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Sulfur Deprivation Results in Oxidative Perturbation in *Chlorella sorokiniana* (211/8k)

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Sulfur deficiency in plant cells has not been considered as a potential abiotic factor that can induce oxidative stress. We studied the antioxidant defense system of *Chlorella sorokiniana* cultured under sulfur (S) deficiency, imposed for a maximum period of 24 h, to evaluate the effect of an S shortage on oxidative stress. S deprivation induced an immediate (30 min) but transient increase in the intracellular H_2O_2 content, which suggests that S limitation can lead to a temporary redox disturbance. After 24 h, S deficiency in *Chlorella* cells decreased the glutathione content to <10% of the value measured in cells that were not subjected to S deprivation. Consequently, we assumed that the cellular antioxidative mechanisms could be altered by a decrease in the total glutathione content. The total ascorbate pool increased within 2 h after the initiation of S depletion, and remained high until 6 h; however, ascorbate regeneration was inhibited under limited S conditions, indicated by a significant decrease in the ascorbate/dehydroascorbate (AsA/DHA) ratios. Furthermore, ascorbate peroxidase (APX) and superoxide dismutase (SOD) were activated under S deficiency, but we assumed that these enzymes were involved in maintaining the cellular H_2O_2 balance for at least 4 h after the initiation of S starvation. We concluded that S deprivation triggers redox changes and induces antioxidant enzyme activities in *Chlorella* cells. The accumulation of total ascorbate, changes in the reduced glutathione/oxidized glutathione (GSH/GSSG) ratios and an increase in the activity of SOD and APX enzymes indicate that oxidative perturbation occurs during S deprivation.

Keywords: Ascorbate • *Chlorella sorokiniana* • Glutathione • Oxidative stress • Reactive oxygen species • Sulfur starvation.

Abbreviations: ANOVA, analysis of variance; APX, ascorbate peroxidase; AsA, ascorbate; DHA, dehydroascorbate; DTNB, 5,5′ dithiobis(2-nitrobenzoic acid); GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NBT, nitroblue tetrazolium; S, sulfur, SOD, superoxide dismutase; TEMED, tetramethylethylenediamine.

Introduction

Plant exposure to biotic or abiotic stress increases the production of reactive oxygen species (ROS). In plant cells, ROS, such as singlet oxygen, the superoxide anion radical, the hydroxyl radical and hydrogen peroxide ($^1O_2$, $O_2^{-}\cdot$ OH· and H_2O_2, respectively) are normally generated in chloroplasts, mitochondria and peroxisomes as products of aerobic metabolism (Maurino and Flugge 2008). However, excessive production and concentration of these species leads to oxidative damage or programmed cell death (Boonstra and Post 2004, Mullineaux and Baker 2010).

Cells have an antioxidant defense system to remove ROS and avoid oxidative stress (Noctor and Foyer 1998, Tuteja 2007). ROS are scavenged by enzymatic antioxidants including SOD, ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antioxidants such as ascorbate and glutathione (Foyer and Noctor 2005). Antioxidants can inhibit the oxidation of substrates, providing electrons with free radicals and thereby affecting the balance and efficient functioning of cells. Higher antioxidant contents and antioxidant enzyme activities are associated with higher stress tolerance in unicellular algae (Shiu and Lee 2005, Vega et al. 2005).

The primary scavengers in the detoxification of ROS in plant cells are SODs (EC 1.15.1.1), metalloenzymes that produce H_2O_2 as a result of the dismutation reaction of O_2^{-}. SODs are classified into three types based on their metal cofactor: Fe-SOD (chloroplast), Mn-SOD (mitochondria) and Cu/Zn-SOD (chloroplast, cytosol and peroxisome) (Sen 2012). In recent years, the absence of Cu/Zn-SODs has been demonstrated in some eukaryotic algae such as *Chlamydomonas reinhardtii* (Merchant et al. 2007), *Phaeodactylum tricornutum* (Bowler et al. 2008) and *Cyanidioschyzon merolae* (Matsuzaki et al. 2004); however, Cu/Zn-SOD activity was detected in several charophyte algae (Kanematsu et al. 2010).

APX and GR enzymes are important antioxidant components that belong to the ascorbate–glutathione cycle responsible for H_2O_2 removal. APXs (EC 1.11.1.11) are oxidoreductase enzymes that are able to use peroxide as an electron acceptor and ascorbate as a donor. Five APXs have been identified in plants: cytosolic, mitochondrial, peroxisomal/glyoxysomal and chloroplastic isoforms (Dabrowska et al. 2007). In green algae, APX has also been found to have a function as an H_2O_2 scavenger; however, its subcellular localization remains unclear. *Chlamydomonas reinhardtii* and *Chlorella vulgaris* only contain one APX isoform (Takeda et al. 1998), and in *Euglena gracilis*, APX is localized only in the cytosol (Shigeoka et al. 1980).

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Among the enzymatic antioxidants, GR (EC 1.6.4.2) is one of the key enzymes in the active oxygen scavenging system. This enzyme regenerates reduced glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine), which regenerates ascorbate. In the ascorbate–glutathione cycle, GSH is oxidized to GSSG, which is further reduced to GSH by the NADPH-dependent activity of GR. Thus, GR maintains the balance between GSH and the ascorbate pools, which in turn maintain the cellular redox state (Contour-Ansel et al. 2006). Two GR isoforms have been found in the unicellular green alga *C. reinhardtii*, thought to be localized in the cytoplasm and chloroplast (Serrano and Llobell 1993).

Nutrient deficiency in plants cells represents an abiotic stress. Plants frequently grow in soils that contain very low concentrations of macronutrients such as nitrogen, phosphorus, potassium or sulfur (S) (Schachtman and Shin 2007). S deficiency is a recent but serious problem in some European countries (Lehmann et al. 2008). This deficiency can be attributed to the decline in industrial emissions and to less intensive application of mineral fertilizers (Scherer 2009). S is a major macronutrient that is required for optimal plant cell growth and development, and a deficiency of this nutrient leads to visible symptoms such as chlorosis and retarded growth (Nikiforova et al. 2004).

In all plants, S is assimilated into numerous essential compounds such as the amino acids cysteine and methionine, glutathione, hormones (ethylene, polyamines), vitamins and cofactors (biotin, thiamine, CoA), and numerous secondary products (Leustek 2002). In addition, some S-containing compounds are involved in stress responses; for example, glutathione is an important antioxidant (Pócsi et al. 2004) that prevents ROS from damaging critical cellular components and is a substrate for phytochelatin synthesis (Davidian and Kopriva 2010). In combination with GSSG, GSH forms a sensitive redox buffer that allows fine-tuning of the cellular redox environment under normal and external environmental stressed conditions (Meyer and Hell 2005). Consequently, insufficient S nutrition reduces plant growth and resistance to abiotic and biotic stresses (Lewandowska and Sirko 2008, Bashir et al. 2013).

Despite an increasing literature on oxidative stress in plants as a result of abiotic stress (Fan et al. 2012, Farfan-Vignolo and Asard 2012, Choudhury et al. 2013), there is limited knowledge of the role of nutrient deficiency as a factor causing oxidative stress. Recent studies have shown that roots undergo an oxidative burst due to nitrogen, phosphorus or potassium deprivation (Shin et al. 2005, Hernandez et al. 2012). Similarly, an oxidative burst in young leaves has been reported in response to S limitation (D'Hooghe et al. 2013).

In addition, ROS play an important signaling role in the response of plants to external biotic and abiotic stimuli (Gill et al. 2013), allowing the activation of several 'defense genes' (Foyer and Noctor 2012). The ROS appear to act as a metabolic interface between the environment and cellular metabolism and, subsequently, acclimation or death. To adapt and grow in a nutrient-limited environment, plants needs to sense changes in external and internal mineral nutrient concentrations and adjust their growth to match resource availability (Schachtman and Shin 2007).

In this study, we used the unicellular alga *Chlorella sorokiniana* to determine the effects of S starvation on the occurrence of H_2O_2 as a representative of ROS generation. In addition, the activation of antioxidative enzymes and changes in ascorbate and glutathione levels were investigated. *Chlorella sorokiniana* cells rapidly adapt their metabolism in response to changes in the availability of external S by varying fundamental physiological processes (Di Martino Rigano et al. 2000). S starvation strongly reduces growth, decreases photosynthetic capacity, rapidly inhibits ammonium uptake and causes a marked increase in the pool of free non-S amino acids (Di Martino Rigano et al. 2000).

There is no direct evidence that shows that ROS are produced or that antioxidant enzymes are involved in the response to S deficiency. The participation of ROS in S signaling might be more complex than for other nutrients because in the removal of H_2O_2 the ascorbate–glutathione cycle is implicated, which is downstream of S assimilation. In this study, we aimed to determine whether ascorbate was efficiently regenerated at low glutathione levels because S deficiency causes a decrease in the cysteine and glutathione contents in *Chlorella* cells (Carfagna et al. 2011) and glutathione represents a key component of the ascorbate–glutathione cycle in the H_2O_2 detoxification pathway. The results presented are discussed in the context of physiological changes that occur in an S-deficient plant cell and aim to provide new insights on metabolism regulation under S-limited conditions.

Results

Total soluble protein content

The effects of S deprivation on the total soluble protein content (expressed as pg per cell) of *C. sorokiniana* are presented in **Fig. 1**. A marked decrease in the total soluble protein content was measured in cells subjected to S deficiency for 4, 6 and 24 h. The protein content at time zero (at the beginning of S starvation) was 1.96 ± 0.18 pg cell^{-1}, which decreased significantly during the first 6 (1.18 ± 0.05 pg cell^{-1}) to 24 h (0.29 ± 0.07 pg cell^{-1}) of S starvation.

Hydrogen peroxide content

The intracellular H_2O_2 content was measured over time to determine the effect of S starvation on the occurrence of oxidative stress in *Chlorella*. The H_2O_2 levels increased significantly 30 min after the S deprivation was imposed, but by 4 h and throughout the remainder of the experiment (24 h), the H_2O_2 content decreased to a value lower than that at time zero (**Fig. 2**).

Glutathione and ascorbate contents

The glutathione (GSH and GSSG) and ascorbate [oxidized ascorbate (AsA) and dehydroascorbate (DHA)] contents were measured at different times of S starvation to determine whether oxidative imbalance was induced in the cell.

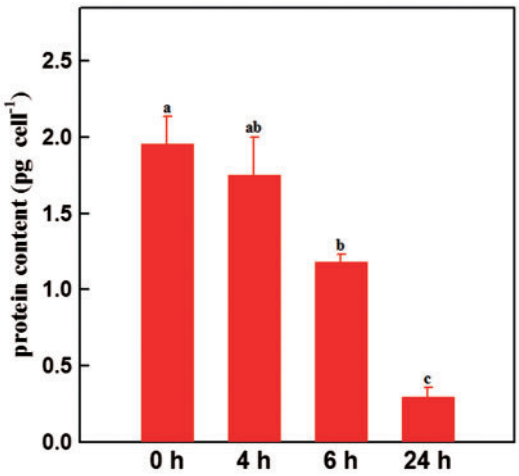

Fig. 1 Effect of sulfur deprivation on total soluble protein levels in *Chlorella sorokiniana* cells. The protein contents (expressed as pg per cell) were determined using the Bradford method 0, 4, 6 and 24 h after the initiation of S starvation. Data are presented as the means ± SE ($n = 5$). Values with the same letter within a column were not significantly different ($P \leq 0.05$, ANOVA, Tukey's multiple comparison).

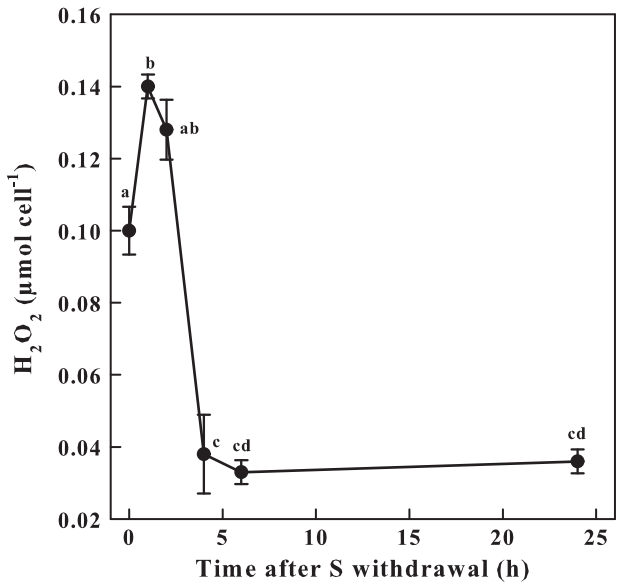

Fig. 2 Time course of intracellular hydrogen peroxide content in *Chlorella sorokiniana* under S deprivation, measured at 0, 0.5, 2, 4, 6 and 24 h after S withdrawal. The H_2O_2 content (expressed as µmol per cell) is the mean of at least three different experiments. Data presented are means ± SE. Different letters indicate statistically significant differences ($P < 0.001$, ANOVA, Tukey's multiple comparison).

The total glutathione content (GSH plus GSSG) was severely affected by S starvation and decreased strongly with increasing time since the beginning of S starvation. The minimum total glutathione content was measured after 24 h (**Fig. 3A**).

The GSSG level increased shortly (2 h) after the S starvation was imposed and then decreased. The GSH level decreased over time, reaching a minimum value after 24 h (**Fig. 3A**). The GSH/GSSG ratio decreased slightly after 2 h and then increased over time (6 h). The intracellular levels of GSH and GSSG were similar after 24 h (**Fig. 3B**).

Total ascorbate (AsA plus DHA) increased significantly within 2 h of the S depletion and remained high until 6 h (**Fig. 4A**). The AsA/DHA ratio decreased strongly under S deprivation, which indicated a conversion of the total ascorbate pool to its oxidized form (**Fig. 4B**).

Antioxidant enzymes

The time course analysis of APX activities (**Fig. 5A**) indicated that these enzyme were strongly affected in the 24 h following S starvation. The increase in the APX activities was not significant during the first 4 h, but there was a considerable increase in the following hours. These results were confirmed using native electrophoresis on crude extracts of *C. sorokiniana* cells collected at different times of S starvation (0, 2, 4, 6 and 24 h). A Native PAGE, which was loaded with the same amount of proteins in each lane, was stained for APX activity (**Fig. 5B**). The protein bands from S-starved cell extracts showed a more intense staining compared with those from the non-starved cells. Furthermore, the protein gel revealed the occurrence of three distinct bands that probably corresponded to different isoforms of the enzyme (**Fig. 5B, C**). However, image analysis of protein migration on Native PAGE did not provide information on the molecular weight of the isoforms or their cellular localization. No significant changes were observed in APX isoenzymatic patterns during the first 6 h of S starvation; however, approximately 2.05-, 2.45- and 3.11-fold increases in APX-1, APX-2 and APX-3, respectively, were observed after 24 h (**Fig. 5C**).

A time course analysis was performed on the activity of SODs subjected to S deficiency (**Fig. 6A**). S starvation caused a significant increase in SOD activities at 4, 6 and 24 h compared with cells that were not S starved (time 0 h). Native protein electrophoresis stained for SOD activity confirmed the increased activity of these enzymes caused by S starvation and suggested the existence of three distinct isoforms with different electrophoretic mobility, referred to as SOD-1, SOD-2 and SOD-3 (**Fig. 6B, C**). There was no significant change in the SOD isoenzymatic pattern up to 6 h of S starvation compared with the cells that were not S starved (time h). Therefore, a significant increase in all SOD isoforms was observed after 24 h of S starvation (**Fig. 6B, C**).

The relative activities of GR decreased approximately 7-fold after 24 h of S starvation compared with the cells that were not S starved (time 0 h) (**Fig. 7**).

Discussion

S limitation is an abiotic stress for plant cells. In this study, the relationship between S deprivation and the antioxidant defense system was investigated in the green microalgae *C. sorokiniana*. Until now, the involvement of S deficiency as a possible abiotic factor causing oxidative stress in plant cells has not been clearly studied or demonstrated. In *Chlorella*, S starvation can stimulate important changes in cell

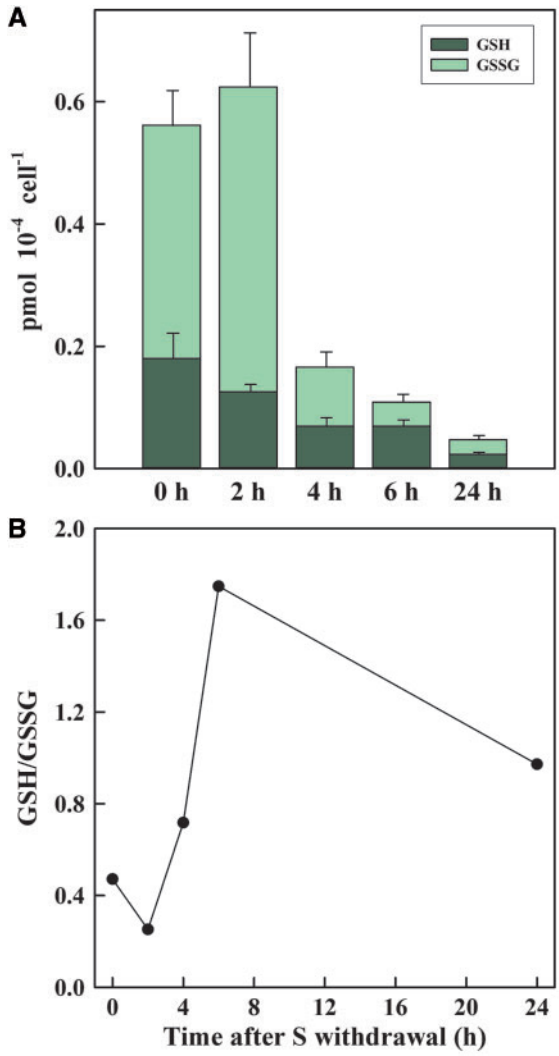

Fig. 3 Total glutathione levels in cells of *Chlorella sorokiniana* measured 0, 2, 4, 6 and 24 h after the initiation of S starvation. The data sets are illustrated using stacked bar graphs to compare the GSH and GSSG contents. Values (expressed as pmol 10^{-4} per cell) are means ± SE ($n = 3$). (B) The GSH/GSSG ratio at the indicated time after S withdrawal. Data presented are the ratios of the means.

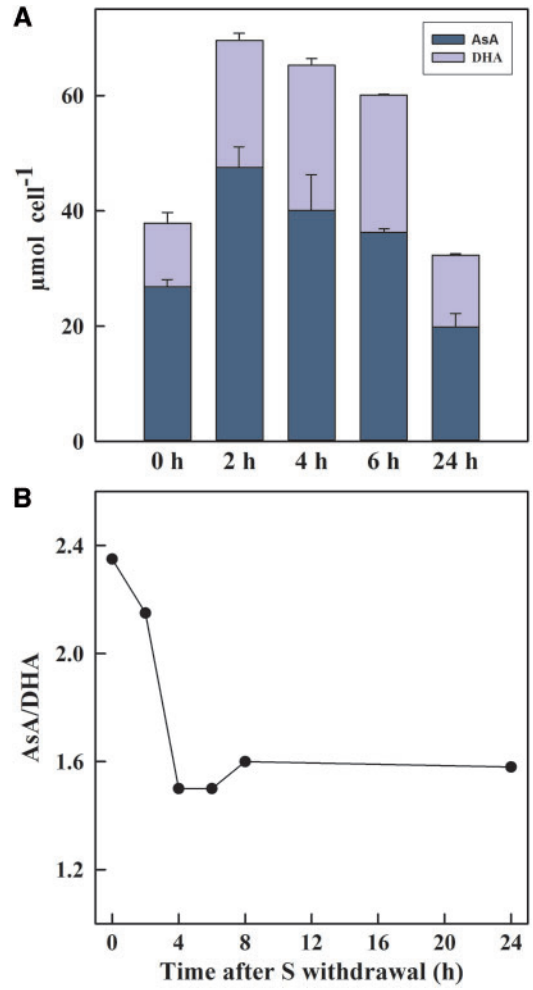

Fig. 4 Total ascorbate levels in cells of *Chlorella sorokiniana* measured 0, 2, 4, 6 and 24 h after the initiation of S starvation. The data sets are illustrated using stacked bar graphs to compare the AsA and DHA contents. Values (expressed as µmol per cell) are means ± SE ($n = 3$). (B) The AsA/DHA ratio at the indicated time after S withdrawal. Data presented are the ratios of the means.

metabolism (Di Martino Rigano et al. 2000, Carfagna et al. 2011). In addition to affecting growth, photosynthesis and respiration, S deficiency increases the demand for S-containing compounds, which enhances the activity of *O*-acetylserine(thiol)lyase enzymes (Salbitani et al. 2014) and provokes a dismantlement of glutathione to obtain the amino acid cysteine (Carfagna et al. 2011).

In addition, S starvation induces an immediate (only 30 min) and transient increase in the intracellular H_2O_2 content, which suggests that S restriction leads to a temporary oxidative imbalance. In the early stages of S starvation, H_2O_2 could act as a putative signaling molecule that induces a cellular response to S deprivation, which commonly occurs in a wide range of abiotic stress responses (Choudhury et al. 2013). Following the early and rapid increase in the intracellular H_2O_2 content in response to S starvation, the H_2O_2 decreased over time to levels lower than those measured in non-starved cells after 24 h.

This reduction in the H_2O_2 content can be explained as follows: (i) the APX activities increased strongly during S deprivation; (ii) cellular respiration and photosynthesis, i.e. the main physiological processes involved in the natural production of H_2O_2, decreased considerably in S-starved cells (Di Martino Rigano et al. 2000, Antal et al. 2011); and (iii) H_2O_2 may have been excreted into the culture medium. This is considered one of the mechanisms for algae to avoid harmful accumulation of cellular H_2O_2 (Shiu and Lee 2005).

The ascorbate–glutathione cycle, which is implicated in the removal of H_2O_2 (Foyer and Noctor 2011), occurs downstream of S assimilation. Consequently, the involvement of ROS signaling or the origin of oxidative stress in S deprivation may be more complex and interesting to study than other nutrient deficiencies.

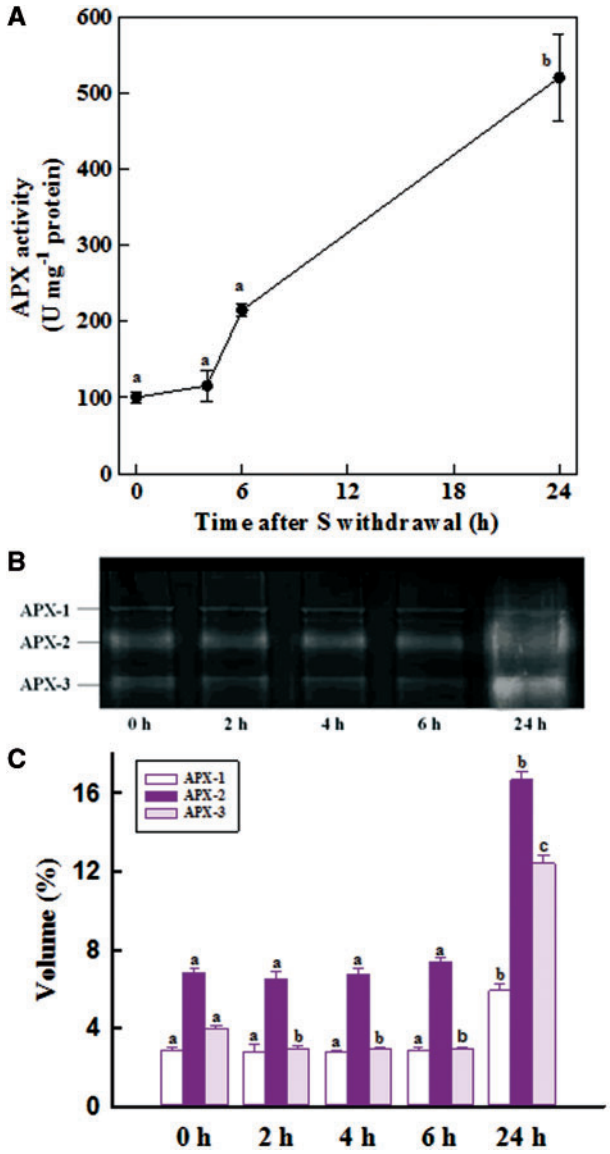

Fig. 5 The time course of APX activities measured at 0, 4, 6 and 24 h after sulfur withdrawal (A). The results are expressed as U mg^{-1} protein. Data presented as the means ± SE ($n = 3$). Values followed by the same letter were not significantly different ($P < 0.001$, ANOVA, Tukey's multiple comparison). APX activity stain on Native PAGE (B) and quantitative protein band analysis (C). Polyacrylamide native gel (10%) electrophoresis, which was stained for APX activities, was carried out on crude extracts of *Chlorella sorokiniana* cells at 0, 2, 4, 6 and 24 h from the beginning of S starvation. Equal amounts (30 μg) of proteins were loaded in each lane (B). Gels were analyzed using the ImageMaster™ 2D Platinum software (GE Healthcare). The presented image represents one electrophoresis experiment; however, at least three gel electrophoresis experiments were performed. Quantitative results are expressed as % ± SE; the protein band volume was normalized to the band volume of the entire gel. Values followed by the same letter are not significantly different ($P < 0.05$ and $P < 0.001$, ANOVA, Tukey's multiple comparison).

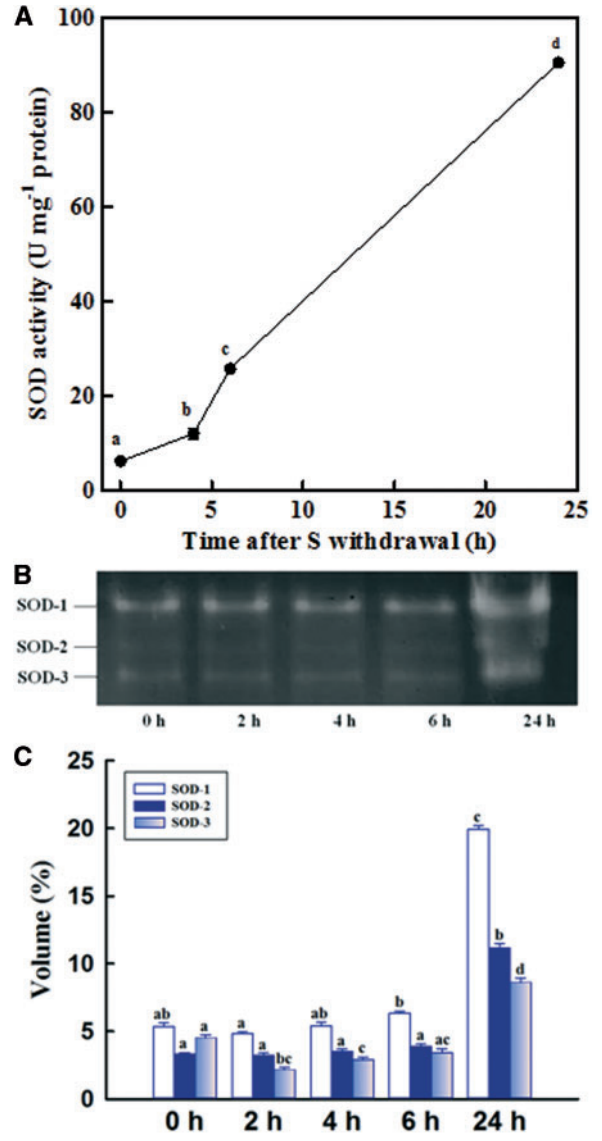

Fig. 6 The time course of SOD activities measured at 0, 4, 6 and 24 h after S withdrawal (A). The results are expressed as U mg^{-1} protein. Data presented are means ± SE ($n = 3$). Values followed by the same letter are not significantly different ($P < 0.001$, ANOVA, Tukey's multiple comparison). SOD activity stain on Native PAGE (B) and quantitative protein band analysis (C). Polyacrylamide native gel (10%) electrophoresis, which was stained for SOD activities, was carried out on crude extracts of *Chlorella sorokiniana* cells at 0, 2, 4, 6 and 24 h from the beginning of S starvation. Equal amounts (30 μg) of proteins were loaded in each lane (B). Gels were analyzed using the ImageMaster™ 2D Platinum software (GE Healthcare). The presented image represents one electrophoresis experiment; however, at least three gel electrophoresis experiments were performed. Quantitative results are expressed as % ± SE; the protein band volume was normalized to the band volume of the entire gel. Values followed by the same letter are not signififlig;cantly different ($P < 0.001$, ANOVA, Tukey's multiple comparison).

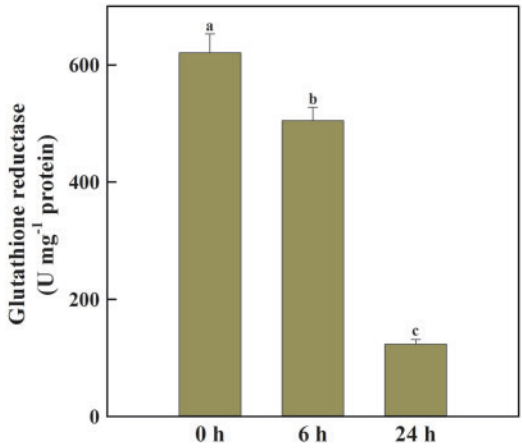

Fig. 7 GR activity (U mg^{-1} protein) was determined in *Chlorella sorokiniana* 0, 6 and 24 h from the beginning of S starvation. Data presented are means ± SE ($n = 3$). Values with different letters within a column indicate significant differences ($P < 0.05$, ANOVA, Tukey's multiple comparison).

In general, glutathione is considered to be the most important storage and transport form of reduced S. Glutathione and ascorbate are essential components of the cellular antioxidative defense system and are directly involved in the maintenance of cellular redox homeostasis to maintain low concentrations of ROS (Noctor and Foyer 1998). In particular, the ratios between the reduced and oxidized forms of ascorbate (AsA/DHA) and glutathione (GSH/GSSG) play an important role in signaling and the activation of various defense mechanisms in plant cells (Latowski et al. 2010, Foyer and Noctor 2012). Ascorbate and glutathione are influenced differently by environmental factors (Foyer and Noctor 2011): glutathione and its redox state are influenced by light, CO_2 or O_2 (Gill et al. 2013), while ascorbate synthesis is particularly sensitive to the light environment (Foyer and Noctor 2011). S nutrition in *C. sorokiniana* directly affects the intracellular glutathione content. Cysteine is a precursor of glutathione and its degradation product. When S supply limits the concentration of this amino acid (Carfagna et al. 2011), the intracellular glutathione level decreases because: (i) its ex novo synthesis is strongly compromised; and (ii) its degradation is promoted to obtain cysteine.

A significant reduction in GR activity was observed in *Chlorella* cells 24 h after the initiation of the S starvation. High GR activity was maintained during the first 6 h after S depletion to preserve the GSH/GSSG ratio even if the level of total glutathione was reduced by a lack of S. After 24 h of S deprivation, low GR activity might slow the reduction of GSSG to GSH, which is the electron donor for reducing dehydroascorbate to ascorbate. There was a strong decrease in the AsA/DHA ratio under S deprivation, which indicated a conversion of the ascorbate pool to its oxidized form. Notably, GR activity increases in various plant species under different types of abiotic stresses (Lascano et al. 1998, Romero-Puertas et al. 2006, Gill et al. 2013).

We hypothesized that the decreased glutathione availability in *Chlorella* S-starved cells would limit the reduction of DHA to AsA, similar to the results presented by Kandlbinder et al. (2004). Although S deprivation resulted in an increase in the total ascorbate pool within 2 h, the AsA content decreased considerably with respect to the DHA contents in the following hours, resulting in a marked decrease in the AsA/DHA ratio.

The decrease in the AsA content under S deprivation could also result from increased APX activities. Interestingly, the increase in the APXs, which are key enzymes in the degradation of H_2O_2, resulted in a concomitant decrease in the intracellular H_2O_2 content during S starvation. While five APX isoforms have been identified in plants (Dabrowska et al. 2007), immunoblot analysis on *C. vulgaris* showed only one isoform of APX (Takeda et al. 1998). However, the gel activity stain of *C. sorokiniana* extracts suggested the existence of multiple APX isoforms. Further studies are needed to clarify their subcellular localizations. Therefore, all detected *C. sorokiniana* APXs appeared to be strongly influenced by S deprivation, especially after 24 h.

These results suggest that in *C. sorokiniana*, the content of AsA and its regeneration are particularly sensitive to S availability. In *Arabidopsis thaliana* leaves, however, S starvation does not affect the AsA status (Kandlbinder et al. 2004).

The total SOD activities in *Chlorella* were increased by S deprivation. In contrast to the APXs, there were significant increases in the activity of the SODs in the early stages (4 h) of S starvation. However, we believe that the rapid H_2O_2 increase following the start of S starvation, in addition to the SOD reaction, may have other origins. Moreover, the fact that native protein electrophoresis stained for the activity of SODs confirmed an increase of these enzymes and suggested the existence of at least three isoforms with different migration rates in *Chlorella* cells. Similar to the APX enzymes, there is no information on the cellular localization of these enzymes. The increase in SOD and APX under S starvation reinforced the idea that an oxidative perturbation occurred in *Chlorella* S-deficient cells. We attempted to complement biochemical evidence with gene expression studies to determine whether the observed changes in APX and SOD activities were under transcriptional or post-translation control. We used a qRT–PCR assay to determine the transcriptional control of the APX and SOD genes using specific primer pairs based on *C. vulgaris* and *Chlorella variabilis* sequences because the genome of *C. sorokiniana* has not been sequenced. We did not obtain threshold cycle values <35 for the two tested genes, which suggests there are differences in the sequence of these genes between various *Chlorella* species.

Under S deficiency conditions, the ascorbate pool remained fully oxidized, whereas the glutathione pool was more reduced and decreased in size. These results suggest that when the glutathione pool is depleted, as occurred in the S-starved cells, the control of the cellular redox state shifts to ascorbate-dependent processes.

In conclusion, tight regulatory cross-talk between glutathione and ascorbic acid metabolism and the activation of

ROS-scavenging enzymes occur in *C. sorokiniana* subjected to S deficiency. The rapid and transient accumulation of H_2O_2, which increased immediately after the initiation of the S deprivation, represents a signaling response that leads to the induction of antioxidant defense systems to remove harmful ROS.

Based on these preliminary findings, we suggest that S starvation in *Chlorella* cells induce a reversible physiological oxidative perturbation.

Materials and Methods

Plant material and culture conditions

Chlorella sorokiniana Shihira & Krauss, strain 211/8k (CCAP of Cambridge University), was grown under controlled conditions in batch culture (2 liters) at 35°C and was continuously illuminated (Philips TLD 30 W/55 fluorescent lamps, 250 μmol photons m^{-2} s^{-1}) and flushed with air containing 5% CO_2 at a flow rate of approximately 80–100 l h^{-1}. The basal medium had the following composition: 13 mM KH_2PO_4, 4.3 mM K_2HPO_4, 0.35 mM NaCl, 1.2 mM $MgSO_4$, 0.35 μM Fe-EDTA, 0.18 mM $CaCl_2$, 5 mM KNO_3 and oligoelements (0.31 μM Cu, 0.1 μM Mo, 9.1 μM Mn, 0.76 μM Zn, 46 μM B). The pH of the basal medium was adjusted to 6.5. Under these conditions, the algal growth rate constant (μ) was 3 d^{-1}. Cells used in the experiments were harvested during the exponential growth phase (culture OD between 0.5 and 1.0). Specifically, an S-starved culture was obtained by harvesting cells from the batch culture and collecting them using low-speed centrifugation (4,000 × *g* for 5 min). Residual sulfate was removed by washing the cells twice in S-free medium [13 mM KH_2PO_4, 4.3 mM K_2HPO_4, 0.35 mM NaCl, 1 mM $MgCl_2$, 0.35 μM Fe-EDTA, 0.18 mM $CaCl_2$, 2.5 mM KNO_3 and oligoelements (0.31 μM Cu, 0.1 μM Mo, 9.1 μM Mn, 0.76 μM Zn, 46 μM B)] using centrifugation (4,000 × *g* for 5 min). Then, the resulting packed cells were suspended in the S-free medium and cultured up to 24 h.

The number of *Chlorella* cells per ml of culture was determined by direct counting of algae in the growth medium using a Bürker chamber. The counting was performed in duplicate.

Total soluble protein determinations

Total soluble proteins (pg $cell^{-1}$) were determined spectrophotometrically using the reagent Protein Assay (Bio-Rad) based on the Bradford method (1976) with bovine serum albumin as the standard.

Reduced (GSH) and oxidized (GSSG) glutathione contents

The GSH and GSSG contents of *C. sorokiniana* were determined following the method of Anderson (1985) with minor adjustments. Specifically, a cellular pellet from 200 ml of algal culture was re-suspended in 3 ml of 5% sulfosalicylic acid. Cells were lysed by passaging at 11,000 p.s.i. through a French pressure cell. Then, 100 μl of crude extract was added to 600 μl of reaction buffer (0.1 M Na-phosphate, pH 7.00, 1 mM EDTA), 40 μl of 0.15% 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) and 400 μl of water. The GSH content was determined at 412 nm after 2 min. Then, 50 μl of 0.4% NADPH and 1 μl of GR (0.5 U) were added to the same reaction mixture. The total glutathione (GSH plus GSSG) content was determined at 412 nm after 30 min at room temperature. The GSSG content was calculated as the difference between the total glutathione and GSH contents.

Determination of hydrogen peroxide content

An aliquot of 300 ml of algal culture was harvested using centrifugation (4,000 × *g* for 5 min). The pellet was re-suspended in 5 ml of 50 mM phosphate buffer (pH 6.8) and then lysed by passaging at 11,000 p.s.i. through a French pressure cell. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C, and the clear supernatant (crude extract) was used for H_2O_2 determination. Crude extract (10 and 20 μl) was added to peroxidase solutions (83 mM phosphate buffer, pH 7.00, 0.005% *o*-dianisidine dichloride, 0.25 mg ml^{-1} horseradish peroxidase) to a final volume of 1 ml, and the reaction mixtures were incubated at 30°C for 10 min. The reaction was stopped by adding 200 μl of 1 N perchloric acid. The H_2O_2 absorbance was measured at 436 nm. The H_2O_2 concentration was calculated using a linear calibration curve of H_2O_2 solutions. The calculated extinction molar coefficient was 0.0024 mM^{-1} cm^{-1}. To prevent the reduction of the protein content, which is typical of S-deprived cells, possibly distorting our final results, the H_2O_2 content was evaluated per cell and not per protein.

Determination of the antioxidant enzyme activities

The APX activity was measured by monitoring the decrease in absorbance at 290 nm for 15 min. Approximately 10 μl of crude extract (see the section 'Determination of hydrogen peroxide content' for the extract preparation) was added to the APX reaction mixture (final volume of 1 ml) containing 50 mM phosphate buffer (pH 7.00), 1 mM ascorbate and 5 mM H_2O_2. The oxidation of ascorbate was followed spectrophotometrically. The molar extinction coefficient was calculated to be 1.3 mM^{-1} cm^{-1}.

An aliquot (10–50 μl) of crude extract (see above) was added to the SOD reaction mixture (final volume of 1 ml) containing 50 mM phosphate buffer (pH 7.7), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT) and 2 μM riboflavin for the SOD assay. The reaction mixture was illuminated for 15 min at a light intensity of 350 μmol m^{-2} s^{-1} and then the absorbance was monitored at 550 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction.

The activity of GR was measured according to Smith et al. (1988). The assay mixture contained 100 mM phosphate buffer (pH 7.5), 0.2 mM diethylenetriamine pentaacetic acid, 0.75 mM DTNB, 0.1 mM NADPH, 0.5 mM GSSG and 50–100 μl of extract in a total volume of 1 ml.

Reduced (AsA) and oxidized (DHA) ascorbate contents

The AsA and DHA contents were measured according to Kampfenkel et al. (1995).

To determine the AsA content, an aliquot of extract (see the section 'Determination of hydrogen peroxide content' for the extract preparation) was added to a solution (final volume of 1.2 ml), containing 0.2 mM phosphate buffer (pH 7.4), 10% trichloroacetic acid (TCA), 42% (v/v) H_3PO_4, 4% (w/v) 2.2-bipyridyl (dissolved in 70% ethanol) and 3% (w/v) $FeCl_3$. The absorbance was measured at 525 nm after 40 min at 42°C.

The total ascorbate (AsA plus DHA) content was determined by adding an aliquot of extract to 0.2 mM phosphate buffer (pH 7.4) and 10 mM dithiothreitol (DTT). After incubation at 42°C for 15 min, 0.5% *N*-ethylmaleimide was added. Then, a solution containing 10 mM DTT, 10% TCA, 42% (v/v) H_3PO_4, 4% (w/v) 2.2-bipyridyl (dissolved in 70% ethanol) and 3% (w/v) $FeCl_3$ was added. The absorbance was measured at 525 nm after 40 min at 42°C.

The content of DHA was calculated as the difference between the total ascorbate and AsA contents.

Detection of antioxidant isoenzymes using Native PAGE

After electrophoresis, the native polyacrylamide gels (10%) were stained for APX and SOD activities. The gels for the APX activity were first equilibrated in 50 mM sodium phosphate buffer (pH 7.00) containing 2 mM AsA for 30 min and then incubated in a solution including 50 mM sodium phosphate buffer (pH 7.00), 4 mM AsA and 2 mM H_2O_2 for 20 min. The gels were washed immediately in 50 mM sodium phosphate buffer (pH 7.00) and then placed in the APX development solutions containing 50 mM sodium phosphate buffer (pH 7.8), 28 mM tetramethylethylenediamine (TEMED) and 2.5 mM NBT, in the presence of light.

The gels for SOD activity were equilibrated for 30 min using 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM TEMED. The gels were washed immediately in distilled water and submerged, in the presence of light, in the SOD development solutions (50 mM potassium

phosphate buffer pH 7.8, 28 μM riboflavin, 28 mM TEMED, 2.5 mM NBT) until the appearance of activity bands. The staining reaction was terminated using 1% glacial acetic acid.

Gel images were acquired using an Image Scanner III flatbed scanner (GE Healthcare). Digitized gel images were analyzed using the ImageMaster™ 2D Platinum software (GE Healthcare), which allowed the detection and quantification of different APX and SOD enzyme isoforms. The band volume was used as the analytical parameter for quantifying the enzymes. The protein band volume was normalized to the band volume of the entire gel.

Statistical analysis

Data analysis was conducted using Sigmaplot 12 software. The data presented are the means ± SE of three independent experiments. The statistical analysis was performed using a one-way analysis of variance (ANOVA) and the Tukey's post-hoc test to determine whether differences between the S treatments were significant at $P < 0.05$ and $P < 0.001$. Data expressed as a percentage were transformed using an arcsin transformation ($y' = \arcsin y$, y = original percentage/100).

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Disclosures

The authors have no conflicts of interest to declare.

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