Role of DNA methylation in the chromium tolerance of Scenedesmus acutus (Chlorophyceae) and its impact on the sulfate pathway regulation

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Role of DNA Methylation in the chromium tolerance of *Scenedesmus acutus* (Chlorophyceae) and its impact on the sulfate pathway regulation

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ABSTRACT

DNA methylation is a very important epigenetic modification that participates in many biological functions. Although many researches on DNA methylation have been reported in various plant species, few studies have assessed the global DNA methylation pattern in algae. Even more the complex mechanisms by which DNA methylation modulates stress in algae are yet largely unresolved, mainly with respect to heavy metal stress, for which in plants, metal- and species- specific responses were instead evidenced. In this work, we performed a comparative Whole-Genome Bisulfite Sequencing (WGBS) on two strains of the green alga *Scenedesmus acutus* with different Cr(VI) sensitivity. The pattern of distribution of 5-mC showed significant differences between the two strains concerning both differentially methylated local contexts (CG, CHG and CHH) and Differentially Methylated Regions (DMRs) as well. We also demonstrated that DNA methylation plays an important role in modulating some genes for sulfate uptake/assimilation confirming the involvement of the sulfate pathway in the Cr-tolerance. Our results suggest that DNA methylation may be of particular importance in defining signal specificity associated with Cr-tolerance and in establishing new epigenetic marks which contribute to the adaptation to metal stress and also to transmit the epigenomic traits to the progeny.

1. Introduction

DNA methylation, generally referred as an addition of a methyl group onto the C5 position of cytosine to form 5-methylcytosine (5-mC), is a conserved epigenetic mechanisms that contributes to modulate gene expression without altering the DNA sequence. DNA methylation, together with other epigenetic marks, such as the histone code (post-translational modifications in histone proteins) and non-coding RNAs (ncRNAs), contributes to establish the "epigenome" that, unlike the stable genome, is dynamically altered during developmental processes and in response to different environmental factors and/or stresses [1,2]. In plants, DNA methylation occurs in symmetric CG and CHG contexts but also in asymmetric CHH contexts (H = A, C, or T) [3]. Albeit DNA methylation has been studied in many plant and animal species and also in unicellular eukaryotes [4–9], it is still poorly understood in microalgae [10–12]. Flowering plants have generally high methylated genomes and extensive variation has been found between species, reflecting their evolution, both in the level and in the patterns of DNA methylation with the greatest variations observed in non-CG context [13]. In contrast in *Chlamydomonas reinhardtii* a very low DNA methylation such as 5.4%, 2.6%, and 2.5% respectively in the CG, CHG and CHH contexts, has been detected [14].

DNA methylation is crucial for various biological processes, including gene and transposon silencing, imprinting and X chromosome inactivation [15,16]. Dynamic DNA methylation is also fundamental in the response to both external and internal stimuli thus playing a relevant role in plant diversity and development [17–21].

Methylation profile can change in response to environmental stressor and it has been widely investigated in plants, but few data exist in algae [22]. It is known that abiotic stress, including heavy metals, drought and cold, can trigger DNA methylation changes at a genome-wide scale in plants [23]. In fact, stresses can induce changes in gene expression through hypomethylation or hypermethylation of DNA [24], especially in genome regions showing an adaptive significance during stress responses and which can direct genome evolution [25]. GC methylation has different effects on gene expression: in promoters

ABBREVIATIONS: WGBS, Whole Genome Bisulfite Sequencing; DMRs, Differentially Methylated Regions; BS-seq, Bisulfite Sequencing.

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and transposable elements (TEs), is correlated to gene and transposon silencing, while in gene body often interfere with gene regulation and is presumably associated to the suppression of spurious transcription from cryptic promoters [14,20,26]. Moreover, in presence of a persistent stress an epigenetic mechanism could establishes a DNA methylation-dependent stress memory, mainly due to GC-rich sequences methylation [27–29]; this mechanism ensures the faithfully transfer of the "stress memory" to the offspring [30]. However, epigenetic factors involved in the stress response and their implications in algae remain poorly understood [31,32].

In this context, the aim of the present work is to increase the knowledge on the epigenetic mechanisms and responses to heavy metals in microalgae. Our attention has been focused on stress induced by chromium (VI), which has been recognized as one of the most toxic and widespread metal in the environment [33–36]. To achieve this aim we used two strains of the unicellular green alga *Scenedesmus acutus* with different chromium sensitivity: the wild type and a Cr(VI) tolerant strain, previously selected by treating the cells culture with a sub-lethal concentration of chromium (1 mg Cr(VI) L^{-1}) for a long period (3 months) [37,38]. In this strain the Cr-tolerance was heritable, since the chromium tolerant strain (Cr-t) was able to grow in the presence of Cr(VI) even after prolonged culturing in Cr(VI)-free medium [37,38]. Previous analysis by 5-mC immunocytochemical localization revealed differences between the two strains in control conditions and changes in the 5-mC methylation patterns/level induced by Cr(VI) exposure [39]. These evidences suggested that epigenetic mechanisms could be at the basis of the Cr-tolerance acquisition in *S. acutus*. The Cr-tolerance could have been established during the selection in Cr (VI) supplemented medium fixed in the algal population, inherited and maintained through the progeny even in absence of the metal. To verify this hypothesis and assess the involvement of some epigenetic information in the transgenerational transfer of the Cr (VI) tolerance, we generated a whole genome bisulfite sequencing (WGBS) in order to analyse methylome of *S. acutus* Cr-tolerant strain (Cr-t) *vs* sensible strain (wt). In addition, since previous works suggest a relationship between chromium tolerance and sulfate reductive assimilation pathway in *S. acutus* [40–42], the methylation of genes involved in S uptake and assimilation was analysed. To this aim, we characterized 30 sulfate pathway related genes and evaluated the expression of the genes that resulted differentially methylated in Cr-t vs wt strain. The role of their differential methylation and expression, as well as their complex regulation network in the sulfate assimilation pathway, are discussed.

2. Materials and Methods

2.1. In vitro culture of Scenedesmus acutus

Synchronized axenic cultures of the wild-type (wt) and Cr-tolerant (Cr-t) strains [38] of the unicellular green alga *S. acutus* were maintained in sterile liquid culture medium (US EPA, 1978; pH 7.7 ± 0.1; modified by dissolving in distilled water both micro and 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, 16:8 h photoperiod). The cultures were continuously aerated with sterile filtered air. Stock cultures were renewed 3 days before starting experiment by adding 900 mL of fresh medium to 100 mL of algal suspension in 1 L Erlenmeyer flasks to collect wt and Cr-t cells in exponential growth phase (3 days). Aliquots of the stock cultures, in exponential growth phase, were collected by centrifugation for 10 min at 2200 × *g*, washed twice with sterile double-distilled water, frozen in liquid nitrogen, lyophilized, mortar grinded in liquid nitrogen and stored at −80 °C before DNA and RNA extraction.

2.2. DNA extraction and library preparation

Genomic DNA was extracted from both wt and Cr-t strain following the procedure of Doyle & Doyle (1990) [43] modified as follow: about 50 mg of frozen samples were treated with 1 mL of extraction buffer (2% CTAB w/v; 100 mM Tris-HCl pH 8; 1.4 M NaCl,; 20 mM EDTA; 2 % beta-mercaptoethanol) and incubated for 50 minutes at 65 °C. After centrifugation at 3000 x *g* for 10 min, the supernatant was collected in a clear tube, supplemented with 50 μg of DNAse free RNAse (Roche, Basel, Switzerland) and incubated at 37 °C for 30 minutes to remove RNA. After RNase digestion, the samples were washed twice with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and precipitated with an equal volume of ice-cold iso-propanol. The DNA pellets were washed twice with 70% ethanol, air-dried and resuspended in sterile distilled water. Integrity and quantity of extracted DNA were evaluated by electrophoresis (0.8% agarose gels containing 5 μg/mL ethidium bromide) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. The DNA was fragmented using a Diagenode sonicator to a mean size of 100–300 bp, followed by DNA-end repair, 3'-dA overhangs and the ligation of methylated sequencing adaptors according to manufacturer's instructions (Paired-End Sample Prep Kit - Illumina, San Diego, CA). DNA samples were treated with bisulfite using the Ovation Ultralow Methyl-Seq with TrueMethyl oxBS (NuGEN, Redwood City, CA) following the manufacturer's instructions. After bisulfite treatment unmethylated cytosine are converted in uracil, whereas methylated cytosines (5-mCs) remains intact. Unmethylated lambda phage DNA was spiked-in to estimate the bisulfite conversion rate. Six final libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Agilent Bioanalyzer DNA assay (Agilent Technologies, Palo Alto, CA). Libraries were pooled at equimolar concentrations and sequenced across a NovaSeq6000 (Illumina, San Diego, CA) to generate paired-end 150-bp reads for each library. For each strain, the sequencing was carried out with three biological replicates.

2.3. Bisulfite sequencing data analysis

Raw data were processed for both format conversion and de-multiplexing by Bcl2Fastq 2.0.2 version of the Illumina pipeline. Adapter sequences was masked with Cutadapt v1.11 from raw fastq data using the following parameters: --anywhere (on both adapter sequences) --overlap 5, --times 2, --minimum-length 35 and --mask-adapter [44]. Lower quality bases and adapters were removed by ERNE software [45]. Alignments of reads were performed on *Tetradesmus obliquus* (synonym of *S. acutus*) UTEX 393 reference genome (accession no. GCA_900108755.1; [46]) with Bismark v 0.19.0 using the parameters --bowtie2 e -N 1 [47]. The system produces directional bisulfite-converted libraries. The forward sequencing reads correspond to a bisulfite-converted version of either the original top or the original bottom strand (the C-to-T reads) and the reverse sequencing reads correspond to the complement of the original top or the complement of the original bottom strand (the G-to-A reads). The nuclear genome annotation of *T. obliquus* was not available and thus we annotated the scaffolds and predicted Gene models by using AUGUSTUS software [48] as reported in Suzuki et al. (2018) [49]. The Sequence Alignment Map (SAM) generated by Bismark were used to identify differentially methylated cytosine bases or regions using the methylKit v 0.9.5 Bioconductor package in R with logistic regression [50]. Cytosines were called with minimum coverage threshold of 10X and three methylation call files for CpG, CHG, and CHH contexts were generated for both Cr-t and control samples (wt). The significance of the differences in methylation percentage of total C analysed and in methylation level (%) in CG, CHG and CHH context between wt and Cr-t strain was evaluated by *t-test* ($p < 0.05$). By sliding window approach, using 500 bp win-

dows size and 250 bp step size, Differentially Methylated Regions (DMRs) were identified. Differentially hyper- and hypomethylated regions in Cr-t *vs* wt were identified using logistic regression (*q value* < 0.01) and a percentage methylation difference larger than 25% as cut-off. DMRs were mapped to various annotated features of hypothetic genic region, including protein-coding genes and their sub-features, such as promoter regions, gene body and 5'- and 3'-untranslated regions, through the genomic coordinates. The promoter region was defined as 2,000 bases upstream of the first base of the mRNA annotated region. KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/) software was used for the statistical analyses of the Gene Ontology (GO) data by Singular Enrichment Analysis (SEA) algorithm and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) pathways [51]. GO term and KEGG enrichment of hyper- and hypo- methylated predicted genes was performed separately using Fisher's exact test (*p value* < 0.05) and *C. reinhardtii* as referent organism.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted as described by Sardella et al. (2019) [42]. RNA extracted was quantified by Nanodrop (ND-1000) and its integrity was checked through an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only RNA samples with an RNA integrity number ≥ 7 were used to first-strand cDNA synthesis. The cDNA synthesis was performed by SuperScript™ III using oligo-dT primers (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

2.5. Characterization of genes involved in S uptake and assimilation

The candidate genes belonging to the sulfate uptake/assimilation pathway were identified by peculiar approach. First, for each gene, a consensus sequence was built by using the gene sequences present in the database (https://www.ncbi.nlm.nih.gov) of the other green algae phylogenetically close to *S. acutus*. Subsequently, the obtained consensus sequences were aligned with *T. obliquus* UTEX 393 genome. On the identified region of *T. obliquus* genome, primers were designed (Tab S.1) and used to amplify the corresponding genomic DNA or cDNA fragments through PCR (Polymerase Chain Reaction). The same approach was utilized to attain the full length of genes previously partially identified. The reaction mixture (50 μL) contained 100 ng DNA, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 2 μM of each primer, and 21.25 U Platinum® Pfx DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed on MWG Biotech Inc Primus 96 Thermal Cycler using the cycling parameters as follows: 94 °C for 5 min followed by 34 cycles of 94 °C for 15 seconds, 58-60 °C for 30 seconds and 68 °C for 1-3 minute. PCR products were separated by agarose gel electrophoresis, and the excided amplification bands were eluted from agarose gel and purified using a QIAquick Gel Extraction Kit (QIAGEN, Crawley, UK). All PCR products were cloned using the pGEM®-T Easy vector system (Promega, Madison, WI). PCR product were sequenced by BMR Genomics (Padua, Italy) using Sanger method.

2.6. RT-qPCR of Differentially methylated Sulfate pathway related genes

Primers used for quantitative real-time PCR analysis (RT-qPCR) were designed using Primer Express™ Software v3.0.1 (Applied Biosystems, Foster City, CA). Primers pairs were selected according to robustness, specificity and consistency. Only the pairs with an average efficiency between 0.90 and 1.0 were used (Table 1). Amplification reactions were performed using a STEP ONE instrument (Applied Biosystems, Foster City, CA) and prepared in a final volume of 20 μL containing: 1X Select SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM each primer and 50 ng cDNA. The reactions were carried out in triplicate in 48-well reaction plates and negative controls were included. The cycling parameters were as follows: 50 °C for 2 min. 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Melting curve analysis was also performed to confirm the existence of a unique PCR product and evaluated by an increase of 0.5 °C every 10 s, from 60 °C to 95 °C. Relative expression and statistical significance of each target gene were calculated using the Relative Expression Software Tool REST 2009, version 2.0.13 [52]. This tool uses the hypothesis test P(H1) to determine significant differences between sample and control groups and provides proper error propagation and robust statistical analysis by using a random reallocation algorithm with 10,000 iterations. A *p*-value lower than 0.05 was considered significant. The RT-qPCR data were normalized using *SaCBLP* (encoding a Guanine nucleotide-binding protein subunit beta-like protein; GeneBank: MT611452) as internal reference [53] after checking its constant expression in the two strains. The results were expressed as $-Log_2$[Fold change(Cr-t/wt)]. Mean expression levels were calculated from three biological replicates obtained from three independent experiments.

Table 1
List of primers used for relative quantification RT-qPCR of differently methylated gene of sulfate pathway in Cr-t vs wt.

Gene	Primer		Tm (°C)
SaARS	FW	5'- TTCACCCTGACAACAGTGTAACC - 3'	58
	BW	5'- GCCTGTAGAAGTCATCGTGCTT - 3'	57
SaSULTR1	FW	5'- TGGCTACCCTTCCAGTATGTTG - 3'	58
	BW	5'- GGACGTGGACTCAAGCATGT - 3'	57
SaSabc	FW	5'- AGTCAGGCAGCCCCAATG - 3'	58
	BW	5'- CCGACGAAGCCCATAACAA - 3'	58
SaOASTL3	FW	5'- GATGGCCGCATCAAGATG - 3'	57
	BW	5'- CAGTGCCCAGCAAACAACTC - 3'	58
SaSAT1	FW	5'- AGCGTGGAAGCCGTGTT - 3'	56
	BW	5'- ACTGTCAGGCGGGTTGTG - 3'	57
SaGSH1	FW	5'- ATGACAGGGACTACCTCAGAAACG - 3'	60
	BW	5'- ACGGTGCCTCCCTTGAAAG - 3'	59
SaCBLP	FW	5'- GTGCTGTCCGTGGCCTTCT - 3'	60
	BW	5'- TGTTCCACAGCTTAATGGACTTGT - 3'	59

3. Results

3.1. Quality control of the Whole Genome Bisulfite Sequencing data

To profile DNA methylation patterns at single-nucleotide resolution in wt *vs* Cr-t strain, WGBS library constructions and sequencing was performed on three biological replicates per strain. Single-base DNA methylation BS-Seq of these libraries generated up to ~ 42 million sequencing reads (2 × 150 bp paired end) for each replicate (Table S.2). For each sample, the bisulfite conversion rate (%) determined using the unmethylated λ phage genome, was higher than 98% (Table S.2). After quality filtering, the reads for all the samples resulted of high quality and so they uniquely mapped to the scaffolds of *T. obliquus* UTEX 393 genome [46]. The genomic mapping rate, the total number of high-quality reads, the number of mapped reads and the total number of C analysed are reported in Table 2 for each replicate. Our data show a relatively low mapping rate (32%), this is probably attributable to the genomic variability existing between our strains and UTEX 393 and to the fact that the reference genome is still in the draft state. In some areas indeed the reads did not align, but we found extensive regions where the coverage was instead excellent and homogeneous. Moreover, the genes we have characterized showed a 99% sequence identity with the corresponding portions of the scaffolds of the genome used as a reference.The percentage of methylated cytosines in CG, CHG and CHH sites was very similar across the three biological replicates, both in Cr-t as well as in wt strain. In particular, roughly 68% of the cytosine positions in the CG context of genome were methylated, around 25% of the

Table 2
Alignment of the reads against to *T. obliquus* UTEX 393 genome.

	Sample name					
	wt_1	**wt_2**	**wt_3**	**Cr-t_1**	**Cr-t_2**	**Cr-t_3**
Total no. of high-quality reads analyzed	17,695,822	19,389,250	18,156,991	19,149,090	21,025,102	18,415,906
No. of mapped reads	5,740,118	6,268,933	5,240,317	6,016,659	7,121,427	6,079,045
Mapping Rate (%)	32.4	32.3	28.9	31.4	33.9	33.0
Total no. of C's analyzed	438,918,968	479,612,354	406,566,138	459,649,334	553,134,264	469,293,558

positions were completely unmethylated and the remaining positions were partially methylated to various degrees in both the strains. On the contrary, roughly 68% of the cytosine positions in the CHG and CHH contexts where unmethylated and no fully methylated positions were observed (Fig. 1). Pairwise Pearson correlation coefficients were calculated among the six samples within two strains, to produce a correlation matrix, which easily compare correlation coefficients between pairs of samples. The analyses clearly showed that samples of the same strain cluster together independently (Fig. 1). As regard the CG methylation, the Pearson's correlation coefficient was constantly 0.98 within each strain and 0.97 in comparisons between samples of different strains. For CHG methylation context, Pearson's correlation coefficient was in the range 0.85-0.86 and 0.87 within Cr-t and wt samples, respectively; whereas the coefficient ranged from 0.79 to 0.84 between strains. In the same way, Pearson's correlation coefficient of methylation in CHH context, was higher within each strain and lower between strains (Fig. 1).

3.2. DNA methylation landscape in wt and Cr-t strains

Our results show that, among the analysed cytosines, 61,754,952 and 74,230,649 were methylated in wt and Cr-t strain, respectively. This corresponds to a significant difference in methylation percentage between strains (14% wt and 15% Cr-t). The overall methylation degree in the CHG and CHH contexts were significantly higher in the Cr-t ($p < 0.05$) compared to wt (Fig. 2). In particular, CHG methylation levels were 4.4 % in the Cr-t and 2.6 % in wt strain, whereas in CHH context, values of 2.7 % and 1.6 % were observed in the Cr-t and in the wt, respectively. On the contrary, in CG context no significant differences in methylation level were observed between strains (Cr-t 76.3 % *vs* wt 76.9 %, $p = 0.65$) (Fig. 2).

A sliding window approach, using 500 bp windows size and 250 bp step size, was employed across the scaffold of *T. obliquus* genome to identify DMRs in the Cr-t strain *vs* wt. The results of DRMs analysis are reported in Fig. 3. The DMRs, detected in Cr-t strain *vs* wt, were assigned to proximal predicted protein-coding genes based on the genomic coordinates. The analysis indicate that 32.25% of hypermethylated and the 34.38% of hypomethylated regions did not overlap with the hypothetical protein-coding genes, suggesting that methylation in these regions putatively occurred into repeats and transposable elements (TEs). Interestingly, 244 hypomethylated and 210 hypermethylated regions were identified in CG context; on the contrary, 106 hypermethylated and only 9 hypomethylated regions were identified in CHG context, whereas only 56 hypermethylated and 2 hypomethylated regions were identified in CHH context (Fig. 3A). The results showed that 62.9%, 56.6% and 64.9% of the hypermethylated regions in CG, CHG and CHH contexts, overlapped with protein-coding genes (131, 60 and 37 genes in each context respectively) (Fig. 3B). Similarly, 68.85% (168 genes), 11.1% (1 gene) and 0% (no gene) of the hypomethylated regions in CG, CHG and CHH contexts respectively, overlapped with protein-coding genes (Fig. 3). As a result, a total number of 229 hypermethylated and 168 hypomethylated genes were identified in Cr-t *vs* wt strain. Interestingly, among these genes, 18 were hypermethylated in more than one context; 17 genes were hypermethylated in both CHH and CHG contexts and only 1 gene was hypermethylated in all contexts (Fig. 3B).

In order to define which genic regions were associated with a specific methylation context, we examined the distribution of differentially hyper- and hypomethylated genes in various annotated features of genic regions, including promoter regions, 2000 bp upstream of the transcription start site, gene body up to 5'- and 3'- untranslated regions. The hypermethylation in CHG and CHH contexts was observed predominantly in the gene body and to a less extent in the flanking regions, whereas CG hypermethylation was mainly located in the gene body and to a less extent in the 5' untranslated region (Fig. 3C). Scarce differences were evidenced in CG hypomethylation between the different genic regions although with a greater extent in both 5'- and 3'- untranslated regions (Fig. 3D).

SEA algorithm was used *via* KOBAS 3.0 to analyse Gene Ontology (GO) term enrichment of hyper- and hypo- methylated hypothetic genes, using *C. reinhardtii* as reference organism and Fisher's exact test (*P value* < 0.05) (Fig. 4). Results of the GO categorization were expressed as three independent hierarchies: Molecular Functions, F (gene product at biochemical level), Biological Processes, P (cellular events to which the gene product contributes) and Cellular Components, C (location or complex of gene/protein). Among the 209 hypermethylated hypothetical genes, 129 genes were successfully blasted in KOBAS 3.0. Regarding Molecular Function, the higher significant term corresponded to helicase activity (GO:0004386) followed by several binding activities, namely ATP (GO:0005524), adenyl ribonucleotide (GO:0032559), adenyl nucleotide (GO:0030554), nucleoside (GO:0001882), ribonucleotide (GO:0032553), nucleotide (GO:0000166) and purine (GO:0017076); correspondence with ATPase (GO:0016887), lyase (GO:0016829) and transcription regulator (GO:0030528) activities were also identified. As regard as Biological Processes, 37 differentially hypermethylated genes were significantly enriched: i.e metabolic process (GO:0008152), regulation of transcription (GO:0006351), RNA metabolic process (GO:0051252), protein metabolic process (GO:0019538) and regulation of several process (GO:0050794, GO:0051171, GO:0031326, GO:0009889, GO:0010556, GO:0010468, GO:0050789). No significant Cellular Components were found in hypermethylated gene fraction (Fig. 4A).

Among the hypomethylated predicted genes, 103 were successfully blasted in KOBAS 3.0. For these genes, all functional groups have been identified, although the Biological Process consisted only of GO:0008152 metabolic process. Molecular function was costituited by ATPase activity coupled (GO:0042623), transporter activity (GO:0005215), active transmembrane transporter activity (GO:0022804), nucleoside-triphosphatase activity (GO:0017111), hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides (GO:0016818), pyrophosphatase activity (GO:0016462), hydrolase activity, acting on acid anhydrides (GO:0016817), substrate-specific transmembrane transporter activity (GO:0022891), ATPase activity (GO:0016887), hydrolase activity (GO:0016787) and substrate-specific transporter activity (GO:0022892). Finally, Cellular Component GO included: cell (GO:0005623), cell part (GO:0044464), mem-

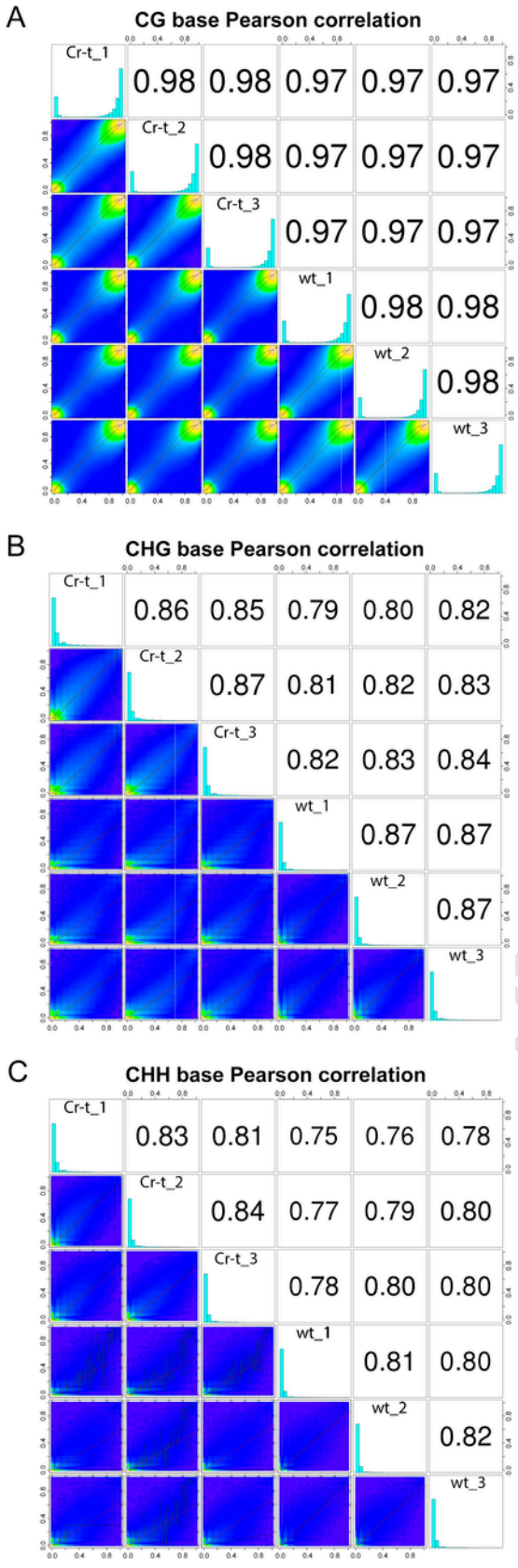

Fig. 1. Methylation distribution and Pearson's correlation between the replicate. The histograms on the diagonal and the scatter plots denote respectively the percent methylation distribution and Pearson's correlation on base resolution, between the replicate in CG (A), CHG (B) and CHH (C) context. Numbers on upper right corner denote pair-wise Pearson's correlation scores.

brane (GO:0016020), intracellular (GO:0005622) and intracellular part (GO:0044424) categories (Fig. 4B).

In order to unveil the involvement of different life processes or metabolisms between strains, hyper- and hypo- fractions of differentially methylated genes were blasted with KEGG data base by using KOBAS 3.0 software (Fig. 5). Data analyses showed that Cr-t hypermethylated genes were involved in important pathways as biosynthesis of secondary metabolites, metabolic pathways, biosynthesis of amino acids, pentose phosphate pathway, glycerophospholipid metabolism, RNA transport, cysteine and methionine metabolism, mRNA surveillance pathway, glycolysis/gluconeogenesis, ubiquitin mediated proteolysis, spliceosome, sulfur metabolism, glycerolipid metabolism, 2-oxocarboxylic acid metabolism, carbon metabolism, glutathione metabolism and glycine, serine and threonine metabolism (Fig. 5A). The significantly enriched KEGG pathways of hypomethylated fraction were riboflavin metabolism, metabolic pathways, pantothenate and CoA biosynthesis, photosynthesis - antenna proteins, phagosome, DNA replication, pyrimidine metabolism and carbon fixation in photosynthetic organisms (Fig. 5B).

3.3. Analysis of Sulfate pathway

In order to better understand the relationship between Cr-tolerance and differential methylation of the sulfate reductive pathway genes, we identified and characterized 30 genes involved in this metabolism; their sequences have been submitted and are available in GenBank. In particular, for the genes coding for ATP sulfurylase 1 (*SaATS1*; GeneBank: KJ187406.2), ATP sulfurylase 2 (*SaATS2*; GeneBank: KJ187408.2), chloroplast sulfate permease SULP1 (*SaSULP1*; GeneBank: JN903531.2), chloroplast sulfate transporter (*SaSabc*, cysA; GeneBank: KJ130520.2), adenylylphosphosulfate reductase (*SaAPR*; GeneBank: KT768122.2), cysteine synthase 1 (*SaOASTL1*; GeneBank: KJ130518.2), cysteine synthase 3 (*SaOASTL3*; GeneBank: KJ130519.2) and Snf1-like kinase (*SaSAC3*; GeneBank: KJ187407.2), partially identified previously, we attained their full-length. Besides we completely identified and characterized the genes encoding periplasmic arylsulfatase (*SaARS*; GeneBank: MT611454), H^+/sulfate transporter 1 (*SaSULTR1*; GeneBank: MG969380.2), H^+/sulfate transporter 2 (*SaSULTR2*; GeneBank: MF457897.2), sulfate permease SULP2 (*SaSULP2*; GeneBank: MT611471), adenosine 5'-phosphosulfate kinase (*SaAPK*; GeneBank: MT611453), ferredoxin-sulfite reductase (*SaSIR1*; GeneBank: MT611464), sodium sulfate co-transporter (*SaSLTa*; GeneBank: MT611469), cysteine synthase 2 (*SaOASTL2*; GeneBank: MT611460), cysteine synthase 4 (*SaOASTL4* GeneBank: MT611461), serine acetyl transferase 1 (*SaSAT1*; GeneBank: MT611462), serine acetyl transferase 2 (*SaSAT2*; GeneBank: MT611463), cystathionine gamma-synthase (*SaCGS1*; GeneBank: MT611456) cystathionine beta-lyase (*SaMETC*; GeneBank: MT611458), threonine synthase (*SaTHS*; GeneBank: MT611466), cobalamin-independent methionine synthase (*SaMETE*; GeneBank: MT611459) and gamma-glutamylcysteine synthetase (*SaGSH1*; GeneBank: MT611456). Finally, we partially identified the genes encoding a sodium sulfate co-transporter (*SaSLTb*; GeneBank: MT611470), sulfite reductase (*SaSIR3* GeneBank: MT611465), chloroplast sulfate-binding protein (*SaSBP*; GeneBank: MT611472) cobalamin-dependent methionine synthase 1 (*SaMETH1*; GeneBank: MT611467) cobalamin-dependent methionine synthase 2 (*SaMETH2*; GeneBank: MT611468) and glutathione synthetase (*SaGSH2*; GeneBank: MT611457). All these genes were mapped against the scaffolds of *T. obliquus* genome, and then, each DMRs was assigned to proximal identified genes based on the genomic coordinates. The gene methylation levels were investigated from upstream, gene body, up to the downstream regions. Six of these genes resulted differently

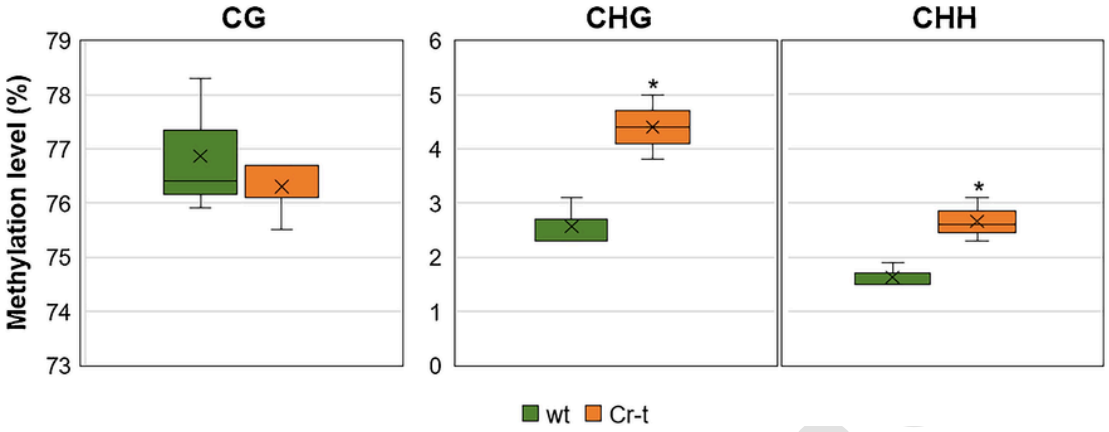

Fig. 2. Methylation level in wt and Cr-t strain. Percentage of methylation level in CG, CHG and CHH context in wt and Cr-t strain. Asterisk above the bars indicates the significant difference between strains performed by *t-test* ($p < 0.05$).

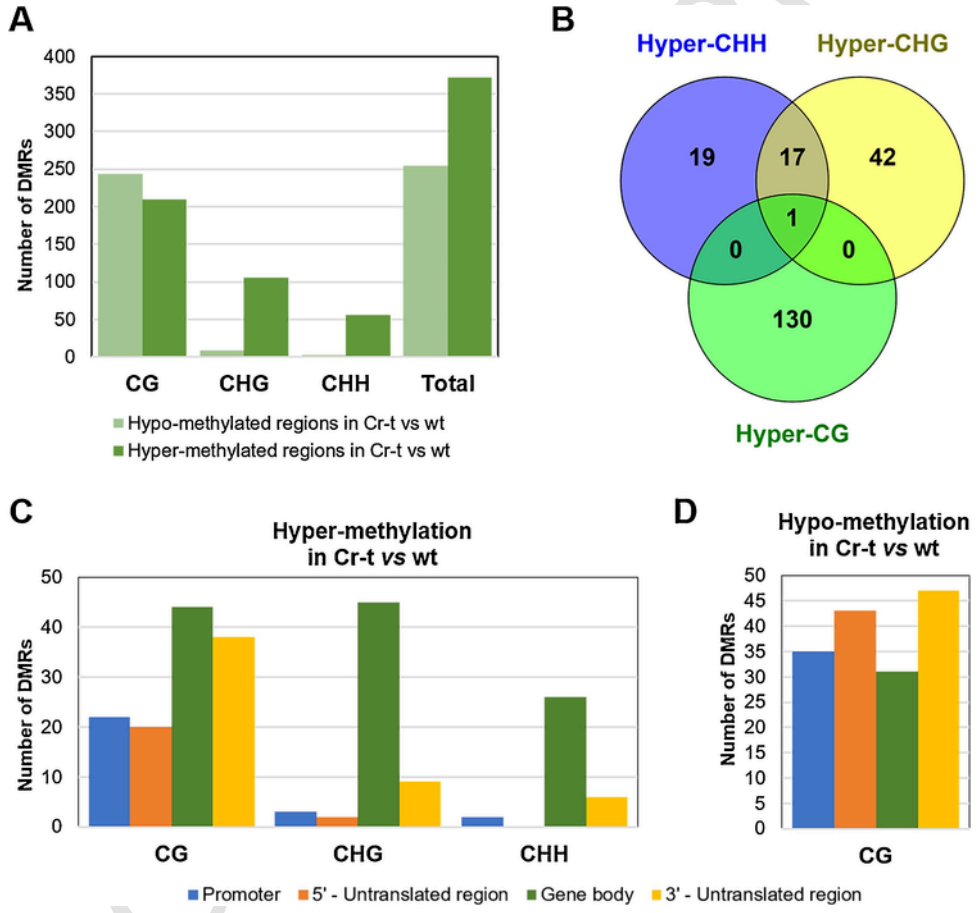

Fig. 3. Analysis of differentially methylated regions in Cr-t *vs* wt strain. A, Number of differentially hyper- and hypo-methylated regions in CG, CHG and CHH contexts in Cr-t strain vs wt. B, Number of protein-coding genes overlapping with differentially hypermethylated regions in CG, CHG and CHH contexts. C and D, Distribution of differentially hypermethylated (C) and hypomethylated regions (D) to the annotated features of predicted protein-coding genes.

methylated in Cr-t *vs* wt. More precisely, *SaSULTR1* were hypomethylated in CG context on the promoter region whereas *SaARS* and *SaOASTL3* were hypomethylated in CG context on gene body. *SaSabc* and *SaSAT1* were hypermethylated in CG context on gene body and *SaGSH1* was hypermethylated in CHG context on gene body (Fig. 6).

The RT-qPCR showed that the two hypomethylated genes, *SaARS* and *SaSULTR1*, were expressed exclusively in Cr-t strain. All the hypermethylated genes were instead significantly ($p < 0.001$) down-regulated in this strain respect to wt. Overall, the analysed genes showed a negative association between DNA methylation and expression level, with the exception of *SaOASTL3*, that albeit hypomethylated is down regulated in the Cr-t strain (Fig. 7).

4. Discussion

In order to give additional information on the involvement of DNA methylation mechanisms in heavy metal tolerance of the freshwater green alga *S. acutus*, a WGBS was performed in the two strains with dif-

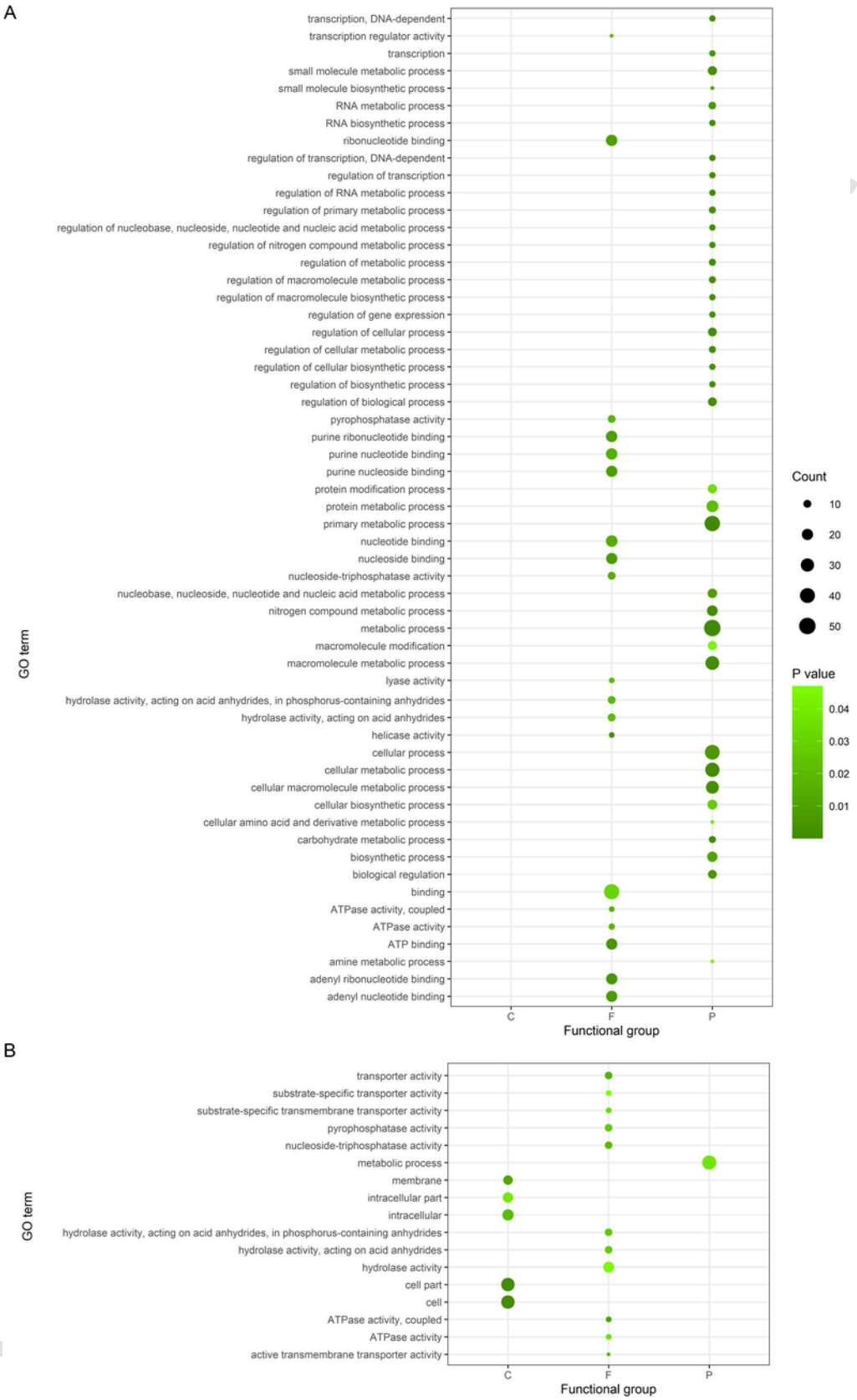

Fig. 4. GO-term enrichment of differently methylated predicted genes in the Cr-t strain. Fisher's exact test for the significant enrichment analysis ($P < 0.05$) of GO terms of the cellular component (C), biological process (P) and molecular function (F) categories of the hypermethylated (A) and hypomethylated (B) predicted genes. The size of the circles represents the number of genes in the test set belonging to each GO category.

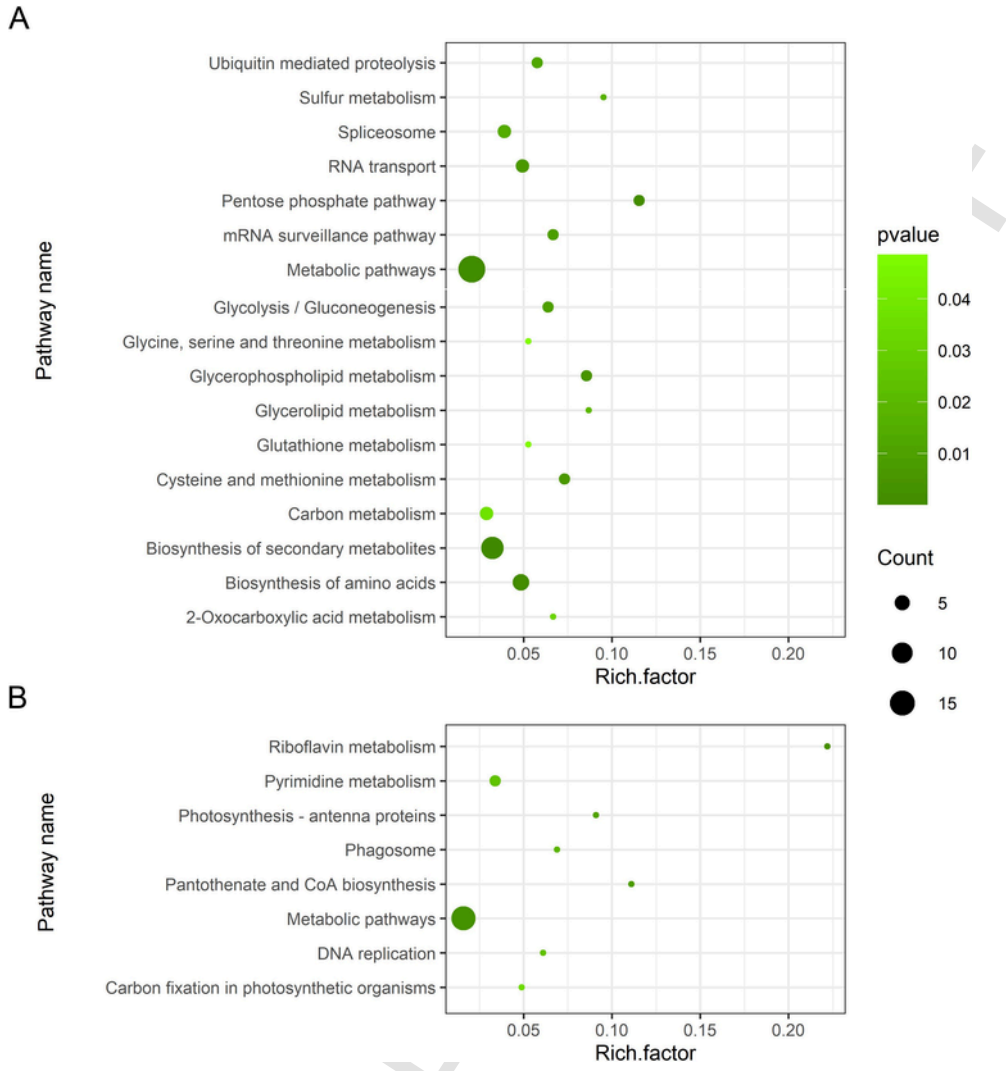

Fig. 5. KEGG Pathway analysis of differently methylated predicted genes in Cr-t strain. Fisher's exact test for the KEGG pathway enrichment analysis ($P < 0.05$) of hypermethylated (A) and hypomethylated (B) genes in the Cr-t. The size of the circles represents the number of genes belonging to each pathway.

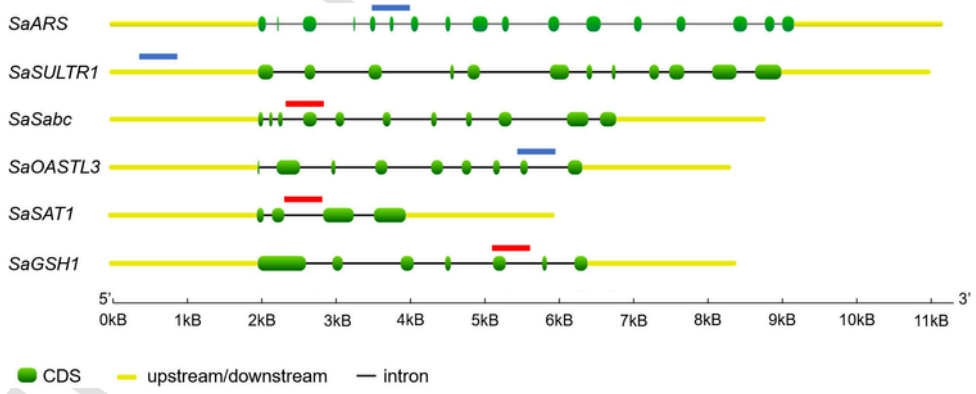

Fig. 6. Features of the differently methylated genes belonging sulfate pathway in Cr-t *vs* wt. Distribution of DMRs throughout the genes: the blue and red rectangles respectively represent the hypomethylated and hypermethylated regions.

ferent Cr(VI) sensitivity (Cr-t *vs* wt) of this unicellular microalga. Notwithstanding the studies on stress-responsive epigenomes and transcriptomes are important to understand the evolution of mechanisms related to stress adaptation in photosynthetic organisms, very few data have indeed been reported in algae [11,14,54]. The comparison of genome-wide DNA methylation profiles under the two different biological contexts, Cr-t *vs* wt, suggests that an epigenome reprogramming occurred as a result of chromium stress in *S. acutus* and strengthens the hypothesis of a strain-dependent DNA methylation pattern.

Our data show that DNA methylation in *S. acutus* is low and comparable to the levels seen in other green algae (*C. reinhardtii* and *Chlorella sp.)*, diatoms and brown algae [9–11,14]. In both *S. acutus* strains, cy-

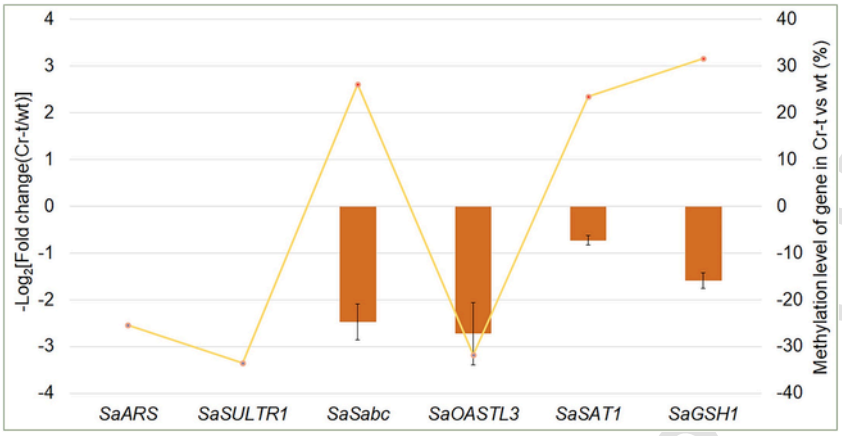

Fig. 7. Relative expression analysis of differentially methylated genes in Cr-t *vs* wt strain. The RT-qPCR data were normalized using *SaCBLP* as reference gene. Relative quantification was obtained using REST 2009 [48] and the results represent the mean value (± standard deviation) of triplicate of three biological replicates. The bars represent the -Log_2[fold change (Cr-t/wt)] and the line represents the difference in methylation level (%) between Cr-t and wt in the considered genes. All the differences observed between strains in the analyzed genes were highly significant ($p < 0.0001$). *SaARS* and *SaSULTR1* were expressed only in Cr-t strain and are thus not reported in the chart.

tosine methylation occurred principally in the symmetric CG rather than in CHG and CHH contexts. This confirms what was found in *C. reinhardtii* where, although in an overall low level of methylation, CG is the mainly methylated context (5.4%, 2.6%, and 2.5% in CG, CHG and CHH contexts, respectively) [14]. Also in *Chlorella sp.* genome methylation occurs mainly in CG context (80.5%, 2.2%, and 0.25% in the contexts CG, CHG and CHH, respectively), while *Volvox carteri* genome is much less methylated and almost exclusively in the CG context (2.6%, 0.08% and 0.08% in CG, CHG and CHH contexts, respectively) [54]; this is not unusual since prevalent methylation in CG context is an evolutionary conserved features in angiosperms as well [13].

Albeit the overall CG methylation degree was similar between *S. acutus* strains, the Cr-t showed a higher number of hypomethylated regions in CG context and *vice versa* higher hypermethylated regions in CHG and CHH contexts than wt. In agreement with the previous data on the immunolocalization of 5-mC in CG context [39], these results indicate that Cr-tolerance is related to hypomethylation in CG context. In *S. acutus* DNA methylation occurs in all the genic regions, in contrast to what reported for other algae and plants in which methylation/demethylation preferentially occur in specific genic regions. Methylation of repeats has also been reported in the green algae *C. reinhardtii*, *Chlorella sp.* and *V. carteri* [14,54]. In the green alga *Chlorella sp.*, CHG methylation is confined to repeats and in the spike moss *Selaginella moellendorffii* very little methylation occurs in gene bodies, whereas transposable elements are methylated in the CG and to a lesser extent in the CHG and CHH context [54]. In soybean and *Arabidopsis* plants exposed to various biotic stress, methylation/demethylation occurs predominantly in the gene body [55,56], while in rice non-CG methylation is essentially absent from genes, and methylation in all contexts is abundant in transposable elements [54]. Changes in DNA gene body methylation, following metal exposure were also observed in genes of rice plants exposed to cadmium [15]. These findings would suggest that methylation or demethylation occur not only at gene level and mainly in the gene body and exons, which is considered the ancestral condition [14], but also in TEs. In *A. thaliana* a distinct chromatin state was adopted by silent TEs, due to combination with demethylation of the repressive histone H3 lysine 9 (H3K9me2) and elevated DNA methylation levels and other histone modifications [4,57]. Moreover, TEs, as well as genes, participate in environmental stress responses through epigenetic variations [58]. Likewise, we can hypothesize that in *S. acutus* TEs could dynamically regulate the transcription of transposons and proximal genes involved in Cr-tolerance through a chromatin remodelling, which is in turn regulated by the DNA methylation state. This hypothesis is strengthened by previous evidences revealing a significantly lower level of heterochromatin in Cr-t than in wt strain and a change in the euchromatin percentage after 72 h Cr(VI) exposure, when became similar in the two strains and significantly higher than heterochromatin [39].

The GO and KEGG enrichment analyses of the genes overlapping with DRMs reveals many different biological processes in *S. acutus*, putatively related to the Cr-tolerance. Many of the functional groups identified in this study are indeed in common with those involved in Cr(VI) stress response in other plants [59–61]. For example, photosynthesis - antenna proteins, carbon fixation in photosynthetic organisms, biosynthesis of secondary metabolites, pentose phosphate pathway, glycolysis/gluconeogenesis and glycerophospholipid metabolism were hypo- or hyper-methylated in Cr-t strain. The same pathways were negatively regulated after Cr(VI) exposure in *Brassica napus* [59] and in rice [61]. Interestingly in Cr-t strain, photosynthesis and chlorophyll content were not altered after 1 mg Cr(VI) L^{-1} treatment, whereas wt decreased its photosynthetic activity [38]. The genes involved in riboflavin metabolism and amino acid biosynthesis, differentially methylated in the two *S. acutus* strains, resulted as well differently regulated in chickpea during chromium exposure [62]. Besides, differentially methylated genes in Cr-t strain *vs* wt belongs to pathways involved in RNA transport, mRNA surveillance, spliceosome, phagosome, ubiquitin mediated proteolysis, glycerolipid metabolism and pyrimidine metabolism, the same genes that Gill et al. (2016) [59] found modulated in the transcriptome profile of two cultivars of *B. napus* with different Cr-tolerance response. Our data, supported by literature, thus suggest that in *S. acutus* DNA methylation may have been of particular importance in reprogramming primary metabolism during prolonged Cr(VI) exposure and defining signal specificity associated with the resistance mechanism to metal stress.

In our study particular attention was focused on the sulfate uptake/assimilation pathway, previously indicated as putatively involved in Cr(VI)-tolerance in *S. acutus* and showing differential methylation between the two strains. This pathway is fundamental since its final products (cysteine and GSH and their derivative phytochelatins) are directly involved in the detoxification of heavy metals (chromium included) both through direct chelating action and through mitigation of oxidative stress [36]. Higher levels of cysteine, GSH and S content were previously observed in Cr-t strain than in wt [40–42]; moreover in both strains a period of S-starvation induced a transient increase in Cr(VI) tolerance [40,41]. Six of the thirty characterized genes of S-pathway resulted differentially methylated and transcribed in Cr-t, indicating that epigenetic mechanisms are involved in the basal regulation of this pathway. In Cr-t strain, we indeed observed hypomethylation associ-

ated to the expression of two genes involved in S-uptake, namely a periplasmatic arylsulfatase (*SaARS*) and a sulfate transporter (*SaSULTR1)*; four genes involved in sulfate assimilation resulted instead down-regulated and hypermethylated, with the exception of *SaOASTL3*. *SaSULTR1* is the only gene in which we observed differential methylation in the promoter region, whereas the others showed differential gene body methylation. In agreement with literature data [63], hypomethylation in the promoter region enhanced *SaSULTR1* expression in Cr-t. A dynamic DNA methylation, particularly in the promoter region, has been proposed as an important mechanism in the regulation of the expression of nutrient-deficiency-responsive genes [64]. The DNA methylation in the promoter could indeed repress transcription in two way: i) inhibiting the binding of transcriptional activators (thus hindering the activation of gene expression); ii) recruiting the binding of transcriptional repressors to the promoter (thus repressing gene expression) [65]. The demethylation in *SaSULTR1* promoter region could thus activate gene expression either allowing binding of a transcriptional activator or releasing a transcriptional repressor.

Our data show also a methylation/demethylation in gene body correlated with the gene expression. *SaSabc, SaSAT1* and *SaGSH1* were indeed hypermethylated in gene body and downregulated in Cr-t; whereas *SaARS* was hypomethylated in gene body and expressed only in Cr-t strain. Intriguingly, *SaOASTL3* was instead hypomethylated and downregulated. Variable gene body methylation has been demonstrated as a general feature of plant and animal genomes, although the functions of this kind of methylation are not fully understood. It has been suggested that it might regulate alternative splicing efficiency, prevent aberrant transcriptional initiation from cryptic sites within genes impeding transcriptional elongation [63,66–69]. Moreover, CG gene body methylation is usually absent in inducible and developmentally regulated genes [70,71]; on the other hand, positive correlation between gene expression and gene body methylation was also reported [14,54,72]. A complex relationship between gene body methylation level and gene expression was highlighted in rice: apparently gene expression increases with methylation levels up to a certain degree, whereas it is repressed by heavy gene body methylation and genes with moderate levels of body methylation show the highest expression levels [68]. Moderate methylation tends to enhance gene expression, whereas low or high methylation tends to inhibit gene expression [73]. All things considered, the heterogeneous effect of body methylation on gene expression could be linked to the extent of methylation in the target genes.

The different methylation and expression level of genes involved in sulfate uptake/assimilation in the Cr-t strain, suggest that epigenetic mechanisms play an important role in the modulation of sulfate pathway. We may hypothesize that the heritable methylation patterns, occurred in the gene body and promoter regions throughout prolonged Cr(VI) exposure during the Cr-t strain selection, gave rise to new gene combinations with the consequent variation in regulation of gene expression driving to Cr(VI)-tolerance acquisition.

The expression of *SaARS* and *SaSULTR1* only in Cr-t cells, suggest a constitutive higher sulfate uptake in this strain than in wt. Literature data show that some genes involved in sulfate uptake (e.g. encoding for Arylsulfatases and high-affinity sulfate transporters) are not expressed in non-limiting sulfate condition but strongly induced by sulfur (S) limitation in *C. reinhardtii* [74]. In *A. thaliana* the hyper methylation in the flanking regions of the sulfur responsive element (SURE) in the promoter of *SULTR1;1*, which is essential for the S-deficiency response [75], determines a low expression of this transporter under S-sufficient condition. Conversely, the hypo-methylation of SURE under S-deficiency condition induces a strong *SULTR1;1* expression [75]. Besides, in *Arabidopsis* more sulphur accumulation1-1 (*msa1-1*) mutant, the flanking sequence of SURE in the promoter of *SULTR1;1* is hypo-methylated and associated with the increase of this transporter expression with the consequent increase of S uptake in the plant [75]. The low expression of genes involved in sulfur assimilation such as *SaSabc*, *SaOASTL3*, *SaSAT1* and *SaGSH1* would indicate a putative reduced sulfur assimilation in Cr-t cells. This hypothesis is apparently in contrast with data already reported showing in Cr-t strain a higher Cys content [40] and a higher Cys production efficiency [42] than in wt. Nevertheless, Cys synthesis can occur in different cell compartment; indeed, different isoforms of the enzymes (SAT and OASTL) involved in the formation of the Cysteine Synthase Complex (CSC) were found configuring a complex regulation network of the assimilation pathway [76–80]. Many evidences indicate that Cys biosynthesis is regulated in a compartment-specific manner: the mitochondrion would be responsible for providing most of the O-acetyl-l-serine in the cell, while cytosol would represent the major site of cysteine production [76–80]. Multiple isoforms of OASTL with a possible different cell localization were found in algae [81] and the recent paper of Sardella et al. (2019) [42] suggests that a cytosolic mechanism, probably involving the OASTL isoform located in this compartment, may be involved in the higher Cys production/S assimilation of the Cr-t strain. Four OASTL isoforms are present in *S. acutus* genome and the differently methylated *SaOASTL3* isoform is homologous to OASTL3 of *C. reinhardtii* (XP_001703301.1) which is ambiguously predicted to be chloroplast targeted by homology and mitochondrial targeted by target-P analysis. We are however not aware of which role the differently methylated *SaSAT1* and *SaOASTL3* play in sulfate assimilation in *S. acutus*. It is thus difficult to argue from the analysis of single isoforms the functioning and regulation of such a complex pathway and this aspect should deserve further investigation.

5. Conclusions

In conclusion, we provide a high-resolution methylation status of individual cytosines throughout the genome of two strains of *S. acutus* with different sensitivity to Cr (VI). The results bring substantial first information about the *S. acutus* methylome and show that the two strains have a different 5-mC distribution pattern regard to both methylated local contexts (CG, CHG and CHH) and differentially methylated regions. The significant differences in methylation-mediated gene expression involved in sulfate pathway between Cr-t and wt cells, suggests that cytosine methylation is involved in Cr-tolerance. The emerging picture suggests that, under a prolonged heavy metal exposure, algae activity is directed to enhance and/or maintain the signal levels and response that are relevant during stress to sustain the growth, through a different DNA methylation pattern as "stress memory". In this context, sulfate uptake/assimilation pathway plays a pivotal role and the increase in sulfur uptake results in an improved cell defence capacity. This study has the potential to greatly enhance our understanding of links between DNA methylation and the Cr tolerance in *S. acutus* and provides a means to explore the basis of metal resistance in the microalgae.

CRediT authorship contribution statement

Michele Ferrari: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-Review&Editing. **Anna Torelli:** Conceptualization, Visualitazion, Writing- Review&Editing. **Matteo Marieschi:** Investigation, Resource. **Radiana Cozza:** Conceptualization, Writing-Review&Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110680.

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