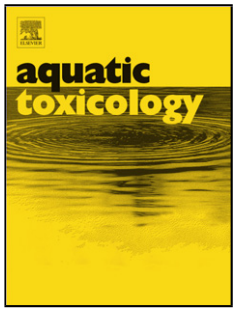
The relationship between sulfur metabolism and tolerance of hexavalent chromium in Scenedesmus acutus (Spheropleales): Role of ATP sulfurylase



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THE RELATIONSHIP BETWEEN SULFUR METABOLISM AND TOLERANCE OF HEXAVALENT CHROMIUM IN *SCENEDESMUS ACUTUS* (SPHEROPLEALES): ROLE OF ATP SULFURYLASE

RUNNING TITLE:

ROLE OF ATS IN CR(VI) TOLERANCE

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Highlights

- S-starvation induces the increase in the cell S/Cr ratio
- Inner detoxification rather than Cr exclusion determines Cr-tolerance in *S. acutus*
- Two ATP sulfurylases are differentially expressed in realation to S availability
- Higher *SaATS2* transcription in S sufficient conditions marks the Cr-tolerant strain
- The higher cys level in the Cr-tolerant strain seems due to cytosolic cys synthase

ABSTRACT

Sulfur availability and the end products of its metabolism, cysteine, glutathione and phytochelatins, play an important role in heavy metal tolerance, chromium included. Sulfate and chromate not only compete for the transporters but also for assimilation enzymes and chromium tolerance in various organisms has been associated to differences in this pathway. We investigated the mechanisms of Cr(VI)-tolerance increase induced by S-starvation focusing on the role of ATP sulfurylase (ATS) in two strains of *Scenedesmus acutus* with different chromium sensitivity. S-starvation enhances the defence potential by increasing sulfate uptake/assimilation and decreasing chromium uptake, thus suggesting a change in the transport system. We isolated two isoforms of the enzyme, *SaATS1* and *SaATS2*, with different sensitivity to sulfur availability, and analysed them in S-sufficient and S-replete condition both in standard and in chromium supplemented medium. *SaATS2* expression is different in the two strains and presumably marks a different sulfur perception/exploitation in the Cr-tolerant. Its induction and silencing are compatible with a role in the transient tolerance increase induced by S-starvation. This enzyme can however hardly be responsible for the large cysteine production of the Cr-tolerant strain after starvation, suggesting that cytosolic rather than chloroplastic cysteine production is differently regulated in the two strains.

KEYWORDS:

ATP sulfurylase; Cr-tolerance; Cr-uptake; Cysteine; GSH; Sulfur starvation.

LIST of ABBREVIATIONS:

AAS: atomic absorption spectrophotometry

APR: APS reductase

APS: adenosine-5'-phosphosulfate

ATP: adenosine-triphosphate

ATS: ATP- sulfurylase

CSC: cysteine synthase complex

cys: cysteine

dNTP: deoxinucleotide triphospate

DTPA: diethylene triaminopentaacetic acid

DW: dry weight

GSH: reduced glutathione

ISO: International Organization for Standardization

LOEC: Lowest Observed Effect Concentration

OAS: O-acetyl serine

OAS-TL: O-acetyl serine (thiol)lyase

Pi: inorganic orthophosphate

PVP: Poly-Vinyl-Pyrrolidone

RT-PCR: Reverse Transcriptase-PCR

SAT: serine acetyl transferase

SED: Sulfur Enhanced Defence

SIR: Sulfur Induced Resistance

SSA: 5-sulfo salicylic acid

STAS: Sulfate Transporter and AntiSigma factor antagonist

1. INTRODUCTION

In photosynthetic organisms, the capacity to overcome biotic and abiotic stresses is supported by processes known as Sulfur Induced Resistance (SIR) or better as Sulfur Enhanced Defence (SED) (Rausch and Wachter 2005), that relies on sulfur availability. Various end-products of sulfur assimilation (cysteine, glutathione and phytochelatins) are involved in counteracting negative impacts of different stressors. Many studies demonstrated that cys and GSH have a role in heavy metal tolerance (Hu et al. 2001, Pawlik-Skowrońska et al. 2004, Torricelli et al. 2004, Le Faucher et al. 2006), chromium included (Shanker et al. 2004, Foyer and Noctor 2005, Gorbi et al. 2006). The reduction of Cr(VI) by thiols reductants, through the formation of instable Cr(VI)-thiolate complex, is regarded as a key reaction in chromium metabolism inside the cells (Brauer et al. 1996). It has been shown that the reaction between GSH and dichromate leads to the formation of 1:1 GS-CrO_3^- thiolate complex in aqueous solution (Brauer and Wetterhahn 1991) and that the same mode of action is displayed by other biological thiols, e.g. cysteine (Brauer et al. 1996). According to the authors, only one Cr(VI) per $Cr_2O_7^{2-}$ molecule forms thiolate complex, the other Cr(VI) species being released as $HCrO_4^-$ in equilibrium with the original chemical form and chromate CrO_4^{2-} anion. The competitive interaction between sulfate and chromate uptake is a well known process in plants, algae, fungi and yeast: chromate anions can easily cross cell membranes by means of the active sulfate transporters because of the similarity with the sulfate anion structure (Cervantes et al. 2001). However, it seems that the interaction between sulfur and Cr(VI) is not limited to the competition in the uptake process but also involves reductive assimilations steps. After uptake, sulfate anions undergo activation by ATP-sulfurylase (ATS) enzyme, the reaction yielding adenosine-5'-phosphosulfate (APS). Afterwards, reduction processes, catalysed by APS-reductase and sulfite-reductase in plastids, cause the reduction to sulfide (S^{2-}) which is incorporated in cysteine (cys). Various evidences suggest that sulfate and chromate may compete for the same reduction pathway inside the cell and that responses to chromate often imply an enhancement of S

uptake/assimilation pathway; many Authors indeed report an ATS up-regulation at the gene or protein level in various bacteria and yeast exposed to chromate (Ackerley et al. 2006; Brown et al. 2006;, Chourey et al. 2006; Pereira et al. 2008; Henne et al. 2009; Thompson et al. 2010; Monsieurs et al. 2011). In addition, Schiavon et al. (2008) observed in *Brassica juncea*, the induction of genes involved in sulfate assimilation pathway (i.e. ATP-sulfurylase *atps6*; APS-reductase *apsr2*; Glutathione synthethase *gsh2*) and the accumulation of cysteine (cys) and glutathione (GSH) after treatment with chromate.

Wilson and Bandurski (1958) showed that ATS can use chromate and other anions (i.e. selenate, sulfite, molybdate and tungstate) as a substrate instead of sulfate. However, only the reaction with sulfate and selenate leads to the formation of stable adenylic acid-anion anhydrides; the reaction with all other anions leads instead to free adenylic acid (AMP) formation, as demonstrated by the failure to obtain coupling between sulfurylase and APS-kinase and the absence of Pi exchange into ATP. Many evidences underline a key role of ATS in the response to sulfur-limited conditions with a further possible implication of this enzyme in metal tolerance.

Changes in regulation of ATS have been reported both in algae and vascular plants in response to different nutrient limitation (Brunold and Suter 1984; Lappartient and Touraine 1996; Lappartient et al. 1999; Song et al. 2013; Liu et al. 2014). Literature data attribute a negative feedback regulative role respectively for GSH and L-cysteine on ATS activity and expression (Lappartient et al. 1997; Bolchi et al. 1999), but recently also a redox regulative mechanism targeting the cysteine residues of the enzyme has been proposed (Prioretti et al. 2014; Prioretti et al. 2016). In flowering plants and algae, ATS activity and/or expression is also modulated in response to oxidative stress, for instance a great activity increase was reported in *Lemna gibba* and *Salvinia natans* exposed to arsenic (Leao et al. 2014) and in *Brassica juncea* exposed to cadmium (Lee and Leustek 1999). All these evidences indicate that ATS is involved in different cellular functions and plays a central role in

different metabolic adjustments likely associated to the maintenance of cell homeostasis (Montechiaro and Giordano 2010; Giordano 2013).

A previous study by Gorbi et al. (2007) indicated that after a period of S-deprivation a transient increase in chromium tolerance occurred in the wild-type and a Cr-tolerant strains of the freshwater green alga *Scenesdesmus acutus* (Chlorophyceae). After S-starvation both strains were indeed able to multiply at Cr(VI) concentrations normally inhibiting their growth. After medium renewal and S-resupply, an elevated S-uptake and a consequent increase in cysteine biosynthesis, particularly elevated in the Cr-tolerant strain, were observed. Studies on *Chlamydomonas reinhardtii* acclimation to sulfur deprivation, indicate that arylsulfatase, high affinity sulfate transporter, as well as many enzymes of the S assimilation pathway, ATSs included, are specifically induced by S-deprivation (Zhang et al. 2004; González-Ballester et al. 2010; Aksoy et al. 2013). The up-regulation of the sulfur uptake/assimilation pathway in the two strains of *S. acutus* was consequently hypothesized to explain the tolerance transient increase, which indeed was lost after two days of recovery in sulfate-supplemented medium, and the different cys production was associated to the difference in the degree of tolerance between strains. The Authors suggested that the down regulation of the sulfate uptake/assimilatory process in the wild type occurs at levels of the intracellular negative regulator end-products lower than in the Cr-tolerant strain, which in turn activates an earlier up-regulation of sulfur assimilation processes. Further researches by Marieschi et al. (2015) suggested that the increase in Cr-tolerance in the two strains following sulfur starvation could relies on overlapping mechanisms, lowered chromium accumulation and enhancement of the sulfur uptake/assimilation pathway, which act differently in the two strains

However, which step(s) of sulfur uptake/assimilation pathway and metabolism are involved in Cr-tolerance is still unclear.

The aim of the present study was to investigate the implication of ATS in the tolerance of chromium in the unicellular alga *S. acutus*, taking advantage from the availability of two strains

with different sensitivity to Cr(VI) and sulfur deprivation. The difference in response of the two strains to chromium poisoning and S-starvation can indeed constitute a good premise for elucidating the mechanisms that link chromium tolerance and sulfur metabolic pathways. To this aim various parameters were analysed just after 7-day pre-culture in standard and S-deprived medium and during recovery in sulfate-supplemented medium, also in the presence of Cr(VI): i) sulfur and chromium content, ii) cysteine and GSH levels and net efficiency production, iii) ATS transcription and activity.

2. MATERIALS AND METHODS

2.1. In vitro culture of Scenedesmus acutus.

Synchronized axenic cultures of the wild-type and Cr-tolerant strains (Corradi et al. 1995) of the green unicellular alga *Scenedesmus acutus* were maintained in sterile liquid culture medium (Miller et al. in US EPA, 1978; pH 7.7±0.1; modified by dissolving in distilled water both the micro and the macronutrients to obtain a final concentration double of that indicated), in a climate-controlled chamber (23±1 °C, 230 μmol m^{-2} s^{-1} light intensity irradiance, white-cool fluorescent lamps, 16 h photoperiod). No organic matter was present in the medium at the beginning of the culture. The pH values were lowered to 7.5 in chromium supplemented medium. This pH value was in the range of the pH found in freshwater systems (pH 6.5-8.5). The cultures were continuously aerated (sterile filtered air). To perform the experiments with algae in exponential growth phase (in the range $1E^6$-$6E^6$ cells/ml), culture medium was always renewed 3 days before starting each experiment by adding 900 ml of fresh medium to 100 ml of algal suspension in 1 l Erlenmeyer flasks (Stock Culture).

2.2. Sulfur starvation and response to Cr-poisoning.

Aliquots of the stock cultures, in exponential growth phase, were collected by centrifugation for 10 min at 2200×*g* and washed with sterile double-distilled water. The pellets were suspended at 3×10^6 cells ml^{-1} density in 200 ml of standard culture medium (+S) containing $MgSO_4$ (29.4 mg l^{-1}), or in sulfate-deprived medium (-S). Since $MgSO_4$ is the only source of sulfur in the standard medium, the amount of $MgCl_2$ was simultaneously increased to restore the standard magnesium concentration in the S-deprived medium. After a 7-day pre-culture in +S (un-starved cells) or in -S medium (S-starved cells) at the same conditions as above, cells of both strains were collected by centrifugation (as described above), washed and treated with Cr(VI) supplied as potassium dichromate ($K_2Cr_2O_7$; Merck, analytical grade) in +S medium. Previous experiments indicated a different cys production in the two strains both in S-sufficient and in S-replete condition (Gorbi et al. 2007). In order to compare basal differences between the strains, different parameters were measured in S-sufficient and S-replete untreated cells and in cells from the same nutritional conditions but exposed to Cr(VI) during recovery. In order to get more insight on the role of sulfur metabolism in the transient Cr(VI) tolerance increase after S-starvation and compare cell response to chromium poisoning, the two strains were exposed to a Cr(VI) concentration that, as established in previous experiments (Gorbi et al. 2007) was growth-inhibiting in S-sufficient cells but tolerated after S-starvation. To this aim starved and un-starved cells were exposed to Cr(VI) at the Lowest Observed Effect Concentration (LOEC, endpoint=population growth), , namely 1 mg l^{-1} for the wild-type and 2 mg l^{-1} for the Cr-tolerant strain in standard culture medium. Metal solutions were sterilized through 0.22 μm nitrocellulose membranes (Millipore). Treatments and controls had three replicates. The initial cell density (3×10^6 cells ml^{-1}) was chosen according to previous studies (Gorbi and Corradi 1993; Corradi et al. 1995; Torricelli et al. 2004; Gorbi et al. 2007) to ensure comparison of the response to metal poisoning. From now on, the term “S-replete cells” will be used to indicate S-starved cells which were transferred to +S medium, while the term “S-sufficient cells” will be used to indicate un-starved cells transferred to and maintained in +S medium. To further ascertain if the different chromium sensitivity was based on a different chromium uptake, the two strains were also cultured

in medium supplemented with both 1 and 2 mg Cr(VI) l^{-1} in S-sufficient and in S-replete conditions. Cells cultured for 24h in standard and S-deprived medium were also analysed to check gene inducibility in response to S-status.

2.3. Determination of dry weight free cysteine (cys), reduced glutathione (GSH), sulphur and chromium content.

For dry weight determination, aliquots (100 mL) of the cultures were collected by centrifugation, washed three times with sterile double-distilled water and filtered on pre-weighed mixed cellulose ester filters with pore size of 0.45 µm (Millipore). The filters were dried at 95°C for at least 3 h and weighed. Dry weight was determined in S-replete and S-sufficient cells at the beginning (time 0) of the experiments and after 1, 24 and 48 h in standard medium with or without chromium. Other aliquots of the same cultures were used to determine cell density by cell counting by means of a Neubauer haemocytometer. For chromium determination, cells of 100 ml of each culture were collected by centrifugation as above and washed two times in Tris-HCl buffer (50 mM, pH 7.5). The obtained pellets were disrupted using "One Shot" Cell Disrupter (Constant Systems Ltd., England) at 270 Mpa, three times. Homogenates of whole cells were completely digested with HNO_3:H_2O_2 (3:1) in a microwave mineralisator (MLS 1200 Mega; Milestone) and the amount of chromium was determined using a flameless atomic absorption spectrophotometer Zeeman/3030 (Perkin Elmer, Waltham, MA, USA) at λ 357.9 nm. Calibration curve was obtained by diluting the Cr reference standard solution for AAS (purchased by ISO certified companies) in the digestion solution at the final concentrations of 0, 20, 60 and 100 ng ml^{-1}. The digestion solution was used as blank. The detection limit of the method was 2 ng ml^{-1}. For free cys and GSH determination, cells were harvested by filtration on a Whatman GF/C filter and extracted using a mortar in ice-cold 5% (w/v) 5-sulphosalicylic acid solution (SSA) with 6.3 mM diethylenetriaminopentaacetic acid (DTPA). After centrifugation at 14,000 x g for 30 minutes, total non-protein thiol compounds were

determined spectrophotometrically at 412 nm in a mixture of 300 µl of the supernatants, 1200 µl potassium-phosphate buffer (pH 7.6) and 25 µl Ellman's reagent. For free cys determination, 100 µl of phosphate buffer were replaced by 0.1 M methyl glyoxal which binds cys and, after 10 min reaction, absorbance was determined at the same wave length as above. Cysteine concentration was calculated by difference between total SH and GSH concentration. A calibration curve for standard SH groups (L-cysteine, Merck) was used for quantitative determination of cys and GSH in the extracts. The efficiency of methyl glyoxal in masking cys was checked on mixtures of cys and GSH in different proportions. For sulfur determination, 1500 ml of each culture were collected by centrifugation for 10 minutes at 2200×*g* and washed 3 times with distilled water. The pellet was dried overnight at 95°C and then turned to powder in a mortar. Sub samples of 15 mg were weighed in tin cups and, after the addition of 10 mg of vanadium pentoxide (V_2O_5), subjected to controlled combustion at 900°C. Total sulfur in the combustion products was determined by gas chromatography (EA1110 CHNS-O; ThermoQuest) (carrier gas = He). All data were referred to the dry weight (milligrams) of the total biomass in 1 ml culture: this parameter takes into account the contribution of both cell number and cellular weight increase to the biomass of the culture, thus allowing a better comparison between treatments (Marieschi et al 2015).

2.4. RNA extraction and purification

RNA was extracted from the algae of both strains cultured in the following conditions:

a) 24h in standard (+S) and S-deprived medium(-S)
b) 7-day pre-culture in +S (un-starved cells) or in -S medium (S-starved cells) (time 0)
c) 24 and 48h in S-sufficient conditions either in absence (control) or in presence of chromium
d) 24 and 48h in S-replete conditions either in absence (control) or in presence of chromium

Culture aliquots were collected by centrifugation, twice washed with double distilled water, frozen in liquid nitrogen, lyophilized, mortar grinded in liquid nitrogen and stored at -80°C before RNA

extraction. Aliquots of the powdered algae were resuspended in 3 mL extraction buffer (25mM Tris-HCl pH 7.5, 75mM NaCl, 2.5mM EDTA, 0.5% SDS, 75mM β-mercapto ethanol) and disrupted using "One Shot" Cell Disrupter at 270 Mpa, 1 time. Homogenates were adjusted to 5ml with extraction buffer and extracted with equal volume of pH 7.5 saturated phenol/chloroform. Total RNA samples were routinely lithium chloride precipitated, analyzed and quantitated by denaturing formaldheyde-agarose gel electrophoresis (Sambrook et al. 1989) and spectral analysis.

2.5. Relative quantification of mRNA by RT- Competitive Multiplex PCR

10 µg aliquots of total RNA for each sample were treated with RNase free DNase (Promega Corporation, Madison, WI, USA). After DNAse digestion RNA was further quantitated and first-strand cDNA synthesis was performed starting from 5 µg of total RNA for each sample. The reaction was carried on in a final volume of 50 µl in the presence of both oligo$(dT)_{20}$ and random hexamers (Invitrogen Life Technologies) with 200U MMLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) as described by Ansari-Lari and Co-workers (1996). Reverse transcriptase was inactivated by heating at 95 °C for 5 min and chilling on ice. 0.5 µl of the reverse transcriptase reactions were used for subsequent amplification with *SaATS1* or *SaATS2* primers designed on the specific sequences in *S. acutus* (*SaATS1* For 5'-CGCCCAACGTGCAGGAGG-3', *SaATS1* Rev 5'-TGTCGCCAGCCAGGTTCTG-3'; *SaATS2* For 5'-TCGGGGGCAAGATCTTCGG-3', *SaATS2* Rev 5'-GGTGCAGCCGTAGTTCTTG-3'), these couple of primers gave rise to amplicons of 321 and 389 bp respectively. To avoid false negatives, we performed a "Competitive Multiplex PCR" (Chamberlain et al. 1988) using Rubisco small subunit (*rbcS*) as internal reference (Chen and Melis 2004) to monitor the amplification reaction. The amplification of *rbcS* was carried out using a primer pair (*SarbcS* For 5'-CCCCTCTCAGCGATGACC-3'; *SarbcS* Rev 5'-GGTCCAGTACCTGTTGTCG-3'**)** constructed on the sequence of *S. acutus* gene and chosen to give an amplification length (167bp) clearly

distinguishable from that obtainable for the target genes. Both the couple of primers worked at an annealing temperature of 60°C and both amplicons were obtainable with 40 sec time extension. To overcome the hindrances due to i) the very different relative abundance of target and reference genes which would easily lead *rbcS* to overwhelm *ATS*s amplification (as indicated by preliminary reactions conducted on single genes), we set up a series of preliminary multiplex PCR reactions in which the ratio between *ATSs* and *rbcS* primer pair changed from equimolar condition (12.5:12.5 picomoles each) to 12.5:5 picomoles. We finally chose this latter ratio in which the primer for *rbcS* were in limiting condition and gave rise to a constant amplification intensity allowing the comparison of the *SaATS1* and *SaATS2* amplification between different experimental condition. The optimal number of PCR cycles was established by analyzing the amplified product on the agarose gel and 35 cycles of amplification was finally chosen, in the considered conditions DNA amplification of the target was still in exponential phase, as determined in preliminary tests. PCRs were performed in 25 µl volume containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 3%DMSO, 2 mM $MgCl_2$, 0.2 mM dNTPs in equimolar ratio, 1U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA, USA). The intensity of the target amplified band was assessed as described below, and reported as a percentage value referred to the sum of the amplified bands' intensity obtained for both internal reference and target in each lane. Mean and standard deviation were calculated for each experimental point.

2.4. Determination of ATP sulfurylase activity.

Culture aliquots of S-sufficient and S-replete cells of both strains cultured in absence or in presence of chromium for 1, 24 and 48h, as well as of S-starved and S-unstarved algae (time 0), containing $2x10^8$ cells, were collected by centrifugation, twice washed with double distilled water and re-suspended in 2 ml of extraction buffer according to the method by Lappartient and Touraine (1996). The buffer composition was 10 mM Na_2EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM 1,4-Dithiothreitol

(DTT). The obtained suspensions were subjected to cell disruption by "One Shot" Cell Disrupter at 270 Mpa (only one time). Insoluble PVP was omitted to avoid nozzle obstruction. Immediately after cell rupture, 20 µM Phenylmethylsulfonyl fluoride (PMSF) was added to the homogenate to prevent protein degradation. The extract was centrifuged at $2x10^4$ x g for 5 min at 4°C and subsequently refrigerated in ice. Protein content was determined in the supernatant according to the Biorad assay method (Bradford 1976) using a BSA standard solution as a reference. The extracts were then diluted with the extraction buffer to obtain a concentration of 20 µg protein per 100 µL of final solution (i.e. diluted extract). Afterward, 100 µL of the diluted extracts were singularly added to microcentrifuge tubes (1.5 mL) containing 500 µL of reaction solution prepared according to Lappartient and Touraine (1996): 7mM $MgCl_2$, 5 mM Na_2MoO_4, 2mM Na_2ATP and 0.032 units ml^{-1} of pyrophosphatase. All the steps were performed maintaining diluted extracts and reaction solutions in ice. The reaction tubes were then transferred into a thermostatic bath at 37°C (10 min) and subsequently at 65°C (5 min) to stop the reaction. Each reaction solution was finally diluted 1:100 with double distilled water and phosphate concentration determined according to the method by Valderrama (1977).

2.7. Other procedures

RNA formaldehyde-agarose gels and RT- multiplex PCR agarose gels, were stained with ethidium bromide, analyzed and quantified with a Kodak DC40 camera (Kodak) using the Kodak digital science 1D Image analysis software (Eastman Kodak Company, Rochester NY)

2.8. Statistical analysis

Data analysis was conducted using SPSS 25 12 software. All experiments were repeated at least three times. Data presented are the mean ± SD of 3-5 independent experiments. The significance of the differences among mean values of the checked different parameters (Cr, S, cysteine and GSH

content as well as ATP sulfurylase transcription and activity) was tested by one-way ANOVA after verification of variance homogeneity (Levene's test). Duncan's test was applied as post-hoc. Before statistical analysis, angular transformation was conducted on data expressed as percentage (RT QC multiplex PCR).

3. RESULTS

3.1. Algae growth and sulfur and chromium accumulation.

In S-sufficient conditions, 24 h after medium renewal, sulfur content showed a level significantly higher in the tolerant strain (62.53 nmol S ml^{-1} culture biomass) than in the wild type (53.98 nmoles S ml^{-1} culture biomass) ($p<0.05$) (Fig.1). In Cr(VI)-treated algae, chromium content was nearly double in the Cr-tolerant strain (7.08 nmoles Cr ml^{-1} culture biomass) than in the wild type (4.18 nmol Cr ml^{-1} culture biomass) and sulfur uptake was lowered by an amount equal to the uptaken chromium (-4.2 and -7.1 nmol S ml^{-1} culture biomass for the wt and the Cr-tolerant strain respectively). Albeit the difference in uptaken chromium was significant ($p<0.05$), it merely reflected the difference in chromate concentration in the culture medium which each strain had been exposed to (19.2 nmol Cr(VI) ml^{-1} for the wild type and 38.4 nmol Cr(VI) ml^{-1} for the Cr-tolerant strain), indicating that Cr uptake in these conditions keeps up with the external concentration.
In S-replete conditions a significant increase in S uptake was observed in both strains with respect to S-sufficient conditions (1.5 and 2 fold in the wild type and Cr-tolerant strain respectively), with highly significant differences between strains (85.29 vs 119.92 nmol S ml^{-1} culture biomass for the wt and the Cr-tolerant strain respectively) ($p<0.01$). Beside enhancing sulfur uptake, S-replete cells nearly halved their chromium uptake which was lowered to 2.33 nmol Cr ml^{-1} culture biomass for the wild type and to 4.94 for the Cr-tolerant strain. As a consequence, the ratio $(S/Cr)_{in}$ (expressed as nanomoles) increased significantly ($p<0.01$) (34.88 and 19.26 for the wt and the Cr-tolerant

respectively) and the ratio between $(S/Cr)_{in}$ and $(S/Cr)_{out}$ nearly triplicated respect to S-sufficient conditions in both strains (Tab1). In S-replete cells indeed the stoichiometric ratio between the two elements changed and the transporters system allowed the entrance of one Cr(VI) nmole every 6 sulfur nmoles.

As shown in figure 2, both strains at the respective LOEC had a similar behaviour and reduced metal uptake in S replete condition, while at 1 mg Cr(VI) l^{-1} this decrease was significant for the wild type but not for the Cr tolerant. In addition, chromium uptake in the Cr-tolerant strain seemed dependent on the outer Cr(VI) concentration, while in the wild type the uptake capacity seemed to be saturated at the concentration of 1 mg l^{-1}, thus resulting in similar amounts of uptaken chromium in S-sufficient and S-replete cells exposed to 2 mg Cr(VI) l^{-1}.

It is however interesting to note that, in S-sufficient condition, the wild type growth was inhibited by an inner chromium amount that did not affect the tolerant strain (at 1 mg Cr(VI) l^{-1}: 4.18 nmol Cr ml^{-1} culture biomass and 4.33 nmol Cr ml^{-1} culture biomass respectively) while it was not inhibited by a similar amount (4.35 nmol Cr ml^{-1} culture biomass) after S-starvation (S-replete conditions at 2 mg Cr(VI) l^{-1}) (Fig. 2).

3.2. Free cysteine (cys) and reduced glutathione (GSH) content.

Since S-starvation seemed to induce in the two strains the same variations in the uptake system (S/Cr ratio varied in the same manner) (Tab1), we investigated if the difference in Cr-tolerance between the strains, and the more efficient detoxifying capacity acquired following S-starvation, could depend on differences in sulfur assimilation.

-*S-sufficient conditions.* As shown in Fig.3, at the end of the pre-culture in +S medium (T0) and at each recovery time, cys content in control algae was significantly higher in the Cr-tolerant strain (nearly double) than in the wild type, with the only exception of 1h recovery. In the wild type, cys level did not significantly change during recovery and, 24h after medium renewal onward, the

highest difference between strains were observed, maybe as a consequence of a sulfur amount significantly higher in the Cr-tolerant strain. In this strain indeed cys level was high even in the control and not significantly enhanced by chromium treatment; in the wild type, cys production increased only in Cr(VI)-supplemented medium after 24h culture reaching levels similar to the ones observed in the Cr-tolerant strain. After 48h recovery, cys amount decreased in both strains treated with chromium, reaching the levels observed in the untreated wild type, though remaining significantly higher in the untreated Cr-tolerant strain.

At the end of pre-culture, the two strains showed similar levels of GSH. During the recovery, GSH followed the same trend as cys, reaching amounts significantly higher in the Cr-tolerant strain than in the wild type even in absence of Cr(VI). Similar amounts were reached by the wild type only after 24h exposure to chromium. However, differently from cys, GSH increased after chromium exposure in both strains reaching significantly higher levels than in the respective controls ($p<0.01$). Also in this case, a decrease in GSH content, below the levels of the wild type control, was observed in both strains after 48h exposure to Cr(VI).

S-replete conditions: At the end of pre-culture in –S medium (T0 in Fig.3), a strong cys depletion occurred in both strains (0.034 ± 0.024 and 0.031 ± 0.022 nmol SH ml^{-1} culture biomass in the wt and in Cr-tolerant strain respectively), but cys rapidly increased to significantly higher level than in S-sufficient conditions within 1h recovery ($p<0.01$), reaching a peak within 24h from medium renewal (Fig.3). In S-replete cells, cys production was strongly enhanced in both strains even in control condition. In the wild type cys reached its maximum at 24h recovery showing a level almost equal to that observed in S-sufficient Cr-treated cells; no further increase was induced by Cr(VI) exposure. In the Cr-tolerant cys production was even more elevated and reached levels four times as great as in S-sufficient cells at 24h recovery. Astonishingly, in this strain chromium exposure drastically reduced cys accumulation, leading to the same time course of cys production observed in the wild type.

A strong increase in GSH levels was observed in the two strains, that showed concentrations far above S-sufficient cells, and no significant differences were observed between Cr-treated and control cells. The comparison between the two strains suggests that the pathway of GSH production in the Cr-tolerant strain is saturated at significantly higher level.

3.3. Cysteine (cys/S) and GSH (GSH/S) net production efficiency:

The efficiency of free cys net production, referred to intracellular sulfur nmoles after 24h recovery in +S medium was significantly higher in the Cr-tolerant strain than in the wild type ($p<0.01$), both in S-sufficient and in S-replete control conditions (Fig. 4). In the wild type, efficiency comparable to that of the Cr-tolerant strain was observed only following chromium exposure. The cys net production after S-starvation significantly increased ($p<0.01$) in untreated cells of both strains. Albeit the GSH content was significantly higher in the Cr-tolerant strain, the efficiency of GSH net production (nmol GSH/nmol S) was similar in the two strains, not affected by sulfur availability during pre-culture and significantly increased ($p<0,05$) by chromium exposure (Fig 4), with the only exception of the S-replete wild type.

3.4. ATP-sulfurylase (ATS) transcript analysis and activity

Since differences between strains were mostly observed in cys amounts and S-starvation strongly enhanced the efficiency in cys net production, the activity and transcription of ATS was analysed, considering that this enzyme catalyzes the first committed step of sulfate assimilation pathway and can use chromate as a substrate. In *S. acutus* we have identified two DNA sequences coding for enzymatic isoforms *SaATS1* and *SaATS2* (*GenBank: KJ187406.1* and *GenBank: KJ187408.1* respectively), highly homologous to *C. reinhardtii* ATP-sulfurylases. The encoded proteins were very similar but the nucleotidic sequences contained some traits of disomogeneity on which discriminating primers have been constructed.

The two isoforms resulted differently inducible by S-starvation: while *SaATS1* is more abundant and constitutively expressed at relatively high levels, *SaATS2* is more sensitive to the S-status of the cells being expressed at low levels in standard medium and significantly induced ($p<0.01$) by 24h S-starvation (Fig.5).

At the end of pre-culture the transcripts of the two isoforms were relatively abundant in algae grown both in +S and –S medium. During recovery however relevant differences in their regulation were observed. *SaATS1* expression appeared to be constant and not significantly affected by the different sulfur availability during pre-culture nor by chromium exposure (Fig. 6): no differences were observed between strains in all conditions. Significant differences ($p <0.05$) were instead observed between strains in *SaATS2* expression: transcript levels were higher in the Cr-tolerant than in the wild type at the end of the pre-culture in +S medium and throughout the subsequent recovery and was not affected by chromium exposure. In the wild type, 24h after medium renewal, *SaATS2* transcription significantly decreased ($p<0.05$) in untreated cells, while it increased in Cr-exposed algae, reaching a level similar to the ones observed in the Cr-tolerant strain (control and Cr-exposed cells). A different behaviour was observed after S-starvation: *SaATS2* transcription, high and similar in the two strains at t0, significantly decreased within 24h from S-resupply (when GSH and cys reached a peak), unresponsively to chromium exposure, and increased again during the subsequent 24 h (when GSH and cys levels dropped).

Steady state levels of ATS activity in stock cultures and 1 h and 24 h after medium renewal both in presence and absence of chromium are reported in Fig. 7; data are referred to the sum of the two isoform activities, since they could not be distinguished by enzymatic assay. Initial levels were not significantly different in the two strains and increased 1 h after medium renewal, though remaining significantly lower ($p < 0.05$) in the Cr-tolerant strain in the absence of chromium. After 24h enzymatic activity returned to the basal levels found in stock cultures.

In S-sufficient cells ATS activity was similar to that of stock cultures, normally S-supplied, while in S-starved cells of both strains it was significantly lower (Fig. 8 time 0) notwithstanding the

transcripts at this time were relatively abundant (Fig 7). The activity remained unchanged over one hour recovery in +S medium both in absence and in presence of Cr(VI) but strongly increased within 24 h after medium renewal both in S-sufficient and in S-replete cells. No significant difference was observed at this time, neither between strains nor between control and Cr-exposed cells. After 24 h recovery, the curve slowed down and tended to a plateau, restoring ATP-sulfurylase activity steady state levels found in stock culture (Fig. 7).

4. DISCUSSION

S-replete cells of both strains were able to grow in presence of Cr(VI) concentrations inhibiting growth in S-sufficient cells (1 and 2 mg l^{-1} for the wild type and Cr-tolerant strain respectively), as already described in previous studies by Gorbi et al. (2007) and Marieschi et al. (2015).

In S-sufficient conditions the two strains accumulated a similar amount of chromium when exposed to 1 mg Cr(VI) l^{-1}; a higher amount than the wild type was instead accumulated by the Cr-tolerant strain when exposed to 2 mg Cr(VI) l^{-1} thus confirming that tolerance in this strain is not due to chromium exclusion. In addition Cr(VI) uptake in the Cr-tolerant strain seems related to the outer Cr(VI) concentration. On the contrary, the uptake capacity in the wild type seems to be saturated at the concentration of 1 mg l^{-1} in accordance with the significantly higher amount of Cr found in its membrane fraction (Marieschi et al. 2015).

In agreement with previous data indicating that Cr(VI) uptake in *S. acutus* is linked to energy dependent processes (Gorbi et al. 2001), the ratio S/Cr inside the cells of both strains (S/Cr $nmoles_{in}$), grown in S-sufficient conditions at the respective LOEC, was nearly double the ratio S/Cr in the medium (S/Cr $nmol_{out}$), indicating that the uptake is due to active transport against concentration gradient. The decrease in sulfur uptake, observed in Cr-supplemented medium and perfectly matching with the corresponding uptaken chromium, evidences the competition between chromate and sulfate for the same transporters. This competition was however more evident in the

Cr-tolerant since only in this strain, both in S-sufficient and in S-replete conditions, S-uptake was significantly decreased by exposure to chromium. The significantly higher levels of sulfur and chromium found in the Cr-tolerant strain suggest a difference in the transport system expressed by the two strains both in S-sufficient and S-replete condition, despite the same values of the ratio $(S/Cr)_{in/out}$. The stoichiometric ratio $(S/Cr)_{in/out}$ indeed varied from two sulfur nmoles every one nmole of chromium in S-sufficient cells to six nanomoles in S-replete cells of both strains, suggesting that the higher sulfur accumulation during recovery after starvation is due to the induction of higher affinity sulfate transporters rather than to a simple increase in transporter number. This suggestion is confirmed by the contemporary reduction in chromium uptake observed in the two strains at the respective LOEC. The induction of high affinity sulfate transporters (Hawkesford 2003; Gigolashvili and Kopriva 2014; and references therein; Yildiz et al. 1994; Pootakham et al. 2010; Zhang et al. 2004) and the increase in sulfate transporter number (Green and Grossman 1988; Appenroth et al. 2008) during acclimation to S-starvation are well documented both in vascular plant and in algae.

Since chromium determines effects mimicking S-starvation and modifies the expression of sulfate transporters (Schiavon et al. 2007; Pereira et al. 2008; Schiavon et al. 2008), alteration in sulfur uptake/assimilation pathway could have been at the basis of the Cr-tolerant strain selection in chromium supplemented medium (Corradi et al. 1995).

The difference in S-uptake between strains and the capacity of the Cr-tolerant strain to overcome an inner chromium amount toxic for the wild type in S-sufficient conditions but tolerated after starvation indicate that the difference between strains relies on a different ability in detoxification and that the detoxifying capacity increases following the changes in sulfur uptake/assimilation triggered by S-starvation.

In S-sufficient conditions, where the sulfur uptake and GSH production were lower and cys flow through to GSH retained a margin of modulation, both strains responded to chromium exposure by increasing the GSH level to counteract oxidative stress induced by the metal, the Cr-tolerant strain

being able to reach a higher level than the wild type as already reported in Gorbi et al. 2006. In S-replete conditions GSH levels were instead strongly enhanced even in control cells and did not further increase following chromium exposure, indicating that the pathway was already carried to its maximum level. This increase could be a consequence of the high cys production after S-starvation and/or a response to oxidative stress caused by S-starvation as reported in *Chlorella sorokiniana* (Salbitani et al.2015). By considering also the GSH net production efficiency, the pathway leading from cys to GSH seems regulated in the same manner in the two strains but saturated at different levels, likely as a consequence of their different cys production which was higher in the Cr-tolerant strain. A strong increase in cys level was indeed observed after 1h recovery from S-starvation in both strains, with a huge accumulation in the Cr-tolerant strain in which most likely cys production is too elevated to be rapidly conveyed in GSH production. This increase is not justified by the necessity to detoxify chromium, whose uptake was lowered after starvation, but seems rather a consequence of the high sulfur uptake.

The reduced chromium uptake after starvation, coupled with the speeding up of the whole assimilation pathway, explains the acquired capacity to overcome exposure to otherwise toxic concentrations of chromium. In this context, the excess of cys produced by the Cr-tolerant strain did not seem directly responsible for the transient tolerance increase induced by S-starvation, since the wild type became more tolerant as well without reaching such cys levels; it however might contribute to an enhanced generalized defence capacity.

The significant increase in cys net production induced in both strains by S-starvation indicates the activation of enzymes of the involved pathways. ATS, the first enzyme of sulfur assimilation pathway is present in a variable number of isoforms in different photosynthetic organisms (Koprivova and Kopriva 2014; Prioretti et al. 2014). Albeit literature data indicate the presence of a unique gene coding for ATS in green algae, with the exception of *C. reinhardtii* for which two isoforms with chloroplastic localization are known (Yildiz et al. 1996; Allmer et al. 2006; Patron et al. 2008; Prioretti et al. 2014), at least two aminoacidic accessions, sharing homology with ATS in

various groups of green algae, are often present in data banks. In *S. acutus* we have identified two DNA sequences coding for the enzymatic isoforms *SaATS1* and *SaATS2*. Since the most popular bioinformatics tools are trained on sequence from higher plants, translocation signal peptides in algae are still misinterpreted. Notwithstanding recent studies (Tardif et al. 2012) based on annotated sequences from *C. reinhardtii* genome have improved the discrimination capacity, few algal genomes are completely annotated and prediction capacity in algae is still far to be exhaustive. Due to this limitation, subcellular localization for SaATS1 and SaATS2 are not known and we could only infer their chloroplastic localization from hortology to *C. reinhardtii* ATS proteins (Allmer et al 2006). *SaATS2* resulted transcriptionally regulated by S-starvation while *SaATS1* is constitutively transcribed in all tested condition.

The higher *SaATS2* transcription in the Cr-tolerant strain at the end of pre-culture in standard medium and its increase in the wild type only upon chromium exposure matches with the differences observed in cys production between the two strains in S-sufficient condition and could partly intervene in determining their different chromium sensitivity.

Since *SaATS2* is constitutively expressed at low level and is inducible by S-starvation, its higher transcription in the Cr-tolerant strain in S-sufficient condition strengthens the hypothesis that this strain has a different perception or exploitation of inner sulfur respect to the wild type. This hypothesis is also consistent with the exceeding cys production and the significantly higher amount of up-taken sulfur in S-replete control cells in this strain, uptake and assimilation processes being regulated in a coordinate manner (Davidian and Kopriva 2010 and references therein).

Despite the relatively high level of ATSs transcripts, ATS activity after S-starvation was low. This is apparently in contrast with literature data in higher plants (Lappartient and Touraine 1996; Lappartient et al. 1999; Bolchi et al. 1999) which indicate an increase of activity after S-deprivation. This behaviour is however not so surprising since in green algae ATSs are rich in cys residues (~10). It is thus possible that after 7-day S-starvation, despite the importance of this enzyme for sulfur assimilation, cells reduce their pool to economize sulfur, or that the ATSs

proteins hemi-life is shorter than 7-days and new enzyme has to be translated from the transcripts to reactivate the pathway. A strong increase of ATS activity was actually observed within 24h recovery during which cell sulfate content was replenished and likely new enzyme had been translated by the transcripts, with a sharper slope in S-replete cells most likely due to the overlapping of the two isoform activities. In this context, the induction of SaATS2 could support SaATS1 activity, thus contributing to the enhancement of cys synthesis during the first hours of recovery. Contrasting results have been described for ATS in different species of algae in response to sulfur limitation. The transcription of both genes *ATS1* and *ATS2* of *C. reinhardtii* was strongly enhanced by sulfur starvation (Yildiz et al.1996, Zhang et al. 2004), as well as the enzyme activity in *Dunaliella salina* (Giordano et al. 2000), *Thalassiosira pseudonana* and *Tetraselmis suecica* and *Synechococcus* sp. (Prioretti et al. 2016) subject to sulfur limitation. On the contrary in *Emiliania huxleyi* ATS1 expression was not affected by sulfur deprivation (Bochenek et al. 2013) and a decrease of activity was observed in *Amphidinium klebsii* (Prioretti et al. 2016 29) in response to S-limitation. These contradictory data on ATSs regulation in algae are probably due to a lack of information about the presence of different isoforms in most of the species and to the difficulty in distinguishing them by RT-PCR and in evaluating their individual activities by enzymatic assays. The decrease of *SaATS2* transcript and the decline in ATS activity, similar in the two strains, 24h after nutrient resupply following S-starvation suggest a feedback regulation by GSH, that reached its maximum level at the same recovery time, in accordance with results reported by studies on *Brassica napus* and *Arabidopsis thaliana* (Lappartient and Touraine 1996, Lappartient et al. 1999). A possible role of cys as negative feedback regulator on ATS (Bolchi et al. 1999) in *S. acutus* is questionable since both the *SaATS2* transcription and ATSs activity were similar in cells with extremely different cys levels. ATS activity seemed however more related to pre-culture conditions than to chromium exposure or strain.

The high levels of transcripts at the end of pre-culture both in +S and –S medium and the increment in ATS activity during recovery of unstarved cells suggest that, even after 7-day culture in +S medium, cells suffered a slight S-shortage.

Despite the lower ATS activity after S-starvation (recovery time 0), a significant increase in cysteine was observed 1h after S-resupply, well higher than that shown by S-sufficient cells. At this time, cys production through the sulfate reductive assimilation pathway was presumably strongly impaired by the low ATS activity and the extremely low level of GSH which is required, as reducing agent, for the reaction catalysed by APR reductase.

Cys synthesis occurs through a two-step reaction, the first catalysed by SAT (associated with OAS-TL in the cysteine synthase complex (CSC)) which leads to OAS production, the second, catalysed by OAS-TL as free dimer or, in the cytosol, linked to the STAS domain of H^+/SO_4^{2-} transporters (Shibagaki and Grossman 2010), which incorporate sulfide produced in the chloroplast, onto OAS (Hell 1997; Droux et al. 1998; Hell and Hillebrand 2001; Hell et al. 2002). Cys synthesis is regulated by the cytosolic OAS which negatively affects the stability of the heterocomplex OAS-TL/SAT (CSC), stabilized in turn by sulfide (Hell 1997; Droux et al. 1998; Hell and Hillebrand 2001; Hell et al. 2002). The CSC is thus regarded as a sensor responsible for the regulation of cys synthesis and homeostasis (Haas et al. 2008; Heeg et al. 2008; Krueger et al.2009; Wirtz et al. 2010; Feldman-Salit et al. 2012). Albeit ATSs and sulfate reductive assimilation in green algae are apparently exclusively localized in chloroplasts (Allmer 2006; and Ravina et al 2002) in plants the enzymes responsible for the formation of CSC complex (namely OASTL and SAT) are present in different cell compartments (plastid, cytosol and mitochondria) and the major amount of cys seems to be produced in the cytosol (Haas et al 2008, heeg et al 2008, Krueger et al.2009). Also in algae the existence of multiple isoforms of OASTL with a possible different cell localization and a cytosolic cys production have been demonstrated (Carfagna et al 2011). Moreover, in addition to the chloroplastic reductive assimilation pathway, sulfide can also be produced in cytosol and mitochondria through cys degradation or cys-consuming processes through the action of cys

desulfydrase DES1 which has been proven to contribute to maintenance of cys homeostasis under environmental stress conditions in *Arabidopsis* (Alvarez et al. 2010). It is conceivable that this enzyme acts downstream the proteases, involved in cell protein rearrangements to economize sulfur during S-starvation, on cys residues derived either from proteins or from GSH pool. A significant decrease in GSH (Gorbi et al 2007, Carfagna et al. 2011 and present study) and in soluble protein in S-starved cells of *C. reinhardtii* (Takahashi et al 2001; Zhang et al. 2004) and *C. sorokiniana* (Carfagna et al 2011) was indeed reported. Protein rearrangement has been interpreted as a mechanism of the cells to cope S shortage and redistribute amino acids to satisfy their nutritional requirements and economyze internal S resources. The presence of cys biosynthetic enzymes in different cell compartments had been initially suggested as a consequence of a scarce permeability of endomembranes to this aminoacid (Lunn et al. 1990). Subsequently, specialized functions of the different cell compartments (assimilation in plastids trough the reductive pathway, regulation in the cytosol by the association/dissociation of CSC complex and degradation of sulfur containing aminoacids in mitochondria) have been proposed (Hell 1997). This hypothesis has been supported by many evidences showing that cys biosynthesis is regulated in a compartment-specific manner: the mitochondrion is responsible for providing most of the OAS in the cell, while cytosol is the major site of cysteine production (Wirtz and Hell 2007, Haas et al. 2008, Heeg et al. 2008, Watanabe et al. 2008a, Watanabe et al. 2008b). These observations raised the hypothesis that cys can move across the organelle membranes. Recently has been reported that cys is transported across the mitochondrial membrane by multiple facilitated mechanisms that operate in a concentration gradient-dependent manner (Lee et al. 2014)

Carfagna and coworkers (2011) reported the activation of a cytoplasmic OAS-TL in *C. sorokiniana* when subjected to S-starvation and hypothesized the involvement of this isoform, more sensitive to cell sulfur, OAS and cys levels, in the rapid cys synthesis upon S-resupply. Cytosolic cys production has been proposed to have an essential role in the hypersensitive response (Alvarez et al 2012) and in plant immunity reactions (Thair et al 2013; Romero et al 2014). The activation of

cytosolic OAS-TLs could be hypothesized in *S. acutus* to sustain cys production after S-starvation as long as new ATS enzymes are synthesized and the chloroplastic pathway is restored. It is thus possible that initial cys accumulation is sustained by cytoplasmic SAT and OASTL and sulfide derived by desulfidrathion of cys residues of degraded proteins during starvation. Cys production, in S-replete control cells of the Cr-tolerant strain, appeared somehow de-regulated and paradoxically turned to the "regular" behaviour in the presence of chromium. The free cys disappearance caused by chromium, suggests either a reduction in the production of the aminoacid or its disappearance as free form (through conjugation with chromium, GSH or phytochelatin production). However a reduced necessity to conjugate chromium, as a consequence of its reduced uptake after starvation, favours the first hypothesis. Moreover the perfect overlapping with the curves obtained in the wild type, supports the involvement of the cytosolic cys production, more directly exposed to chromium effect, and suggests that the time courses of cys levels in the wild type describe the chloroplastic cys production. Since a decrease in cys production between control and chromium-exposed cells was not evidenced in the wild type, the cytosolic rather than chloroplastic cys production seems differently regulated in the two strains.

This would indicate that the higher cys production in the Cr-tolerant during recovery may rely on i) a higher level of cytosolic OAS after S-starvation, ii) a faster sulfide production upon S-resupply iii) a greater affinity of one OAS-TL isoform for sulfide resulting in the speeding up of cys synthesis once this substrate become available. Only the hypothesized "supplementary" cytosolic cys synthesis of the Cr-tolerant strain was affected by chromium with an apparent "switching off" mechanism. However, to our knowledge, no information is available in the literature about a possible interaction between chromium and cytosolic mechanism of cys production. The process by which this mechanism could be silenced by chromium in *S. acutus* was not cleared in the present study. A possible explanation of this sudden decrease in cys production may come from the studies of Berkowitz et al. (2002), indicating that the equilibrium of CSC association/dissociation can be shifted like a switch by OAS concentration. If this mechanism is true, even little changes in OAS

concentration, caused by the speeding up (S-resupply) or slowing down (when exposure to Cr(VI) reduce S uptake) of sulfide production, could determine a threshold effect rather than a proportional response, thus causing the cytosolic cys production switching off.

5. CONCLUSIONS

The different Cr(VI) sensitivity of the two strains is due to a better detoxifying capacity of the Cr-tolerant strain that it is able to cope with a higher Cr(VI) inner content than the wild type. The enhanced sulfur uptake and assimilation, which result in an improved cell defence capacity, and the contemporary decrease in chromium uptake suggest that the transient tolerance increase observed after 7-days of S-starvation involves changes in the transport system.

Even if the excess of cys production in the Cr-tolerant strain can not be regarded as responsible for the Cr(VI) transient increase in tolerance after S-starvation, it can confer a higher defence capacity and contribute to the more elevated production of GSH. The pathway leading from cys to GSH appears indeed regulated in the same manner in the two strains but saturated at different levels. We isolated, for the first time in *S. acutus*, two genes codifying for isoforms of *ATS* (*SaATS1* and *SaAts2*) homologous to the *C. reinhardtii* enzymes. The inducibility of *SaATS2* transcription after S-starvation in both strains, as well as the silencing after S-resupply, are compatible with a role of this isoform in the transient increase of tolerance after S-starvation and may contribute to a rapid recover. Its higher transcription in the Cr tolerant in S-sufficient condition can also mark a different inner sulfur perception or exploitation in this strain. After S-starvation however, the cumulative activity of the two enzymes was lower than in S-sufficient cells and not different between strains thus suggesting that ATSs can hardly be regarded as responsible for the rapid increase of cysteine production observed within 1h from medium renewal nor for the massive cysteine production observed in the S-replete control cells of the Cr-tolerant strain. As the different cys production was not coupled with a difference in ATS activity between the strains, we hypothesized that, during

recovery after S-starvation, the Cr-tolerant strain activates cytosolic processes of cys synthesis, preponderant respect to chloroplastic production and enhancing net production efficiency, that does not occur (or occurs at very limited extent) in the wild type and in S-sufficient conditions.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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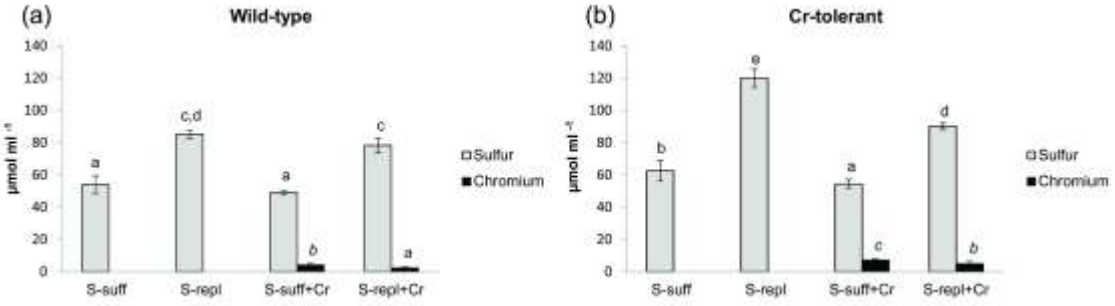

Fig. 1. Sulfur and chromium content in the dry biomass (milligrams) of 1 ml culture. Total sulfur and chromium content in the wild-type (a) and Cr-tolerant (b) strains after 24h recovery in +S medium or in +S medium supplemented with Cr(VI) (+Cr) following a 7-day pre-culture in +S (S-suff) or -S medium (S-repl). Each strain was exposed to the proper LOEC, i.e. 1 mg and 2 mg Cr(VI) l^{-1} for the wild-type and the Cr-tolerant strain respectively. Error bars = standard deviation. Statistical analyses were conducted cumulatively on the two strains and independently on sulfur and chromium content. Different letters (plain text for sulfur, italics for chromium) label significantly different values ($p < 0.05$). Data are mean of 3 independent experiments.

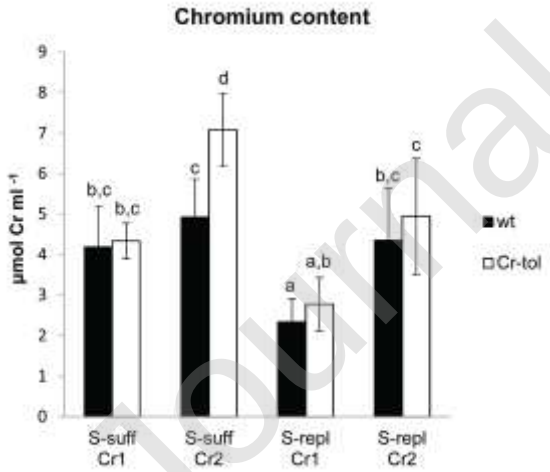

Fig. 2. Chromium content in the dry biomass (milligrams) of 1 ml culture. Total chromium content in the wild-type and Cr-tolerant strains after 24h exposure to 1(Cr1) or 2(Cr2) mg Cr(VI) l^{-1} in +S medium, following a 7-day pre-culture in +S (S-suff) or -S medium (S-repl); Error bars = standard

deviation. Different letters label significantly different values ($p < 0.05$). Data are mean of 3 independent experiments.

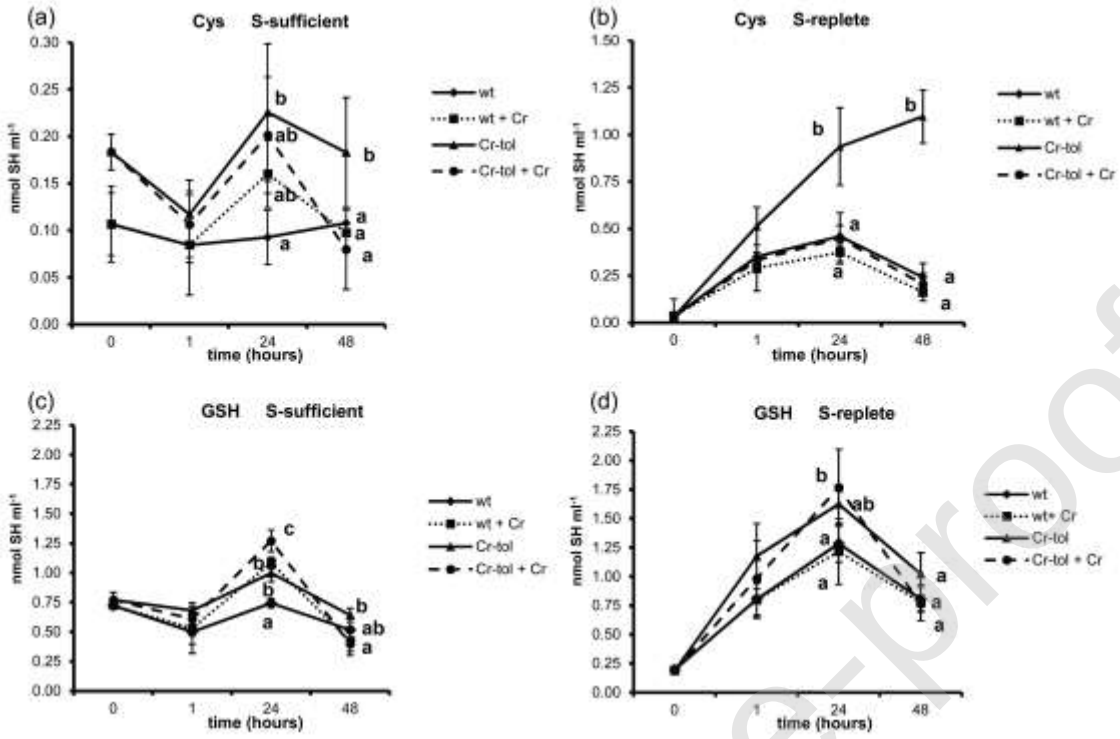

Fig. 3. Free cysteine and reduced glutathione content in the dry biomass (milligrams) of 1 ml culture. Free cysteine and reduced glutathione content in the wild-type and Cr-tolerant at the end of 7-day pre-culture in +S (S-sufficient a,c) or in -S medium (S-replete b,d) (time 0 h) and after 1, 24 and 48 h recovery in +S medium or in +S medium supplemented with Cr(VI) (+Cr). Each strain was exposed to the proper LOEC, i.e. 1 mg and 2 mg Cr(VI) l^{-1} for the wild-type and the Cr-tolerant strain respectively. Error bars = standard deviation; Different letters label significantly different values obtained among treatments and between strains at the considered experimental time point ($p < 0.01$) within each condition (S-sufficient, S-replete). Data are mean of 5 independent experiments.

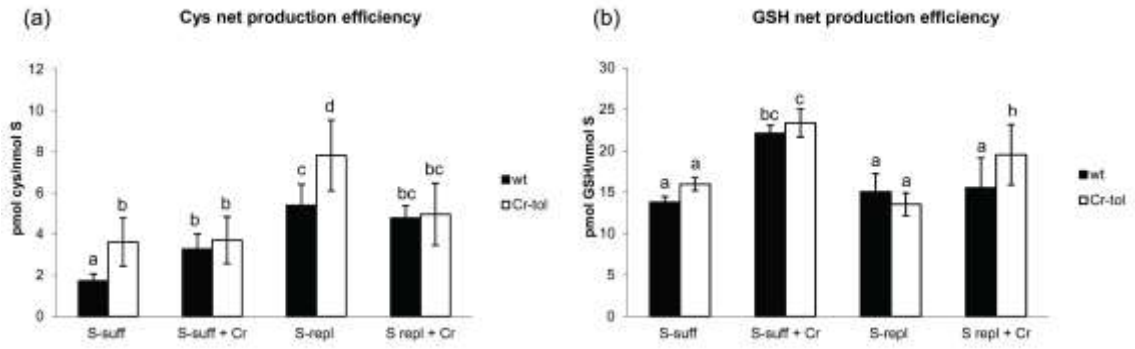

Fig. 4. Cysteine (cys) and reduced glutathione (GSH) net production efficiency. Cys (a) and GSH (b) net production efficiency after 24h recovery in +S medium or in +S medium supplemented with chromium (+Cr), following a 7-day pre-culture in +S (S-suff) or -S medium (S-repl). Each strain was exposed to the proper LOEC, i.e. 1 mg and 2 mg Cr(VI) l^{-1} for the wild-type and the Cr-tolerant strain respectively. Error bars = standard deviation; Different letters label significantly different values (cys: $p < 0.01$; GSH: $p<0.05$).

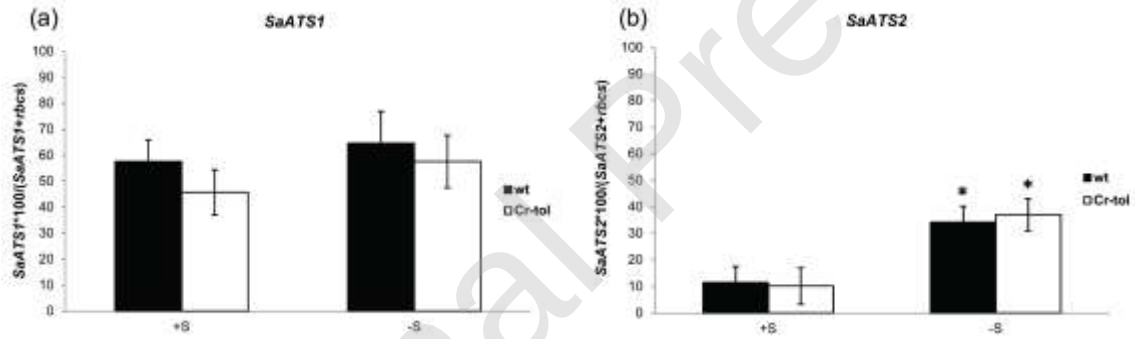

Fig. 5. *SaATS1* and *SaATS2* expression after 24h culture in +S and -S medium. Data are derived from a RT-multiplex PCR and are expressed as intensity percentage of the target band, *SaATS1* (a) or *SaATS2 (b)*, referred to the sum of the intensity of target and standard bands (represented by *rbcS*) in each gel lane. Data are mean of 3 replicates; Error bars = standard deviation; asterisks mark a significant difference between amplification obtained in +S and –S medium within strain ($p<0.01$).

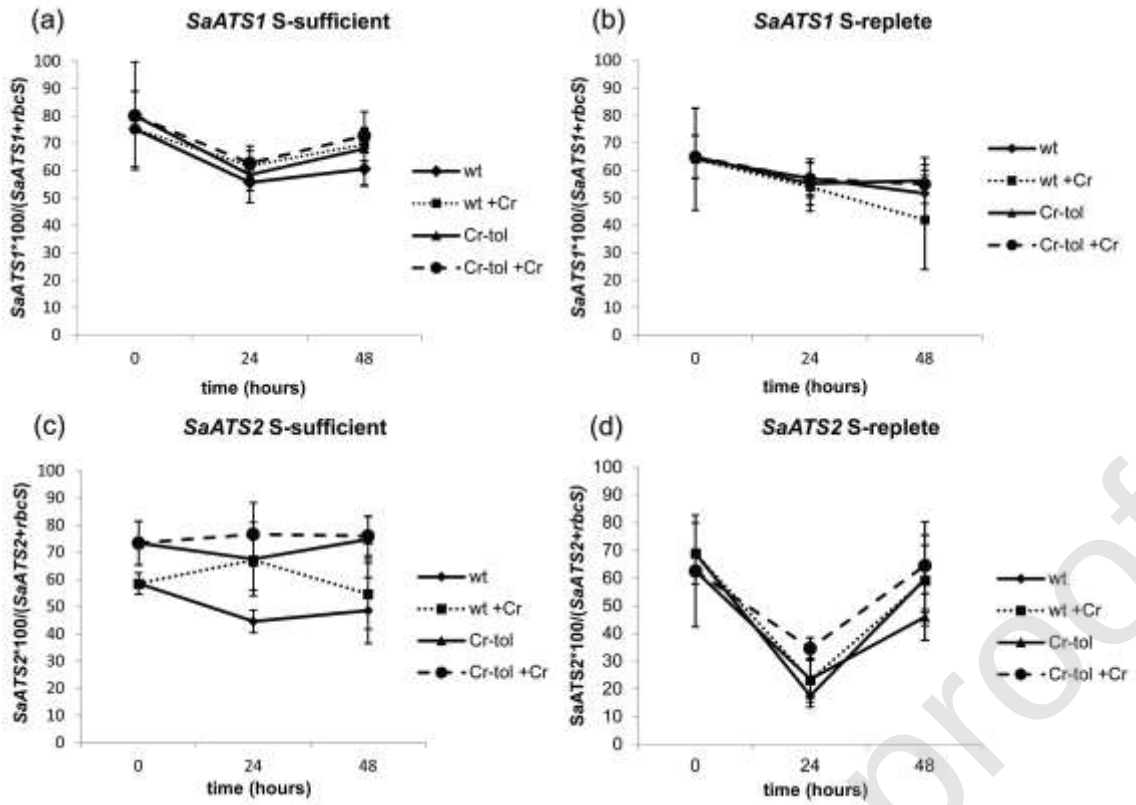

Fig. 6. Time course analysis of *SaATS1* and *SaATS2* expression. Time course analysis of SaATS1 (a, b) and SaATS2 (c, d) expression during recovery in +S medium or in +S medium supplemented with Cr(VI) (+Cr) following a 7-day pre-culture in +S (S-sufficient) (a, c) or -S medium (S-replete) (b, d). Each strain was exposed to the proper LOEC, i.e. 1 mg and 2 mg Cr(VI) L^{-1} for the wild-type and the Cr-tolerant strain respectively. Data are derived from RT-multiplex PCR and are expressed as percentage of intensity of the target band (*SaATS1* or *SaATS2*) referred to the sum of the intensity of target and standard bands (represented by *rbcS*) in each lane. Data are mean of 5 replicates; Error bars = standard deviation.

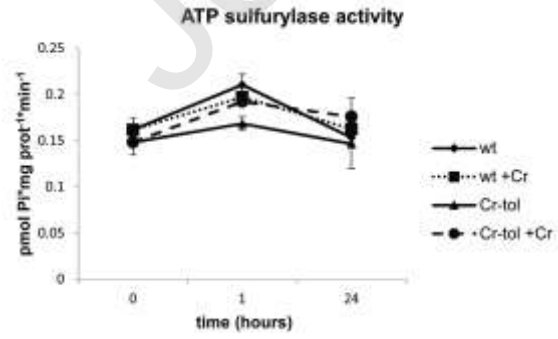

Fig. 7. Steady state levels of ATS activity. Steady state levels of ATS activity in the two strains, after 1h and 24h from medium renewal in absence or presence of chromium (+Cr). Data are expressed as pmoles of Pi produced by 1 mg protein per min. Data are mean of 5 independent experiments. Error bars = standard deviation.

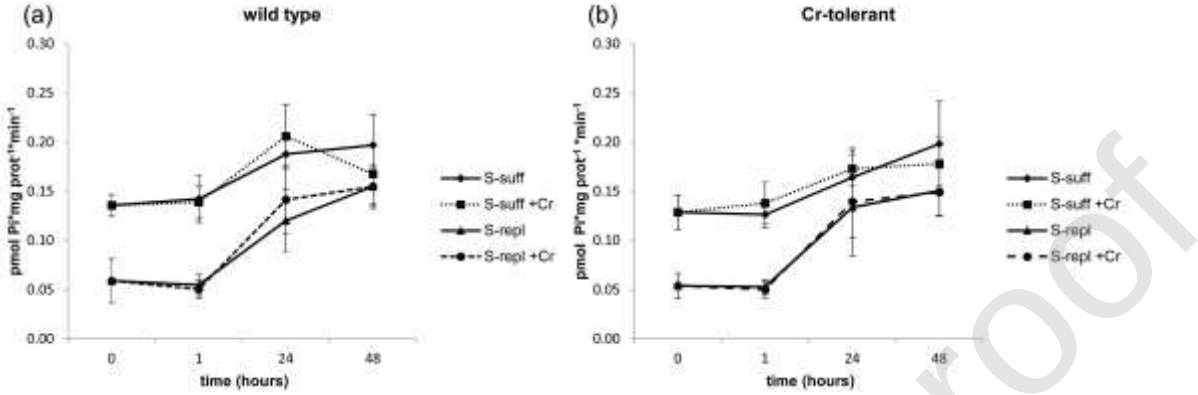

Fig. 8. Time course analysis of ATS activity. Time course analysis of ATS activity in the wild type (a) and Cr tolerant strain (b) during recovery in +S medium or in +S medium supplemented with Cr(VI) (+Cr) following a 7-day pre-culture in +S (S-suff) or -S medium (S-repl). Each strain was exposed to the proper LOEC, i.e. 1 mg and 2 mg Cr(VI) L^{-1} for the wild-type and the Cr-tolerant strain respectively. Data are expressed as pmoles of Pi produced by 1 mg protein per min. Data are mean of 5 independent experiments. Error bars = standard deviation.

Table 1

Ratio between S and Cr nanomoles in the medium ($(S/Cr)_{out}$), in the cells ($(S/Cr)_{in}$) and between medium and cells (in/out). Different letters label significantly different values ($p < 0.05$).

	$(S/Cr)_{out}$	$(S/Cr)_{in}$	$(S/Cr)_{in/out}$
		Wild type	
S sufficient	6.21±0.0	$12.12\pm2.51^{a,b}$	1.95 ± 0.40^{a}
S replete replete	6.21±0.0	34.88 ± 8.90^{c}	5.62 ± 1.43^{b}
		Cr tolerant	
S sufficient	3.10±0.0	7.75 ± 0.81^{a}	2.50 ± 0.26^{a}
S replete	3.10±0.0	19.26 ± 5.32^{b}	6.21 ± 1.71^{b}