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ORIGINAL ARTICLE

Hsu-Ching Chen · Anastasios Melis

Localization and function of SulP, a nuclear-encoded chloroplast sulfate permease in *Chlamydomonas reinhardtii*

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Abstract Recent work [H.-C. Chen et al. (2003) Planta 218:98–106] reported on the genomic, proteomic, phylogenetic and evolutionary aspects of a putative nuclear gene (*SulP*) encoding a chloroplast sulfate permease in the model green alga *Chlamydomonas reinhardtii*. In this article, evidence is provided for the envelope localization of the SulP protein and its function in the uptake and assimilation of sulfate by the chloroplast. Localization of the SulP protein in the chloroplast envelope was concluded upon isolation of *C. reinhardtii* chloroplasts, followed by fractionation into envelope and thylakoid membranes and Western blotting of these fractions with specific polyclonal antibodies raised against the recombinant SulP protein. The function of the SulP protein was probed in antisense transformants of *C. reinhardtii* having lower expression levels of the *SulP* gene. Results showed that cellular sulfate uptake capacity was lowered as a consequence of attenuated *SulP* gene expression in the cell, directly affecting rates of de novo protein biosynthesis in the chloroplast. The antisense transformants exhibited phenotypes of sulfate-deprived cells, displaying slow rates of light-saturated oxygen evolution, low levels of Rubisco in the chloroplast and low steady-state levels of the photosystem-II D1 reaction-center protein. The role of the chloroplast sulfate transport in the uptake and assimilation of sulfate in *C. reinhardtii* is discussed along with its impact on the repair of photosystem-II from a frequently occurring photo-oxidative damage and potential use for the elucidation of the H_2-evolution-related metabolism in this green alga.

Keywords *Chlamydomonas* · Chloroplast envelope · Green algae · Sulfate transport · *SulP* gene

H.-C. Chen · A. Melis (✉)
Department of Plant and Microbial Biology,
111 Koshland Hall, University of California, Berkeley,
CA 94720-3102, USA
E-mail: melis@nature.berkeley.edu
Fax: +1-510-6424995

Abbreviations Chl: Chlorophyll · D1: Photosystem-II reaction-center protein · LHCII: Chl *a*/*b* light-harvesting complex of PSII · PS: Photosystem · QRT–PCR: Quantitative reverse transcription–polymerase chain reaction · RbcL: Large subunit of Rubisco · RbcS: Small subunit of Rubisco · Rubisco: Ribulose 1,5-bisphosphate carboxylase–oxygenase · SulP: Sulfate permease

Introduction

In plants and algae, sulfate nutrients are transported from the medium in the environment through the cytosol to the chloroplast, where they are assimilated to form cysteine and methionine, leading to the biosynthesis of protein (Hell 1997 and references therein; Leustek et al. 2000; Saito 2000). Chloroplasts are pivotal in the primary assimilation of sulfate, since photosynthetic reductant in the form of reduced ferredoxin and ATP are needed for the reduction of sulfate and the biosynthesis of cysteine and methionine (Hell 1997). Sulfur nutrient deprivation impacts de novo protein biosynthesis, protein turnover and cell growth. Of recent interest in this respect was the effect of S-deprivation (Wykoff et al. 1998) on the repair of photosystem-II (PSII) from frequently occurring photodamage (Melis 1999). Sulfur deprivation, and the ensuing inhibition of protein biosynthesis in chloroplasts, prevents the frequent turnover of the PSII D1 reaction-center protein of PSII (Wykoff et al. 1998), thus causing a specific inhibition in the rate of photosynthetic oxygen evolution (Melis et al. 2000). In sulfate-deprived and sealed cultures of *C. reinhardtii*, cellular respiration quickly consumes all dissolved oxygen, leading to anaerobiosis in the culture, expression of the [Fe]-hydrogenase pathway in the chloroplast, and H_2 evolution by the culture in the light (Melis et al. 2000; Ghirardi et al. 2000). In consequence, chloroplast sulfate transporters are of interest

because they have the potential of affecting both the repair of PSII from frequently occurring photodamage and the ability of unicellular green algae to perform a sustained H_2 photoproduction.

In *Chlamydomonas reinhardtii*, sulfate transport has been studied under sulfur-replete and sulfur-deprivation conditions (Yildiz et al. 1994). It was shown that, upon S-limitation, *C. reinhardtii* synthesized a high-affinity sulfate transport system, and that sulfate uptake is an energy-dependent process. Until recently, however, genes encoding for the sulfate transporters in *C. reinhardtii* were not identified. In recent work, we reported the identification and characterization of *SulP* (GenBank Accession numbers AF467891 and AF481828), a novel and unusual nuclear gene encoding a putative chloroplast-targeted sulfate permease (Chen et al. 2003). The deduced amino acid sequence of the SulP protein had a substantial homology with the *CysT* gene product of cyanobacteria and with the chloroplast-encoded sulfate permease in many green algae (Chen et al. 2003). Unlike the prokaryotic gene structure of the sulfate permease in many green algae, the *SulP* gene contained four introns and five exons, and showed no sequence similarity with its prokaryotic relatives at the DNA level. Genomic, proteomic, phylogenetic and evolutionary aspects of the *SulP* gene were reported in detail (Chen et al. 2003). Based on the *C. reinhardtii* genomic DNA sequence data, recently made available by the DOE's Joint Genome Institute (JGI), the occurrence of this unique *SulP* gene was confirmed. Moreover, the *SulP* gene was recently mapped onto the *C. reinhardtii* genome linkage group VII (Kathir et al. 2003).

A plasma membrane sulfate transporter has not yet been identified in *C. reinhardtii*. In higher plants, evidence was presented for the existence of several plasma membrane-localized H^+/SO_4^{-2} co-transporters (Grossman and Takahashi 2001). These transporters were classified into four groups based on their protein sequences, kinetic properties and tissue-specific localization. A proposed chloroplast sulfate transporter from *Arabidopsis thaliana*, which belongs to the H^+/SO_4^{-2} co-transporter type, was initially reported (Takahashi et al. 1999). However, in spite of a previous report indicating a plastid localization (Takahashi et al. 2000), it was later suggested that this sulfate transporter isoform is actually targeted to the vacuole of the plant cell (Takahashi et al. 2003). As such, this protein may not be responsible for the import of sulfate into the chloroplast. To date, the nature and function of the chloroplast sulfate transporters in higher plants remain unknown.

In an effort to elucidate the localization and function of the SulP protein, specific polyclonal antibodies were raised against the recombinant protein. These were used in Western blot analysis of isolated chloroplast fractions to test for the localization of the SulP protein. Moreover, antisense technology with the *SulP* gene and sulfate-deprivation studies were employed as tools in combination with measurements of photosynthesis, rates of sulfate uptake and properties of the photosynthetic apparatus in an effort to investigate functional aspects of the *SulP* gene. Evidence is presented for the role of *SulP* in sulfate uptake and assimilation in *C. reinhardtii*. The role of the chloroplast sulfate transport system in *C. reinhardtii* is discussed in the context of the frequent turnover of the PSII D1 reaction-center protein. The potential application of the *SulP* gene in green-alga H_2 research is also addressed. To the best of our knowledge, this is the first functional characterization of a chloroplast sulfate transporter in a eukaryotic organism.

Materials and methods

Cell growth and measurement

The green alga *Chlamydomonas reinhardtii* was grown mixotrophically in a Tris–Acetate–Phosphate (TAP) medium, pH 7 (Gorman and Levine 1965), either in liquid cultures or on 1.5% agar plates. Liquid cultures were grown on TAP, or TAP with a modified sulfate concentration, at 25°C in flat Roux-type culture bottles with stirring, or in flasks with shaking, under continuous illumination at ca. 40 μmol photons m^{-2} s^{-1}. Culture density was measured by cell counting using a Neubauer ultraplane hemacytometer and a BH-2 light microscope (Olympus, Tokyo). Cells were grown to the early exponential growth phase (about 1×10^6–2×10^6 cells ml^{-1}) for all photosynthesis measurements. For chlorophyll (Chl) measurements, cells or thylakoid membranes were extracted in 80% acetone and debris removed by centrifugation at 10,000 *g* for 5 min. The absorbance of the supernatant was measured with a Shimadzu UV-160U spectrophotometer. The Chl (*a* and *b*) concentration of the samples was determined according to (Arnon 1949).

Isolation and fractionation of chloroplast proteins

Chloroplasts were isolated from the cell wall-less strain *cw15* and envelope membranes were prepared according to the methods described in Mason et al. (1991) and Mendiola-Morgenthaler et al. (1985), respectively, with the following modifications. Cells were grown under a 12 h light/12 h dark cycle in TAP medium to early log phase of a cell density of 1×10^6–2×10^6 cells ml^{-1}. For sulfate-deprivation treatment, cells were transferred into TAP-S medium for 24 h before the preparation. Chloroplasts were isolated from the Percoll gradients according to Mason et al. (1991), except that both the 20–45% and 45–65% interfaces were collected and combined. After washing twice with buffer [300 mM sorbitol, 50 mM Hepes–KOH (pH 7.5), 2 mM Na-EDTA, 1 mM $MgCl_2$], the chloroplasts were lysed hypotonically by resuspension in 50 mM Hepes–KOH (pH 7.5), 2 mM $MgCl_2$, and fractionated through step sucrose gradients (20.5% and 31.8% sucrose) as described by Mendiola-Morgenthaler et al. (1985). The

envelope fraction was collected from the interface and diluted 4 times with lysis buffer [50 mM Hepes–KOH (pH 7.5), 2 mM $MgCl_2$], and subjected to further ultracentrifugation at 60,000 *g* for 1 h. After the centrifugation, the envelope membranes were recovered from the pellet and resuspended in SDS–PAGE solubilization buffer containing 0.5 M Tris–HCl (pH 6.8), 7% SDS, 20% glycerol, 2 M urea, and 10% β-mercaptoethanol. The thylakoid membranes were collected from the pellets at the bottom of the sucrose gradient, and were solubilized prior to SDS–PAGE analysis.

Thylakoid membrane isolation for photochemical apparatus characterization

Cells were harvested by centrifugation at 1,000 *g* for 3 min at 4°C. Pellets were resuspended in ice-cold sonication buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone-40, 0.2% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 μM phenylmethylsulfonylfluoride (PMSF). Cells were broken by sonication in a Branson 250 Cell Disrupter operated at 4°C for 30 s (pulse mode, 50% duty cycle, output power 5). Unbroken cells and starch grains were removed by centrifugation at 3,000 *g* for 4 min at 4°C. The thylakoid membranes were collected by centrifugation of the supernatant at 75,000 *g* for 30 min at 4°C. The thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ for spectrophotometric measurements. The concentration of the photosystems in thylakoid membranes was estimated from the amplitude of the light *minus* dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII (Melis and Brown 1980; Melis 1989).

Oxygen-exchange measurements

Oxygen-evolution activity of the cultures was measured with a Clark-type oxygen electrode illuminated with a slide-projector lamp. Yellow actinic excitation was provided by a CS 3-69 Corning cut-off filter. An aliquot of 5 ml cell suspension was transferred to the oxygen-electrode chamber. To ensure that oxygen evolution was not limited by the carbon source available to the cells, 100 μl of 0.5 M sodium bicarbonate solution (pH 7.4) was added to the suspension prior to the oxygen-evolution measurements. Measurement of the light-saturation curve of photosynthesis was implemented with the oxygen electrode, beginning with the registration of dark respiration in the cell suspension, and followed by measurements of the rate of oxygen evolution in steps at 525, 1,150, 1,800, 2,550 and 3,200 μmol photons m^{-2} s^{-1}. Registration of the oxygen evolution rate was recorded for 2.5 min at each one of the light intensity steps. Measurements of the light-saturated rate of oxygen evolution were carried out at 1,500 μmol photons m^{-2} s^{-1}.

Northern blot analyses, SDS–PAGE and Western blot analysis

For Northern blot analyses, total RNA was extracted from 30 ml of cell culture with a cell density of 1×10^6–2×10^6 cells ml^{-1}, using the "Plant total RNA extraction kit" from Qiagen. 30 μg of total RNA was electrophoresed through a formamide–formaldehyde gel (Sambrook et al. 1989). RNA was transferred onto positively charged nylon membrane (PALL, BiodyneB) overnight, through capillary transfer using 10×SSC buffer. RNA–DNA hybridization reactions were carried out with radiolabeled probes (random primed labeling kit; La Roche) according to the manufacturer's specifications.

For total protein extraction, cells were harvested by centrifugation at 3,000 *g* for 5 min at 4°C, and pellets were lysed upon 30 min incubation with solubilization buffer containing 0.5 M Tris–HCl (pH 6.8), 7% SDS, 20% glycerol, 2 M urea, and 10% β-mercaptoethanol. The crude cell extracts were then centrifuged at maximum speed in a microfuge for 3 min to remove cell debris and other insoluble matter. Chlorophyll concentration was determined by measuring the absorbance of a pigment extract obtained in 80% acetone, followed by the spectrophotometry measurement for the determination of Chl (Arnon 1949).

Proteins were resolved by SDS–PAGE using the discontinuous buffer system of (Laemmli 1970) with 12.5% acrylamide and 0.2% bis-acrylamide. The stacking gel contained 4.5% acrylamide. Electrophoresis on 0.75 mm×7 cm×8 cm slab gels was performed at 4°C at a constant current of 10 mA for 2.5 h. After completion of the electrophoresis, proteins on the gel were either stained with Coomassie Brilliant Blue or electro-transferred onto a nitrocellulose membrane. Immunoblot analysis was carried out with specific polyclonal antibodies, followed by chemiluminescence detection (ECL; Amersham-Pharmacia).

A sequence corresponding to amino acids 55 to 160 of the precursor SulP protein was cloned into pQE31 expression vector using the Quia *express* type IV kit (Qiagen). The expression level of the Histidine tag–SulP fusion protein in *Escherichia coli* was relatively low, and the protein was isolated from the insoluble fraction of the total bacterial cell extract. Two liters of *E. coli* culture was used for protein extraction, and the insoluble protein fraction was prepared and then passed through a nickel column according to the manufacturer's specifications. About 2 mg of purified Histidine tag–SulP protein was obtained by this method and used for the generation of polyclonal antibodies.

Construction of anti-SulP plasmid and generation of antisense transformants

The anti-SulP plasmid (pAnti-SulP) employed in co-transformation experiments was constructed by placing

a partial sequence of the *SulP* cDNA (from nucleotide +525 to the end of the coding sequence at nucleotide +1409) downstream of the *RbcS*2 promoter in the reverse orientation, followed by the *RbcS*2 3′-UTR. Both the *RbcS*2 promoter and the 3′-UTR sequences were PCR-amplified from vector pSP124S (Stevens et al. 1996). The pAnti-SulP was linearized and used in the co-transformation of *Chlamydomonas reinhardtii* with the pJD67 plasmid (Davies et al. 1994) that carries the *Arg7* gene in the pBluescriptII KS+ vector (Stratagene). The arginine auxotroph strain CC425 (*arg*7-8 *mt*+ *cw*15 *sr-u*-2-60; *Chlamydomonas* Genetics center, Duke University) was co-transformed by the glass-bead method (Kindle 1990) with the linearized pAnti-SulP and pJD67 plasmids. Transformants were first selected on TAP plates lacking arginine. For the selection of co-transformants, genomic DNA was prepared from arginine prototroph transformants, and used for PCR analysis with primers located at both ends of the *SulP* cDNA. Transformants that yielded positive amplification of a DNA fragment of about 900 bp were considered positive co-transformants.

Quantitative reverse transcription–polymerase chain reaction (QRT–PCR)

QRT–PCR experiments were carried out using the SuperScript One-Step RT–PCR kit from Invitrogen. The optimal number of PCR cycles for each gene was established by analyzing the amplified product on the agarose gel. For the amplification of *SulP* (accession number AF481828) gene transcripts, 0.2 μg of total RNA was used. The primers used were located at the 5′-end of the gene of which the amplified sequence does not overlap the sequence of pAnti-SulP used for the antisense transformation. Their sequences are as follows:

- *SulP* forward 5′-CCTAAGCAAAAATACCAAAGCC-3′,
- *SulP* reverse 5′-CAGTCTTTGCCGTTGATATCTC-3′,

giving a PCR product of 318 bp. For the amplification of ribulose 1,5-bisphosphate–carboxylase (Rubisco) large subunit (*RbcL*, accession number J01399) and small subunit (*RbcS*, accession number X04472) gene transcripts, 0.1 μg of total RNA was employed. Primers used are as follows:

- *RbcL* forward 5′-GAACGAATTACGTCGCCACC-3′,
- *RbcL* reverse 5′-ACGTTGAAAAAGACCGTAGCC-3′,

giving a PCR product of 283 bp.

- *RbcS* forward 5′-CTGGACCCCGGTCAACAACAAG-3′,
- *RbcS* reverse 5′-CCAGGAAGCCCATGATCTGCAC-3′,

giving a PCR product of 359 bp. The same number of 25 cycles was used for both genes.

Sulfate uptake and ^{35}S-pulse labeling

Sulfate uptake experiments were carried out according to the method of Yildiz et al. (1994) with the following modifications. Cells were grown under continuous illumination at ca. 40 μmol photons m^{-2} s^{-1} to a density of 1×10^6–2×10^6 cells ml^{-1}. Cells were pelleted and washed twice with sulfate-replete TAP. Cells were finally suspended in this medium at a cell density of 0.5×10^6 cells ml^{-1}. Samples were placed under illumination for 24 h prior to the experiments on ^{35}S-sulfate uptake. Prior to sulfate uptake experiments, cells were centrifuged and washed twice with TAP−S (Tris-Acetate-Phosphate medium without sulfate) and concentrated by about 10-fold to a density of 2×10^7–3×10^7 cells ml^{-1} in TAP−S medium. 5 ml of the concentrated cell suspension was then transferred into a glass vial, stirred under continuous illumination at 200 μmol photons m^{-2} s^{-1} for 2 min, followed by addition of 100 μl of $Na_2{}^{35}SO_4$ (NEN; specific activity of 20.7 GBq μmol^{-1}, 37 MBq ml^{-1}; final concentration of sulfate was about 70 μM). An aliquot of 100 μl was removed from the cell suspension at each time point (15, 30, 45, 60, 90 and 120 s) and transferred into a tube containing 1 ml of cold TAP medium. Cells were pelleted by centrifugation, washed twice with 1 ml of TAP and resuspended in 50 μl of TAP, then transferred into a Nano-Sep column (PALL). Following centrifugation at 10,000 *g* for 2 min, the filter of each Nano-Sep column containing the cells was removed and the radioactivity of the sample was measured using the Beckman Liquid Scintillation System (LS6000IC). When the experiment was carried out at 400 μM sulfate concentration, a Mg_2SO_4 solution was used to supplement the sulfate in the medium to a 400 μM final concentration. The longer-term ^{35}S-labeling experiments were carried out essentially in the same way as described above for the sulfate uptake experiments, except that, aliquots were removed at 0, 15, 30, 45, 60 and 90 min. After washing twice with TAP medium, cells were suspended in solubilization buffer and subjected to SDS–PAGE analysis.

Results

Induction of SulP gene expression upon S-deprivation

Sulfur deprivation has been shown to induce the expression of different sulfate transporters in both higher plants and cyanobacteria (Laudenbach and Grossman 1991; Shibagaki et al. 2002; Yoshimoto et al. 2002; Hirai et al. 2003; Nikiforova et al. 2003). The high similarity of the SulP protein to chloroplast-encoded sulfate permeases from other organisms suggested the possibility of a SulP function in the transport of sulfates

from the cytosol into the chloroplast, where sulfate assimilation exclusively takes place. To examine the effect of sulfate deprivation on the expression of the *SulP* gene, levels of *SulP* gene transcripts in *C. reinhardtii* were measured in cells grown under control (400 μM sulfate) and sulfur deprivation conditions. Figure 1a shows a Northern blot analysis in which levels of the ca. 2-kb *SulP* transcripts were relatively low under control conditions (Fig. 1a, 0 h). Upon a 6-h S-deprivation, transcripts increased substantially and remained at this high level for at least 24 h in the absence of sulfate (Fig. 1a, 6 h and 24 h, respectively). It was determined that other environmental stresses (e.g. light intensity changes in the 5–200 μmol photons m^{-2} s^{-1} range) did not bring about induction in the expression of *SulP* (not shown).

To investigate whether induction of *SulP* expression at the transcript level is accompanied by a corresponding increase at the protein level, efforts were made to raise specific polyclonal antibodies against the SulP protein. The SulP protein contains seven transmembrane helices with two sizable hydrophilic loops that extend into the aqueous space between inner and outer envelope (Chen et al. 2003). An attempted over-expression of the hydrophilic region located close to the C-terminus of the protein (from A285 to N360, including two of the transmembrane helices) in *E. coli* was not successful.

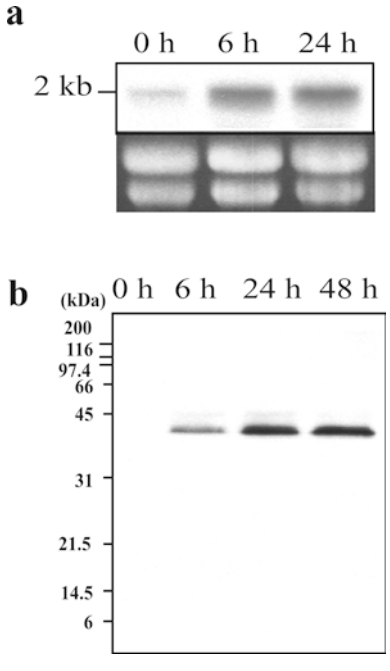

Fig. 1a,b Induction of *SulP* gene expression upon sulfate nutrient deprivation in *Chlamydomonas reinhardtii*. Wild-type cells were incubated in TAP growth medium, in the absence of sulfate nutrients for 0, 6 or 24 h. **a** Equal amounts of total RNA (30 μg) from each sample were loaded in the agarose gel lanes prior to electrophoresis. *Upper panel* Northern blot analysis; *lower panel* ethidium bromide staining of rRNA. **b** Western blot analysis. Lanes were loaded on equal-Chl basis (2 nmol of total Chl). Specific polyclonal anti-SulP antibodies were used at 1:1,000 dilution

These *E. coli* cells ceased to grow upon isopropyl β-D-thiogalactopyranoside (IPTG) induction, and significant protein expression could not be detected. Alternatively, efforts were made to over-express the first hydrophilic region located close to the N-terminus of the SulP protein (A55 to L160 of the precursor SulP protein). These efforts proved successful in terms of *E. coli* cell growth; however, the expression level of the oligopeptide was low, requiring sizable volumes of bacterial culture as the starting material in order to obtain enough purified His-tag–SulP fusion protein for the raising of antibodies (Materials and methods). Figure 1b shows a Western blot analysis of a *C. reinhardtii* crude extract of total protein, in which the anti-SulP immune serum specifically cross-reacted with a single *C. reinhardtii* protein band. The apparent size of this polypeptide was estimated to be about 38 kDa, i.e., somewhat smaller than the deduced size of the precursor SulP protein (43.7 kDa). However, the apparent size of the cross-reacting protein corresponds well with the predicted size of the mature protein (357 amino acids, 37.8 kDa), following cleavage of the predicted chloroplast transit peptide of 54 amino acids (Chen et al. 2003). This result is consistent with the notion of a SulP protein import process from the cytosol into the chloroplast, where the mature protein probably functions in the transport of sulfates (see below).

The level of the SulP protein was low under control growth conditions (TAP medium containing 400 μM sulfate, Fig. 1b, 0 h), which correlates with the relatively low *SulP* transcript level under these conditions (Fig. 1a, 0 h). A substantial induction in the level of the protein was observed upon 6 h of sulfate deprivation, and maximal induction was detected after 24 h of S-deprivation (Fig. 1b). The elevated SulP protein level was maintained for at least 48 h under S-deprivation conditions. These results clearly show a coordinate induction of gene expression at both transcript and protein levels during S-deprivation, and provide evidence that the level of sulfate in the growth medium regulates *SulP* gene expression in *C. reinhardtii*.

Localization of the SulP protein in the *C. reinhardtii* chloroplast envelope

The presence of a predicted chloroplast transit peptide at the N-terminus of the SulP protein, as well as the size of the mature protein based on the Western blot analysis of Fig. 1b, suggested that SulP is imported and probably localized in the chloroplast envelope membrane. In order to test this hypothesis, a cellular fractionation experiment was undertaken, followed by Western blot analysis of the various chloroplast compartments with specific polyclonal anti-SulP antibodies. Intact chloroplasts were isolated and both envelope and thylakoid membranes were prepared from sulfate-replete and sulfate-deprived cell cultures (see Materials and methods). In the course of this experimentation, it was observed

that chloroplast envelope fractions from sulfate-replete cultures appeared yellowish in coloration, whereas those isolated from sulfate-deprived cells appeared to be slightly green. Figure 2a shows the SDS–PAGE Coomassie staining profile of the resolved proteins from control cells grown in the presence of 400 μM sulfate (lane 1, T), total protein extract of cells incubated in the absence of sulfate for 24 h (lane 2, T), the envelope fraction (lane 3, Env), and isolated thylakoid membrane proteins (lane 4, Thy). The samples in lanes 2–4 were isolated following 24 h incubation under sulfate deprivation conditions. Noteworthy in the results of Fig. 2a is the lower amount of Rubisco in sulfate-deprived cells (ca. 56 kDa, lane 2, T) and the absence of both Rubisco and the LHCII (31–26 kDa) from the chloroplast envelope fraction (lane 3, Env). Figure 2b shows the corresponding Western blot analysis with specific anti-SulP, anti-LHCII and anti-RbcL polyclonal antibodies, respectively. The anti-SulP antibodies specifically cross-reacted with a ca. 38-kDa protein band in the chloroplast envelope fraction (Env), but no cross-reaction could be detected with the thylakoid membrane fraction (Thy). Similarly, the anti-SulP antibodies cross-reacted with a ca. 38-kDa protein band in the total cellular protein extract of sulfate-deprived *C. reinhardtii* (lane 2, T). The cross-reaction with the total cellular protein extract of S-replete *C. reinhardtii* was too faint to be seen in this experiment (lane 1, T).

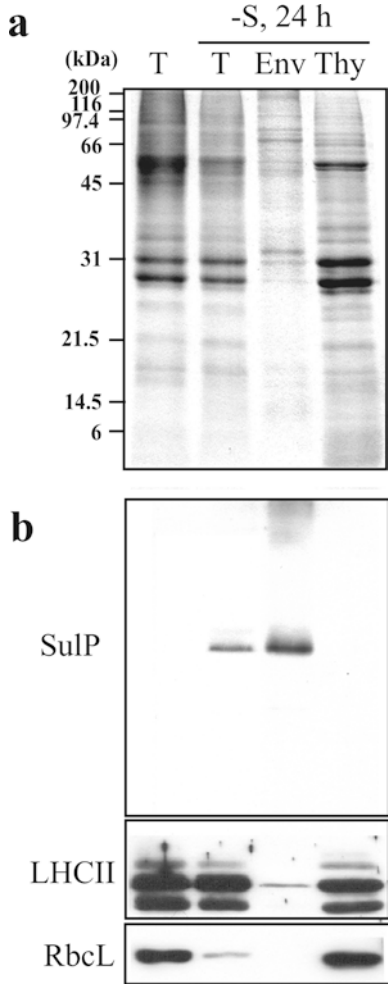

Fig. 2a,b Localization of the SulP protein based on fractionation and Western blot analysis of isolated chloroplasts of *C. reinhardtii*. **a** Coomassie-stained SDS–PAGE gels loaded with total cellular protein extract (*T*), isolated chloroplast envelope membrane proteins (*Env*) and isolated thylakoid membrane proteins (*Thy*). Total and thylakoid membrane lanes were loaded on equal-Chl basis (1 nmol of total Chl). For the envelope membrane lane, 6.25 μg of protein was loaded. **b** Western blot analysis of the samples shown in **a** with specific polyclonal antibodies raised against SulP, the LHCII and RbcL. Samples were isolated from sulfate-replete (*lane 1*, *T*) and S-deprived cells (*lanes 2–4*, *T*, *Env*, *Thy*)

Figure 2b also shows a Western blot analysis of the above-mentioned fractions with specific polyclonal anti-LHCII and anti-RbcL antibodies. LHCII proteins were abundant in the total cell and thylakoid membrane fractions, but were absent from the envelope fraction, suggesting that the latter is relatively free of thylakoid membrane contaminants (Fig. 2b, LHCII panel). As revealed by specific polyclonal anti-RbcL antibodies, Rubisco large subunit could be detected in the total cell extract and thylakoid membrane protein fraction, but not in the envelope fraction (Fig. 2b, RbcL panel), suggesting that the latter is relatively free of stroma contaminants. High levels of RbcL in the thylakoid membrane fraction suggest that pyrenoids, chloroplast structures where Rubisco is known to be concentrated in *C. reinhardtii* (Moroney and Somanchi 1999), may have co-purified with the thylakoids. Lower levels of Rubisco in the S-deprived extract are consistent with the loss of Rubisco upon S-deprivation (Zhang and Melis 2002; Zhang et al. 2002). These results provided evidence for a chloroplast envelope localization of the SulP protein in *C. reinhardtii*.

Generation and screening of *SulP* antisense transformants

Expression of the *SulP* gene is regulated by the amount of sulfate nutrients in the growth medium (Fig. 1), indicating a possible link between gene function and sulfate transport. To investigate the function of the *SulP* gene, antisense technology was employed to down-regulate the *SulP* expression and, subsequently, to test for the functional impact of such interference. An antisense construct of the *SulP* gene was made by fusing the *RbcS2* promoter to a partial sequence of the *SulP* cDNA (in the reverse direction), followed by the *RbcS2* 3′-UTR. Co-transformation of the arginine auxotroph CC425 strain of *C. reinhardtii* (*arg*7-8 *mt*+ *cw*15 *sr-u*-2-60; *Chlamydomonas* Genetics Center, Duke University) was implemented with this anti *SulP* (pAnti *SulP*) construct and the pJD67 plasmid containing the *Arg7* gene (Davies et al. 1994). Transformants were selected first on the basis of arginine prototrophy. Out of 120 arginine prototrophic transformants, co-transformants (containing both pJD67 and pAnti *SulP*) were selected upon

screening with genomic DNA PCR, testing for the presence of the inserted anti *SulP* cDNA sequence. From this secondary screening, 31 co-transformants were isolated (co-transformation efficiency of about 26%).

Functional properties of the isolated anti *SulP* antisense transformants were first tested by measuring light-saturated rates of oxygen evolution. Analysis showed that about 50% of these transformants had rates of O_2 evolution that were slower by 20%, or more, compared to that of the CC425 wild type (Fig. 3). Among the antisense transformants that showed significantly slower rates, we selected *asulp29* for further analysis. More-detailed measurements of the light saturation curve of photosynthesis were performed with *asulp29* in comparison to the wild type. Figure 4 shows that *asulp29* had a light-saturated rate of photosynthesis (P_{max}) that was slower by about 30% relative to the wild type. The absolute rate of photosynthesis, obtained after correction for respiration, was slower by about 40% in *asulp29* compared to that in the wild type. These results are consistent with the notion that the *asulp29* phenotype is a consequence of the pAnti *SulP* expression, which lowered levels of the SulP sulfate permease, causing slower rates of sulfate uptake by the chloroplast, resulting in slower rates of photosynthesis (Zhang et al. 2002).

Down-regulation of *SulP* gene expression in the *asulp29* antisense transformant

A single copy of pAnti *SulP* insertion in the genome of the *asulp29* antisense transformant was confirmed by Southern blot analysis (data not shown). To investigate whether antisense transformation upon insertion of pAnti *SulP* affected the expression of the *SulP* gene, we used QRT–PCR (Materials and methods) to investigate *SulP* transcript levels in both the wild type and *asulp29* antisense transformant. The highly sensitive QRT–PCR method was chosen because the level of *SulP* gene transcripts is quite low under normal growth conditions, for which the Northern blot analysis may not yield clear results. In these QRT–PCR analyses, both *RbcL* and *RbcS* gene transcript levels were also determined in parallel with those of *SulP*, and served as controls. Figure 5a shows the result of a measurement used in the optimization of the amplification cycles for *SulP* transcripts. The amplification appears to be linear from 28 to 32 cycles, as judged by the intensity of the DNA band.

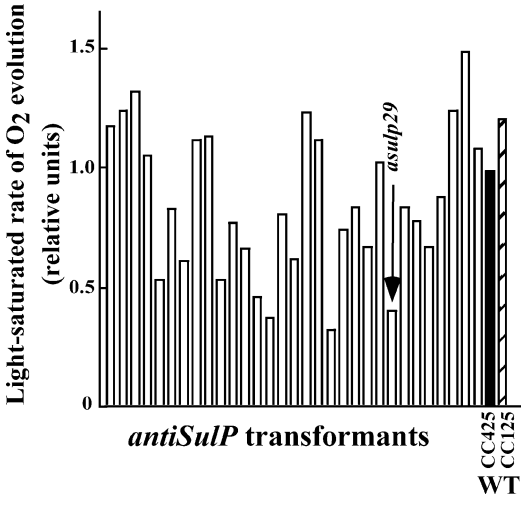

Fig. 3 Light-saturated oxygen evolution in wild type (*WT*) and anti-SulP antisense transformants of *C. reinhardtii*. Light-saturated rates of oxygen evolution were measured in vivo at an actinic light intensity of 1,500 μmol photons m^{-2} s^{-1}. Values are presented as relative rates of oxygen evolution, normalized to that of the CC425 WT control. Shown are 31 antisense transformants and two 'wild type' strains (*black column* CC425, *dashed column* CC125). Antisense transformant *asulp29* is indicated by the *arrow*

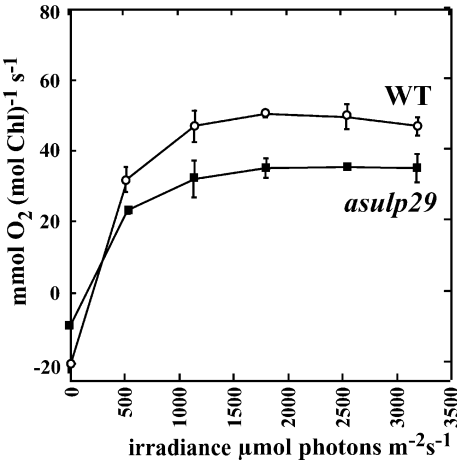

Fig. 4 Light-saturation curves of photosynthesis for the wild type (*open circles*) and *asulp29* antisense transformant (*solid squares*) of *C. reinhardtii*, measured from the rate of oxygen evolution under different actinic light intensities in the laboratory

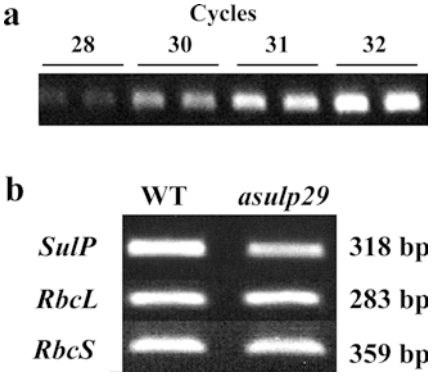

Fig. 5 a QRT–PCR analysis of the wild type and *asulp29* antisense transformant of *C. reinhardtii*. Determination of the cycle number for optimal *SulP* transcript amplification. A repeat of each cycle number is shown. **b** Quantitative analysis of *SulP* gene transcripts was carried out by using 0.2 μg of total RNA and 30 cycles of PCR amplification, whereas 0.1 μg of total RNA and 25 cycles of amplification were employed for the analysis of the *RbcL* and *RbcS* genes. The size of the amplified product from each gene transcript is indicated on the *right*

Accordingly, we opted to use 30 cycles for the comparative determination of *SulP* transcript level in this analysis. Similar measurements were also carried out for both *RbcL* and *RbcS*, in which 25 cycles were determined to be optimal. For the latter samples, however, only half the amount of RNA was loaded as the starting material, compared to that for the *SulP*. The QRT–PCR results (Fig. 5b) showed that the *SulP* gene transcript level in the *asulp29* transformant was about 50% of that in the wild type, whereas similar levels for both *RbcL* and *RbcS* gene transcripts were shown in wild type and mutant. At least three independent experiments for each gene were performed and reproducible results were always obtained. These results suggested that *SulP* gene expression is down-regulated in the *asulp29* transformant, due to insertion of the pAnti *SulP* plasmid and expression of the SulP gene in the antisense direction. Because of the low SulP protein concentration in the wild type (Fig. 1b), and presumably in the *asulp29* transformant, Western blot analysis could not be applied with a resolution sufficient to permit assessment of differential protein concentration between these two strains.

The *asulp29* strain is impaired in sulfate uptake and chloroplast protein biosynthesis

Functionally, a lower level of *SulP* gene expression in the *asulp29* antisense strain should lower the overall sulfate uptake and assimilation capacity of the cell. This was tested in wild type and *asulp29* strains directly upon ^{35}S-sulfate uptake and incorporation measurements (Materials and methods). Two different sulfate concentrations (400 μM and 70 μM) were used for these measurements and the duration of pulsing was either limited to the 0–120 s range (uptake measurements) or extended to 0–90 min (incorporation measurements).

In *C. reinhardtii*, sulfate uptake by the cytosol is followed immediately by sulfate transport to the chloroplast as this unicellular green alga lacks vacuoles in which to store significant amounts of divalent anions. It is thus believed that sulfate uptake by the chloroplast determines the overall sulfate uptake by the organism. Figure 6a shows the kinetics of ^{35}S-sulfate uptake for the wild type in the presence of 400 μM (solid circles) and 70 μM sulfate (open circles). Similar ^{35}S-sulfate uptake kinetics were observed under these two different sulfate concentrations. These results indicated that the cellular sulfate uptake capacity of the wild type was saturated in the presence of 70 μM of sulfate, such that 400 μM sulfate did not bring about a rate increase. Figure 6a also shows the kinetics of ^{35}S-sulfate uptake for *asulp29* in the presence of 400 μM (solid squares) and 70 μM sulfate (open squares). Similar ^{35}S-sulfate uptake kinetics were observed for *asulp29* under these two different sulfate concentrations. Here again, the results indicated that the cellular sulfate uptake capacity of *asulp29* was already saturated in the presence of 70 μM of sulfate, such that 400 μM sulfate did not bring about a rate increase. The quantitative difference in the sulfate uptake rate between the wild type and *asulp29* in this experiment is noteworthy and relevant. In *asulp29*, uptake rates were only about 50% of those in the wild type, irrespective of the sulfate concentration used. This quantitative difference is consistent with the notion of fewer (by about 50%) sulfate permeases in the envelope of *asulp29* as compared to the wild type. It is suggested that a lowering of *SulP* gene expression adversely affected the sulfate uptake capacity of the antisense transformant.

The lowered sulfate uptake capacity of the *asulp29* antisense transformant should impact the rate of cellular protein biosynthesis. To address this question, ^{35}S-labeling experiments were carried out over a longer period of 0–90 min. Figure 6b shows the kinetics of ^{35}S-sulfate label incorporation in the wild type and the *asulp29* antisense transformant. Results from this comparative analysis also showed a difference between the wild type and *asulp29*. The rate of incorporation and absolute incorporation of sulfate into *asulp29* proteins was about 50% of that in the wild type. Figure 6c shows an SDS–PAGE and autoradiographic analysis of the ^{35}S-sulfate labeled proteins. Rates of RbcL, RbcS and D1 protein biosynthesis in the *asulp29* transformant were slower, by about 40%, relative to the wild-type control. Slower rates of Rubisco and D1 protein biosynthesis are consistent with a limitation in the supply of sulfates to the chloroplast.

Measurements of photosystem concentration, protein composition and chloroplast size

Earlier work from this laboratory has shown that sulfate deprivation brings about substantial changes in thylakoid membrane protein composition and function in green algae (Zhang et al. 2002). The level of Rubisco declined rapidly (halftime about 15 h) upon S-deprivation and oxygenic photosynthesis was inhibited with about similar kinetics (Wykoff et al. 1998; Melis et al. 2000). It was of interest, therefore, to find out how parameters of the photosynthetic apparatus might be affected by the *asulp29* antisense transformation. Analysis of the photochemical apparatus organization in the wild type and *asulp29* antisense transformant was conducted, results of which are shown in Table 1. It can be seen that the Chl content of the *asulp29* transformant was about 70% of that in the wild type. Similarly, the PSI (P700) and PSII (Q_A) contents of the *asulp29* were about 70% of those in the wild type. However, the photosystem PSII/PSI ratio was similar in the two strains. Figure 7 shows the Coomassie-stained profile of proteins (Fig. 7a) and the corresponding Western blot analysis (Fig. 7b) from the wild type (WT) and the *asulp29* antisense transformant. Loaded on an equal-cell basis, a lower level of RbcL was detected in the *asulp29* strain by both Coomassie staining and Western blot

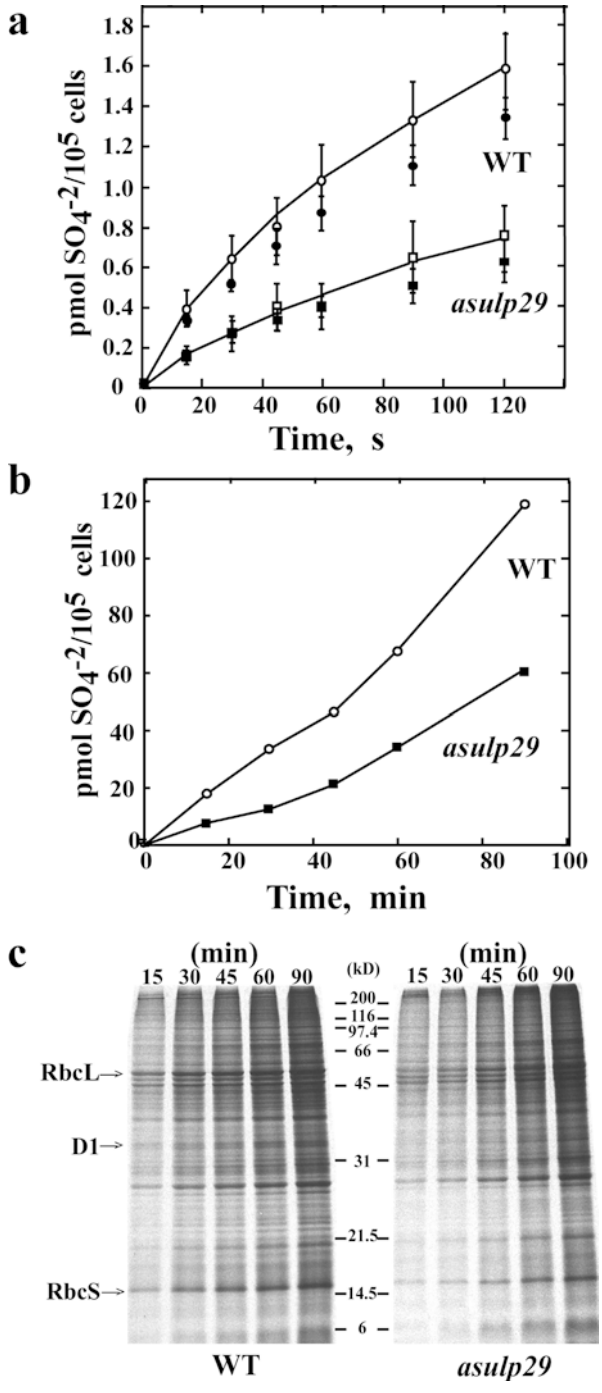

Fig. 6a–c Sulfate uptake and ^{35}S-pulse labeling measurements for the wild type and *asulp29* antisense transformant of *C. reinhardtii*. **a** Cells were grown in TAP in the presence of 400 μM sulfate in the medium. Sulfate uptake was measured at two different sulfate concentrations, 400 μM (*solid symbols*) and 70 μM (*open symbols*), for the wild type (*circles*) and *asulp29* (*squares*). Three independent experiments were carried out for each set of measurements. Means ± SE. **b** ^{35}S-pulse labeling of *C. reinhardtii* proteins in the presence of 70 μM sulfate. Aliquots were removed from the labeling reaction mixture at 0, 15, 30, 45, 60 and 90 min, respectively. **c** Radiolabeled (^{35}S-sulfate) proteins as revealed by SDS–PAGE and autoradiography. Total cellular proteins were extracted and loaded on equal-cell basis. Air-dried polyacrylamide gels were exposed to X-ray film and the autoradiography of the ^{35}S-label of proteins was recorded. The protein bands corresponding to RbcL, RbcS and D1 are indicated by *arrows*

analysis. Because the steady-state level of the RbcL gene transcripts was not affected in the *asulp29* antisense transformant (shown by the QRT–PCR analysis in Fig. 5b), it would appear that the lower amount of RbcL in *asulp29* is the result of slower rates of protein biosynthesis, apparently a consequence of the slower rates of sulfate uptake (Fig. 6). Further, this SDS–PAGE and Western blot analysis showed that levels of the PSII reaction-center D1 protein and the LHCII proteins were also lower in the *asulp29* antisense transformant relative to the wild type (Fig. 7b, D1 and LHCII panels). These results are consistent with the Chl and photosystem per cell contents shown in Table 1.

The above analyses showed that the basic photochemical apparatus and electron-transport chain organization are about the same in the wild type and *asulp29* antisense transformant. However, the number of electron-transport chains, including that of the two photosystems, was lowered in *asulp29*. Similarly, the amount of LHCII and the concentration of Rubisco were lowered in *asulp29*. These results suggested that antisense transformation of *C. reinhardtii* with the *SulP* gene might have resulted in an overall smaller chloroplast size in this green alga. This hypothesis was investigated by high-resolution confocal microscopy. Figure 8 shows images of cell and chloroplast outlines obtained either by combined transmission and Chl fluorescence microscopy (DIC + Fluor) or enhanced fluorescence microscopy (Fluor) of the same wild type and *asulp29* single cells. In the case of fluorescence measurements (Fluor), only the parts of the cell that contain Chl appear bright red. This comparative microscopic analysis helped to delineate the outline of the cells versus that of the red-fluorescing thylakoid membranes in the wild type and *asulp29*. It is clearly seen that the *asulp29* has a diminished cross-sectional area of Chl fluorescence relative to that of the wild type. These microscopy results are consistent with the biochemical quantitative analyses

Table 1 Steady-state concentrations of Chl, P700 (PSI) and Q_A (PSII) in the thylakoid membrane of wild-type *Chlamydomonas reinhardtii* and the *asulp29* antisense transformant

Strain	Chl/cell (mol×10^{-15}/cell)	Chl/P700 (mol:mol)	Chl/Q_A (mol:mol)	P700/cell (mol×10^{-18}/cell)	Q_A/cell (mol×10^{-18}/cell)	PSII/PSI (mol:mol)
Wild type	1.9 ± 0.22	816 ± 91	739 ± 46	2.1 ± 0.1	2.8 ± 0.3	1.3/1
asulp29	1.3 ± 0.1	823 ± 22	635 ± 12	1.62 ± 0.2	2.1 ± 0.4	1.3/1

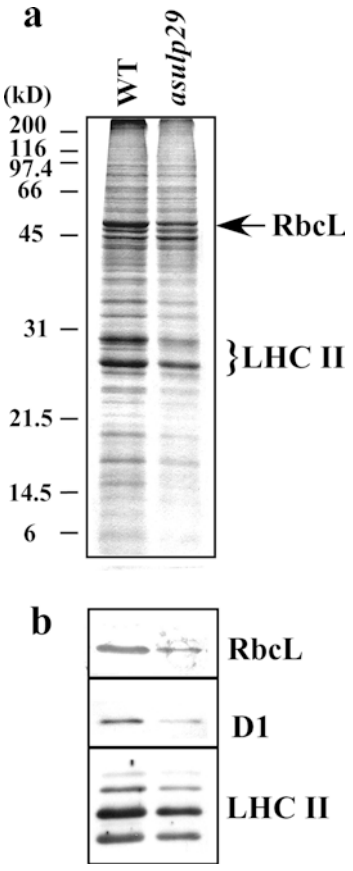

Fig. 7a,b Electrophoretic separation and quantitative Western blot analysis of proteins from wild-type and *asulp29* *C. reinhardtii*. **a** Coomassie-stained SDS–PAGE profile of total protein extracts from the wild type and *asulp29* transformant. Samples were loaded on an equal-cell basis. The protein bands corresponding to RbcL and LHCII are indicated. The molecular weights of the protein markers are indicated on the *left*. **b** Western blot analysis using specific polyclonal antibodies against RbcL (large subunit of Rubisco), D1 (PSII reaction center protein) and LHCII (Chl *a*/*b* light-harvesting complex of PSII)

of Table 1 and Fig. 7 and show less Chl and fewer thylakoid membranes in the *asulp29* antisense transformant relative to that in the wild type.

Discussion

Cellular localization of the *SulP* gene product

Recently, a novel nuclear-encoded sulfate permease gene (*SulP*) was cloned in the green microalga *Chlamydomonas reinhardtii* (Chen et al. 2003). Genomic, proteomic, phylogenetic and evolutionary analyses suggested a sulfate permease gene of prokaryotic origin. It was postulated that this novel gene probably migrated from the chloroplast to the nuclear genome during the evolution of *C. reinhardtii* (Chen et al. 2003). Neither the *SulP* gene, nor any of its homologues, has been retained in vascular plants, e.g. *Arabidopsis thaliana*, although it is encountered in the chloroplast genome of several

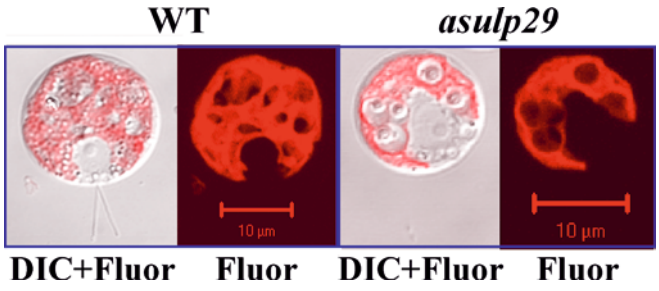

Fig. 8 In vivo differential interference contrast (*DIC*) and Chl fluorescence (*Fluor*) microscopy of the *C. reinhardtii* wild type (*WT*) and *asulp29* transformant. Zeiss 510 confocal scanning laser microscopy was operated in the transmission and epi-fluorescence mode (*DIC + Fluor*) or in the epi-fluorescence mode (*Fluor*). For the fluorescence measurement, excitation was provided by a Helium Neon Laser at 543 nm, while Chl fluorescence emission was detected in the red region of the spectrum with a 560-nm long-pass cut-off filter in front of the detector. Bars = 10 µm

green algae and even in the liverwort *Marchantia polymorpha* (Chen et al. 2003). This nuclear-encoded sulfate permease contains a putative chloroplast transit peptide, while the deduced amino acid sequence of the mature protein contains seven transmembrane helix domains and two large hydrophilic loops. These properties of the SulP suggested a membrane-integral protein. Localization and functional properties of this sulfate permease were investigated in this work.

Foremost, it was shown that a specific *SulP* gene induction occurs under conditions of sulfate nutrient deprivation of the cells, evidenced by the accumulation of gene transcripts (Northern blot analysis) and accumulation of the mature protein (Western blot analysis with specific polyclonal antibodies). Cellular fractionation approaches permitted isolation of a chloroplast envelope membrane fraction. Western blot analyses clearly showed presence of the SulP protein in this chloroplast envelope fraction and absence of the SulP protein from the thylakoid membrane fraction. These analyses provided evidence for the localization and function of the SulP protein in the chloroplast envelope of *C. reinhardtii*.

Chloroplast sulfate transport and regulation of photosynthetic gene expression

Antisense transformation of *C. reinhardtii* with the *SulP* cDNA provided evidence of a substantial impact of this gene on chloroplast function. Specifically, sulfate uptake and assimilation capacity were lowered as a consequence of attenuated *SulP* gene expression in the cell, directly affecting rates of de novo protein biosynthesis, and causing a lower photosynthetic capacity of the cell. Results in this work are consistent with the notion that sulfate uptake by the cell is quickly followed by transport into the chloroplast where sulfate assimilation takes place. This assertion is supported by the fact that *C. reinhardtii* lacks sizable vacuoles in which to store sulfate anions.

That the SulP protein functions in sulfate uptake and chloroplast protein biosynthesis is evident in the *asulp29* antisense transformant by the overall slower rates of RbcL, RbcS and D1 protein biosynthesis. Slower rates implied a reduction in the translation of the corresponding genes. However, QRT–PCR analysis showed that transcript levels of these genes are not significantly affected in the *asulp29* antisense transformant under control growth conditions. Therefore, reduction of *RbcL*, *RbcS* and *PsbA* (D1) gene expression in *asulp29* must be regulated at the translational and/or post-translational level. Several studies have reported on the translational regulation of *PsbA* (D1) and *RbcL* gene expression. In particular, the translational control of the *PsbA* gene expression in *C. reinhardtii* is well documented (Danon and Mayfield 1994; Kim and Mayfield 1997). A model for the light-activated translational regulation of the *PsbA* transcript in the chloroplast was proposed (Yohn et al. 1998). It was suggested that a reducing environment is generated by PSII during the light reactions of photosynthesis, which triggers the translation of the *PsbA* gene. Considering the impaired PSII function and slower rates of light-saturated O_2 evolution of the *asulp29* antisense transformant, it is possible that a diminished amount of reductant in the latter is unable to promote maximal rates of *PsbA* mRNA translation. Moreover, it was reported that the glutathione redox potential regulates the translation of *RbcL* gene expression (Irihimovitch and Shapira 2000). Glutathione is generated through the cysteine biosynthetic pathway and its function is subject to an oxidation/reduction equilibrium between GSH and GSSG (for a review, see Leustek et al. 2000). A lower level of cysteine biosynthesis, due to limitation in sulfate supply, might affect the pool of glutathione as well as its redox potential, which could then slow-down the rate of translation for the *RbcL* gene. Results in this work showed that synthesis of RbcS is also affected in the *asulp29* antisense transformant, resulting in lower levels of Rubisco in the latter, consistent with findings in sulfate-deprived *C. reinhardtii* (Zhang et al. 2002), *Dunaliella salina* (Cao et al. 2001; Giordano et al. 2000), *Lemna* (Ferreira and Teixeira 1992), and wheat (Gilbert et al. 1997). The concomitant lowering of the D1, RbcL and RbcS proteins demonstrates a coordinate regulation in the acclimation of cells to a suboptimal nutrient environment. This regulation evidently involves both the light and the carbon reactions of photosynthesis.

Sulfate transport systems

The *C. reinhardtii* chloroplast sulfate permease (SulP) belongs to the ABC (ATP-binding cassette) transporter superfamily. We have recently cloned three additional genes coding for putative components of the envelope-localized sulfate permease holocomplex:

i. The *SulP2* gene (GenBank Accession # AY536251) encodes the second chloroplast sulfate permease which, along with SulP, probably forms a heterodimeric inner envelope transmembrane complex.
ii. The *Sabc* gene (GenBank Accession # AY536252) encodes an ATP-binding protein, probably localized on the stroma-side of the chloroplast envelope.
iii. The *Sbp* gene (GenBank Accession # AY536253) encodes a putative sulfate-binding protein, which is probably localized on the outer chloroplast envelope, and whose function we postulate to be linkage of the cytosolic compartment with the inner chloroplast envelope. These new findings significantly strengthen the notion that an ABC-type sulfate transport system in the chloroplast of *C. reinhardtii* functions in the process of import of sulfate from the cytosol into the chloroplast of this green alga.

To the best of our knowledge, no other sulfate transport gene has been identified in *C. reinhardtii*. The *SacI* gene, which encodes a regulatory protein involved in cell acclimation to sulfate deprivation, has some similarity to transporters with 12 membrane helices (Davies et al. 1996). However, no evidence has been provided for the direct function of this protein in sulfate transport. Further, the nature and function of chloroplast sulfate transporters in higher plants also remains unknown. Leustek et al. (2000) have identified in *Arabidopsis* a number of sequences similar to ABC transporter genes. However, sequences similar to the *SulP* gene have not been identified in higher plants. Although gene sequences homologous to *SulP* have been retained in the chloroplast genome of the liverwort *Marchantia polymorpha* (Ohyma et al. 1986), it is possible that these prokaryotic-type sulfate permease genes were not retained during the evolution of higher plants (Chen et al. 2003) In the cyanobacterium *Synechococcus* sp. strain PCC 7942, evidence was provided for the function of *CysA*, *CysT* and *CysW* in sulfate transport (Laudenbach and Grossman 1991). Mutant strains with an interruption in any of these genes exhibited essentially no sulfate uptake and were not viable when grown with sulfate as the sole sulfur source (Laudenbach and Grossman 1991). However, genes encoding both H^+/SO_4^{-2} co-transporters and ABC transporters could be identified in the genome of *Synechocystis* sp. PCC6803 (Kohn and Schumann 1993; Kaneko et al. 1996). It would be interesting to know how these two types of sulfate transporter coordinate with each other in terms of regulation of sulfate uptake and assimilation in this cyanobacterium. Our current investigation on the function of three other genes, *SulP2*, *Sabc* and *Sbp* (see Introduction), encoding putative components of the chloroplast ABC transport system in *C. reinhardtii*, should lead to a better understanding of the chloroplast sulfate transport mechanism in this green alga.

Sulfate uptake, the PSII damage and repair cycle and H_2 evolution in green algae

The D1/32-kDa reaction-center protein of PSII accounts for less than 1% of the total thylakoid membrane protein. Yet, the rate of its biosynthesis is comparable to or exceeds that of the abundant large subunit of Rubisco in the chloroplast (Bottomley et al. 1974; Eaglesham and Ellis 1974; Mattoo and Edelman 1987). The reason for the high rates of de novo D1 biosynthesis is the frequent turnover of this protein, which is a consequence of photo-oxidative damage in chloroplasts (Melis 1999). Investigations on the PSII damage and repair cycle (Adir et al. 1990; Guenther and Melis 1990) revealed that a constant supply of sulfate nutrients to the chloroplast is needed to sustain D1 biosynthesis and recovery of PSII from this photo-oxidative damage (Ohad et al. 1984; Vasilikiotis and Melis 1994; Wykoff et al. 1998). Upon S-deprivation, D1 biosynthesis slows-down and the PSII repair process is impeded. A gradual loss of PSII activity and loss of oxygen evolution is then manifested as photodamaged PSII centers accumulate in the chloroplast thylakoids (Wykoff et al. 1998). Rates of photosynthetic oxygen evolution drop below those of mitochondrial respiration in the green algae (Melis et al. 2000), causing anaerobiosis in a sealed culture (Ghirardi et al. 2000). It was shown that this condition is necessary and sufficient for the induction of the [Fe]-hydrogenase pathway in green algae (Melis and Happe 2001), leading to sustained rates of H_2 photoproduction by the culture. Thus, S-deprivation proved to be a useful tool in studying both the PSII repair-process in chloroplasts and the induction of the [Fe]-hydrogenase pathway and H_2 photoproduction in *C. reinhardtii*. In this respect, attenuation in the expression of the *SulP* gene may serve to down-regulate sulfate nutrient uptake by the chloroplast in *C. reinhardtii*, leading to sustained rates of H_2 photoproduction in sulfate-replete media of this green alga. The latter can be achieved through antisense technology of the *SulP* gene, which lowers the sulfate uptake capacity of the cell. The successful application of *SulP* antisense technology in *C. reinhardtii* was demonstrated in this work. Accordingly, efforts currently focus on establishing the relationship between the sulfate uptake capacity of the chloroplast in *C. reinhardtii* and PSII repair process, as well as H_2 evolution in *SulP* antisense transformants.

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