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Disruption of the plastid *ycf10* open reading frame affects uptake of inorganic carbon in the chloroplast of *Chlamydomonas*

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The product of the chloroplast *ycf10* gene has been localized in the inner chloroplast envelope membrane (Sasaki *et al.*, 1993) and found to display sequence homology with the cyanobacterial *CotA* product which is altered in mutants defective in CO₂ transport and proton extrusion (Katoh *et al.*, 1996a,b). In *Chlamydomonas reinhardtii*, *ycf10*, located between the *psbI* and *atpH* genes, encodes a putative hydrophobic protein of 500 residues, which is considerably larger than its higher plant homologue because of a long insertion that separates the conserved N and C termini. Using biolistic transformation, we have disrupted *ycf10* with the chloroplast *aadA* expression cassette and examined the phenotype of the homoplasmic transformants. These were found to grow both photoheterotrophically and photoautotrophically under low light, thereby revealing that the Ycf10 product is not essential for the photosynthetic reactions. However, under high light these transformants did not grow photoautotrophically and barely photoheterotrophically. The increased light sensitivity of the transformants appears to result from a limitation in photochemical energy utilization and/or dissipation which correlates with a greatly diminished photosynthetic response to exogenous (CO₂ + HCO₃⁻), especially under conditions where the chloroplast inorganic carbon transport system is not induced. Mass spectrometric measurements with either whole cells or isolated chloroplasts from the transformants revealed that the CO₂ and HCO₃⁻ uptake systems have a reduced affinity for their substrates. The results suggest the existence of a *ycf10*-dependent system within the plastid envelope which promotes efficient inorganic carbon (Ci) uptake into chloroplasts.

Keywords: *Chlamydomonas reinhardtii*/CO₂ uptake/light sensitivity/mass-spectrometry/*ycf10*

Introduction

The complete DNA sequences of a dozen plastid genomes have been determined and they have revealed the existence

of many open reading frames of unknown function (for review, see Reardon and Price, 1995). Those that are conserved in algae and in higher and lower plants have been designated *ycf* for 'hypothetical chloroplast open reading frame'. The *ycf10* gene codes for a putative polypeptide of 229–231 amino acids. This ORF was located upstream of *petA* (encoding apo-cytochrome *f*) in tobacco (Shinozaki *et al.*, 1986), rice (Hiratsuka *et al.*, 1989) and *Marchantia* (Ohyama *et al.*, 1986). In pea, *ycf10* has been shown to be cotranscribed with *petA* (Willey and Gray, 1990; Nagano *et al.*, 1991). The putative *ycf10* product was first called HBP (for Heme-Binding Protein) because of a short region of homology, around a conserved histidine residue, to the heme-binding domain of two cytochrome *b* polypeptides (Willey and Gray, 1990). The Ycf10 product was immuno-localized in the inner membrane of the pea chloroplast envelope (Sasaki *et al.*, 1993; Gray, 1996). Accordingly, *ycf10* was renamed *cemA* (chloroplast envelope membrane).

Cyanobacterial mutants affected in CO₂ transport have been shown to contain mutations within the *cotA* gene which displays significant sequence identity to *ycf10* (Katoh *et al.*, 1996a). More recently a role for the *cotA* product in light-induced proton extrusion has been proposed (Katoh *et al.*, 1996b). This raises the possibility that Ycf10 may have a role in CO₂ assimilation. To overcome the low affinity of ribulose 1,5-bisphosphate carboxylase (Rubisco) for its substrate, CO₂, green algae like *Chlamydomonas reinhardtii* have developed a carbon concentrating mechanism (CCM) which elevates the CO₂ concentration available to Rubisco in order to favour the carboxylation reaction at the expense of the oxygenase reaction (Badger and Price, 1994). Perhaps the most important components of the CCM are carbonic anhydrase and the transport systems for inorganic carbon (Ci). It has been shown that wild-type cells of *C.reinhardtii* have the capacity to utilize both CO₂ and HCO₃⁻ for photosynthesis (Moroney *et al.*, 1985; Williams and Turpin, 1987; Sültemeyer *et al.*, 1991; Palmqvist *et al.*, 1994). Periplasmic carbonic anhydrase facilitates CO₂ acquisition as it rapidly converts HCO₃⁻ to CO₂ which in turn is then taken up by the cells (Badger and Price, 1994). Besides the presence of external carbonic anhydrase, the presence of different intracellular isozymes has been demonstrated in mitochondria and chloroplasts (Badger and Price, 1994; Husic and Marcus, 1994; Amoroso *et al.*, 1996; Eriksson *et al.*, 1996). These enzymes are induced 4- to 10-fold when cells are cultivated under limiting CO₂ concentrations. However, their exact physiological role is unknown. A cytosolic carbonic anhydrase has been proposed (Sültemeyer *et al.*, 1991; Badger and Price, 1994) but its presence has not yet been conclusively demonstrated. In addition, a Ci transporter in the chloroplast envelope is also induced in cells grown in high-salt-minimum (HSM)

medium with air, but not in cells grown in Tris-acetate-phosphate (TAP) medium or in HSM medium with CO₂-enriched air (Moroney *et al.*, 1987; Sültemeyer *et al.*, 1988). However, it is not known which Ci species is transported into the plastid.

To gain further insights into the function of *ycf10*, we have disrupted this chloroplast gene in *C.reinhardtii*, using biolistic transformation. Here we show that the Ycf10 product is not essential for cell viability and photosynthetic function, provided cells are grown under low light. However, the *ycf10*-deficient mutants are very sensitive to high light, a property which cannot be related to altered chlorophyll, carotenoid or quinone metabolism. Instead, it appears to be a consequence of a limitation in photochemical energy utilization and/or dissipation which correlates with a decrease in CO₂-dependent photosynthesis and a reduced affinity of the CO₂ and HCO₃⁻ uptake systems for their substrates.

Results

Localization and unusual structure of the *ycf10* gene of *C.reinhardtii*

Using a *ycf10* tobacco probe, we mapped *ycf10* by Southern hybridization on the adjacent chloroplast *EcoRI* fragments R7 and R8 of *C.reinhardtii* between *rbcL-atpA-psbI* and *atpH* (cf. Figure 3). While still associated with other photosynthetic genes, the *ycf10* gene of *C.reinhardtii* is found in a different region of the chloroplast genome as compared with higher plants. This result is not surprising, due to the considerable variation in chloroplast genome organization in *C.reinhardtii* compared with land plants (see Boudreau *et al.*, 1994).

The chloroplast DNA region containing *ycf10* was sequenced and the deduced amino acid sequence of the Ycf10 product is shown in Figure 1. The *ycf10* open reading frame is oriented in the same direction as the upstream *atpA* and *psbI* genes and the downstream *atpH* gene (cf. Figure 3). The putative *ycf10* ATG initiation codon is preceded by a 40 nucleotide sequence which is highly AT rich (93%) and which lacks any apparent ribosome binding site. However, only 40% of chloroplast genes examined contain a potential Shine–Dalgarno sequence within the 15 nucleotides upstream of the initiation codon (Bonham-Smith and Bourque, 1989).

The *C.reinhardtii ycf10* open reading frame encodes a putative polypeptide of 500 amino acids with a molecular weight of 57 818, which greatly exceeds the size of its higher plant homologues usually consisting of ~230 residues. The size of Ycf10 of *Marchantia polymorpha* (ORF 434) is closer to that of *C.reinhardtii*. The Ycf10 protein can be divided into several domains based on its sequence conservation in *C.reinhardtii*, *M.polymorpha* and higher plants. The N-terminal 80 amino acid region (IS1 in Figure 2) is very basic in *C.reinhardtii* and followed by a 45 amino acid region, CR0, partially conserved between *C.reinhardtii* and *M.polymorpha* (45% sequence identity and 60% sequence similarity). This N-terminal domain is absent from Ycf10 in higher plants. The two conserved regions, CR1 and CR2 are separated by a poorly conserved domain, IS2, which is considerably larger in *C.reinhardtii* (150 residues, Figure 2). Regions IS1, CR1 and CR2 together contain five hydrophobic domains of

which domains 2, 3 and 5 (indicated by black boxes in Figure 2A) are predicted to be transmembrane segments (Persson and Argos, 1994; Rost *et al.*, 1995). In *C.reinhardtii* and *M.polymorpha* the CR1 regions (68 amino acids) are 50% identical and 54% similar and the CR2 regions (138 amino acids) are 44% identical and 60% similar. Comparison of 10 known Ycf10 protein sequences reveals poor conservation: 3% identity (11% similarity) for CR1 and 14% identity (22% similarity) for CR2 (Figure 2B).

Besides the reported homology between Ycf10 and the *CotA* gene product from *Synechocystis sp.* 6803 (37% sequence identity, 59% sequence similarity; Katoh *et al.*, 1996a), we found that another gene from *Synechocystis* (accession number sl11685) encodes a protein related to Ycf10 (23% sequence identity, 48% sequence similarity, Figure 2B).

The chloroplast *ycf10* gene is not required for cell viability

To investigate the function of *ycf10* in *C.reinhardtii*, this ORF was disrupted independently at two unique sites (*NdeI* within the CR1 domain and *EcoRI* within the CR2 domain, cf. Figures 1 and 3) with the *aadA* expression cassette (Goldschmidt-Clermont, 1991). Since this cassette contains the *rbcL* 3' end which is known to exert a polar effect on transcription and/or RNA accumulation of the downstream region, it was inserted in the antisense direction at the two sites indicated relative to the transcription of *ycf10*. The corresponding constructs were introduced into a wild-type strain of *C.reinhardtii*, using biolistic transformation and selecting for spectinomycin resistance. In transformants 2D and 13A the *aadA* cassette was inserted at the *EcoRI* and *NdeI* sites of *ycf10*, respectively. Hybridization of *PstI*-digested DNA of transformant 2D with an *aadA* probe revealed as expected a 6.8 kbp fragment (Figure 4A). However, further analysis of this transformant revealed loss of *atpA*, *psbI* and part of *ycf10* (Figure 4A and data not shown). This deletion was created through homologous recombination between the 3' *rbcL* end of the *aadA* cassette and the authentic 3' *rbcL* end which are oriented in the same direction 6 kbp apart (Figure 3, data not shown). A similar deletion also occurred in transformant 13A in which additional DNA rearrangements were observed (Figure 4A). Since the homoplasmic transformant 2D was still able to grow on a medium containing acetate (cf. Figure 5), it can be concluded that the entire chloroplast DNA region between *rbcL* and *ycf10* is not essential for cell viability.

To avoid chloroplast DNA rearrangements, another cassette in which *aadA* is driven by the promoter and 5' untranslated region of *psbD*, but lacking a chloroplast termination or processing site, was used for transformation. Transformants T1 and N3-1 were obtained with the cassette inserted at the *EcoRI* site in the same orientation as *ycf10* (Figure 3). Transformants T2 and T3 had the cassette inserted at the *NdeI* site in the same and opposite orientation as *ycf10*, respectively (Figure 3). As shown in Figure 4B hybridization of the *ycf10* probe to *HindIII* digested DNA yields a 1.72 kbp fragment in the wild type. The corresponding fragments of the transformants are 2.45 (T1) and 2.62 kbp (T2 and T3) as expected from a homologous recombination event at the *EcoRI* and *NdeI* sites (including the 0.17 kbp *EcoRI*–*HindIII* deletion in

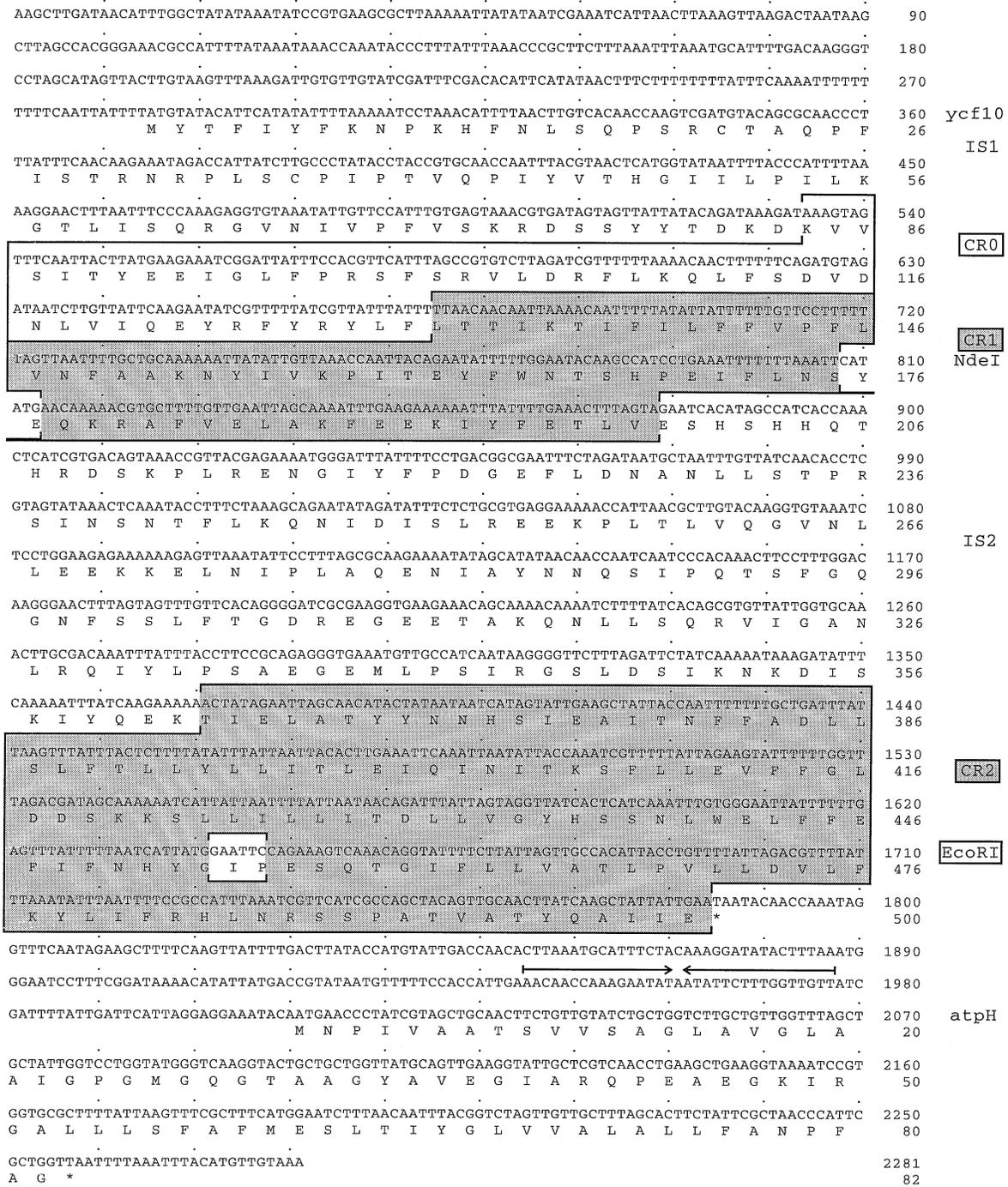


Fig. 1. Nucleotide sequence of the *ycf10* region of the chloroplast DNA of *C.reinhardtii*. The *ycf10* and *atpH* DNA and the deduced amino acid sequences are shown. For Ycf10, the amino acids are numbered from the first putative initiating methionine. The two additional in-frame sequences IS1 and IS2 are indicated as well as the more conserved domains CRO, CR1 and CR2. A 16 nucleotide inverted repeat in the *ycf10-atpH* intergenic region is marked with arrows. The accession number of this sequence in the DDBJ/EMBL/GenBank nucleotide sequence database is X90559.

T1), respectively. Although the *ycf10* and *atpH* genes are oriented in the same direction and separated by only 223 bp, disruption of *ycf10* did not significantly affect the level of *atpH* mRNA (data not shown).

The chloroplast *ycf10* gene is not essential for the photosynthetic reactions

Fluorescence transients of six independently generated *ycf10::aadA* transformants were undistinguishable from those of wild type, thus indicating that photosystem I and

II and the cytochrome *b6f* complex are not significantly affected in these mutants (data not shown).

The growth pattern of the *ycf10* transformants was compared with several control strains including wild type, Fud50, a mutant lacking *atpB*, a control strain (Ct) containing the *aadA* cassette in a region of the chloroplast genome which does not encode any essential or photosynthetic gene and the transformant 2D with a deletion of *ycf10*, *psbI* and *atpA* (see above). These strains were tested under photoheterotrophic conditions (TAP medium),

and photoautotrophic conditions (HSM medium) under high (90 $\mu\text{E}/\text{m}^2/\text{s}$) or dim light (3 $\mu\text{E}/\text{m}^2/\text{s}$) in the presence or absence of spectinomycin (Figure 5). As expected, the control transformant (Ct) grew under all conditions tested, and the wild-type and Fud50 strains did not grow on spectinomycin plates. Fud50 and strain 2D were unable to grow on HSM medium and their growth was impaired in high light on TAP medium because of their photosensitivity (Figure 5), due to the lack of ATP synthase (Woessner *et al.*, 1982). The transformants T1, T2 and T3 grew as fast as wild type and the control strain on TAP medium in dim light. On HSM medium in dim light the growth patterns of the transformants were the same as for wild type, although growth was considerably slower than on TAP medium. Hence no essential photosynthetic function is affected in the transformants lacking functional *ycf10*.

Loss of *ycf10* leads to increased light sensitivity

On TAP medium in high light (90 $\mu\text{E}/\text{m}^2/\text{s}$) growth of the *ycf10* mutants was considerably impaired as compared with wild type and the control strain Ct in the presence of spectinomycin (Figure 5). These mutants appeared to be even more photosensitive than the ATP synthase lacking mutant Fud50. Growth of the *ycf10* mutants was undetectable on HSM medium in high light (Figure 5). Interestingly, the ability of the *ycf10* mutants to grow in HSM medium in high light was partially restored in the presence of spectinomycin.

Since carotenoid deficiency is known to confer light sensitivity, the carotenoid and chlorophyll content of wild-type and the *ycf10*-deficient mutants were determined and compared. No significant difference was detected for wild-type cells grown in dim light or grown in dim light and transferred to high light for 24 h (Table I). Furthermore, we observed no significant difference in carotenoid composition between wild-type and mutant cells grown in dim light. This observation appears to rule out a direct role of

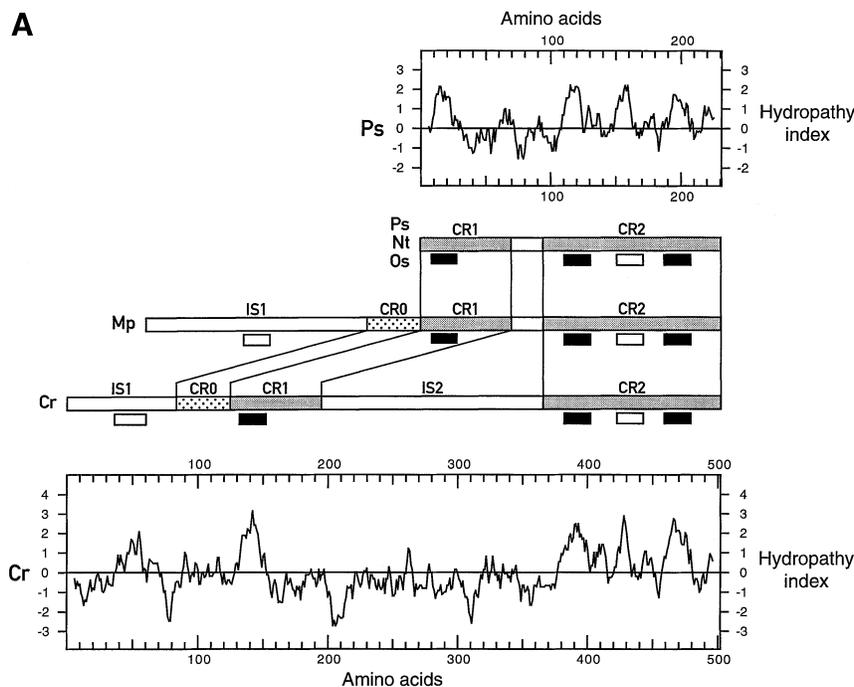
the *ycf10* gene product in the carotenoid biosynthetic pathway.

However, upon transfer to high light, the carotenoid composition was markedly modified in the *ycf10* mutants (Table I). α - and β -carotene were reduced two-fold and lutein was increased >2 -fold in the mutants. Other pigments were observed in mutant cells that were undetectable in wild-type cells grown under the same conditions. In particular one of the major pigments accumulating in high light was identified as zeaxanthin, a pigment directly involved in energy dissipation. Evidence has been presented that one major pathway for zeaxanthin synthesis occurs through hydroxylation of β -carotene via β -cryptoxanthin (Demmig-Adams, 1990). The 50% reduction of β -carotene in the mutant cells (Table I) could be directly correlated to the accumulation of zeaxanthin. The latter was not detectable in wild-type cells grown under the same conditions. It is an excess of light, and not high light *per se*, that induces the increased accumulation of zeaxanthin (for review see Demmig-Adams, 1990). These results suggest that disruption of *ycf10* lowers the threshold level of light perceived as excessive. However, similar changes in carotenoid composition upon exposure to high light as observed for the *ycf10*-deficient strains were also found in FuD50, a mutant strain that is light-sensitive because it lacks ATP synthase (data not shown).

The quinone (phylloquinone, plastoquinone and α -tocopherol) content was not found to be affected in the mutants when compared with wild-type cells (not shown), thus excluding a role for the *ycf10* product in the biosynthesis or regulation of these compounds.

Loss of *ycf10* strongly limits the apparent photosynthetic affinity for CO_2 when mutant cells have reached the CO_2 compensation point

A significant sequence homology has been noticed between Ycf10 and the cyanobacterial CotA protein which has



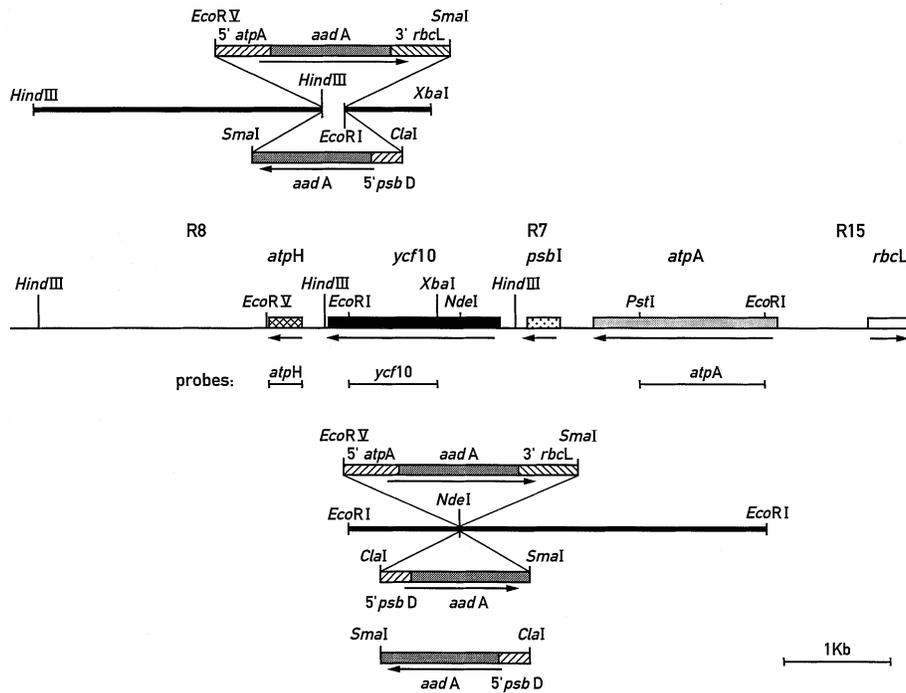


Fig. 3. Disruptions of the *ycf10* gene of *C.reinhardtii*. The map of the region of the chloroplast genome containing *atpH*, *ycf10*, *psbI*, *atpA* and *rbcL* is shown. Except for *rbcL* transcription of the other genes proceeds from right to left. The two *aadA* cassettes used for *ycf10* disruption, 5' *atpA*-*aadA*-3' *rbcL* and 5' *psbD*-*aadA*, were inserted in the orientations shown. The transformants obtained with the corresponding constructs are indicated in the legend of Figure 4.

been shown to be required for CO₂ uptake and proton extrusion (Kato et al., 1996a,b) thus raising the question whether Ycf10 may be involved in CO₂ assimilation. Several mutants of *C.reinhardtii* affected in this process have been shown to be light-sensitive (Spreitzer and Mets, 1981; Spreitzer and Ogren, 1983a).

To test whether the apparent photosynthetic affinity for CO₂ is affected in the *ycf10*-deficient mutants, cells from wild type and the T1 mutant were grown either in HSM medium in dim light or in TAP medium in the dark. Cells were then collected and resuspended in 10 mM HEPES pH 7.15 in an illuminated O₂ electrode chamber (872 μE/m²/s) for 1.5 h to deplete their internal Ci pool ('low Ci' cells). At this stage cells had reached their CO₂ compensation point (i.e. the concentration of CO₂ at which the CO₂ fixed by photosynthesis is equal to the CO₂ released by respiration) which is near zero for *C.reinhardtii* (Spalding et al., 1983b and Figure 6, as deduced from the O₂ evolution curves). CO₂-dependent photosynthetic O₂ evolution of the cells was measured after addition of increasing amounts of Ci. To test for photoinhibition under these conditions, Fv/Fm fluorescence measurements were performed. The T1 mutant was found to be slightly more photoinhibited than wild type as the Fv/Fm value decreased from 0.77 ± 0.02 before, and to 0.37 ± 0.02 after the light treatment whereas the corresponding wild-type values were 0.78 ± 0.03 and 0.56 ± 0.05, respectively.

Figure 6A shows the results obtained with 'low Ci' cells from wild type and the T1 mutant pregrown in TAP medium in the dark and transferred for 36 h in HSM in dim light. It can be seen that while the photosynthetic affinity for Ci was unaffected in the mutant, CO₂-dependent photosynthetic O₂ evolution at saturation was significantly reduced (by 20%) in the mutant as compared with

wild type. Figure 6B shows the results obtained when the cells were pregrown in TAP medium in the dark. Under these conditions, the efficiency for inorganic carbon transport was diminished and the wild-type cells had a lower photosynthetic affinity for Ci. While the rate of light-induced O₂ evolution in wild-type cells increased as Ci was added, no corresponding increase was observed in the T1 mutant cells even when the concentration of the exogenous Ci exceeded 100 times the normal level of Ci (Figure 6B). It should be noted that while O₂ evolution remained unchanged in the mutant cells after addition of exogenous Ci, the photosynthetic and respiratory systems were still functional. Upon addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) the respiration rate of wild-type cells was biphasic with a high initial rate followed by a slower phase (Figure 6A and B). Only the latter was observed with the mutant cells.

Loss of Ycf10 increases the sensitivity of mutant cells to inhibitors of carbonic anhydrase

The data described above suggest that loss of Ycf10 affects the photosynthetic affinity for CO₂. To further confirm these observations, *C.reinhardtii* cells were treated with ethoxzolamide (EZ), a membrane-permeable inhibitor of extracellular and intracellular carbonic anhydrases shown to reduce the apparent photosynthetic affinity for Ci in *C.reinhardtii* (Moroney et al., 1985).

Figure 6D shows the results obtained with cells pregrown in TAP medium in the dark. After reaching the CO₂-compensation point, cells were transferred to the dark. Respiration was active in both wild-type and mutant cells, indicating that the cells did apparently not suffer from the previous light regime. Upon return to the light, wild-type cells photosynthesized and quickly reached the

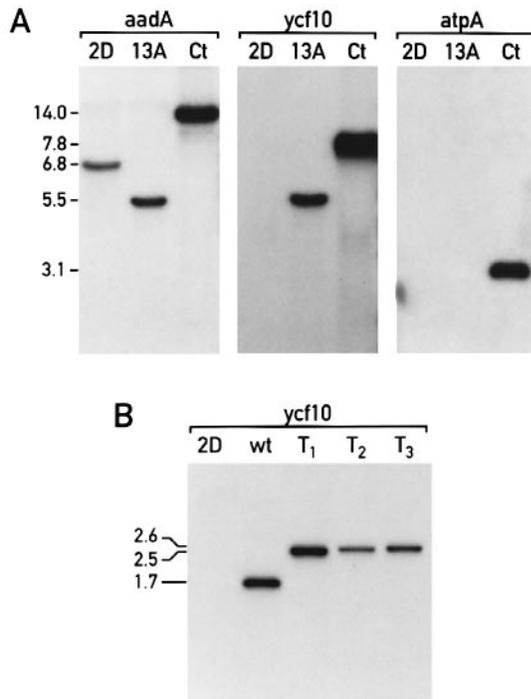


Fig. 4. Southern analysis of the *ycf10::aadA* transformants. (A) Transformants 2D and 13A were obtained with the 5' *atpA*-*aadA*-3' *rbcL* cassette inserted at the *EcoRI* and *NdeI* sites, respectively. Ct is a control transformant in which the *aadA* cassette was inserted near *atpB*. Total DNA was digested with *PstI* and hybridized with the probes *aadA* (0.7 kbp *NcoI*-*SphI* fragment, corresponding to the coding sequences of *aadA*), *ycf10* (0.7 kbp *XbaI*-*EcoRI* fragment of R7 corresponding to part of the *ycf10* coding regions, residues 226-453) and *atpA* (1 kbp *PstI*-*EcoRI* fragment of R7 corresponding to an internal portion of *atpA*). (B) Transformants T1, T2 and T3 were obtained with the 5' *psbD*-*aadA* cassette. Total DNA was digested with *HindIII* and hybridized with the *ycf10* probe. The 1.7 kbp band corresponds to the wild-type DNA and the 2.5 and 2.6 kbp bands correspond to the transformant DNA after integration of the *aadA* cassette in the *EcoRI* (T1 same orientation as *ycf10*) and *NdeI* site (T2, T3, same and opposite orientation as *ycf10*, respectively).

original CO₂-compensation point (Figure 6D). Under the same conditions, mutant cells were also able to photosynthesize although a longer time was needed to reach the compensation point (Figure 6D). This shows that the low O₂ evolution in the T1 mutant observed in Figure 6B is not due to an inhibition of the primary photosynthetic reactions. Addition of EZ (Figure 6D) to wild-type cells altered neither the CO₂-compensation point nor the response to addition of exogenous Ci. In contrast, addition of EZ to mutant cells (Figure 6D) led to an immediate reduction of photosynthetic activity that was not relieved by addition of exogenous Ci.

Similar patterns were obtained with cells pregrown in HSM medium under dim light (Figure 6C). In this case the effect of the carbonic anhydrase inhibitor EZ was even more pronounced since under these conditions the mutant cells still had the capacity to photosynthesize when exogenous Ci was added in the absence of EZ (Figure 6A). After addition of EZ, photosynthesis was almost completely abolished, and even addition of large amounts of exogenous Ci could not relieve this inhibition (Figure 6C).

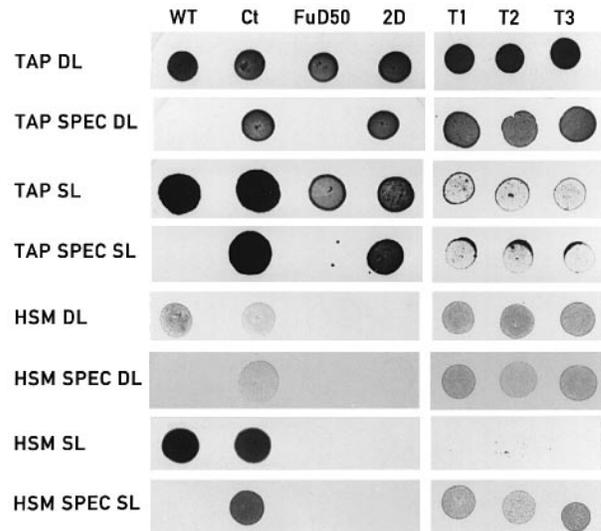


Fig. 5. Growth patterns of the *ycf10*-deficient transformants. Equal number of cells were plated either on TAP medium (photoheterotrophic growth) or on HSM medium (photoautotrophic growth) in the presence or absence of 150 µg/ml spectinomycin (spec). Plates were photographed after 10 days of growth under strong light (SL; 90 µE/m²/s) or dim light (DL; 3 µE/m²/s). HSM plates exposed to dim light were photographed after 18 days. Controls included cells from wild type (WT); Ct; T FuD50 lacking ATP synthase and transformant 2D.

Table I. Pigment composition of wild-type and *ycf10*-deficient mutant cells

	Wild-type DL	Mutants DL	Wild-type SL 24 h	Mutants SL 24 h
Chla/Chlb	2.44	2.59	2.42	2.31
Car/Chla+b	0.27	0.26	0.20	0.22
Carotenoid composition (% of total carotenoids):				
α- and β-carotene	40.5	42.1	36.5	20.4
Lutein	18.6	15.8	20.0	35.6
Violaxanthin	12.6	11.0	10.0	8.7
Loroxanthin	15.3	13.6	8.4	4.8
Neoxanthin	8.9	10.1	21.0	13.0
Zeaxanthin/Cryptoxant.	n.d.	n.d.	n.d.	10.0
Others	4.6	4.5	4.2	6.9

Chla/b, chlorophyll a, b; Car, carotenoids. The values presented for the mutants correspond to the average obtained from the three *ycf10::aadA* transformants (T1, T2 and T3). The cultures (400 ml) were grown in dim light (DL; 2 µE/m²/s) until the mid-log phase (2 × 10⁶ cells/ml). Half of the culture was kept in dim light and the other half was transferred to high light (SL: 90 µE/m²/s) for 24 h. n.d., not detected. Chlorophyll content per 10⁶ cells was 4.13 µg for wild type and 3.7 ± 0.3 µg for the three mutants. Carotenoid content per 10⁶ cells was 1.01 µg for wild type and 1.03 ± 0.1 µg for the mutants. Light treatment did not affect these values significantly.

Mass spectrometric measurements of CO₂ and HCO₃⁻ uptake in intact cells and isolated chloroplasts

The experiments described above show that Ci-dependent oxygen evolution in intact cells is severely reduced in the *ycf10*-deficient strains grown in TAP medium once cells have reached the CO₂ compensation point (Figure 6B). These measurements provide only an indirect estimate of Ci uptake. To obtain more insights into this process we used a recently developed mass spectrometric disequilibrium technique which allows the quantitative determination of

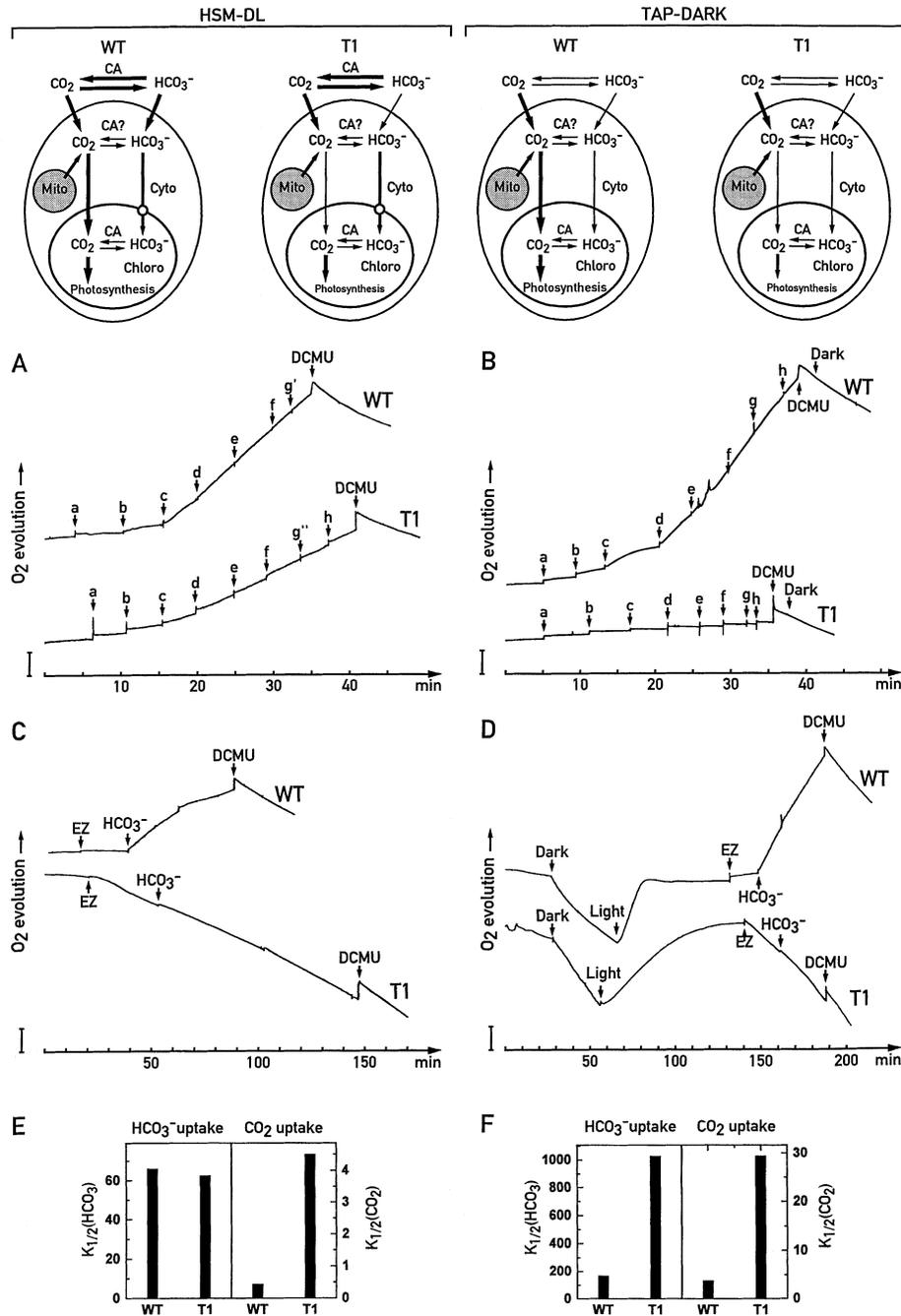


Fig. 6. Upper part: scheme of inorganic carbon fluxes in the wild-type and the *ycf10*-deficient T1 mutant of *C.reinhardtii* grown on HSM or TAP medium. Under the former growth conditions, a bicarbonate transporter is induced in the chloroplast envelope. The periplasmic, putative cytosolic and chloroplast carbonic anhydrases (CA) are indicated (Moroney *et al.*, 1987; Husic *et al.*, 1989). Chlo, chloroplast; Mito, mitochondria; Cyto, cytosol. Lower part: response of photosynthesis to external $[\text{CO}_2 + \text{HCO}_3^-]$ in the wild-type (WT) and *ycf10*-deficient mutant (T1) cells pregrown in HSM in dim light ($3 \mu\text{E}/\text{m}^2/\text{s}$, left panels) or TAP in the dark (right panels). Prior to all measurements, cells were transferred into 10 mM HEPES pH 7.15 for 90 min in high light ($872 \mu\text{E}/\text{m}^2/\text{s}$) until cessation of O_2 evolution. In panels A–D photosynthetic oxygen evolution was measured continuously with increasing amounts of C_i which was added in the form of NaHCO_3 (a, 0.25 nmol; b, 2.5 nmol; c, 12.5 nmol; d, 25 nmol; e, 62.5 nmol; f, 187.5 nmol; g, 1.25 μmol ; g', 1.25 μmol ; h, 2.5 μmol). (A) Cells pregrown in HSM medium. The chlorophyll concentration for wild-type and T1 cells was 4.5 and 4 $\mu\text{g}/\text{ml}$, respectively. DCMU was added to a final concentration of 5 μM . (B) Same as (A), except that the cells were pregrown in TAP medium. The chlorophyll concentration for the wild-type and T1 cells was 5.7 and 5.4 $\mu\text{g}/\text{ml}$, respectively. (C) Effect of EZ: cells pregrown in HSM medium. After cessation of O_2 evolution, the cells were transferred to the dark and later to the light as indicated. EZ (at a final concentration of 100 μM), HCO_3^- (2.5 mM) and DCMU (5 μM) were added as shown. The chlorophyll concentration for the wild-type and T1 cells was 5 and 4.4 $\mu\text{g}/\text{ml}$, respectively. (D) Same as in C for cells pregrown in TAP medium. The chlorophyll concentration for the wild-type and T1 cells was 5 and 4 $\mu\text{g}/\text{ml}$, respectively. The vertical scale represents 20 nmol O_2 . (E and F) Estimation of the affinities ($K_{1/2}$) for HCO_3^- and CO_2 from mass-spectrometric measurements of HCO_3^- and CO_2 uptake in cells grown in HSM (E) or TAP (F) medium. The $K_{1/2}$ values were determined from response curves similar to those shown in Figure 7.

Table II. Values for $K_{1/2}(\text{Ci})$ (μM) for O_2 evolution, HCO_3^- and CO_2 uptake in cells and chloroplasts from wild-type and the *ycf10*-deficient mutant

		Cells							
		Wild-type				Mutant			
		TAP	TAP ^a	HSM	HSM ^a	TAP	TAP ^a	HSM	HSM ^a
O_2 evolution	$K_{1/2}(\text{Ci})$	230 ± 37	211 ± 25	738 ± 8.0	62.8 ± 6.1	1642 ± 192	1512 ± 211	3300 ± 382	48.6 ± 5.2
HCO_3^- uptake	$K_{1/2}(\text{HCO}_3^-)$	168 ± 21	179 ± 17	48 ± 6.0	66.1 ± 8.2	1133 ± 186	1089 ± 153	1970 ± 232	62.6 ± 6.8
CO_2 uptake	$K_{1/2}(\text{CO}_2)$	3.8 ± 0.2	4.5 ± 0.3	2.5 ± 0.3	0.43 ± 0.05	32 ± 3.9	28 ± 4	37.4 ± 5.2	4.5 ± 0.6

		Chloroplasts	
		Wild-type	Mutant
		TAP	TAP
O_2 evolution	$K_{1/2}(\text{Ci})$	457 ± 71	3731 ± 839
HCO_3^- uptake	$K_{1/2}(\text{HCO}_3^-)$	453 ± 64	2481 ± 771
CO_2 uptake	$K_{1/2}(\text{CO}_2)$	6.7 ± 0.8	34.3 ± 5.3

^aRefers to cells preilluminated for 90 min at $870 \mu\text{E}/\text{m}^2/\text{s}$ to deplete their internal Ci pool ('low Ci' cells, cf. text).

The numbers ± SD represent mean values from three separate experiments. Similar values for $K_{1/2}$ were obtained for the wild-type walled and cell-wall-deficient cw-15 strains with intact cells. Similar values for $K_{1/2}$ were also obtained for the *ycf10*-deficient walled T1 and T3 and the cell-wall- and *ycf10*-deficient N3-1 mutant strains with intact cells. Chloroplasts were isolated from cw-15 and N3-1 cells grown in TAP medium.

O_2 and HCO_3^- uptake in suspensions of aquatic microorganisms or isolated chloroplasts (Badger *et al.*, 1994). When cells grown in HSM were used, the values for $K_{1/2}(\text{HCO}_3^-)$ were similar in wild type and T1 indicating that the mutant has the ability to fully induce the high-affinity HCO_3^- transporter (Figure 6E). There is also a significant induction of CO_2 uptake in phototrophically grown mutant cells although the affinity for CO_2 is 10-fold lower than in wild type (Figure 6E and F, and Table II). This induction of CCM in the mutant is not caused directly by the preillumination since no increase in affinity was observed when the preillumination was performed with cells in TAP- rather than in acetate-free medium (data not shown). The substrate affinities were reduced for cells grown under mixotrophic conditions. However, this decrease was considerably more pronounced in the mutant (Figure 6F). It is noteworthy to mention that similar uptake characteristics were observed with other *ycf10*-deficient mutants such as T3 and N3-1.

Figures 7 and 8 show the estimated uptake of CO_2 and HCO_3^- concomitantly with O_2 evolution as a function of external Ci concentration for whole cells and isolated chloroplasts (for details see Materials and methods). Cell wall-deficient strains containing either the wild-type (cw15) or disrupted *ycf10* gene (N3-1) were used to facilitate the isolation of intact chloroplasts. Cells were grown in TAP medium under low light ($25 \mu\text{E}/\text{m}^2/\text{s}$) and transferred to the dark for 12 h before the measurements ('high Ci' cells). It can be seen that wild-type cells (cw15) have the ability to take up both Ci species, but predominantly HCO_3^- (Figures 7 and 8). In contrast, HCO_3^- transport activity is markedly reduced in the mutant N3-1 and CO_2 is the major Ci species taken up. The CO_2 uptake and O_2 evolution curves from the mutant can be nearly superimposed (Figure 8). Although the mutant cells are able to transport HCO_3^- , it appears that all of the CO_2 fixed by photosynthesis can be accounted for by the CO_2 taken up. This uptake system has a low affinity for CO_2 which is similar to that of Rubisco ($K_m \sim 29 \mu\text{M}$, Chen

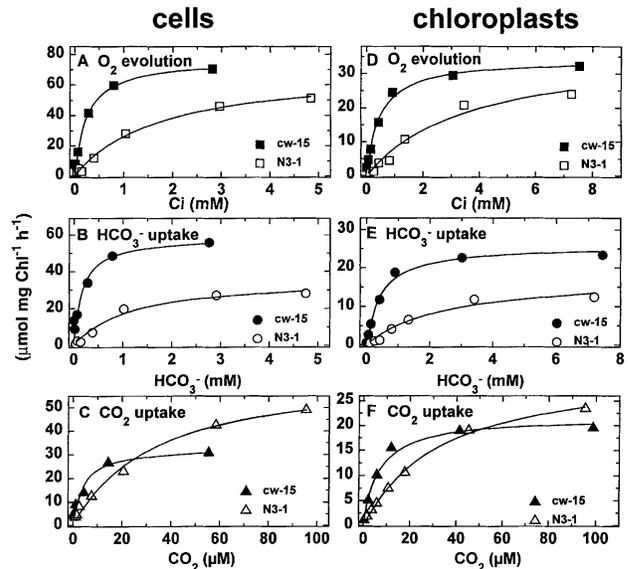


Fig. 7. Substrate dependence of O_2 evolution, HCO_3^- and CO_2 uptake in intact cells and chloroplasts. The cell wall-deficient strain and the N3-1 mutant (CW15, *ycf10::aadA*) were used. Cells were grown in TAP medium under low light ($25 \mu\text{E}/\text{m}^2/\text{s}$) and transferred to the dark for 12 h. For measurements with whole cells, cells were centrifuged and resuspended in 25 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP)-HCl, pH 8.0 at 25°C under strong light ($850 \mu\text{E}/\text{m}^2/\text{s}$). Chlorophyll concentrations ranged between 8 and $15 \mu\text{g}/\text{ml}$. For measurements with chloroplasts, plastids were resuspended in assay buffer containing 25 μM BTP-HCl, pH 8.0, 300 mM mannitol, 2 mM MgCl_2 , 0.2 mM KH_2PO_4 , 25 mM KCl and 2 mM PGA. Illumination was $850 \mu\text{E}/\text{m}^2/\text{s}$ and chlorophyll concentration ranged between 15 and $30 \mu\text{g}/\text{ml}$.

et al., 1988), suggesting that CO_2 uptake in the mutant is driven by Rubisco acting as a sink. A striking feature is that the CO_2 uptake in the mutant is lower than in wild type at low concentrations of CO_2 , but exceeds that of wild type at CO_2 concentrations above $30 \mu\text{M}$ (Figure 7C). These Ci response curves from 'high Ci' cells indicate that the affinities for HCO_3^- and CO_2 are reduced 7- to

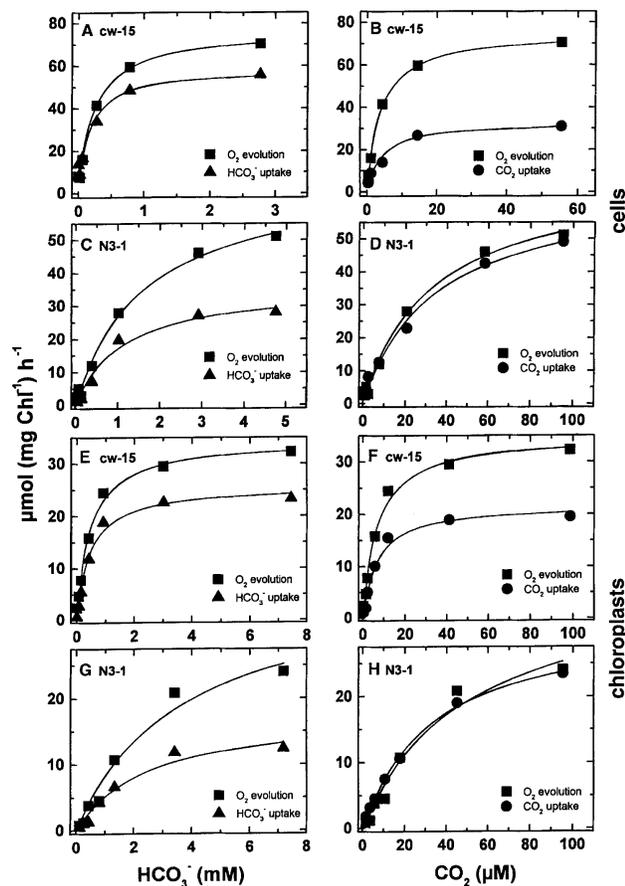


Fig. 8. Comparison of O_2 evolution with Ci uptake with intact cells and chloroplasts. The data of Figure 7 have been redrawn for comparing O_2 evolution with inorganic carbon uptake. Panels A, B, C and D refer to whole cells, panels E, F, G and H refer to isolated chloroplasts.

8-fold in the *ycf10*-deficient mutant as compared with wild type (Table II). Table II shows that while, as expected, there is an induction of the HCO_3^- transport activity and, to a lesser extent, of the CO_2 uptake system in wild-type 'high Ci' cells grown in HSM, no increase in Ci affinity occurs in the mutant cells grown under the same conditions in marked contrast to 'low Ci' mutant cells (compare TAP^a/HSM^a in Table II).

Efficient Ci-dependent oxygen evolution was observed with isolated chloroplasts from wild-type and, to a lesser degree, from mutant cells (Figure 7D). HCO_3^- and CO_2 uptake were very similar to those obtained with intact cells except that the measured fluxes were reduced ~2-fold and that the contributions of HCO_3^- and CO_2 were more balanced for wild-type chloroplasts. An important finding is the significant decrease in affinity for CO_2 in the mutant as compared with wild type (Figure 7F and Table II). This points to the existence of an additional component of CO_2 uptake in wild-type chloroplasts that cannot be explained by simple diffusion.

Mutants of *C.reinhardtii* affected in one of the components of the CCM can be rescued on HSM medium by aeration with CO_2 -enriched air (Spalding *et al.*, 1983a,b; Moroney *et al.*, 1989). If Ycf10 is involved directly in the Ci concentrating process one would expect that the high light sensitivity of the *ycf10*-deficient mutant would

also be overcome by growing the mutant cells in high CO_2 . However, contrary to this expectation, the mutant remained light-sensitive even when aerated with 5% CO_2 -air.

Discussion

Unusual size of *ycf10* from *C.reinhardtii*

The *ycf10* gene has been identified in all chloroplast genomes examined, with exception of *Euglena* (Hallick *et al.*, 1993). Its sequence is considerably less conserved than the sequences of chloroplast genes encoding subunits of photosynthetic complexes. This suggests that Ycf10 has been subjected to lower evolutionary constraints. The C-terminal 140 residues of Ycf10 are the most conserved and could form two to three transmembrane domains (CR2 in Figures 1 and 2a). In *C.reinhardtii* the other less conserved regions CR0 and CR1 are flanked by two segments IS1 and IS2 of 80 and 165 amino acids respectively, that are poorly conserved or absent from the other *ycf10* sequences, and that form a continuous open reading frame with the more conserved regions. Together IS1 and IS2 comprise 46% of the residues of Ycf10. They do not appear to be introns, since the size of the *ycf10* transcript corresponds to the entire *ycf10* coding region (data not shown). Further, these additional sequences do not include any typical group I or group II intron motifs. No significant sequence similarity between these sequences and other known protein sequences could be detected. However, the motif YNN X SI (11X)SLFT was found in both IS2 (aa 278–304) and CR2 (amino acids 369–390) (cf. Figure 1). It is therefore possible that some parts of IS2 were created through sequence duplication. The chloroplast *rpoC2* and *clpP* genes of *C.reinhardtii* also contain large additional translated regions that form a contiguous ORF with the conserved upstream and downstream domains (Fong and Surzycki, 1992; Huang *et al.*, 1994). Inspection of the Ycf10 sequence reveals that the next putative initiation codons, GTG, are at positions 41 and 71 (cf. Figure 1). It is not yet known at which of the three putative initiation codons translation of Ycf10 starts.

Ycf10 is not essential for cell viability

The Ycf10 protein has been previously localized in the inner membrane of the chloroplast envelope, a unique feature amongst chloroplast-encoded polypeptides (Sasaki *et al.*, 1993; Gray, 1996). It could therefore be suspected to be involved in subcellular communication, and thus vital for the cell. However, we have shown here that homoplasmic transformants with disrupted *ycf10* can be obtained readily indicating that this chloroplast gene is not essential for cell viability, even under conditions where photosynthesis is required. Four chloroplast encoded polypeptides of 61, 54, 30 and 25 kDa were found to be associated with the chloroplast envelope of *C.reinhardtii* (Clemetson *et al.*, 1992). The latter two were found to be located within the outer envelope membrane. It remains to be seen whether any of the two larger polypeptides corresponds to the Ycf10 protein.

The putative *ycf10* gene product was previously identified in pea chloroplasts as HBP (for Heme-Binding Protein, Willey and Gray, 1990) based on sequence conservation around a conserved histidine residue shared with two

cytochromes *b*. Since this residue is not conserved in the primary sequence of Ycf10 from *C.reinhardtii*, this possibility appears less likely. Further, no cytochrome could be detected in purified chloroplast envelope membranes from higher plants (Jäger-Vottero *et al.*, 1997).

In all species studied, *ycf10* is included in a cluster of genes functionally related to photosynthesis. In *C.reinhardtii*, *ycf10* is flanked by *atpA*, *psbI* and *atpH*. In higher plants, *ycf10* is in close vicinity to *petA* and the two genes have been shown to be cotranscribed in pea (Willey and Gray, 1990; Nagano *et al.*, 1991).

Loss of *ycf10* leads to increased light sensitivity

The *ycf10*-deficient *C.reinhardtii* strain obtained in this study has a functional photosynthetic apparatus as it is still capable of photoautotrophic growth in dim light. However, a striking feature of the transformants is their light sensitivity. Several light-sensitive mutants of *C.reinhardtii* have been described. They include mutants defective in the photosynthetic complexes PSI, ATP synthase and in the dark reactions of photosynthesis (Spreitzer and Mets, 1981; Spreitzer and Ogren, 1983a). All of these mutants are acetate-requiring. Some non-acetate-requiring pigment mutants have also been shown to be light-sensitive (Spreitzer and Ogren, 1983b). Finally, carotenoid deficiency in higher plants induced either through herbicide treatment or mutation is known to confer sensitivity to high light irradiation. The carotenoid composition of the *ycf10*-deficient strains was found to be the same as for wild type in cells grown under low light, but not under high light. The differences observed under these conditions are also seen, however, in ATP synthase-deficient light-sensitive mutants and are therefore not directly linked to the *ycf10* deficiency.

The *ycf10::aadA* transformants are significantly more light-sensitive than strain 2D, which lacks *atpA*, *psbI* and *ycf10*. Lack of *psbI* has been shown to reduce the level of PSII to 15% as compared with wild type (Künstner *et al.*, 1995) and thus to limit the overall PSII activity, a condition which can be considered advantageous when light intensity is excessive, or when dissipation or utilization of photochemical energy is limited. Restoration of photoautotrophic growth in the presence of spectinomycin is most likely also due to the reduced level of PSII complex under these conditions. Although the transformants with the chloroplast *aadA* cassette are resistant to the drug, synthesis of chloroplast proteins is diminished and leads to a drop in PSII content (Monod *et al.*, 1994). Taken together, these observations suggest that *ycf10* is involved in a process essential for adaptation of the cells to high photosynthetic growth rate. Thus *ycf10* function is essential only when the chloroplast electron transfer chain is highly active.

Loss of Ycf10 affects CO₂-dependent photosynthesis and inorganic carbon uptake in *C.reinhardtii*

Our results show that the loss of *ycf10* greatly affects the response of the photosynthetic activity to external Ci and that this response varies depending on whether the cells have reached the CO₂ compensation point after preillumination with high light in acetate-free medium ('low Ci' cells) or not ('high Ci' cells). Although the 'low Ci'

mutant cells were slightly more photoinhibited than wild-type cells after this preillumination, the effect on photosynthetic activity of HSM-grown cells appears to be minimal (Figure 6A and E) and photosynthetic activity is clearly maintained in TAP-grown mutant cells (Figure 6D). However, significant differences between TAP-grown 'low Ci' mutant and wild-type cells were observed. The *ycf10*-deficient cells lack efficient Ci transport and appear to be limited in assimilation of CO₂ supplied exogenously (Figure 6B and F). In contrast phototrophically grown *ycf10*-deficient cells are able to induce a CCM with an affinity for HCO₃⁻ similar to that of wild-type cells although the maximum photosynthetic rate obtained is 20–30% lower than in wild type (Figure 6A and E). Remarkably, there is a 17-fold increase in affinity for HCO₃⁻ in mutant cells grown in HSM as compared with cells grown in TAP medium (Figure 6E and F, and Table II). The apparent affinity of photosynthesis for exogenous Ci is strongly affected in mutant cells when EZ, blocking the carbonic anhydrase activity, is used (Figure 6C and D). In contrast, this inhibitor does not markedly affect the photosynthetic activity of wild-type cells under the conditions used. Hence the photosynthetic activity in the mutant is fully dependent on the activity of carbonic anhydrase whereas the wild type promotes Ci uptake into the chloroplast through a different *ycf10*-dependent mechanism which is insensitive to EZ.

In the case of 'high Ci' cells, the mass spectrometric measurements with intact cells and isolated chloroplasts reveal a considerable decrease of HCO₃⁻ uptake in the *ycf10*-deficient mutant both with regard to affinity and the maximal rate of uptake (Figures 7 and 8, and Table II). These measurements also show that in contrast to 'low Ci' mutant cells, 'high Ci' cells are unable to induce either the HCO₃⁻ or CO₂ uptake systems when grown in HSM (Table II). Taken together these observations suggest that Ycf10 may also be involved in the regulation of the induction process of the carbon concentrating system.

The nature of the CO₂ uptake system in cells and chloroplasts remains unknown. However, the small $K_{1/2}$ (CO₂) estimated for 'low Ci' wild-type cells (0.43 μM) indicates that this process is active. If uptake from external medium is due to passive diffusion, the $K_{1/2}$ (CO₂) should be determined to a large extent by the $K_{1/2}$ (CO₂) of Rubisco which is 29 μM for green algae (Chen *et al.*, 1988). A similar value for $K_{1/2}$ (CO₂) was indeed found in 'high Ci' cells and chloroplasts from the *ycf10*-deficient mutant even after growth on HSM medium in dim light (Table II). These results can be explained by the existence of a *ycf10*-dependent active CO₂ uptake mechanism. In this respect, it is noteworthy that a correlation exists between the lack of *ycf10* in the chloroplast genome of *Euglena* (Hallick *et al.*, 1993) and the absence of a CO₂ concentrating mechanism in this organism (Yokota *et al.*, 1989).

The deficiency of CO₂ uptake, but not of HCO₃⁻ transport, is overcome in the mutant at higher concentrations of exogenous CO₂ (Figure 7). CO₂ uptake in the mutant even exceeds that of wild type at very high concentrations of CO₂ (>30–40 μM) and can account for most of the photosynthetic rate (Figure 7C and F). These observations are compatible with a limitation in CO₂ diffusion through the chloroplast envelope in the *ycf10*-

deficient mutants at low levels of CO₂ and with the idea that the active component of HCO₃⁻ and CO₂ uptake is abolished. Under these conditions CO₂ diffusion is mainly governed by the activity of Rubisco so that the important passive CO₂ uptake observed at very high CO₂ concentrations in the mutant can only be observed because the active component of Ci transport has been lost. It is also possible that the loss of Ycf10 from the inner envelope membrane could alter the response of this membrane at high levels of CO₂.

The results obtained with intact cells and isolated chloroplasts show unambiguously the occurrence of HCO₃⁻ and CO₂ uptake even in TAP-grown wild-type cells (Figure 7). In particular, the demonstration of a HCO₃⁻ transporter in the chloroplast envelope under mixotrophic growth conditions was surprising as it was concluded previously that HCO₃⁻ transport is induced only during acclimation to low CO₂ concentrations (Moroney *et al.*, 1987). Our data with wild-type cells and chloroplasts are more consistent with a constitutive HCO₃⁻ transporter which undergoes quantitative rather than qualitative changes during adaptation to low Ci (Palmqvist *et al.*, 1994).

The observation that the *ycf10* deficiency affects both CO₂ and HCO₃⁻ uptake raises several possibilities. Ycf10 could be associated with a system in the chloroplast envelope involved in uptake of both HCO₃⁻ and CO₂ into the chloroplast. In this respect it is of interest that in *Synechocystis sp.* 6803 two genes, *cotA* and *sll1685* (cf. Figure 2B), homologous to *ycf10* have been identified. Mutants affected in *cotA*, are deficient in CO₂ uptake, presumably through an indirect effect (Katoh *et al.*, 1996a,b). In contrast, HCO₃⁻ uptake appears to be unaffected in these mutants. An intriguing possibility is that the second *ycf10*-like gene is involved in active HCO₃⁻ uptake. In *C.reinhardtii* *ycf10* would combine the functions of both genes.

Another possibility is that the effect observed on Ci uptake in the *ycf10*-deficient mutant is indirect. Ycf10 might be involved in pH regulation and could be associated with the recently proposed redox chain in the chloroplast (Jäger-Vottero *et al.*, 1997). This chain may be involved in the export of photosynthetic reducing power to the cytosol and in particular in proton extrusion. Ycf10 could therefore play a role in such a process in a similar way as proposed for cyanobacteria (Katoh *et al.*, 1996b). Proton extrusion may cause a local acidification near the intermembrane space of the chloroplast envelope and thereby stimulate conversion of HCO₃⁻ into CO₂ which could readily diffuse into the chloroplast. This mechanism could explain why HCO₃⁻ uptake is also reduced in the mutant. The observation that the high light sensitivity of the *ycf10*-deficient mutant cannot be rescued by aeration of the cells with CO₂-enriched air also suggests that Ycf10 may not be involved directly in the Ci uptake system but rather that it plays a role in a primary process which could regulate the Ci transport system.

Materials and methods

Strains, media and growth conditions

The wild-type and mutant strains were grown on TAP medium (Gorman and Levine, 1965), and they were tested for growth on HSM medium

(Rochaix *et al.*, 1988) and on TAP medium containing 150 µg/ml spectinomycin to test for resistance to the drug. The mutant strain Fud50 (Woessner *et al.*, 1984) was used. The Ct strain used is a wild-type strain in which the *aadA* cassette was inserted downstream of *atpB*. This strain has wild-type photosynthetic activity and is spectinomycin resistant.

Cloning of the chloroplast *ycf10* gene from *C.reinhardtii*

The plasmid pTB22 of the tobacco clone bank, containing the *ycf10* gene in a 4.8 kb *EcoRI* chloroplast DNA fragment, was provided by M.Sugiura. The 690 bp DNA fragment corresponding to the coding sequence of the *ycf10* gene was amplified from the pTB22 plasmid by PCR, using two oligonucleotide primers (5'-CCATGGCAAAAAGAAAGCATTCACTCC and 5'-GCATGCGGATCCTTTATCAGTC) flanking the coding sequence. The amplified fragment was used as a probe for identifying the *EcoRI* chloroplast DNA restriction fragment from *C.reinhardtii*, containing the *ycf10* gene. Conditions for DNA restriction and Southern analysis were as described below, except that hybridization was performed at 42°C.

Sequencing of the chloroplast DNA region containing *ycf10* and *atpH*

The 5' and 3' regions of *ycf10* are located on the chloroplast *EcoRI* fragments R7 (3.5 kbp) and R8 (3.6 kbp) of *C.reinhardtii*, respectively. Various regions of these fragments were subcloned and the *ycf10* and *atpH* regions were sequenced on both strands using the sequenase version 2.0 kit from United States Biochemical (Cleveland, OH).

Plasmid construction

Recombinant plasmids were prepared as described by Sambrook *et al.* (1989). Two different disruptions of *ycf10* were performed. In the first, the R7 chloroplast DNA fragment, subcloned in pBluescript KS⁻, was digested with *NdeI*, blunted with Klenow fragment and used to insert an *aadA* expression cassette conferring spectinomycin resistance in either orientation. In the second, the 0.7 kbp *XbaI*-*EcoRI* fragment of R7 was subcloned in pBluescript KS⁻. The 2.5 kbp *HindIII* fragment of R8 was then inserted in the correct orientation at the *HindIII* site of this plasmid, resulting in the loss of the 0.17 kbp *HindIII*-*EcoRI* fragment of R8 encoding the last 50 amino acids of the *ycf10* product. The unique *EcoRI* site of this new plasmid present within *ycf10* was used for inserting an *aadA* expression cassette in both orientations.

Two *aadA* cassettes were used (cf. Figure 3). In one construction, the *aadA* cassette was excised from the plasmid pUC-atpX-AAD (5' *atpA*-*aadA*-3' *rbcl*; Goldschmidt-Clermont, 1991) by digesting with *SmaI* and *EcoRV*. In the other construct, the *aadA* cassette was excised from the plasmid *patp*-*intcg11* (Nickelsen *et al.*, 1994) with *ClaI* and *PstI* and blunted with Klenow fragment. In this case the *aadA* sequence was fused to the *psbD* promoter and 5' untranslated region and it lacked any specific 3' untranslated region. Restriction analysis confirmed that these *aadA* cassettes were inserted in both orientations at the different insertion sites. The chloroplast DNA region flanking the *aadA* cassette inserted at the *EcoRI* and *NdeI* sites were 2.5 and 0.7 kbp, and 2.7 and 1 kbp, respectively, in the plasmids used for transformation.

Chloroplast transformation

Recombinant plasmids with the *aadA* cassette in both orientations were used to transform wild-type *C.reinhardtii* cells with a particle gun as described (Zumbrunn *et al.*, 1989; Goldschmidt-Clermont, 1991) using resistance to spectinomycin for selection.

Hybridizations

The DNA used for Southern analysis of the transformants was prepared from cells grown in TAP medium and dim light, according to the procedure described by Weeks *et al.* (1986). DNA from the transformants was digested with various restriction enzymes, according to Sambrook *et al.* (1989). Southern analysis was performed with Amersham Hybond-N⁺ membrane according to the manufacturer's instructions. Prehybridizations and hybridizations were performed in 6× SSC, 5× Denhardt's solution, 0.5% SDS and 0.1 mg/ml salmon sperm DNA at 65°C. The final wash was at 65°C in 0.5× SSC and 0.1% SDS.

Determination of pigment and quinone content

Pigment content of wild type and transformants was determined according to Krinsky and Levine (1964) with the correction of Francis *et al.* (1973). Phylloquinone, plastoquinone and α-tocopherol contents were determined according to Barr and Crane (1971).

Table III. Photosynthetic properties of isolated chloroplasts from cw-15 and N3-1

	cw-15	N3-1
Buffer A	15.8	16.9
Buffer A + 2 mM PGA	32.8	24.8
Buffer A + 2 mM PGA, 2 mM OAA	60.1	50.7
Buffer A + 10 mM P _i	0	0

Standard test medium consists of buffer A, containing 0.1% BSA and 7 mM HCO₃⁻. Photosynthetic rates are given in μmol O₂/(mg Chl)/h; PGA, 3-phosphoglycerate; OAA, oxaloacetic acid.

Photosynthetic measurements

Fluorescence transients were recorded as described (Monod *et al.*, 1994) and O₂ evolution activity was measured with a Clark-type oxygen electrode or by mass spectrometry (see below).

For measurements of photosynthetic activity, cells were prepared as described by Badger *et al.* (1980). The cell cultures were centrifuged at 1000 g for 5 min. The cells were washed and the isolated pellet was resuspended in 50 mM HEPES pH 7.15. This cell suspension was stored on ice in the dark, for the duration of the experiment. This treatment does not alter the photosynthetic performance of the cells (Badger *et al.*, 1980). Prior to incubation of the cells with NaHCO₃, an aliquot of concentrated cell suspension was diluted into 1 ml of 50 mM HEPES pH 7.15 in an illuminated O₂ electrode chamber (Hansatech, 872 μE/m²/s), with a final chlorophyll concentration of 4–6 μg/ml. The chamber was closed and the cells were allowed to deplete their internal Ci pool and the medium of endogenous CO₂ (Badger *et al.*, 1980).

Isolation of chloroplasts

For the isolation of chloroplasts from cw-15 and the mutant N3-1, the cells were grown in 500 ml TAP-medium under continuous light (25 μE/m²/s) at room temperature on a rotary shaker until the cell density was ~10⁶ cells/ml. Prior to plastid isolation the cells were darkened for 12–14 h. Thereafter, cells (1l) were centrifuged (600 g; 10 min) and washed once with 15 mM HEPES–KOH pH 7.2. All further steps were carried out at 4°C. The pellet was resuspended in 10 ml buffer A containing 0.3 M sorbitol, 50 mM HEPES–KOH pH 7.8, 2 mM EDTA, 5 mM MgCl₂ and 1% BSA. The suspension was introduced into a pre-chilled Yeda Press chamber and incubated at 4×10⁴ Pascal for 3 min. The pressurized cells were released slowly and the lysate was placed on top of a 45%/75%-Percoll step gradient in buffer A with only 0.1% BSA. After centrifugation in a swing-out rotor at 4000 g for 20 min, chloroplasts were collected from the interface, diluted with 10 ml buffer A (0.1% BSA) and centrifuged at 600 g for 3–5 min. The pellet was resuspended in 100–500 μl buffer A (0.1% BSA) and stored on ice until used in the experiments. The isolated chloroplasts were tested for their photosynthetic activity, integrity and purity by measuring O₂ evolution in the presence of 3-phosphoglycerate (PGA), PGA and OAA, and P_i (Table III). Uptake of PGA, an intermediate of the Calvin cycle, into chloroplasts stimulates this cycle and leads to the oxidation of NADPH and to Ci-dependent O₂ evolution (Walker, 1990). After uptake into chloroplasts OAA is converted into malate, a reaction which consumes NADPH, activates the electron transport chain and leads to O₂ evolution. P_i is taken up into chloroplasts through the phosphate translocator in exchange of intermediates of the Calvin cycle which causes their depletion in the chloroplast and the loss of photosynthetic activity. Since PGA, OAA and P_i have no effect on the photosynthetic activity of whole cells, the results of Table III demonstrate that the isolated chloroplasts used were functional and not significantly contaminated with whole cells.

Mass spectrometric measurements of CO₂ and O₂ gas exchanges

CO₂ and O₂ gas exchange measurements by cells and chloroplasts were performed at 25°C in a closed system consisting of a stoppered reaction chamber and a mass spectrometer which are connected via a semipermeable membrane inlet system (Fock and Sültemeyer, 1989). To analyze whole cells, the chamber was filled with 10 ml HEPES–KOH pH 7.8 with a chlorophyll concentration of 10–15 μg/ml. Chloroplasts (20–30 μg/ml) were used in 10 ml buffer A (0.1% BSA) containing 2 mM 3-phosphoglycerate (PGA). In addition, the buffer contained 25 mM acetazolamide to inhibit surface carbonic anhydrase activity. Changes in the concentrations of CO₂ (*m/z* = 44) and O₂ (*m/z* = 32)

were continuously and simultaneously measured in the dark and during illumination (850 μE/m²/s). The rates of net O₂ evolution, HCO₃⁻ and CO₂ uptake were calculated from these measurements using the equations described in detail by Badger *et al.* (1994). For the calculations, the rate constants for the spontaneous hydration and dehydration of CO₂ and HCO₃⁻, *k*₁ and *k*₂, were experimentally determined as 2.0 and 0.042 min⁻¹, respectively. The efflux of CO₂ was estimated from the rate of CO₂ increase in the incubation medium in the dark immediately following the illumination according to Badger *et al.* (1994) and added to the net CO₂ uptake measured in the light to estimate the gross CO₂ uptake.

Accession number

The sequence data has appeared in the DDBJ/EMBL/GenBank Sequence Data Library under accession number X90559.

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