Investigations on the CO2 concentrating mechanism of two green algae
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Chapter 4

Identification and localization of a thylakoid bound carbonic anhydrase from the green algae *Tetraedron minimum* (Chlorophyta) and *Chlamydomonas noctigama* (Chlorophyta)

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Abstract

In order to broaden our understanding of the CO₂ concentrating mechanism in eukaryotic phytoplankton we studied the occurrence and localization of a thylakoid-associated carbonic anhydrase (EC 4.2.1.1) in the green algae Tetraedron minimum and Chlamydomonas noctigama. Both algae induce a CO₂ concentrating mechanism when grown under limiting CO₂ conditions. Using mass spectrometric measurements of ¹⁸O exchange from doubly labelled CO₂, the presence of a thylakoid-associated carbonic anhydrase was confirmed for both species. From purified thylakoid membranes the photosystem I (PSI), photosystem II (PSII) and light harvesting complex were isolated by mild detergent gel electrophoresis. The protein fractions were identified by 77 K fluorescence spectroscopy and immunological studies. A polypeptide was found to immunoreact with an antibody raised against thylakoid carbonic anhydrase (CAH3) from Chlamydomonas reinhardtii. It was found that this polypeptide was mainly associated with PSII, although a certain proportion was also connected with the light harvesting complex II. This was confirmed by activity measurements of carbonic anhydrase in isolated bands extracted from the electrophoresis gel. The thylakoid carbonic anhydrase isolated from T. minimum had an isoelectric point between 5.4 and 4.8. Together the results are consistent with the hypothesis that thylakoid carbonic anhydrase resides within the lumen where it is associated with the PSII complex.

Abbreviations: CA = carbonic anhydrase; CCM = CO₂ concentrating mechanism; Ci = inorganic carbon (CO₂ + HCO₃⁻); PSI (II) = photosystem I (II); LHC = light harvesting complex; RC = reaction centre
**Introduction**

Many aquatic photosynthetic micro-organisms, including cyanobacteria and eukaryotic algae, concentrate inorganic carbon (Ci) in response to varying external Ci levels. This CO\textsubscript{2} concentrating mechanism (CCM) has been well studied both on a molecular and on a physiological level in cyanobacteria and eukaryotic unicellular algae (Badger et al. 1980, 1998; Raven 1997; Kaplan and Reinhold 1999). Its function is to increase the partial CO\textsubscript{2} pressure at the CO\textsubscript{2} fixing site of Rubisco in order to ensure efficient CO\textsubscript{2} fixation even when CO\textsubscript{2} concentrations in the surrounding aquatic environment are low.

Numerous evidence indicates that a CCM is the result of a close interaction between Ci transport systems and a compartmentation of Rubisco and carbonic anhydrase (CA; Sültemeyer et al. 1993, 1998; Badger and Price 1994; Bonfil et al. 1998; Klughammer et al. 1999). The eukaryotic algae and cyanobacteria studied so far have the capacity to take up CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} simultaneously from the surrounding medium (Sültemeyer et al. 1993; Palmqvist et al. 1994; Matsuda and Colman 1995) and this appears to be also true for isolated chloroplasts (Amoroso et al. 1998), indicating that an important component of the Ci-transport system is located at the chloroplast envelope in eukaryotic algae. In cyanobacteria, most, if not all, of the cell's Rubisco is located within the carboxysomes and a carboxysomal CA ensures rapid dehydration of accumulated HCO\textsubscript{3} to provide Rubisco with CO\textsubscript{2} (Price at al. 1998; Kaplan and Reinhold 1999). In green algae, a functional homologue of the carboxysomes may be the pyrenoid because it contains major proportion of Rubisco (Morita et al. 1997, 1999; Moroney and Chen 1998). However, so far no direct evidence exists for the presence of a CA within the pyrenoid.

There is physiological and molecular evidence for at least one chloroplastic CA in the green alga *Chlamydomonas reinhardtii* (Karlsson et al. 1995; Amoroso et al. 1996; Funke et al. 1997). Its gene was cloned and the protein identified as a 29,5 kDA polypeptide (CAH3) which might be attached to the lumen side of the thylakoid membranes. CAH3 was shown to be constitutively expressed with only an 1.5 – 2 fold increase after acclimation of the cells to low-Ci. Its exact physiological role is unknown but is has been speculated that it catalyses the formation of CO\textsubscript{2} from HCO\textsubscript{3} within the lumen by using the light-depending accumulation of protons (Pronina and Borodin 1993; Raven 1997). The released CO\textsubscript{2} could diffuse out of the lumen and serve as a substrate for Rubisco.
So far, detailed investigations of this CAH3 have been restricted to the green alga *Chlamydomonas reinhardtii*. In order to broaden our knowledge of the CCM in algae, we investigated whether CAH3-like CA also occurs in other green algae such as *Tetraedron minimum* and *Chlamydomonas noctigama*, which were shown previously to possess an inducible CCM (van Hunnik et al. 2000). We present physiological and immunological data that these species do possess a thylakoid-bound CA which is closely associated with PS II at the lumen side of the thylakoid.
Materials and methods

Algal strains and culture conditions. *Chlamydomonas noctigama* (no. 33.72), and *Tetraedron minimum* (no. 44.81, both from the Sammlung von Algenkulturen, Universität Göttingen, Germany) were grown as described previously (van Hunnik et al. 2000). Cultures were bubbled with air (low-Ci cells) or with air enriched with 5% CO₂ (high-Ci cells).

Isolation of thylakoid membranes. Cells were harvested at the end of the exponential growth phase and broken by potter homogenisation in a Tris-glycine buffer (6.2 mM Tris, 48 mM glycine, pH 7.8) containing 0.3 M sucrose until a final yield of >80% of broken algal cells was obtained. Thylakoid membranes were prepared as previously described (Sukenik et al. 1987) and the photosynthetic complexes were isolated by mild detergent gel electrophoresis, using 7.5% PAGE with 0.05% deoxycholic acid, as described by Sukenik et al. (1992). Contamination of the thylakoid preparation was checked by measuring activities of a marker enzyme for cytosol (phosphoenol pyruvate carboxylase) and mitochondria (succinate dehydrogenase) according to Amoroso et al. (1998). In addition, possible contamination by periplasmic CA, which occurs in *C. noctigama*, was checked with antibodies raised against CAH1, the periplasmic CA from *C. reinhardtii*. The marker enzyme measurements revealed less than 2% contamination by cytosol and mitochondria while no indications for the presence of periplasmic CA were obtained in isolated thylakoids (data not shown).

Protein electrophoresis and immunoblotting. Proteins were separated with SDS-PAGE described previously (Leammli 1970). The proteins were detected by staining the gel with coomassie blue R 350 (Pharmacia). Immunoblotting was preformed as described by the protocol from Bio Rad Laboratories. The antibody against the thylakoid CA was a kind gift of Dr. G. Samuelsson (Umeå University, Sweden).

77 K Spectrum. The bands of interest were cut out from the mild detergent gel and reduced to small pieces. After extraction with 200 μl 20 mM Hepes (pH 8.0) for 30 min at 4°C, the samples were centrifuged in an Eppendorf centrifuge. From the supernatant were sucked into a clear glass pipette, 2 mm diameter, and frozen in liquid nitrogen. The samples were analyzed using an Anminco-Bowman series 2 Luminescence spectrometer equipped with a Dewar flask in the light
beam. The samples were illuminated with a laser with a wavelength of 440 nm, the fluorescence emission was recorded from 650 to 770 nm. For excitation and emission a 4 nm bandwidth was used. The fluorescence emission was corrected for the photomultiplier response according to the instructions of the manufacturer.

**Mono-P column chromatography.** A sample of 0.5 ml of isolated thylakoid membranes was loaded on a mono-P column (Pharmacia). The column was washed, and subsequently the proteins were eluted with a flow of 0.5 ml/min as described in the instructions from Pharmacia. Two runs were performed, from pH 9 to pH 6 and pH 7 to pH 4. Two ml samples were collected and freeze dried. The samples were resuspended in 50 µl distilled water and dialyzed against distilled water. From the samples 10 µl was used for dot blot analysis using thylakoid CA antibodies.

**Measurements of CA activity.** CA activity was measured by mass spectrometry by monitoring $^{18}O$ depletion from doubly labeled CO$_2$ ($^{13}$C$^{18}$O$_2$) at 30°C in a buffer containing 25 mM KCL, 1 mM MgCl$_2$, 0.3 M Sucrose, 30 mM HEPES/KOH, 30 mM, pH 8.0. No detergent was added. The $^{18}O$ exchange was analysed with a quatro pole mass spectrometer (MSD 5970, Hewlett Packard, Waldbronn, Germany) as described by Sültemeyer and Rinastr 1996. Changes of the signals m/z = 45 ($^{13}$C$^{16}$O$_2$), m/z = 47 ($^{13}$C$^{18}$O$^{16}$O) and m/z = 49 ($^{13}$C$^{18}$O$_2$) were recorded and calculated as log enrichment = log (((49)*100)/(45+47+49)) according to Amoroso et al. 1996. This technique allows the detection of compartmented CA (eg. CA within thylakoids) without destroying the membrane structure (Silverman et al. 1976; Silverman 1982) and has been applied to examine internal CA activity in intact cells and chloroplast (Palmqvist et al. 1994; Sültemeyer et al. 1990, 1995; Amoroso et al. 1996) as well as in isolated plasma membranes (Sültemeyer and Rinastr 1996). For routine measurements of CA activity from gel slices a colorometric assay according to Pocker and Stone (1967) was used. The bands of interest were cut from the mild detergent gel, homogenized and resuspended in a veronal buffer (10 mM, pH 8.0) including 0.05% deoxycholic acid (Biomol). The fragments were analyzed spectrophotometrically using p-nitrophenylacetate as a dye (Pocker and Stone, 1967). The method was calibrated using commercial carbonic anhydrase (1mg/ml, 5.800 Wilbur-Anderson-units/mg protein, Sigma).
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Results

In Fig. 1 the changes in the log enrichment of $^{18}$O before and after the addition of isolated thylakoids from low-Ci cells of *T. minimum* and *C. noctigama* are shown. For both preparations three phases can be distinguished. In the absence of thylakoids (phase I) the decrease in log enrichment was rather slow. Addition of thylakoid membranes caused an initial rapid decrease (phase II) which lasted only about 30 seconds. It was followed by a slow change nearly comparable with the curve before the addition of thylakoids (phase III). According to Silverman et al. (1976), phase I is attributed to a non-catalyzed (spontaneous) chemical reaction, phase II is the result of intravesicular CA activity and phase III is caused by non-catalyzed chemical reactions and extravesicular CA activity. Consequently, these data indicate that CA is closely associated with thylakoid membranes of *T. minimum* and *C. noctigama* and that most of it is located within the thylakoids. Note that a higher chlorophyll content was used with thylakoids from *C. noctigama* than from *T. minimum*, indicating lower levels of thylakoid-associated CA in the former cells.

To study the localization of the thylakoid-associated CA in more detail, we decided to separate various components of thylakoid membranes. Gel electrophoresis of thylakoid membranes from high-Ci cells of *T. minimum* and *C. noctigama* under mild denaturing conditions resulted in a number of bands (Fig. 2A) consisting of the complexes of the photosynthetic apparatus (Sukenik et al. 1987). Staining of the gel with coomassie blue did not reveal more bands, indicating that we had only isolated the different parts of the photosynthetic apparatus. The second, fourth, fifth and seventh band were cut out of the gel and analyzed by 77 K fluorescence spectra (Fig. 3). The second band showed a fluorescence maximum at 714 nm and another minor peak at 682 nm which is typical of PSI (Marquadt and Rehm 1995). Band four and five had a maximum at around 680 nm and a small shoulder between the range of 690-700 nm which were reported to be characteristic for PSII (Satoh 1980). In addition, 77K fluorescence analysis of band 7 showed spectra typical for LHC (Marquadt and Rehm 1995). Different subunits of the various parts of the photosynthetic apparatus were also identified by antibodies raised against RC I, for PSI, RCII for PSII and a polyclonal antibody against LHCII (data not shown, Sukenik et al. 1987).
Fig. 1 Changes in the log enrichment $^{18}$O in absence and presence of isolated thylakoids from low-Ci cells of *T. minimum* and *C. noctigama*. Chlorophyll content was 20 µg/ml, *T. minimum*, and 30 µg/ml, *C. noctigama*. For a better comparison the spontaneous decline in the log enrichment has been extrapolated.

Each isolated band from the mild detergent gel was tested for CA activity. When the activities in total thylakoid extracts were compared, CA activity was found to be generally lower in *C. noctigama* than in *T. minimum* (Tab. 1) which is consistent with the data obtained from Fig. 1. The results showed that for both species CA activity was mainly associated with the PSII fraction where 55% (*T. minimum*) and 40% (*C. noctigama*) could be detected. For both species less than 20% of the total CA activity was recovered in the LHC fraction while PSI and free pigments, which consist of pigments not bound to any of the photosynthesis complexes but which are attached to proteins dissociated from the photosynthesis complexes, retained only minor proportions of CA activity. The dominant association of CAH3 with PSII was also confirmed by Western Blot analysis of the mild detergent gel using a CAH3 antibody (Fig. 4). CAH3 binding was confined to the PSII region of *T. minimum* while of *C. noctigama* both PSII and
Fig. 2 A-C Mild detergent gel obtained from thylakoid membranes of high-Ci cells from *Tetraedron minimum* (A), *Chlamydomonas noctigama* (B) and Coomassie stained mild detergent gel from high-Ci cells of *Chlamydomonas noctigama* (C). The bands are labeled from the top to the bottom by increasing numbers.

Fig. 3 Fluorescence spectra at 77K obtained of various bands extracted from the mild detergent from both high-Ci cells from *Tetraedron minimum* and *Chlamydomonas noctigama*. The bands 2, 5 and 7 corresponds to PSI, PSII and LHC as indicated in parentheses, respectively. Labeling of the bands is equivalent to Fig. 2.
Identification and localization of thylakoid CA

**Tetraedron minimum**

**Fluorescence (rel. units)**

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<th>720</th>
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<td>3</td>
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**Chlamdomonas noctigama**

**Fluorescence (rel. units)**

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<tr>
<th>nm</th>
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**Detection of 7 and 2 (PSI)**

**4+5 (PSII)**
Fig. 4 A,B Immuno blot of a mild detergent gel from *T.minimum* (A) and *C.noctigama* (B). Thylakoid preparations were separated on a mild detergent gel as shown in Fig. 2 and polypeptides were subsequently transferred on a nitrocellulose membrane. The immuno signals were obtained by probing the membrane with anti CAH3. Note that the top band showing the most intense signal corresponds to PSII in both algae. For a better comparison the identified protein complexes (Figs. 2, 3) are indicated on the right.

LHC exhibited an immuno reaction. The total signal obtained in *T. minimum* was stronger than the signal obtained in *C. noctigama*. The various bands from the mild detergent gel (Fig.2) were cut out and analyzed by 7.5% SDS-PAGE electrophoresis and Western Blot analysis (Fig. 5). As shown for *T. minimum*, the CAH3 antibody showed an immuno signal in the lane obtained from the PSII band (no. 5), while the antibody showed no cross reaction with polypeptides from the other bands (data not shown). When using the thylakoid preparation directly for Western blot analysis, a much stronger reaction, in the same part of the gel, could be seen in both algae (data not shown). This can be explained by assuming that during each step of the isolation procedure for thylakoids protein is lost.
Identification and localization of thylakoid CA

Fig 5 A,B SDS-PAGE obtained from the bands of the mild detergent gel from *Tetraedron minimum* (A). The bands were cut out of the mild detergent gel, incubated with 10% SDS heated to 70°C and put on top of a 7.5% SDS gel. Lane MW, molecular weight markers in kDa. Lane 1-8 are numbered corresponding to the bands from Fig. 2. (B) Western blot obtained from a 7.5% SDS-PAGE from the PSII fraction obtained from a mild detergent gel from *Tetraedron minimum* incubated with antibodies against CAH3. The arrow indicates the labeled band.

Table 1 Activity measurements of CA in total thylakoid preparations and excised bands 2, 5, 7 and 8 from high-Ci cells of *Tetraedron minimum* and *Chlamydomonas noctigama*. For each fraction the absolute activities are shown, the numbers in parentheses represent the relative activities as compared to the total extract which was applied to the gel. The activity of the total extract was measured in the same detergent concentrations as the bands. The results are the mean values of at least 3 separate measurements with SD smaller than 12%. The photosynthetic components to which the bands correspond are indicated (see also Fig. 2).

<table>
<thead>
<tr>
<th>CA activity (units/fraction)</th>
<th>Tetraedron minimum</th>
<th>Chlamydomonas noctigama</th>
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</thead>
<tbody>
<tr>
<td>Total</td>
<td>158 (100)</td>
<td>86 (100)</td>
</tr>
<tr>
<td>Band 2 (PSI)</td>
<td>3.5 (2.2)</td>
<td>9.5 (11)</td>
</tr>
<tr>
<td>Band 5 (PSII)</td>
<td>86.8 (55)</td>
<td>34.2 (40)</td>
</tr>
<tr>
<td>Band 7 (LHC)</td>
<td>30.1 (19)</td>
<td>16.0 (18.6)</td>
</tr>
<tr>
<td>Band 8 (Free pigments)</td>
<td>3.7 (2.3)</td>
<td>2.6 (3.0)</td>
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</table>
To determine the isoelectric point of the CA, thylakoid membrane extracts of *T. minimum* were analyzed on a mono-P anion exchange column. The column was washed and proteins were eluted by applying a pH gradient, from pH 7.0 to pH 4.0 (Fig. 6A). Fractions were freeze dried, resuspended in 50 µl H₂O and subjected to dot blotting (Fig. 6B). Fractions 27 to 32 gave positive immuno signals with anti CAH3, indicating an isoelectric point between pH 5.4 to 4.8. Similar results were obtained with *C. noctigama* (data not shown).

Fig. 6 A,B Elution profile from pH 7.0 to pH 4.0 (upper curve) of a mono-P column which was loaded with isolated thylakoid membrane from high-Ci cells of *Tetraedron minimum* (A). The protein content was measured photometrically at 280 nm (lower curve in relative absorbance units (AU)). Indicated are the fractions collected which gave a positive signal with the CAH3 antibody in a dot blot. (B) Dot blot from the fractions obtained from the mono-P column incubated with antibodies against the thylakoid CA. The numbers indicate the fractions indicated in Fig. 6A
Identification and localization of thylakoid CA

A

B

29 30 31 27 28
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Discussion

The results obtained from the 77 K fluorescence spectra of the fractions obtained by gel electrophoresis under mild-detergent conditions, and SDS-PAGE of these fractions, clearly indicated that this method resulted in the separation of the two photosystems and LHC. The isolation and subsequent electrophoresis of the thylakoids apparently did not destroy the complexes, allowing detailed study of their composition. SDS-PAGE of the fractions showed that all the different parts of the complexes were present. There were some minor bands in the mild-denaturing gel, which consisted of components from both photosystems, indicating a contamination in these bands. We could demonstrate that there were some slight differences in the composition of each of the complexes of the photosynthetic apparatus between *T. minimum* and *C. noctigama* (Fig. 2) which could be due to species variations.

In this study the localization of the thylakoid CA was investigated by activity measurements and by using a specific antibody, CAH3 (Tab. 1; Figs. 2, 4). The thylakoid CA did not react with antibodies against periplasmic, mitochondrial or bovine CA (data not shown). Using soluble proteins only we could not detect any cross-reaction with CAH3, neither did we see any cross-reactions when using isolated cell membrane (data not shown). Both facts indicate to specific binding of the CAH3 to thylakoid CA in both algae used in this study. Both approaches revealed that most of the CAH3 was located in the PSII complex in both *T. minimum* and *C. noctigama*. The fact that *C. noctigama* contained relatively less CA activity within PSII may indicate either species variations with respect to the distribution of CAH3, or a different chemical association with PSII resulting in a different release from PSII during the extraction procedure. The dominant localization of the CAH3 in PSII in *T. minimum* and *C. noctigama* is consistent with earlier studies from *C. reinhardtii* (Park et al. 1999). In addition to PSII association of CAH3 we presented evidence that CAH3 is located to the lumen side of PSII (Fig. 1).

Mass spectrometric measurements of thylakoid CA activity show a biphasic decrease in log enrichment after the addition of thylakoids. Similar slopes have been obtained with red blood cells (Silverman et al. 1976, Silverman 1982), cells and protoplasts from eukaryotic algae (Tu et al. 1986; Sültemeyer et al. 1990, 1995; Palmqvist et al. 1994; Amoroso et al. 1996), isolated chloroplast from green algae (Amoroso et al. 1996) and CA-loaded plasma membranes from *C.
reinhardtii (Sültemeyer and Rinast 1996). The rapid decline in log enrichment during phase II (Fig. 1) is caused by the loss of label from dissolved CO₂ which diffuses into the lumen space where its ¹⁸O label is exchanged to ¹⁶O due to CA located within the lumen. Interestingly, the slope of phase III is very similar to the spontaneous decline of log enrichment (phase I) indicating that basically no CA activity occurs at the surface of the thylakoids from both *T. minimum* and *C. noctigama*. Consequently, the mass spectrometric measurement of CA activity in thylakoid preparations are the most direct evidence for the almost exclusive presence of CA activity in the lumen. This conclusion is supported by the published sequence of CAH3 from *C. reinhardtii* which contains signal domains that would target the protein to the thylakoid lumen side (Karlsson et al. 1998).

A possible localization within the lumen may also be inferred from the rather acid isoelectrical point of the thylakoid CA from *T. minimum* and *C. noctigama* (Fig. 6), because thylakoids may reach pH values of around 5 during illumination. However, the latter conclusion should be taken with care because from the predicted amino acid sequence an isoelectric point of 9.05 is calculated for CAH3 from *C. reinhardtii* (Karlsson et al. 1998).

The physiological function of the luminal CA within the CCM remains unknown. The possibility that it catalyses the formation of CO₂ from HCO₃⁻ using light accumulated protons has been suggested earlier (Pronina and Borodin 1993) and was theoretically supported recently (Raven 1997). According to these models, HCO₃⁻ is transported into the lumen during illumination and converted to CO₂ which diffuses out of the lumen where it serves as the substrate for Rubisco, thus making the luminal CA the final and most important step within the CCM of micro-algae. Studies using fluorescence techniques on whole cells appear to support this model (Park et al. 1998; Park et al 1999). In contrast, we were unable to detect the predicted light dependent HCO₃⁻ uptake and CO₂ evolution by mass spectrometric analysis of thylakoid preparations from *C. reinhardtii* although the thylakoids exhibited high rates of coupled (to ATP formation) electron flow (van Hunnik and Sültemeyer, unpublished results). This could indicate that CAH3 might be involved in the eukaryotic CCM in other ways than predicted by the models of Pronina (Pronina & Borodin 1993) and Raven (1997).
The close association of CAH3 with PSII may also allow speculation about an alternative function of this enzyme. The protein may be involved in the regulation of electron flow either by controlling PSII activity, protonation of plastoquinone or adjusting a H\(^+\) gradient (Stemler 1997; Moskvin et al. 2000). Support for this hypothesis comes from the observation that even high-Ci-grown cells of *C. reinhardtii* possess luminal CA, although the CCM in these cells is completely suppressed (Badger et al. 1980; Karlsson et al. 1995; Sultemeyer et al. 1995; Amoroso et al. 1996). In addition, luminal CA activity has also been reported for higher C3 plants like pea (Vaklinova et al. 1982; Moskvin et al. 2000), which also lack a CCM. These facts indicate that luminal CA may be involved in other processes than CCM.

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References


Palmqvist K, Yu JW, Badger MR (1994) Carbonic anhydrase activity and inorganic carbon fluxes in low and high Ci of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*; Physiol. Plant 90: 537-547
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