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Changes in cysteine and O-acetyl-L-serine levels in the microalga *Chlorella sorokiniana* in response to the S-nutritional status

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ABSTRACT

We analyzed the effects of deprivation and subsequent restoration of sulphate (S) in the nutrient solution on cysteine (Cys) and O-acetyl-L-serine (OAS) levels in *Chlorella sorokiniana* (211/8k). The removal of S from the culture medium caused a time-dependent increase in O-acetyl-L-serine(thiol)lyase (OASTL) activity and a decrease in soluble proteins content. The protein gel blot analysis was used to show that OASTL isoforms are located in the chloroplast and in the cytoplasm of S-starved cells. S-deprivation caused a decrease in the intracellular levels of Cys and glutathione (GSH) and an increase in serine (Ser) and OAS, reflecting an imbalance between sulphur and nitrogen assimilation. Re-supplying of sulphate to S-starved cells produced a decrease in OAS levels and concomitant rapid increase in Cys and GSH concentrations. The simultaneous addition of OAS and sulphate to S-starved cells did not further increase the concentration of Cys, suggesting the existence of a threshold level of intracellular Cys that is independent of the cellular concentration of OAS. Our findings that OAS is stored during S-starvation and that its quick decrease appears to be coupled with the increase of Cys levels upon re-supply of sulphate, imply that the central role that these two compounds play is in the regulation of sulphur-assimilating enzymes in response to the S status of the cell.

Introduction

In higher plants, sulphate is taken up from the soil by specific root membrane transport systems, then stored in the vacuoles or transported via xylem and plasmodesmata to the leaves. In the chloroplasts, SO_4^{2-} is reduced and then assimilated into cysteine (Cys). Specific signaling mechanisms control, regulate and coordinate sulphate uptake in the roots, transport through the plant, and assimilation, which occurs mainly in the leaves. When plant cells are subjected to a low sulphur regime, they exhibit a suite of responses, including the synthesis of extracellular arylsulphatases in algae (Pootakam et al., 2010), elevated sulphate transport activity (Gojon et al., 2009; Pootakam et al., 2010), and an increase in the levels of transcripts and activities of enzymes associated with S-assimilation (Davidian and Kopriva, 2010) in higher plants. These responses allow plant cells to efficiently scavenge and assimilate sulphate available in the environment.

The utilization of unicellular algae as a model system to study enzymes involved in plant metabolism is advantageous because their metabolism depends only on the nutrients that each cell takes up from the medium and not from a metabolite exchange between cells. The unicellular alga *Chlorella sorokiniana* (211.8k) represents a suitable experimental system to study phenomena that occur in plant cells as a consequence of sulphur shortage or S supply. *C. sorokiniana* reproduces faster (approximately 6 h) (Janssen et al., 1999) than *Chlamydomonas reinhardtii*, the algal model organism (Janssen et al., 1999), which provides an opportunity to introduce a wide range of nutritional changes in culture to study their effects on cellular metabolic processes in a short span of time. There has been increasing interest in microalgae recently, especially in the large-scale cultivation of *C. sorokiniana* for biotechnology and biofuel applications (Zijffers et al., 2010).

C. sorokiniana exhibits an ability to rapidly adapt its metabolism in response to changes in the availability of external SO_4^{2-} by varying some fundamental physiological processes, such as photosynthesis and nitrogen assimilation (Di Martino Rigano et al., 2000). Specifically, it has been shown in *C. sorokiniana* that SO_4^{2-} removal strongly reduces growth, decreases photosynthetic capacity, provokes a rapid inhibition of ammonium uptake and elicits a

Abbreviations: Cys, cysteine; GSH, glutathione; Met, methionine; OAS, O-acetyl-L-serine; OASTL, O-acetyl-L-serine(thiol)lyase; PCV, packed cell volume; Ser, serine.

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substantial increase in free non-sulphur amino acids (Di Martino Rigano et al., 2000).

Cys synthesis in higher plants occurs in three cellular compartments: mitochondria, cytosol and plastids (Heeg et al., 2008), whereas in the eukaryotic alga *Chlamydomonas reinhardtii*, Cys biosynthetic enzymes appear to be localized only in the chloroplasts (Ravina et al., 2002).

In algae, as in higher plants, L-Cys represents the first amino acid regarded as the terminal metabolite of sulphur assimilation. The free Cys content in plant cells of *Arabidopsis* is maintained at low basal levels (Krueger et al., 2009) because it is rapidly incorporated into proteins and GSH, or serves as a sulphur donor for the synthesis of methionine (Met) and many other secondary compounds.

The biosynthesis of Cys is carried out by two sequential reactions that are catalyzed by two different enzymes: serine acetyltransferase (SAT, EC 2.3.1.30) and O-acetyl-L-serine(thiol)lyase (OASTL, EC 4.2.99.8). SAT acetylates L-serine (Ser) in the presence of acetyl-CoA to form O-acetyl-L-serine (OAS), and OAS is then used as a substrate in the reaction catalyzed by the OASTL enzyme. The latter inserts a sulphide into the carbonic skeleton of OAS to constitute the sulphur containing amino acid L-Cys. Both enzymes, SAT and OASTL, interact with each other, forming a hetero-oligomeric complex called the cysteine synthase complex (CSC), which was first described in *Salmonella typhimurium* by Kredich et al. (1969) and later in plants (Ruffet et al., 1994; Droux et al., 1998; Jost et al., 2000). The CSC plays a fundamental role in the rate of Cys synthesis (Wirtz and Hell, 2006): the association of SAT with OASTL is controlled by OAS, Cys and inorganic sulphur, and the ratio between free and linked SAT and OASTL control the rate of Cys biosynthesis, and therefore, that of other sulphurylated compounds.

The aim of this study was to investigate the mechanisms that control the physiological changes in response to the deprivation and re-supply of SO_4^{2-}. Measuring of the intracellular levels of SH-related compounds in algal cells grown under different sulphur nutritional conditions will allow us to ascertain their roles as substrates, intermediary metabolites or as effector molecules in sulphur assimilation.

Materials and methods

Organism and growth conditions

C. sorokiniana Shihira and Krauss, strain 211/8k (CCAP of Cambridge University) was grown in batch culture at 35 °C, under continuously light (Philips TLD 30W/55 fluorescent lamps, 250 μmol photons m^{-2} s^{-1}), and flushed with air containing 5% CO_2 at a flow rate of about 80–100 L h^{-1}. The composition of the basal medium and the growth procedure were previously reported (Di Martino Rigano et al., 2000). Sulphate was supplied in the medium as 1.2 mM $MgSO_4$. The N source was supplied as 5 mM KNO_3. Under these conditions, the growth rate constant (μ) was 3 d^{-1}. For experiments, cells were collected 2 d after the start of the culture when they were in exponential growth (S-sufficient cells). S-starved cells were obtained collecting batch grown cells by low speed centrifugation, washed two times and re-suspended in a S-free medium under the same conditions as for growth.

The effects of addition of a OAS exogenous sterile solution to the nutrient medium of S-starved cells for 24 h were analyzed after supplying OAS at a final concentration of 1 mM.

Chlorophyll analysis

Chlorophyll content of cells was estimated spectrophotometrically after extraction into N,N-dimethylformamide according to Inskeep and Bloom (1985).

PCV determination

The packed cell volume (PCV) was estimated by centrifuging 10 mL of cell suspension in a haematocrit tube at 4000 × *g* for 5 min. At the end of centrifugation the packed cell volume at the bottom of the tube was determined and expressed in μL.

Content of Ser, OAS and Met

Samples of the cell suspensions (about 10 mL) were collected at the given times by centrifugation (4000 × *g* for 15 min). The packed cells were treated with 1 mL of cold 80% EtOH and vortexed. The amino acids were extracted for 10 min at 4 °C and finally clarified by centrifugation. The supernatant was filtered using Waters Sep-Pak Cartridges C18 light (Milan, Italy), and utilized for amino acid analysis as previously described (Di Martino Rigano et al., 2000). Quantification of Ser and OAS was made against a relative calibration curve and expressed as μmol mL^{-1} PCV.

Content of thiols

Samples of the cells (about 50 mL) were collected by centrifugation (4000 × *g* for 15 min), the packed cells frozen with liquid nitrogen and then treated with 2 mL of ice-cold 0.1 N HCl–1 mM EDTA buffer. The homogenates were centrifuged at 15,000 × *g* at 4 °C for 15 min. Thiols were reduced at room temperature for 1 h by mixing 400 μL of the supernatants with 600 μL of 200 mM 2(N-cyclohexylamino)-ethanesulfonic acid (CHES) (pH 9.3) and 100 μL of 3 mM dithiothreitol (DTT). Aliquots (330 μL) were derivatized in the dark for 15 min by adding 20 μL of 15 mM monobromobimane in acetonitrile. The reaction was stopped by adding 250 μL of 0.25% (v/v) methanesulfonic acid. Samples were finally centrifuged for 15 min (14,000 rpm). Derivatized thiols were separated and quantified by reverse-phase HPLC using the method described by Newton et al. (1981). Quantification was made against a calibration curve for Cys and GSH. Thiol levels were expressed as μmol mL^{-1} PCV.

Assay of OASTL activity

C. sorokiniana cells (500 mL of suspension), harvested by low-speed centrifugation (4000 × *g* for 5 min), were re-suspended in cold extraction buffer: 50 mM potassium phosphate buffer (pH 7.5), 1 mM DTT, 10 μM pyridoxal 5′-phosphate (PLP) and broken by passing twice through a French pressure cell (11,000 psi). The homogenate was centrifuged at 16,000 × *g* for 20 min at 4 °C, and the clear supernatant was used as crude extract. Enzymatic OASTL activity was determined colorimetrically measuring the Cys formed in a reaction mixture containing in a final volume of 100 μL: 100 mM Hepes/KOH (pH 7.5), 5 mM DTT, 10 mM OAS, 5 mM Na_2S and an aliquot of the crude extract. The reaction was started by the addition of Na_2S, and after 5 min at 50 °C, was stopped by adding 50 μL of a 20% (v/v) TCA. Cys was determined according to Gaitonde's procedure (1967) using the acid ninhydrin reagent. OASTL activity was expressed in μkat which corresponds to the formation of 1 μmol of Cys min^{-1}.The OASTL activity was related to the soluble protein content of the samples. Protein amounts were determined using the Bio-Rad protein assay based on the Bradford method (1976) with bovine serum albumin as the standard.

Protein gel blot analysis

Protein electrophoresis was carried out according to Laemmli (1970), using 4.5% acrylamide as stacking gel and 12% acrylamide as resolving gel. Electrophoresis was carried out at constant current of 45 mA for 1.5 h. Protein bands were blotted onto Hybond-ECL membrane (0.2 μm) (Amersham) in the Mighty Small transphor blot

system (Ge-Healthcare, USA). Membranes were blocked in 20 mM Tris/HCl (TBS buffer, pH 8.4) with 5% BSA for 90 min, washed with TBS containing 0.1% of Tween-20 (TBS-T buffer) and incubated for 90 min in TBS buffer with 0.2% BSA containing rabbit primary antibody against the purified OASTL proteins from *Arabidopsis thaliana*. OASTL antibodies were produced against cytosolic (A), plastidial (B) and mitochondrial (C) isoforms.

The membrane was finally washed two times for 5 min with TBS-T buffer and incubated for 90 min with TBS buffer with 0.2% BSA containing anti-rabbit IgG horseradish peroxidase conjugate (Sigma). After rinsing in TBS buffer for 5 min (two times), membranes were developed with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and Lumifilm chemiluminescent detection film (Roche, Basel, Switzerland) according to the manual.

Statistical analysis

Experimental data analyses were conducted using Sigmaplot 11 software. Data of means ± SE of three independent experiments were presented. The statistical analysis was performed by one-way ANOVA to determine differences between the S-sufficient and S-starved algae, with $P < 0.05$ as significant. The Pearson correlation coefficient was used to assess the correlation between the OASTL activity and the Cys content during the S-deprivation.

Results

Effects of S removal on soluble protein content

In *C. sorokiniana* cells, the soluble protein contents strongly decreased during S-starvation, on a PCV basis. During the first 6 h of S-starvation, the protein content decreased from 11.5 ± 1.1 to 4.3 ± 0.9 μg μL^{-1} PCV. After 24 h of S depletion, the protein content was estimated to be 1.8 ± 0.5 μg μL^{-1} PCV, which is approximately 15% of the control value (Fig. 1).

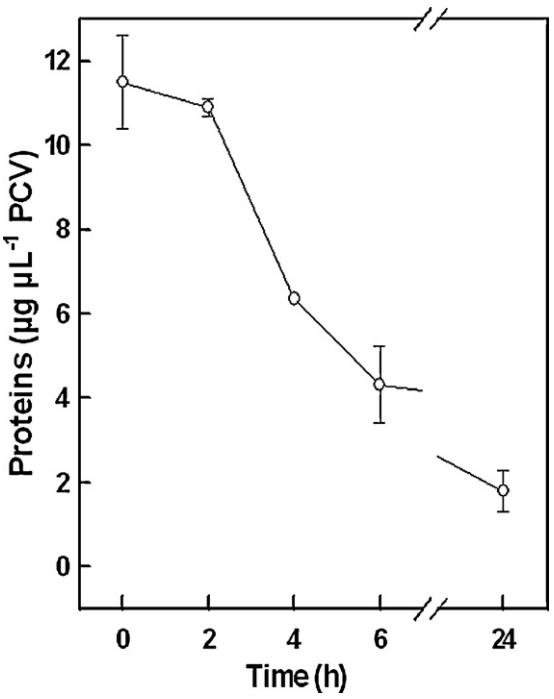

Fig. 1. Changes in total soluble protein concentration (μg μL^{-1} PCV) upon sulphate starvation in cells of *C. sorokiniana* S-starved cells were obtained collecting S-sufficient cells by a low speed centrifugation, washed two times and re-suspended in a S-free medium (zero time). At the indicated times, cells were assayed for total soluble protein content. The values reported are means ± SE ($n = 3$). Error bars smaller than symbols are not shown.

Effects of S removal on intracellular concentrations of GSH, Cys, Met, OAS and Ser

The intracellular levels of OAS, Ser, Met, Cys and GSH were determined in *C. sorokiniana* S-sufficient cells and in 24 h-S-starved cells to investigate the effects of sulphate deficiency on nitrogen and sulphur metabolism. A limited supply of sulphate caused a decrease in the levels of sulphur-containing metabolites, such as Cys and GSH. In S-sufficient cells, the concentration of Cys was lower than other amino acids (Di Martino Rigano et al., 2000) and was slightly, but significantly, reduced in cells starved for S for 24 h; GSH concentrations were 1.24 ± 0.04 μmol mL^{-1} PCV in S-sufficient cells and 0.19 ± 0.04 μmol mL^{-1} PCV in cells after 24 h of S-starvation (Fig. 2). The Met level was not significantly influenced by the S nutritional status of the cells (Fig. 3).

S-starved cells contained levels of Ser that were 3.6-fold higher than S-sufficient cells after 24 h (Fig. 3). Similarly, the levels of OAS, the immediate N-precursor of Cys, increased during S-starvation, reaching a value that was 3-fold higher than that of S-sufficient cells (Fig. 3).

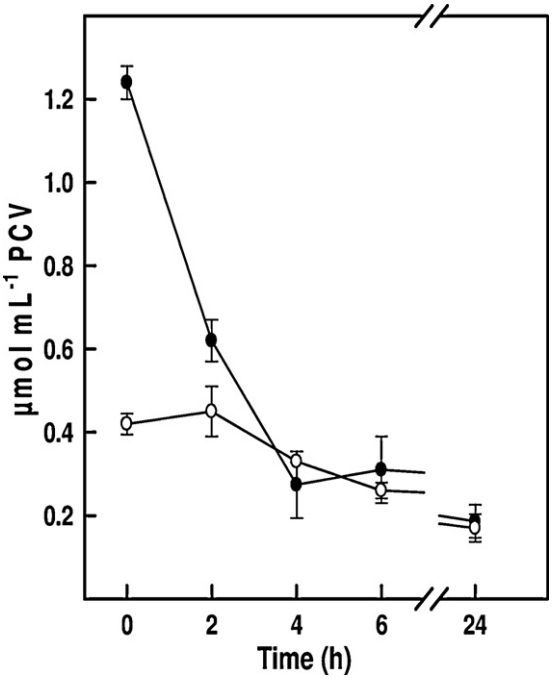

Fig. 2. Contents of cysteine (○-○) and glutathione (●-●) (μmol mL^{-1} PCV) upon sulphate starvation in cells of *C. sorokiniana* S-starved cells were obtained collecting S-sufficient cells by a low speed centrifugation, washed two times and re-suspended in a S-free medium (zero time). At the indicated times, cells were assayed for cysteine and glutathione contents. The values reported are means ± SE ($n = 3$).

Short-term variations in the intracellular concentration of Cys, GSH, OAS and Ser upon S re-supply to S-starved cells

The intracellular concentration of Cys rapidly increased in S-starved cells upon sulphate re-supply, from 0.13 ± 0.01 μmol mL^{-1} PCV to 3.00 ± 0.07 μmol mL^{-1} PCV in the first 20 min, and followed by a slight increase to 4.1 ± 0.21 μmol mL^{-1} PCV in the following 3 h (Fig. 4A). The level of GSH suddenly increased in S starved

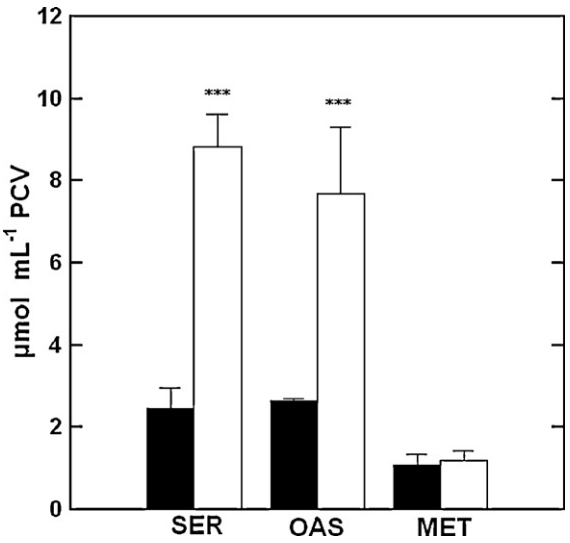

Fig. 3. Contents of serine, OAS and methionine (μmol mL^{-1} PCV) in S-sufficient and S-starved *C. sorokiniana* cells. The dark histograms represent S-sufficient cells, white histograms represent S-starved cells. The values reported are means ± SE (n = 3). Significant differences, using one-way ANOVA, were analyzed in S-starved cells with respect to S-sufficient cells (P < 0.001, ***).

cells within 1 h, changing from 0.082 ± 0.006 μmol mL^{-1} PCV to 0.81 ± 0.05 μmol mL^{-1} PCV; GSH content then decreased slightly after 8 h, reaching a value similar to S-sufficient cells (Fig. 4D).

After S re-supply, the intracellular concentration of OAS decreased from the initial 8.7 ± 1.15 to 3.6 ± 1.10 μmol mL^{-1} PCV in the first 10 min. After 6 h of the sulphate addition, the OAS content reached a value similar to S-sufficient cells (Fig. 4B). The level of Ser rapidly decreased from 8.8 ± 0.9 to 1.7 ± 0.36 μmol mL^{-1} PCV within 20 min, similar to the amount found in S-sufficient cells (Fig. 4C).

Effects of sulphate plus OAS addition on the cellular OAS and Cys content of S-starved cells

A re-supply of 1.2 mM sulphate plus 1 mM OAS to 24 h S-starved *C. sorokiniana* cells produced a rapid increase in the internal OAS concentration from 8.7 ± 1.15 up to 15 ± 1.00 μmol mL^{-1} PCV in 10 min, followed by OAS promptly returning to its starting level within 50 min (Fig. 5).

The re-supply of 1.2 mM sulphate plus 1 mM OAS to 24 h S-starved *C. sorokiniana* cells produced a rapid increase in Cys content, from 0.3 ± 0.03 to 3.9 ± 0.09 μmol mL^{-1} PCV in 2 h, which then remained constant over the following 4 h (Fig. 6). The amount of Cys formed was similar to that formed in S-starved cells re-supplied with sulphate alone (Fig. 4A) and was independent of the different intracellular concentrations of OAS.

S availability and OASTL activity

The OASTL activity was measured in *C. sorokiniana* S-sufficient and S-starved cells. S starvation caused a substantial time-dependent increase in OASTL specific activity from 3.7 ± 0.2 up to 49.8 ± 9.9 μkat mg^{-1} protein after 24 h (Fig. 7).

Due to the reduction in the protein content caused by S-deprivation, OASTL activity was also expressed with respect to the chlorophyll content (Fig. 7 panel A) and to PCV (Fig. 7 panel B). These data confirm the actual induction of OASTL activity upon S-starvation.

Occurrence of different OASTL isoforms

The OASTL from crude extracts of S-sufficient or S-starved *C. sorokiniana* cells cross-reacted with antibodies against *Arabidopsis* OASTL. The protein gel blot analysis of crude extracts from *C. sorokiniana* (Fig. 8), probed with three different *Arabidopsis t.* OASTL isoforms (cytosolic, chloroplastic and mitochondrial), suggests the occurrence of a chloroplastic isoform in both S-sufficient and S-starved cell. However, the presence of a cytosolic isoform was detected only in S-starved cells.

The cytosolic isoform of OASTL had a subunit size of 31 kDa, which is slightly smaller than that of the chloroplastic OASTL (34.3 kDa) (Fig. 8).

Discussion

Sulphate uptake and assimilation in plant cells are regulated at different key points. The assimilation pathway is regulated by sulphur demand. Specifically, it is repressed when reduced sulphur or thiols are available, and it is promptly activated by sulphur deficiency. Different regulatory mechanisms of sulphur metabolism have been recently described in plants, such as transcriptional, post-transcriptional, protein-protein interaction and feed-back control (Davidian and Kopriva, 2010; Yi et al., 2010). In the study of the regulation of Cys synthesis, it is important to consider the different cellular locations of the enzymes operating in this pathway and the cellular distribution of the metabolites involved. The exchange mechanisms among the different cellular compartments (cytosol, plastids and mitochondria) of the metabolites related to Cys synthesis are as yet unknown. The assimilatory sulphate reduction is located in plastids, suggesting that sulphide is able to diffuse through membranes to reach both the cytosol and mitochondria. Moreover, in *Arabidopsis*, the exchange of sulphur-related metabolites between the cytosol and plastids is required to coordinate the synthesis of Met and GSH (López-Martín et al., 2008; Krueger et al., 2009). It still remains questionable if and how much Cys is actually stored in the cell.

The cytosol is considered the major site of Cys synthesis (Haas et al., 2008). Krueger et al. (2009) demonstrated in *Arabidopsis thaliana* that Cys concentrations in the cytosol are higher than in the other cell compartments. In *Arabidopsis,* cytosolic Cys levels are estimated to be over 300 μM, whereas the other cell compartments contain below 10 μM (Alvarez et al., 2010). As a consequence, Cys homeostasis in the cell should be significantly controlled in the cytosol, which is the main site of Cys biosynthesis.

A previous study by our group (Di Martino Rigano et al., 2000) analyzed some responses of *C. sorokiniana* to sulphur limitation, which included a decrease in growth rate, a reduction in photosynthetic O_2 evolution and an increase in starch content and free amino acids. S-deprivation induced specific responses related to sulphur assimilation in *C. sorokiniana* cell, similar to the response in a number of higher plants (Davidian and Kopriva, 2010). However, the effects in this microalga arose more quickly than in higher plants.

In *C. sorokiniana* cells grown under sulphate deficiency, decreases in both Cys and GSH intracellular concentrations were observed. GSH levels significantly decreased within the first 4 h from the beginning of the S-deprivation state. Upon sulphate deprivation, the degradation of intracellular GSH may have contributed to the in maintenance of Cys homeostasis in the cells. Despite the finding that Cys levels in *Chlorella* cells decrease during S-deprivation, Met levels do not decline.

In S-starved cells, a strong decrease in soluble protein levels was observed as well. It could be assumed that the S-starved cells may utilize sulphur from an internal protein pool to redistribute amino acids to satisfy their nutritional requirements. It has

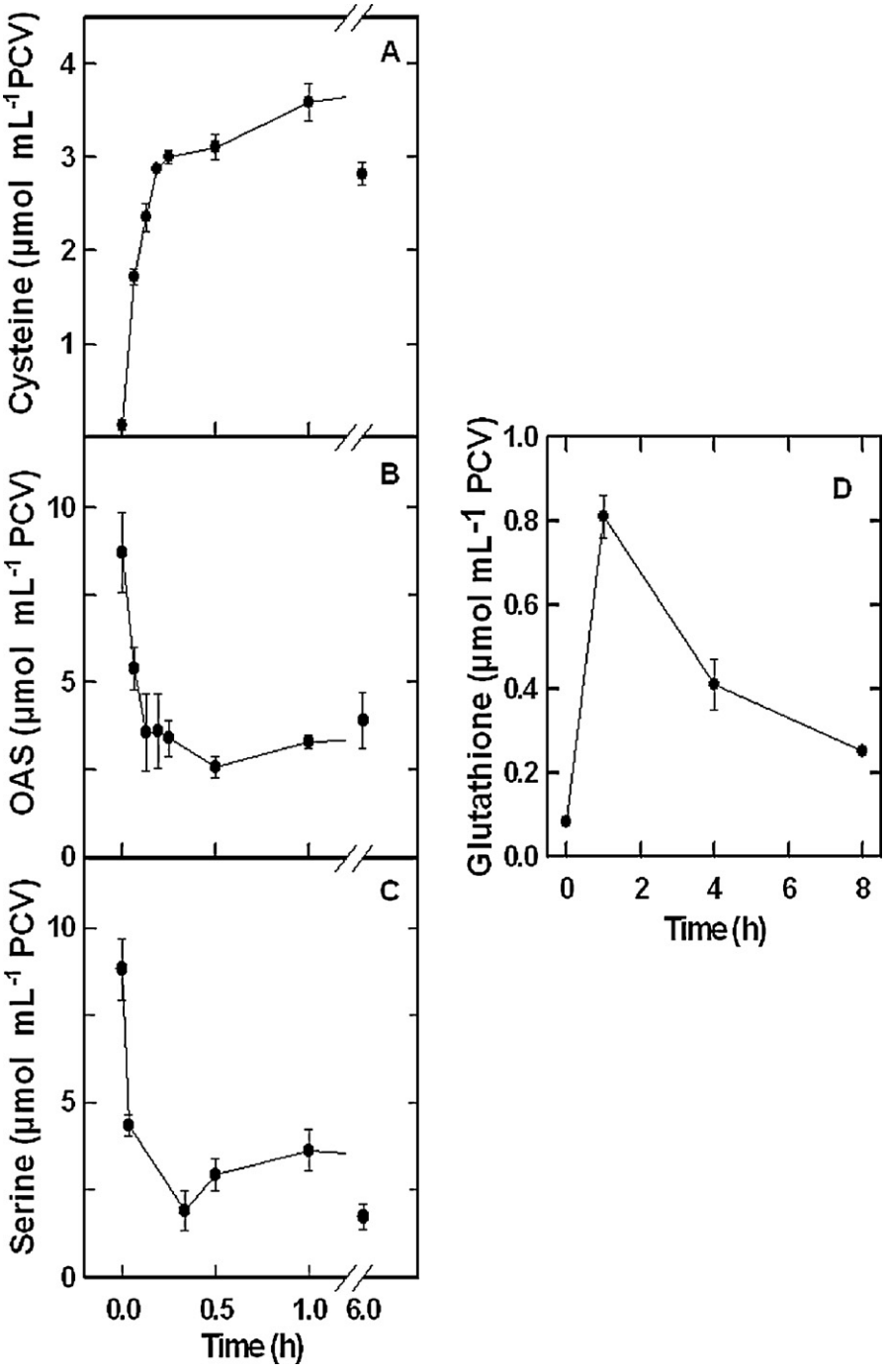

Fig. 4. Variations in the intracellular levels of (A) cysteine, (B) OAS, (C) serine and (D) GSH upon addition of 1.2 mM $MgSO_4$ (zero time) to S-starved cells of *C. sorokiniana*. The values reported are means ± SE ($n = 3$). Bars smaller than symbols are not shown.

previously been shown in tobacco plants (Lewandowska and Sirko, 2008) and in *Chlamydomonas reinhardtii* (Zhang et al., 2004), that prolonged sulphur starvation influences the expression of many genes, including those involved in protein degradation. The results presented here suggest that, in *C. sorokiniana* S-starved cells, the rapid decrease in protein concentration within 4 h could be ascribed to a dilution effect as a consequence of the cellular growth (even if in S-starved cells it has considerably slowed) together with a reduction in the *ex novo* protein synthesis. The decrease in S-compounds caused a reduction in the amount of total proteins, unbalancing nitrogen assimilation, and considerably affecting the overall metabolism and the growth of algae.

During S-deprivation, *C. sorokiniana* accumulated high intracellular levels of OAS and its upstream metabolite Ser, revealing a disturbed interaction between nitrogen and sulphur metabolism. The storage of OAS (and Ser) could be ascribed to the deficiency of sulphur, more than as a consequence of enhanced protease activity.

Nitrogen metabolism and S-assimilation are mutually affected by S deficiencies because the accumulation of the last precursor of Cys (OAS) influences the re-channeling of assimilated nitrogen (Carfagna et al., 2010).

The levels of OAS in *C. sorokiniana* cells remained high during the entire period of S-starvation. This increase may be essential to allow for fast assimilation of sulphur whenever this nutrient becomes

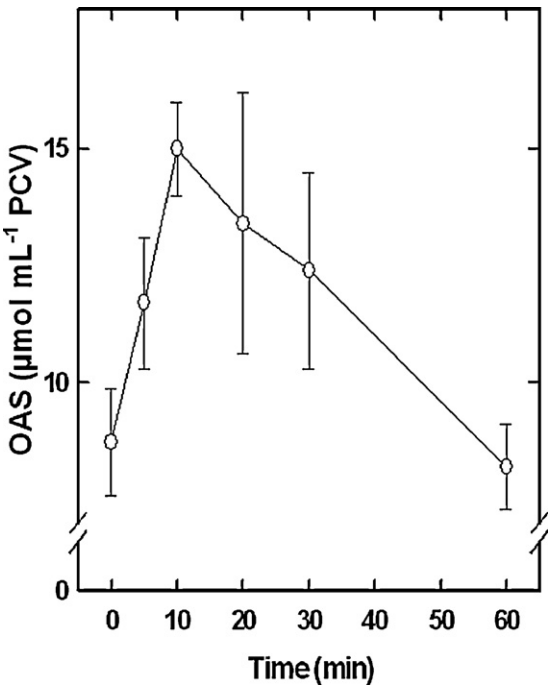

Fig. 5. Variations in the intracellular concentration of OAS upon addition of sterile 1 mM OAS plus 1.2 mM $MgSO_4$ (zero time) to S-starved cells of *C. sorokiniana*. The values reported are means ± SE ($n = 3$).

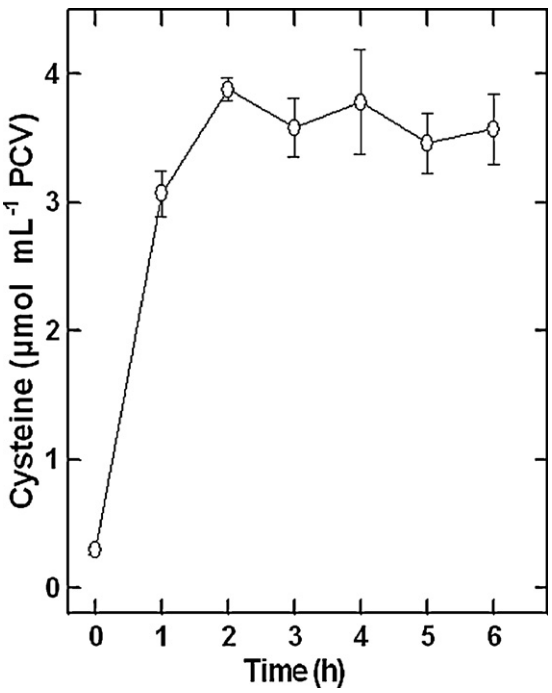

Fig. 6. Increase in the concentration of cysteine upon addition of 1 mM OAS plus 1.2 mM $MgSO_4$ (zero time) to S-starved cells of *C. sorokiniana*. The values reported are means ± SE ($n = 3$).

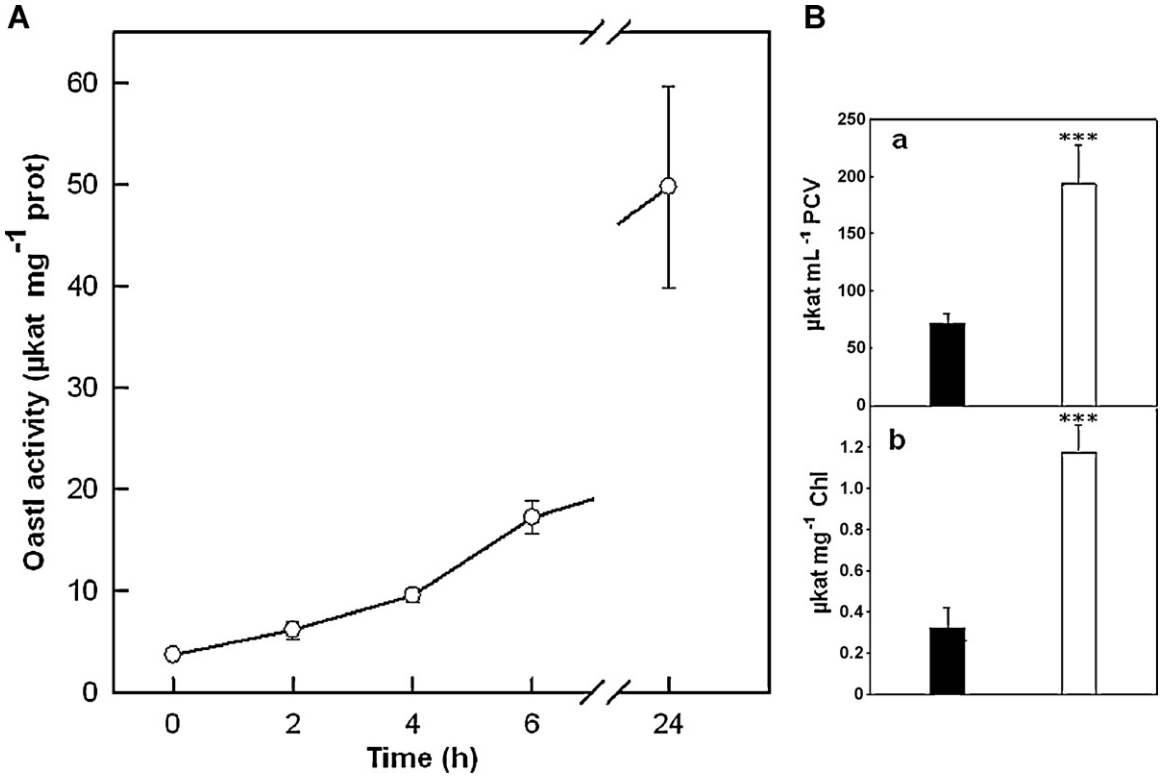

Fig. 7. Effects of sulphate deprivation on OASTL activity in cells of *C. sorokiniana*. S-starved cells were obtained collecting S-sufficient cells by a low speed centrifugation, washed two times and re-suspended in S-free medium for 24 h (zero time). At the indicated times, cells were assayed for OASTL activity. The activity was expressed as µkcat mg^{-1} prot (panel A) and respect to PCV (a) and chlorophyll (b) (panel B). The dark histograms represent S-sufficient cells; white histograms represent S-starved cells. The values reported are means ± SE ($n = 3$). Error bars smaller than symbols are not shown. Significant differences, using one-way ANOVA, were analyzed in S-starved cells with respect to S-sufficient cells ($P < 0.001$, ***).

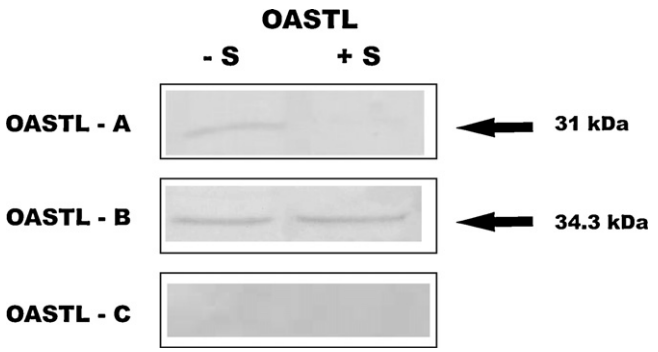

Fig. 8. Protein gel blot analysis of crude extracts from *C. sorokiniana*. Lane +S: OASTL protein analysis of S-sufficient algae crude extract; Lane −S: OASTL protein analysis of S-starved algae crude extract. The protein analysis was made utilizing antibodies raised against the purified OASTL proteins from *Arabidopsis thaliana*. OASTL antibodies were produced against cytosolic (A), plastidial (B) and mitochondrial (C) isoforms. Membranes were developed with Amersham ECL kit. Other details in the section "Materials and methods".

available again. A re-supply of S to S-starved cells rapidly decreased intracellular OAS and Ser to low levels that are typical of S-sufficient cells. A concomitant increase in Cys levels, up to 7.5-fold higher than S-sufficient cells, was also observed within 30 min following re-supply. The promptness of the Cys levels increase upon sulphate re-supply to S-starved cells, confirms the strict relationship between the OAS decrease and Cys synthesis. The GSH levels also increased after sulphate re-supply, but this increase was only transient (1 h) with the level returning to a value similar to the S-starved cells over time.

In S-starved cells, the external supply of OAS rapidly doubled the high internal OAS concentration within 10 min, suggesting that these cells could actively uptake OAS. It is relevant that, in spite of this noticeable increase in internal OAS levels, Cys levels did not exceed those occurring in cells with no OAS added upon S and OAS re-supply. These results support the hypothesis of the existence of a threshold level of Cys that would inhibit its further synthesis.

O-acetyl-L-serine(thiol)lyase is the key enzyme involved in Cys biosynthesis. In recent years, many researchers have purified and characterized OASTL enzymes from higher plants (Wirtz et al., 2004), algae (Ravina et al., 1999) and bacteria (Zhao et al., 2006). Two to four isoforms of OASTL, located in three different cellular compartments, cytosol, chloroplast/plastid and mitochondria, have been isolated in higher plants (Warillow and Hawkesford, 1998; Jost et al., 2000).

As previously observed in higher plants (Carfagna et al., 2010) and in algae (Ravina et al., 2002), levels of OASTL activity are strongly dependent on the sulphur nutritional status of cells. In *Chlamydomonas* a strong increase in transcript levels of both chloroplastic OASTL and SAT, upon sulphur starvation has recently been demonstrated (González-Ballester et al., 2010). After 24 h of S-starvation, the total OASTL activity in *C. sorokiniana* was strongly increased (over 10-fold). Protein gel blot analysis revealed the presence of at least two OASTL isoforms, one localized in the cytosol and one localized in the chloroplast, but the cytosolic isoform was only detected in S-starved cells. The data presented here indicate that activities (and the occurrence) of different OASTL isoforms were regulated by the sulphur status of *C. sorokiniana*: specifically, that the cytosolic isoform could be more susceptible to changes in the levels of sulphur, OAS and Cys. The increase in OASTL activity during S-deprivation could be ascribed to an increase in the cytosolic OASTL isoform.

In *C. sorokiniana* cells, during S-deprivation, both Cys decreased and OASTL activity increased in a time dependent manner. The levels of intracellular Cys correlate ($P = 0.0225$) with the OASTL activity (Supplementary Fig. 1).

In conclusion, these results suggest that *C. sorokiniana* cells have suitable mechanisms to maintain viability under S-starvation. A lack of sulphate unbalances nitrogen metabolism, eliciting an accumulation of the nitrogen precursors of Cys (Ser and OAS) and a decrease in the total soluble protein concentration. The accumulation of OAS caused the release of OASTL from the CSC and induced high-affinity sulphate transporters (Yi et al., 2010), thus activating S assimilation and promoting the synthesis of cysteine. OAS and Cys, located in the cytosol or in the chloroplast, may therefore enhance OASTL isoforms, resulting in the activation of the sulphur assimilating processes.

The Cys level in the cell is finely regulated and never exceeds a threshold level. However, rapid and significant fluctuations in both Cys and GSH levels were measured in cells shifting from an S-starved to an S-sufficient state.

These small changes in the intracellular Cys levels may affect the rate of sulphur assimilation. Therefore, Cys (and possibly GSH) could be a major "sensor" of the S status of the cell, regulating the rate of its own synthesis. Further studies are required to understand the roles of these S-compounds in the modulation of S metabolism in plant cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.07.012.

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