RPR mutation, although the exact nature of the mutation is still not determined. There is a possibility that the mutation caused the decrease in the activation state of RuBP carboxylase, which we have not measured, or the rate of RuBP regeneration, because it was reported that the O_2 sensitivity of photosynthesis decreased when these activities became rate-limiting (Schnyder et al. 1986).

We compared chlorophyll fluorescence parameters of the mutant and wild-type strains, since it is very difficult at the very low concentrations of inorganic carbon to obtain the accurate values by measuring the CO_2-dependent O_2 exchange, $^{14}CO_2$ fixation, or inorganic carbon accumulation. The biggest difference in chlorophyll fluorescence parameters between CC125-A and N21 were observed in qP and the $\Delta F/F_m'$ in the cells adapted to different concentrations of CO_2 at the CO_2 compensation point (Fig. 6). These results suggest that the rate of photosynthetic electron flow through PSII and the following utilization of electrons is higher in N21 than in wild type at the CO_2 compensation point. Under these conditions, cells are adapted to limited CO_2, and there is enhanced operation of the CCM in N21.

In spite of the large standard errors, NPQ at the CO_2 compensation point in N21 appears rather higher than that in CC125-A (Fig. 7). The NPQ increased coincidentally with the increase in photosynthetic affinity for inorganic carbon in both CC125-A and N21, when the cells adapted to different concentrations of CO_2 (Fig. 7). NPQ is thought to depend primarily on a ΔpH across the thylakoid membrane that is generated by photosynthetic electron transport (e.g., Niyogi et al. 1997). The increased ΔpH causes an acidification of lumen that in turn leads to conformational changes in light-harvesting chlorophyll–caretenoid–protein complexes that triggers NPQ (Gilmore et al. 1995, 1996). However, the increase in ΔpH does not appear to explain successfully the increase in NPQ at the CO_2-compensation point after adaptation of cells to limited CO_2, because the difference in NPQ between N21 and wild-type strains was much smaller than that in qP or $\Delta F/F_m'$ (Figs. 6 and 7). Besides such a large ΔpH across the thylakoid membrane expected from NPQ might create a back pressure against the plastquinone pool that results in a decrease in qP.

In the cyanobacterium *Synechococcus* PCC7942, it was reported that a form of qN, another parameter for nonphotochemical quenching, was induced by the addition of inorganic carbon at the CO_2-compensation point (Miller et al. 1996). On the other hand, such a phenomenon was not observed in *C. reinhardtii*. qN and NPQ did not increase, but rather decreased, after the addition of inorganic carbon at the CO_2-compensation point. qN at the CO_2-compensation point was almost saturated at a light intensity of 500 μmol photons·m^{-1}·s^{-1} and showed no significant difference between N21 and CC125-A (data not shown).

The response in nonphotochemical quenching is different in a cyanobacterium and *C. reinhardtii*. However, it is likely in *C. reinhardtii* that the CCM contributes to an increase in the NPQ if, as proposed in Fig. 8, bicarbonate, accumulated in chloroplast stroma by the inorganic carbon transporter(s) located in the chloroplast envelope, is co-transported with protons into the thylakoid lumen, where the transported ions are converted to CO_2 and H_2O by a membrane-bound carbonic anhydrase CAH3 (Karlsson et al. 1998; Hanson et al. 2003) and if the inorganic carbon transport into the thylakoid lumen is the rate-limiting step enhanced by RPR1 mutation. The operation of assumed co-transporter can increase ΔpH, by decreasing the proton concentration in stroma, without affecting that in thylakoid lumen. On the other hand, in our preliminary experiments, we observed a much higher NPQ in the CAH3 mutant, *ca1-1*-12-1C, which cannot utilise the over-accumulated inorganic carbon (Suzuki and Spalding 1989), and a much lower NPQ in *pmp1-1*-16-5K, which cannot accumulate inorganic carbon (Suzuki and Spalding 1989), than that observed in wild-type strains when the cells were adapted 24 h to air containing 300–350 μL·L^{-1} CO_2 (data not shown). Thus, the increase in NPQ seems to be a consequence of increased accumulation of inorganic carbon in the chloroplast under these conditions, consistently with the enhanced photosynthetic electron flow in N21. This form of NPQ might be useful to select

CCM-defective mutants of *Chlamydomonas*, if NPQ can be determined more confidently. Indeed, it seems likely that new CCM mutants may be found among low NPQ mutants (Niyogi et al. 1997).

Our present results suggest that both photosynthetic electron flow and CCM function are more active in N21 cells than that in wild-type strains when the cells adapted to limited CO_2 at the CO_2 compensation point. From this point of view, it is likely that the assumed higher CCM activity in N21 is closely related with higher photosynthetic electron flow. However, it is not clear whether higher electron flow is caused by higher CCM activity, or vice versa. Further investigations will be necessary to more clearly define the RPR mutation in N21.

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