

# Rubisco Activase Is Required for Optimal Photosynthesis in the Green Alga *Chlamydomonas reinhardtii* in a Low-CO<sub>2</sub> Atmosphere<sup>1</sup>

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This report describes a *Chlamydomonas reinhardtii* mutant that lacks Rubisco activase (Rca). Using the *Ble<sup>R</sup>* (bleomycin resistance) gene as a positive selectable marker for nuclear transformation, an insertional mutagenesis screen was performed to select for cells that required a high-CO<sub>2</sub> atmosphere for optimal growth. The DNA flanking the *Ble<sup>R</sup>* insert of one of the high-CO<sub>2</sub>-requiring strains was cloned using thermal asymmetric interlaced-polymerase chain reaction and inverse polymerase chain reaction and sequenced. The flanking sequence matched the *C. reinhardtii* Rca cDNA sequence previously deposited in the National Center for Biotechnology Information database. The loss of a functional *Rca* in the strain was confirmed by the absence of Rca mRNA and protein. The open reading frame for Rca was cloned and expressed in pSL18, a *C. reinhardtii* expression vector conferring paromomycin resistance. This construct partially complemented the mutant phenotype, supporting the hypothesis that the loss of Rca was the reason the mutant grew poorly in a low-CO<sub>2</sub> atmosphere. Sequencing of the *C. reinhardtii* Rca gene revealed that it contains 10 exons ranging in size from 18 to 470 bp. Low-CO<sub>2</sub>-grown *rca1* cultures had a growth rate and maximum rate of photosynthesis 60% of wild-type cells. Results obtained from experiments on a *cia5 rca1* double mutant also suggest that the CO<sub>2</sub>-concentrating mechanism partially compensates for the absence of an active Rca in the green alga *C. reinhardtii*.

The green alga *Chlamydomonas reinhardtii* is an excellent model to study photosynthetic processes. Although it is very difficult to maintain higher plant photosynthetic mutants, *C. reinhardtii* cells that are unable to perform photosynthesis can be grown heterotrophically on acetate. Furthermore, the *C. reinhardtii* nuclear, mitochondrial, and chloroplastic genomes can be genetically manipulated to produce mutant phenotypes (Lefebvre and Silflow, 1999; Rochaix, 2002). A random insertional mutagenesis screen was performed to generate *C. reinhardtii* mutants that were unable to grow optimally in a low-CO<sub>2</sub> atmosphere (Colombo et al., 2002). From the mutants generated, some exhibited a high fluorescence phenotype, whereas others were obligate heterotrophs that died in the light. Approximately one-half of the selected transformants required high CO<sub>2</sub> for optimal growth and grew slowly in a low-CO<sub>2</sub> atmosphere. In the higher plant *Arabidopsis*, Somerville and Ogren (1982) performed a similar screen where they isolated mutants that required high levels of atmospheric CO<sub>2</sub> for growth. Several *Arabidopsis* mutants with defects in photorespiratory carbon and nitrogen metabolism were isolated. One *Arabidopsis*

mutant isolated in that screen exhibited a reduced affinity of the carboxylation reaction for CO<sub>2</sub> and a much lower in vivo activity of ribulose 1,5-bisphosphate (RuBP) carboxylase (Somerville et al., 1982). Later studies determined that Somerville et al. (1982) had isolated a Rubisco activase (Rca) mutant that contained a guanine to adenine transition at the 5' splice junction of intron three (Salvucci et al., 1985; Orozco et al., 1993) in the gene encoding Rca.

Rca catalyzes the activation of Rubisco in vivo by removing inhibitory sugar phosphates (Robinson and Portis, 1988; Portis, 1992, 2003). This enzyme is required to maintain Rubisco activity in higher plants grown at ambient CO<sub>2</sub> concentrations. A substantial reduction (Mate et al., 1993, 1996; Jiang et al., 1994; Hammond et al., 1998) or absence of this protein (Portis, 1992) drastically impairs the photosynthetic process in higher plants. It has been postulated independently by different research groups that the activities of Rubisco and Rca are important key regulation points for photosynthesis under different environmental stress conditions (Hammond et al., 1998; Crafts-Brandner and Salvucci, 2000). Rubisco is activated by the binding of CO<sub>2</sub> to form a carbamate on a Lys residue in the active site and by the binding of Mg<sup>2+</sup> (Portis, 1992). Once activated, Rubisco catalyzes the carboxylation of RuBP to form two molecules of 3-phosphoglycerate or the oxygenation of RuBP to form one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate. Thus, the concentration of CO<sub>2</sub> at the active site of Rubisco is important both to the activation of Rubisco and the carboxylation reaction.

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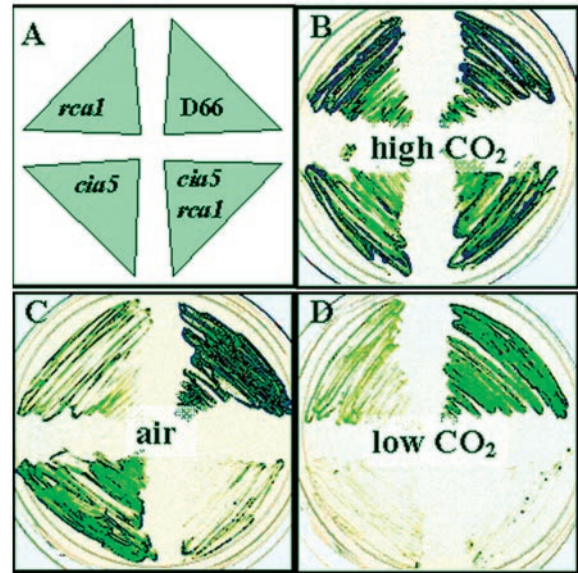
*C. reinhardtii* cells that are grown in air (350 ppm CO<sub>2</sub>) are capable of concentrating CO<sub>2</sub> at the active site of Rubisco. This localized increase in CO<sub>2</sub> concentration allows the algal cell to photosynthesize efficiently at very low levels of external dissolved inorganic carbon (DIC; HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub>). This phenomenon has been termed the CO<sub>2</sub>-concentrating mechanism (CCM; for review, see Kaplan and Reinhold, 1999; Moroney and Somanchi, 1999). The CCM is repressed in cells that are grown in a high-CO<sub>2</sub> atmosphere (1%–5% [v/v] CO<sub>2</sub>). Cyanobacteria also possess a CCM, but it is not clear whether Rca is even present in cyanobacteria. There have been reports of Rca-like activity, yet the cyanobacterial genomes sequenced to date do not appear to have an Rca gene. Therefore, although it is clear that Rca is required for growth in higher plants, it is not clear whether Rca is required for optimal photosynthetic activity in organisms with a CCM.

Recently, several mutants have been isolated that could be defective in one or more components of the CCM (Colombo et al., 2002). These mutants grow more slowly in a low-CO<sub>2</sub> atmosphere but grow normally, in relation to wild-type (WT) cells, in a high-CO<sub>2</sub> atmosphere. In contrast, photosynthesis-deficient mutants exhibit an acetate-requiring phenotype, and many of these mutants must be grown in the dark to avoid chlorophyll (Chl) bleaching caused by elevated levels of photosynthetically generated reactive oxygen species (Rochaix, 1995). This report describes an Rca mutant, *rca1*, in the green alga *C. reinhardtii* that grows normally in a high-CO<sub>2</sub> atmosphere but grows poorly in a low-CO<sub>2</sub> atmosphere. This report also presents data that suggest that although the presence of the CCM partially obviates the need for Rca, in a low-CO<sub>2</sub> atmosphere, *C. reinhardtii* requires both an active CCM and Rca to obtain maximal rates of photosynthesis and growth.

## RESULTS

### *rca1* Has an Insertion in the Gene Encoding Rca

*C. reinhardtii* strain D66 (*nit2*<sup>-</sup>, *cw15*, and *mt*<sup>+</sup>; Schnell and Lefebvre, 1993) was transformed with linearized pSP124s, which contains the modified *Ble*<sup>R</sup> gene (Lumbreras et al., 1998). D66 is a cell wall-deficient strain that exhibits normal photosynthetic characteristics but requires ammonia as a source of nitrogen for growth. Over 42,000 *Ble*<sup>R</sup> insertional mutants were generated and screened for a high-CO<sub>2</sub>-requiring phenotype (Colombo et al., 2002). Figure 1 shows the growth characteristics on minimal media plates in high CO<sub>2</sub> (5% [v/v]; Fig. 1B), air levels (350 ppm CO<sub>2</sub>; Fig. 1C), and low (35 ppm CO<sub>2</sub>; Fig. 1D) CO<sub>2</sub> atmospheres of the WT parent D66, the *rca1* mutant, a CCM-deficient mutant *cia5* (Moroney et al., 1989), and the double mutant *cia5 rca1*. The *cia5* and *rca1* mutants clearly show a reduction in photoautotrophic growth under low-CO<sub>2</sub> conditions com-



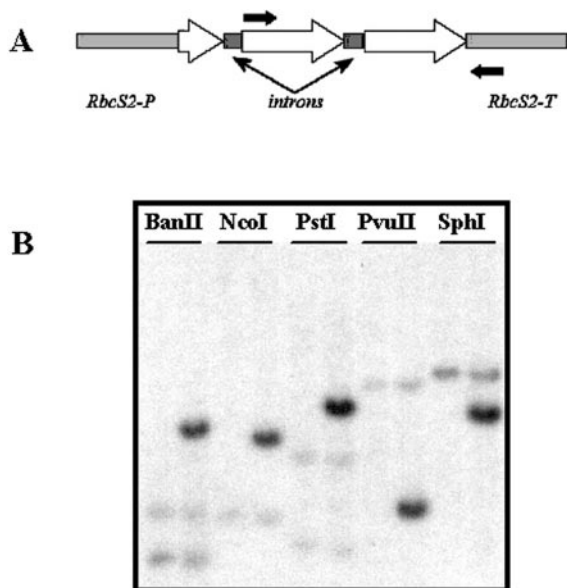
**Figure 1.** Photoautotrophic growth in high CO<sub>2</sub> (5% [v/v]; B), air levels of CO<sub>2</sub> (350 ppm CO<sub>2</sub>; C), and low CO<sub>2</sub> (35 ppm CO<sub>2</sub>; D), for D66, *rca1*, *cia5*, and *cia5 rca1* on solid media (see key in A).

pared with the parental strain D66. The *cia5 rca1* double mutant's growth is severely retarded when the concentration of CO<sub>2</sub> in the environment is at air levels or below.

Southern-blot analysis revealed that a single insertion of the *Ble*<sup>R</sup> cassette was integrated into the genome (Fig. 2). The probe was amplified using PCR and contained the second and third exons of the *Ble*<sup>R</sup> and an intron and 3'-UTR arising from the *C. reinhardtii* *RbcS2* gene (Fig. 2A). The more diffuse bands indicate hybridization of the probe to the endogenous *RbcS2* intron and 3'-UTR that are present in the parent D66 strain and *rca1* (Fig. 2B).

Genetic crosses were performed to determine if the resistance to zeocin was genetically linked to the *rca1* mutation localized in *rca1*. In the cross between *rca1* (*mt*<sup>+</sup>) and the strain *cc124* (*mt*<sup>-</sup>), which grows normally on low-CO<sub>2</sub> concentrations, the segregation of *Ble*<sup>R</sup> resistance showed a ratio of 1:1 in all tetrads examined, and, in all cases, the *Ble*<sup>R</sup> cells required a high-CO<sub>2</sub> atmosphere for optimal growth. In parallel crosses using random spore analysis, all of the *Ble*<sup>R</sup> resistant segregants (approximately 200) exhibited the growth phenotype of *rca1*, further supporting that the pSP124s insertion is the cause of the high-CO<sub>2</sub>-requiring phenotype.

Inverse-PCR and thermal asymmetric interlaced-PCR were used to clone the DNA flanking the *Ble*<sup>R</sup> insert. The sequences of the PCR fragments were identical to the Rca cDNA sequence (Roesler and Ogren, 1990) deposited at the National Center for Biotechnology Information database. The opposite end of the insert was amplified using primers designed to complement the known sequences of the *Ble*<sup>R</sup> insert and the Rca cDNA. Eighty-nine base pairs

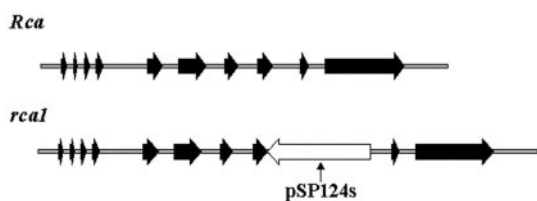


**Figure 2.** Southern-blot analysis of D66 and *rca1* probed with a 588-bp PCR fragment of the *Ble<sup>R</sup>* gene containing two *Ble<sup>R</sup>* exons and part of the *RbcS2* gene. A, Map of the *Ble<sup>R</sup>* portion of the pSP124s plasmid. Bars, *RbcS2*-derived portions of the gene; arrows, coding regions for *Ble<sup>R</sup>*. *RbcS2*-P, *RbcS2* promoter; *RbcS2*-T, *RbcS2* terminator; arrows above and below the map indicate the primers used to make the probe. B, D66 (odd lanes) and *rca1* (even lanes) genomic DNA was digested with the indicated restriction enzymes. The higher intensity bands correspond to the *Ble<sup>R</sup>* insertion, whereas the less intense bands correspond to the endogenous *RbcS2* intron and portion of the *RbcS2* 3'-untranslated region (UTR).

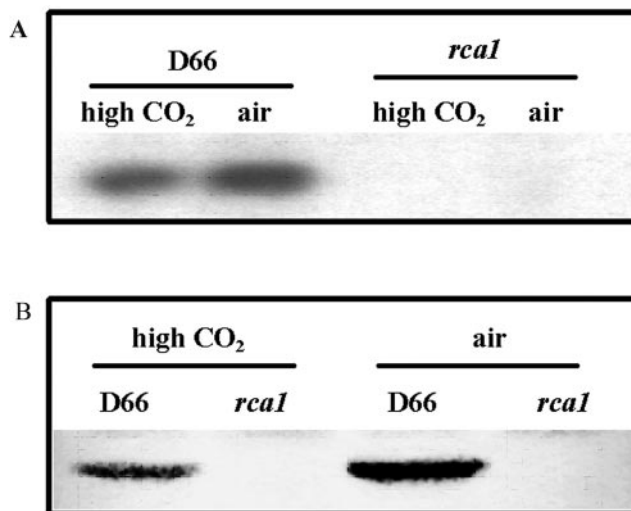
containing the 3' end of exon 8 and the 5' end of intron 8 were deleted upon insertion of the linearized pSP124s. Figure 3 shows the structure of the *Rca* locus and the location of the *Ble<sup>R</sup>* insert in *rca1*. The WT gene was isolated from an indexed cosmid library using a PCR-based technique. The *Rca* gene was completely sequenced and shown to contain 10 exons and nine introns spanning 3 kb (Fig. 3; *Rca*). The deduced structure of the *Rca* gene agrees with the distribution of expressed sequence tags in this region of the *C. reinhardtii* genome (<http://genome.jgi-psf.org/chlre1/chlre1.home.html>; scaffold 1895). In *rca1*, the *Ble<sup>R</sup>* insert was found to be located at the 3' end of exon 8 (Fig. 3; *rca1*).

**Rca Expression in *rca1***

Northern-blot analysis showed that a null mutant for *Rca* had been selected (Fig. 4). In WT cells, a slight



**Figure 3.** Map of the *C. reinhardtii* *Rca* and *rca1* loci. Black arrows, Exons; white arrow, pSP124s insertion.



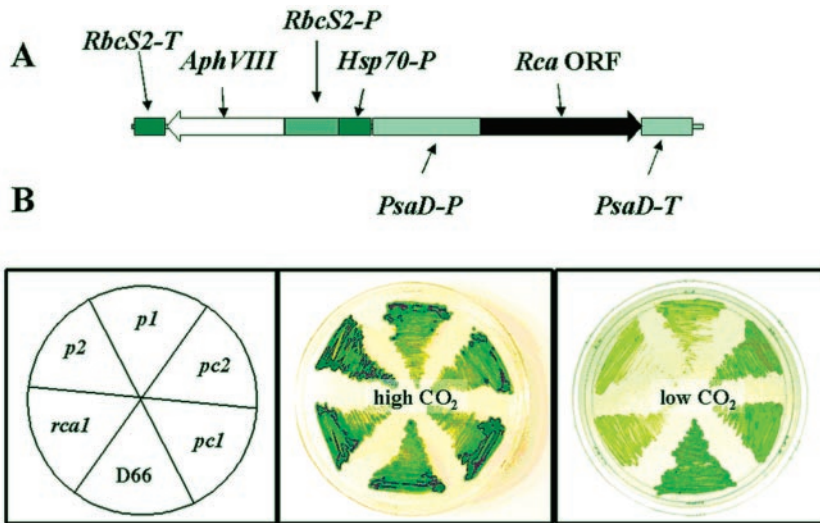
**Figure 4.** Expression of *Rca* in D66 and *rca1* cells. A, Northern-blot analysis of high- $\text{CO}_2$ - and air-grown D66 (WT) cells and *rca1* cells using 10  $\mu\text{g}$  of total RNA per lane and a 1.2-kb *Rca* cDNA PCR product as the probe using a random primer protocol. B, Western-blot analysis of high- $\text{CO}_2$ - and air-grown D66 (WT) cells and *rca1* cells. Cell extracts (50  $\mu\text{g}$  of protein) were probed with a polyclonal antibody raised against *C. reinhardtii* *Rca*. Cells were grown in minimal medium with an atmosphere of 5% (v/v)  $\text{CO}_2$  in air (high  $\text{CO}_2$ ) or 350 ppm  $\text{CO}_2$  (air).

increase in the *Rca* transcript abundance was observed when cells were switched from a high- to low- $\text{CO}_2$  atmosphere (Fig. 4A). No message for *Rca* was detected in the *rca1* mutant (Fig. 4A). Western-blot analysis using polyclonal antibodies raised against *Rca* from *C. reinhardtii* failed to detect the *Rca* protein in the mutant *rca1* cultures (Fig. 4B) and, consistent with the mRNA hybridization results, that the abundance of the *Rca* peptide also increased in the WT cells upon transfer to a low- $\text{CO}_2$  atmosphere. This increase in *Rca* in WT cells under low- $\text{CO}_2$  conditions is in agreement with earlier reports (Rawat and Moroney, 1995; Im et al., 2003).

**Complementation of *rca1***

Attempts to complement the *rca1* strain with the cosmid containing the WT *Rca* gene were unsuccessful. Partial complementation of *rca1* was observed by expressing the *Rca* cDNA under the control of the constitutive *PsaD* promoter and terminator in the vector pSL18 (Fig. 5A; Depege et al., 2003). For this experiment, *rca1* cells were transformed with the *Rca* ORF under the control of the *C. reinhardtii* *PsaD* promoter and terminator linked to a paromomycin resistance cassette. Paromomycin resistance colonies were selected, and a number of these showed improved growth under low  $\text{CO}_2$  as compared with the *rca1* strain (Fig. 5B). Partial complementation was verified by selection on minimal media in 35 ppm  $\text{CO}_2$  in air (Fig. 5B) and confirmed by the reappearance of the 42-kD peptide after transformation (Fig.





**Figure 5.** Partial complementation by the WT *Rca* open reading frame (ORF). A, *rca1* was transformed with a linearized plasmid (pSL18) carrying the paromomycin resistance gene (*AphVIII*) driven by an *RbcS2* and *Hsp70* promoter fusion and the WT *Rca* ORF driven by the *C. reinhardtii* *PsaD* promoter. Colonies resistant to paromomycin were selected, and these transformants were screened on high (5% [v/v]) and low (35ppm CO<sub>2</sub>) CO<sub>2</sub>. Six of 250 (2.5%) of the paromomycin colonies grew on low CO<sub>2</sub> better than the original *rca1* strain but not as well as the WT D66. B, Growth characteristics of D66, *rca1*, two paromomycin-resistant colonies that grew better in low CO<sub>2</sub> (*pc1* and *pc2*), and two colonies that did not show complementation (*p1* and *p2*) on minimal plates in high CO<sub>2</sub> or low CO<sub>2</sub> (35 ppm).

6A). In addition, the strains expressing *Rca* showed rates of photosynthesis that were higher than *rca1*, although not as high as the WT strain (Fig. 6A). The improved growth of the transformants expressing the WT *Rca* protein provides additional support that the poor growth observed in *rca1* is due to the loss of *Rca* (Fig. 6B).

**Pyrenoid Structure**

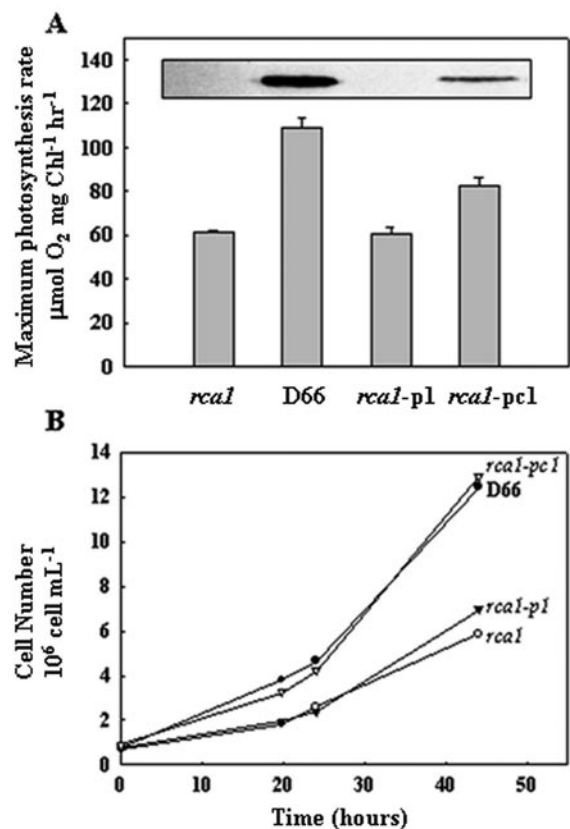
*Rca* is localized to the pyrenoid in *C. reinhardtii* (McKay et al., 1991). The pyrenoid of *rca1* grown overnight at air levels of CO<sub>2</sub> was observed using transmission electron microscopy. No obvious changes with respect to the D66 WT cells were observed in the pyrenoid in mutants cells adapted to a low-CO<sub>2</sub> atmosphere for 12 h (data not shown).

**Characteristics of a *cia5 rca1* Double Mutant**

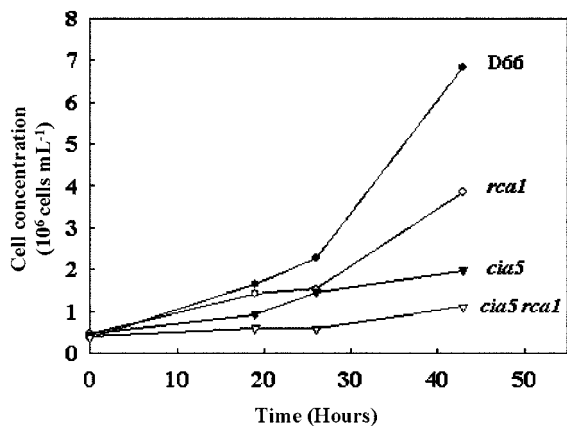
In liquid media bubbled with air levels of CO<sub>2</sub> (0.035% [v/v] CO<sub>2</sub> in air), *rca1* exhibited slower growth rates than the WT D66 (Fig. 7). In higher plants that lack a CCM, an active *Rca* is absolutely required for autotrophic growth in air levels of CO<sub>2</sub> (Somerville et al., 1982). To determine to what extent, if any, the presence of an active CCM compensates for the loss of *Rca*, the CCM-deficient double mutant *cia5 rca1* was constructed. The growth and photosynthetic characteristics of the double mutant were severely reduced as compared with WT or either single mutant (Figs. 1, 7, and 8). Thus, the *cia5 rca1* double mutant phenotype was similar to the *Arabidopsis rca* mutant, suggesting that the CCM does partially compensate for the loss of an active *Rca* in *C. reinhardtii*.

**Photosynthetic Kinetics of *rca1***

Figure 8 shows the rates of photosynthesis versus the DIC concentration for D66, *rca1*, *cia5*, and *cia5*



**Figure 6.** A, Western-blot analysis and maximum rates of photosynthesis of the *rca1* mutant, the WT (D66), and one complemented (*rca1-pc1*) strain. The complemented strain was selected from those colonies shown in Figure 5. An additional paromomycin-resistant strain that did not exhibit complementation (*rca1-p1*) was also tested. Cells were bubbled with air (350 ppm CO<sub>2</sub>) in minimal medium at a constant light intensity of 80 µmol photons m<sup>-2</sup> s<sup>-1</sup>. Error bars = se of two independent experiments. B, Growth curves of the four strains in air levels of CO<sub>2</sub>.



**Figure 7.** Representative experiment measuring growth of D66, *rca1*, *cia5*, and *cia5 rca1* in liquid medium bubbled with air. The cell density is plotted versus time at a constant light intensity of  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

*rca1*. When grown on air levels of  $\text{CO}_2$ , *rca1* exhibited a reduction in the maximum rate of photosynthesis ( $\text{PS}_{\text{max}}$ ) when compared with D66 and *cia5*. After a 6-h acclimation period to low  $\text{CO}_2$ , the apparent affinity of *rca1* for DIC was the same as D66, but the maximum rate of photosynthesis was reduced to 60% of the WT (Fig. 8). In a low- $\text{CO}_2$  atmosphere, the WT cells had a  $\text{PS}_{\text{max}}$  of  $140 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ , *rca1* had a  $\text{PS}_{\text{max}}$  of  $88 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ , *cia5* had a  $\text{PS}_{\text{max}}$  of  $133 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ , and *cia5 rca1* had a  $\text{PS}_{\text{max}}$  of  $17 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ . D66 and *rca1* had similar apparent affinities for DIC, as estimated by the DIC concentration required for half maximal rates of photosynthesis ( $k_{0.5} [\text{DIC}]$ ) suggesting that the CCM was operational in both cell lines. *cia5* and *cia5 rca1* had much lower apparent affinities for DIC, indicating that the CCM was not functioning in these cell lines, consistent with earlier reports (Moroney et al., 1989; Fukuzawa et al., 2001; Xiang et al., 2001).

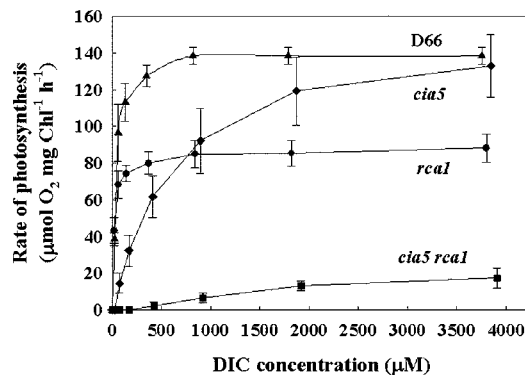
## DISCUSSION

This report characterizes a *C. reinhardtii* mutant that lacks an active Rca. This novel mutant, designated *rca1*, requires a high- $\text{CO}_2$  atmosphere to grow at an optimum rate. This mutant was generated by insertional mutagenesis using the *Ble<sup>R</sup>* gene. Using PCR techniques, the DNA flanking the pSP124s insert was cloned and sequenced. The genomic sequence of the *Rca* gene in *C. reinhardtii* was determined, and this sequence was confirmed by comparison with the first draft of the *C. reinhardtii* genome (<http://genome.jgi-psf.org/chlre1/chlre1.home.html>; scaffold 1895). This sequence also agrees with the *Rca* cDNA sequence (Roesler and Ogren, 1990) except that the 3'-UTR is 512 bp longer than the previously reported *Rca* cDNA. However, the predicted amino acid sequence is identical to that reported previously (Roesler and Ogren, 1990). The *Rca* gene is composed of 10 exons, one of which is only 17

bp long. Conserved splicing sites were located at all of the intron/exon junctions. Another characteristic of the *Rca* locus, shared with several *C. reinhardtii* genes, is the distribution of the largest exons toward the 3'-UTR and the shortest exons toward the 5'-UTR. The *C. reinhardtii* *Rca* was compared with two loci of *Rca* deposited in GenBank from spinach (*Spinacia oleracea*; S45033) and Arabidopsis (M86720). The *Rca* genes in Arabidopsis and spinach have very similar arrangements of their exons and introns. In contrast, the arrangement of exons was completely different in the *Rca* locus of *C. reinhardtii*, suggesting a relatively long evolutionary distance exists between the *Rca* locus of *C. reinhardtii* and the *Rca* loci of higher plants.

Northern and western analyses indicate that *rca1* is a null mutant of Rca because no *Rca* mRNA or Rca protein was detected in the mutant. Genetic analysis and molecular complementation also support the conclusion that the loss of Rca causes a slow growth phenotype in a low- $\text{CO}_2$  atmosphere. If *rca1* was grown on elevated  $\text{CO}_2$  (5% [v/v]  $\text{CO}_2$ ), it had a growth rate indistinguishable from WT cells (Fig. 1) and photosynthesis rates similar to WT cells.  $\text{O}_2$  evolution assays also showed that *rca1* has a WT affinity for DIC. However, a considerable reduction in the maximum capacity to perform photosynthesis is observed when the mutant is grown in air levels of  $\text{CO}_2$  (Fig. 8). Because air-grown *rca1* cultures undergo bleaching of their Chls during long-term growth, it is likely that photoreactive oxygen species are being formed by the inefficient use of energy from excited Chls.

The results in this report suggest that when the  $\text{CO}_2$  level is low and Rca is absent, there is a reduction in the amount of active Rubisco and, thus, a reduction in the maximum attainable rates of photosynthesis. Rubisco can be the limiting step of photosynthesis, and the reduction of the total activity of this protein (physically, by destruction or by inhibition) can cause a reduction in the photosynthetic process. Researchers have reduced the amount of Rca



**Figure 8.** Rates of photosynthesis as a function of the DIC concentration for air grown D66 (▲), *cia5* (◆), *rca1* (●), and *cia5 rca1* (■). Error bars = SE of two or three independent experiments.

in higher plants using antisense silencing. In plants with reduced Rca, a reduction in active Rubisco and photosynthetic capacity has been reported (Mate et al., 1993, 1996; Eckardt et al., 1997; Hammond et al., 1998). The results obtained from *rca1* grown under low-CO<sub>2</sub> conditions are consistent with these earlier reports.

Although *rca1* grown under low CO<sub>2</sub> has reduced photosynthesis, it is still able to grow at air levels of CO<sub>2</sub> in contrast to the original Arabidopsis mutant. An explanation for this difference comes from the observation that a high-CO<sub>2</sub> atmosphere compensates for the loss of Rca in both *C. reinhardtii* (this report) and Arabidopsis (Somerville et al., 1982). *rca1* grown in high CO<sub>2</sub> has rates of photosynthesis and growth that are comparable with those of WT cells (data not shown). These observations suggest that more Rubisco is in its activated form in a high-CO<sub>2</sub> atmosphere even in the absence of Rca. If this were the case, a functional CCM should compensate for the loss of Rca in a low-CO<sub>2</sub> atmosphere.

To test the hypothesis that the CCM partially compensates for the loss of Rca, the *cia5 rca1* double mutant was constructed and analyzed. Growth of the double mutant at air levels of CO<sub>2</sub> was compared with that of WT and the single mutants *cia5* and *rca1*. Both *rca1* and *cia5* can grow on air levels of CO<sub>2</sub> although not WT cells (Fig. 7). In support of the hypothesis that the CCM partially compensates for the loss of Rca, the double mutant did not grow at air levels of CO<sub>2</sub> (Fig. 7) and had a greatly reduced maximal rate of photosynthesis (Fig. 8). Therefore, although an active CCM and a functional Rca are required for maximum rates of photosynthesis, the CCM does compensate partially for the loss of Rca presumably by raising the concentration of CO<sub>2</sub> in the vicinity of Rubisco. Thus, compared with the Arabidopsis *rca* mutant that grows very poorly at air levels of CO<sub>2</sub>, the *C. reinhardtii* mutant is able to sustain appreciable rates of growth in air levels of CO<sub>2</sub> due to the presence of an active CCM. Because the CCM partially compensates for the loss of Rca in an alga with a CCM, a C4 plant deficient in Rca might also be less affected than a C3 plant missing Rca.

The action of Rca should be physically associated with Rubisco. Recently, Borkhsenius et al. (1998) and Morita et al. (1997) have demonstrated that almost 95% of Rubisco is located in the pyrenoid, a characteristic and well-defined structure observed in *C. reinhardtii* chloroplasts when the cells are acclimated to low CO<sub>2</sub>. In addition, McKay et al. (1991) demonstrated that Rca is localized to the pyrenoid in green algae. Therefore, the structure of the pyrenoid in *rca1* was examined to determine whether the pyrenoid structure was disrupted by the loss of Rca. Ultramicroscopic analysis revealed that the pyrenoid structure was unaltered in the *rca1* mutant (data not shown), indicating that Rca is not essential to the

assembly and/or maintenance of the pyrenoid structure.

The *rca1* mutant may be useful in understanding the interaction of Rca with Rubisco. With this mutant, it may be possible to begin to dissect Rca's in vivo function in *C. reinhardtii*. Using site-directed mutagenesis and expression vectors, it may be feasible to manipulate the activity of Rubisco via a genetically modified Rca. In addition, a genetic screen for suppressors and modifiers of the *rca1* mutant phenotype could discover yet uncharacterized genes that may play a central role in the carboxylation reaction of Rubisco.

## MATERIALS AND METHODS

### Strains and Media

*Chlamydomonas reinhardtii* strain D66 (*nit2*<sup>-</sup>, *cw15*, and *mt*<sup>+</sup>) was obtained from Rogene Schnell (University of Arkansas, Little Rock; Schnell and Lefebvre, 1993) and CC124 (*nit1*<sup>-</sup>, *nit2*<sup>-</sup>, and *mt*<sup>-</sup>) from the *C. reinhardtii* Stock Center (Duke University, Durham, NC). The CCM mutant *cia5* (*Cia5* is a putative transcription factor; Fukuzawa et al., 2001; Xiang et al., 2001) was also used in this study because it does not induce the CCM (Moroney et al., 1989). Before transformation, D66 cells were grown in Tris-acetate phosphate (TAP) medium (Sueoka, 1960) under illumination to a density of between 1 and 3 × 10<sup>6</sup> cells mL<sup>-1</sup>. Transformants were selected on TAP plates supplemented with 7.5 μg of zeocin mL<sup>-1</sup> (Invitrogen, Carlsbad, CA). The screening for mutants with phenotype *slc* (sick in low CO<sub>2</sub>) was performed on minimal media (Sueoka, 1960). To determine if the presence of an active CCM could compensate for the loss of Rca, a double mutant was constructed by crossing *rca1* to *cia5* and selecting for the double-mutant progeny. The atmospheric CO<sub>2</sub> levels used in these studies were: high CO<sub>2</sub> (5% [v/v] CO<sub>2</sub> in air), ordinary air (350 ppm CO<sub>2</sub>), and low CO<sub>2</sub> (35 ppm CO<sub>2</sub>). In all cases, the O<sub>2</sub> concentration was 21% (v/v). All strains were maintained axenically on TAP plates.

### *C. reinhardtii* Mutagenesis and Transformation

Strain D66 was mutagenized by introducing the pSP124s plasmid (a gift from Saul Purton, University of London; Lumberas et al., 1998) linearized with the restriction endonuclease *Kpn*I according to the electroporation procedure described by Shimogawara et al. (1998) with modifications described by Colombo et al. (2002).

### Identification of Flanking Regions

Thermal asymmetric interlaced PCR and inverse-PCR were used to obtain the DNA flanking the pSP124s insertion (Liu et al., 1995; Colombo et al., 2002). Homology searches were performed using the BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST>; Altschul et al., 1997), and at the Joint Genome Institute site (<http://aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=chlre1>).

### Nucleic Acid Isolation and Analysis

Total DNA was isolated from mutant cells grown as patches on TAP plates according to Newman et al. (1990; <http://www.biology.duke.edu/chlamy/methods/dna.html>). After digestion with restriction enzymes, DNA (2 μg in each lane) was loaded and separated on a 0.8% (w/v) agarose gel and blotted onto a nylon (Schleicher & Schull, Keene, NH) as described by Sambrook et al. (1989). <sup>32</sup>P-dCTP-labeled probes were prepared using a random primer procedure (Sambrook et al., 1989). Total RNA was isolated from mutant cells grown on TAP liquid media according to the Web site ([http://biosci.cbs.umn.edu/~amundsen/chlamy/methods/RNA\\_prep.html](http://biosci.cbs.umn.edu/~amundsen/chlamy/methods/RNA_prep.html)). Extraction of total RNA and RNA gel-blot analysis were performed by standard procedures (Sambrook et al., 1989). A cosmid containing the full copy of



the gene was isolated from an indexed genomic library using a PCR-based technique.

## Complementation

Partial complementation of *rca1* was observed by expressing the *Rca* ORF under the control of the *PsaD* promoter and terminator in the vector pSL18 (Fig. 6A; Depege et al., 2003; gift from Stephane Lemaire, Université Paris-Sud). pSL18 contains the paromomycin resistance gene under the control of the *C. reinhardtii* *Hsp70* and *RbcS2* promoters (Sizova et al., 2001) linked to the *PsaD* promoter and terminator that can be used to express ORFs in *C. reinhardtii* (Depege et al., 2003). *rca1* was transformed with 1  $\mu$ g of the linearized plasmid using electroporation (Colombo et al., 2002). Transformants were selected on TAP plates containing 10  $\mu$ g mL<sup>-1</sup> paromomycin (Sigma, St. Louis). Partial complementation was verified by the reappearance of the 42-kD peptide recognized by the Rca antibody after transformation and selection on minimal media in 35 ppm CO<sub>2</sub> in air (Fig. 6B).

## Other Methods

Genetic crosses and tetrad analysis were performed as previously described (Sears et al., 1980; Moroney et al., 1986; Harris, 1989). Immunoblots were performed as described earlier (Rawat and Moroney, 1991). Protein extracts were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schull, Keene, NH), and probed with mouse anti-*C. reinhardtii* Rca polyclonal antibodies provided by Archie Portis (University of Illinois, Urbana). The specific protein bands were visualized using a secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). Photosynthesis rates were measured using a Clark-type O<sub>2</sub> electrode as described earlier (Pollock and Colman, 2001). The light intensity used for the photosynthesis measurements was 1,000  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>. Cell density values were determined by direct counting in a hemacytometer chamber. Chl concentrations were estimated using the equations of Holden (1976).

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