Epigenetic regulation based on 6mA is Essential for Cold Stress Acclimation in Chlamydomonas reinhardtii

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Abstract

This study investigates the role of N6-methyladenine (6mA) DNA methylation in the cold stress response of *Chlamydomonas reinhardtii*, the model microalga. While cytosine methylation (5mC) has been extensively studied in various eukaryotes, the role of adenine methylation (6mA) in stress acclimation remains poorly understood, especially in microalgae. Utilizing a chemical approach, we inhibited de novo adenine methylation with pyrimidinedione, a known 6mA methyltransferase inhibitor, and assessed its effects under cold stress conditions (4 °C). Results demonstrated that while 5mC reduction did not significantly impact cell viability, inhibition of 6mA severely impaired the microalga's ability to acclimate to cold stress, ultimately leading to cell death.

To elucidate the molecular mechanisms underlying this phenomenon, we employed Oxford Nanopore Technology (ONT) for genome-wide methylome profiling and Illumina RNA-seq for transcriptome analysis. Data integration revealed that 6mA methylation predominantly occurred near transcription start sites (TSS) and was correlated with the activation of stress-responsive genes critical for cold acclimation. Key regulatory elements, including members of the SnRK family, methyltransferases, and stress-associated enzymes, exhibited differential 6mA methylation patterns and gene expression profiles upon cold exposure. Inhibition of 6mA methylation disrupted these regulatory pathways, underscoring its essential role in gene activation during stress response.

Our findings provide novel insights into the epigenetic regulation of stress acclimation in *Chlamydomonas reinhardtii*, highlighting the pivotal function of 6mA methylation in cold stress survival. This study not only advances our understanding of algal stress biology but also offers potential strategies for engineering microalgae for enhanced stress tolerance and biomolecule production.

Introduction

Microalgae are constantly exposed to a changing environment, as extreme temperature [1,2], desiccation [3] or even the exposure to new pollutants as microplastics [4]. Abiotic stress impairs growth and development, reducing the biomass yield while modulating cell composition [5]. To face abiotic stress, microalgae have developed a complex range of molecular mechanisms for sensing, responding and eventually acclimating to survive. Within the abiotic stresses, the response to low temperature is an excellent model to study the acclimation capacities of microalgae. Cold stress induces in microalgae the growth arrestment and cell morphology change, including the loss of the ciliary apparatus. These changes are associated to a profound proteome and metabolome remodeling, associated to a decrease in the photosynthetic capacity while gluconeogenesis and starch synthesis are activated [1]. Sugars regulate the SnRK signalling pathways, which have an essential role in gene expression regulation through the activation of bZIP transcription factors and SWI/SNF/helicase complexes which modify chromatin structure [5]. For hence, cold stress induces pathways linked to the central metabolism, autophagy, and also epigenetic mechanisms mediated by the SnRKs-bZIPs in *Chlamydomonas reinhardtii* (hereafter Chlamydomonas) [1] while accumulating biomolecules of interest, like starch. The determination of the basic response mechanisms to cold stress in an ancestral organism within Plants like Chlamydomonas would also help to understand the complex responses exhibited by land plants, as cold limits growth and development in all of them [6]. However, the knowledge about how the cell orchestrates a coordinated stress response between the epigenome, the transcriptome and the proteome is far to be understood, as the specific roles of epigenetics are not well-established microalgae [7,8]. Consequently, understanding the role of epigenetic regulation in cold stress will provide novel insights into algal stress biology, and also help to engineer microalgae for producing biomolecules of interest and to understand the evolution of stress response within the Plant kingdom.

The scarce knowledge about the role of epigenetics in stress response in microalgae has been obtained using the model species Chlamydomonas [7,9,10] and is mainly focused on the changes occurring during cell cycle. Among eukaryotes DNA methylation at C is the most studied epigenetic mark. 5mC has a key role in biological processes, such are development, imprinting, flowering and stress response and acclimation [11,12]. In Arabidopsis, 5mC regulatory mechanisms are well-studied and associated to cold stress response, being the epigenetic silencing of *DREB1/CBFs* during cold exposition crucial to control cold response [13]. However,

in Chlamydomonas, this mark occurs at a very low content (0.75 %) in all the three possible contexts (CG, CHG, and CHH), and neither not vary during cell stage neither seems to be correlated with gene transcription repression/activation [14]. Interestingly 5mC was recently defined as necessary in transgenerational long-term stress processes [15], but this mark has not been yet studied during stress acclimation. Chlamydomonas also exhibits specific dynamics in 6mA [7], a mark associated to gene expression recently described in different eukaryotes ranging from plants to humans [16–20]. In Chlamydomonas, this mark constitutes ~0.4 % of total A in the genome, variating its concentration during the cell cycle. This mark is mainly located around TSS, and correlates with nucleosome positioning and marks transcriptionally active genes. As in the case of 5mC, to our knowledge, no information is available about the specific role of 6mA in stress acclimation.

The use of a chemical unmasking approach allowed us to describe the effect in growth of 5mC and 6mA DNA methylation under optimal temperature (25 ºC) and under cold stress (4 ºC). Experimental results demonstrated that reducing 5mC did not have a major impact over cell viability under cold stress. However, reducing A de novo methylation by Pyrimidinedione,1-(4 bromophenyl)-5-(2-furylmethylene)-3-phenyl-2-thioxodihydro-4, 6 (1H,5H)-pyrimidinedione, (thereafter pyrimidinedione) impaired stress acclimation capacity and led to cell death. Therefore, A de novo methylation would be essential in cold stress response in Chlamydomonas, which would be presumably linked with the activation of stress responsive genes, as 6mA has been correlated with gene transcription activation [7]. Determining which genomic regions are regulated by 5mC and 6mA would increase our understanding of the different stress response mechanisms in Chlamydomonas and would provide new insight on the evolution of epigenetic pathways in stress response and acclimation.

The methods for studying DNA methylation differ between the different methylation marks. 5mC site-specific analysis methodologies are well established and there is plethora of protocols for performing wide genome bisulfite sequencing (WGBS) or methylated immunoprecipitation (MeDIP) and for the later bioinformatic analysis. However, site-specific study of 6mA is complicated, as there is no method like the bisulfite conversion of cytosines, and MeDIP approaches do not provide single-base resolution. Recently, the availability of single molecule real time (SMRT) sequencing, as the Oxford Nanopore Technology (ONT), opens the possibility to further study 6mA without extra laboratory techniques by using novel computational methods

including basecalling to predict modified bases from the original signal's information. This approach allows to study both 6mA and 5mC simultaneously.

Taking all of these into account, we chemically manipulated 6mA to decipher the complexity cold stress acclimation in Chlamydomonas we chemically manipulated the 6mA mark. For this, we carried out an experiment employing a 6mA methyltransferase inhibition drug pyrimidinedione [21,22] in combination with cold stress during 120 h. To explore the epigenome and the transcriptionally activated genes by 6mA during this stress we sequenced the DNA employing the SMRT Oxford Nanopore Technologies, combined with Illumina's based RNA-seq analysis. Results showed that adenine methylation gene modulation is key in cold stress acclimation, as its reduction cause Chlamydomonas death during this stress.

Materials and methods

Chlamydomonas culture conditions

Chlamydomonas reinhardtii CC503 (cw92) was obtained from the Chlamydomonas Resource Center (https://www.chlamycollection.org). Cultures were growth in liquid tris-acetate-phosphate (TAP) culture media. Growth conditions were as follows: constant 25 ºC, 180 rpm shaking, and a photoperiod of 16:8 (light:darkness) at 200 µmol photon$\cdot s^{-1} \cdot m^{-2}$ provided by warm white LED.

Pyrimidinedione effect assessment

Seed cultures were kept growing at exponential phase and diluted to $5 \cdot 10^4$ cells/mL in TAP prior to pyrimidinedione treatment. Cultures were grown in liquid Tris-Acetate-Phosphate (TAP) culture media at constant 25 ºC temperature, 180 rpm shaking, and a photoperiod of 16:8 (light:darkness) at 200 µmol photon$\cdot s^{-1} \cdot m^{-2}$ provided by warm white LED.

To demonstrate the effect of pyrimidinedione over 6mA, we attempted to reverse the effect with the hypermethylating agent folic acid (*Figure 1A*). Different treatments were applied (*Figure 1A*): (1) control, with equal amount of DMSO as epi-drug added in cultures; (2) 30 µM pyrimidinedione (Pyr); (3) 50 µM folic acid (Fol); (4) 30 µM pyrimidinedione and 50 µM of folic acid (Pyr+Fol). Three replicates of each treatment were done. Cultures were grown at 4 ºC.

To test pyrimidinedione effect time frame, 30 µM of pyrimidinedione was applied to cultures and same amount of DMSO was added to controls. Two replicates of each treatment were performed (*Figure 1B*) and grown at 4 ºC. Samples of cultures were centrifuged and washed to eliminate pyrimidinedione 1, 3, 6 and 12 h into the cold treatment. Washed samples were resuspended in TAP medium and split into two flasks. One flask was settled to 25 ºC and the other was maintained at 4 ºC. Cell density and Fv/Fm was measured at 0, 24, 48 and 72 h.

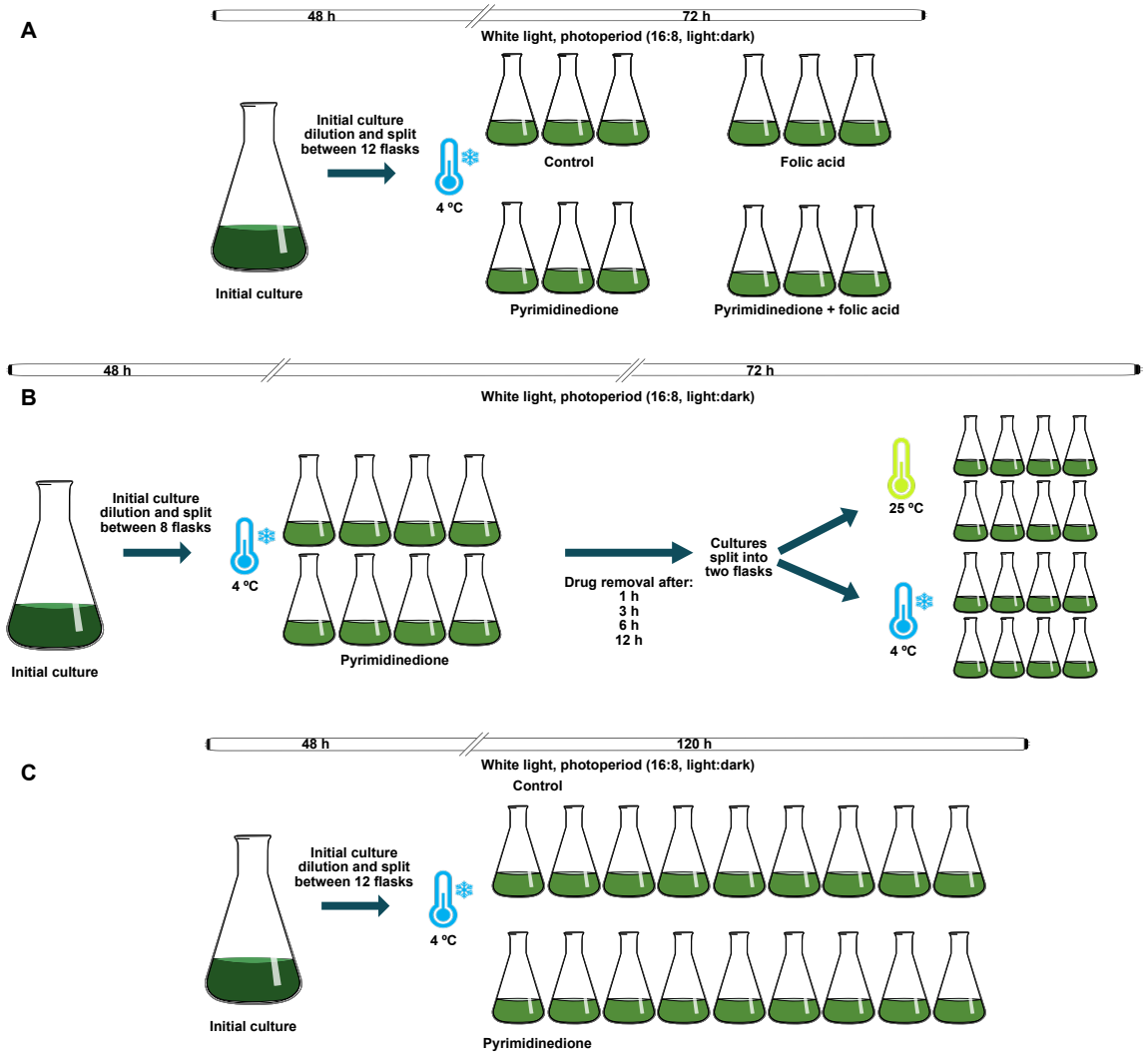


Figure 1. A) Pyrimidinedione effect assessment with the combination with folic acid, together with controls, pyrimidinedione and folic acid treatments. B) Test to know when pyrimidinedione effect under cold can't be reverted. C) Pyrimidinedione and control treatments under low temperature for the omics assay.

Cold and pyrimidinedione treatment for omics assay

Seed culture was growth at exponential phase and diluted to $5 \cdot 10^4$ cells/mL in TAP. Pirymidineone was applied to cultures at a concentration of 30 µM. The same amount of DMSO was added to mock controls. Nine replicates of each treatment were performed (*Figure 1C*). Cultures were grown in liquid TAP, at constant 4 ºC, 180 rpm shaking, and 16:8 (light:darkness) photoperiod at 200 µmol photon$\cdot s^{-1} \cdot m^{-2}$ provided by warm white LED. Samples were taken at 0 h (25 ºC), and 5, 24, 48, 72 and 120 h (4 ºC). Cultures were recovered at 25 ºC during 72 h.

Photosynthesis and growth rate determination

Cell growth was monitored by measuring culture absorbance at 650 and 750 nm with Nabi UV/vis Nano Spectrophotometer (MicroDigital Co., Korea). Photosynthesis rate (Fv/Fm) was measured with a pulse–amplitude modulation fluorimeter (Walz, JUNIOR-PAM) after an acclimation period of 15 min in darkness.

DNA extraction and ONT library preparation

Genomic DNA from 25 ºC control, 5 and 24 h control and pyrimidinedione treatments under cold, and 120 h of control cold stress cultures, was extracted by CTAB method from Gernot Gloeckner lab [23]. Quality and quantity of DNA was evaluated using a UV/vis Nano Spectrophotometer (MicroDigital Co., Korea) and Qubit 3.0 Fluorometer (Invitrogen life Technologies, Carlsbad, CA), respectively.

DNA was processed using the native barcoding (PCR-free) and ligation sequencing Kits (Catalog No. EXP-NBD104 and SQK-LSK109 respectively, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. DNA libraries (approximately 1.2 µg) were constructed and sequenced on the MinION (Oxford Nanopore Technologies).

ONT basecalling and mA detection

Basecalling was performed employing Guppy 6.2.1 [24] and Rerio [25] model "res_dna_r941_min_modbases-all-context_v001". Reads were indexed employing Nanopolish and aligned to Chlamydomonas genome v.6.0 [26,27] employing BWA. Later, reads were resquiggled against Chlamydomonas genome by using Nanopolish Eventalign [28]. Methylation

sites were called using mCaller [29], from which BED files containing site specific 6-mA quantitative values were obtained. A minimum read coverage of 4 was set as a threshold.

ONT basecalling and mC detection

mC calling was performed with Methylation Detection with Nanopore (METEORE [30]) combining the outputs from DeepSignal-plant [31] and Megalodon [32] to produce high-accuracy consensus mC sites. DeepSignal-plant was run over reads basecalled with Guppy 6.2.1 [24] using "res_dna_r941_min_modbases-all-context_v001" model from Rerio [25].

Genome annotation

Gene annotation was performed by using the *Chlamydomonas reinhardtii* genome v6.1 from JGI [26,27].

Methylation analysis

Differential methylation analysis was performed with methylKit (1.22.0) [33] using the BED files obtained from mCaller [29] as an input. Differentially methylated genomic regions were identified by a tiling window analysis using 1000 bp windows and a minimum coverage of 8, and based on a q-value < 0.05 cutoff. Differentially methylated regions were annotated using genomation (1.28.0) [34] and Chlamydomonas genome v6.1 annotations [26,27].

RNA extraction, library preparation, and NGS

RNA was extracted from 25 ºC control, 5 and 24 h control and pyrimidinedione treatments under cold, and 120 h of control cold stress cultures, was extracted following the protocol described by Valledor *et al.* (2014) [35]. Quality and quantity of DNA was evaluated using a UV/vis Nano Spectrophotometer (MicroDigital Co., Korea) and Qubit 3.0 Fluorometer (Invitrogen life Technologies, Carlsbad, CA), respectively. Before library preparation RNA integrity was check in an Agilent Bioanalyzer, with all samples having a RIN > 4.0. Libraries were built after capturing polyA RNAs with a standard oligodT approach. Library preparation and sequencing was outsourced (Novogene, Cambridge, UK). An average of 20 millions of reads were obtained per sample.

Bioinformatics processing analysis of RNA-seq

All reads were trimmed with Trimgalore (0.6.6, Babaham Bioinformatics), and ribosomal reads were removed employing Bowtie2 (2.5.1) and SILVA data base (https://www.arb-silva.de), and independently quantified and mapped to the Chlamydomonas transcriptome (v6.1) with Salmon (1.9).

Biostatistical analysis was performed in R 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) under RStudio IDE (Posit Software, Boston, US). Preprocessing, univariate and multivariate analyses over transcriptomic dataset were performed with bundled core packages and pRocessomics (0.1.13). Physiological and growth measurements were analyzed employing a t-test or Kruskal Wallis followed by Duncan's. The transcriptome dataset was first pre-processed, involving imputation, filtering, and balancing steps. Briefly, missing values were imputed only in those cases that they rep- resented less than 17 % of the samples of a particular treatment (one in three replicates) by using the Random Forests algorithm. After imputation, those transcripts that were not present in all replicates of at least one treatment or in at least three individual replicates, were filtered out, as they were considered inconsistent. Imputed and filtered data was balanced through a sample-centric approach prior to analysis. As most of the preprocessed protein variables failed to comply with the assumption of normality, we tested for the presence of inter-treatment differences by using the Kruskal Wallis test coupled with the Duncan post hoc test. Heatmaps were conducted, grouping proteins according to MapMan onthology, together with Gene Onthology categories enrichment heatmap performed with g:Profiler (https://biit.cs.ut.ee/gprofiler/gost). Principal Component Analysis (PCA) and k-means clustering were also performed.

Transcriptome and methylome correlation

Differentially adenine methylated regions of 25 ºC control and 4 ºC controls and pyrimidinedione 4 ºC treated cultures differences were used to correlate with transcriptome results by obtaining the Gene Onthology categories enrichment using g:Profiler (https://biit.cs.ut.ee/gprofiler/gost).

Code availability

All codes and algorithms used in this chapter are available in https://github.com/Valledor.

Results

Folic acid reverts pyrimidinedione effect on growth at 24 h

To further understand the DNA methylation role during cold stress, we tested for the first time in eukaryotes the drug pyrimidinedione. Chlamydomonas was grown under cold temperature (4 ºC) and 4 treatments: control, Pyr, Fol, and the combination of Pyr+Fol (*Figure 2A; Supplementary table 1*). In the first 5 h, growth showed no differences between treatments. After 24 h, the control showed a higher growth enhancement, followed by Fol. Moreover, Pyr+Fol cultures showed no differences regarding Fol and Pyr, and Pyr cultures had the lowest cell growth values. From 24 to 72 h, control and Fol had a better growth than Pyr and Fol+Pyr.

Pyrimidinedione impairs cold acclimation after 6 h of treatment

To determine when pyrimidinedione effect in growth was irreversible, Chlamydomonas was grown under cold and Pyr during 1, 3, 6, and 12 h. After this time, we removed Pyr by washing and followed cultures growth in parallel at 25 and 4 ºC (*Figure 2B, 2C; Supplementary table 2*). Obtained results showed no recovery under cold after 6 and 12 h of treatment of Pyr, inducing the cell death. All cultures recovered when grown at optimal temperature (25 ºC).

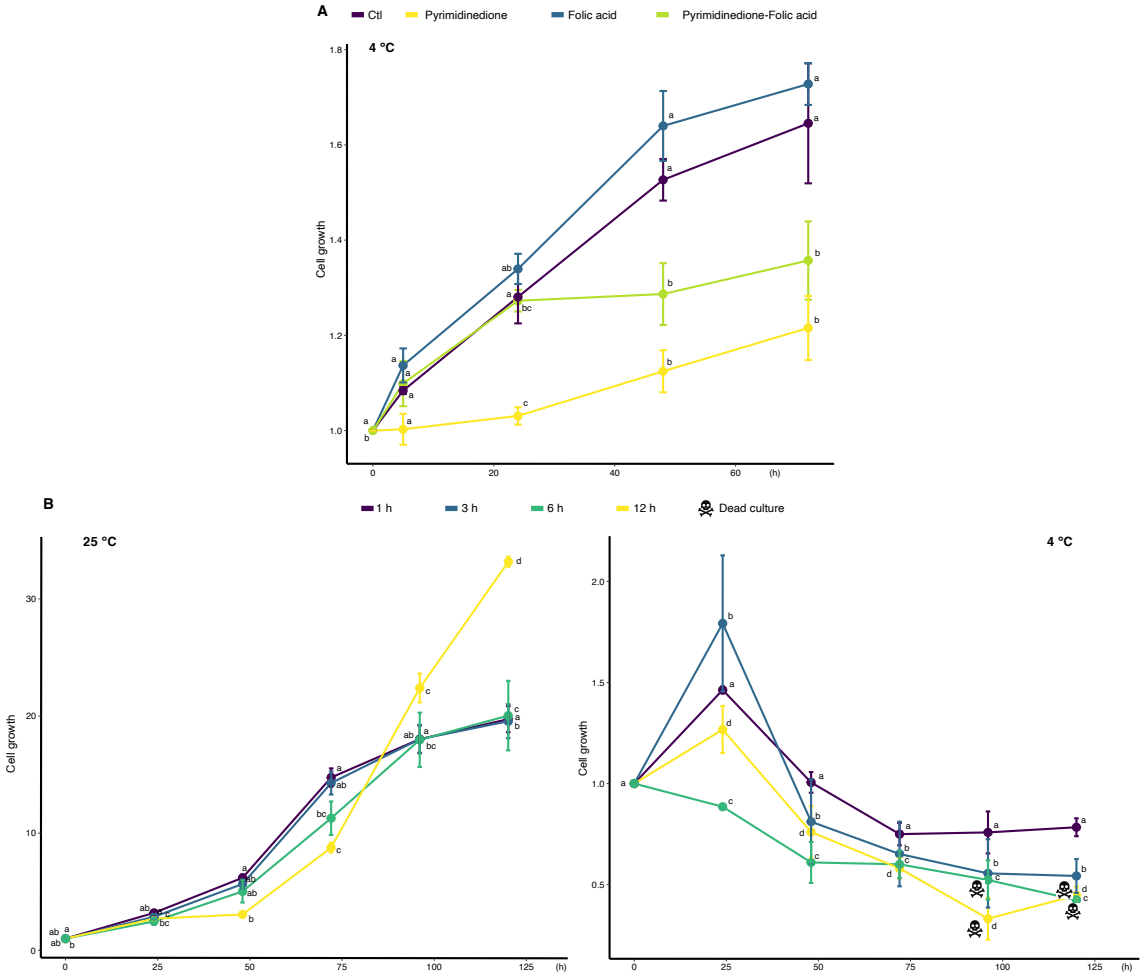


Figure 2. Effects of pyrimidinedione in Chlamydomonas. Letters mean significance ($p<0.05$). A) Growth curve showing Chlamydomonas treatment of control, folic acid, pyrimidinedione and pyrimidinedione combined with folic acid during 72 h under 4 ºC. B) Growth of Chlamydomonas under 25 ºC and 4 ºC after eliminating pyrimidinedione of the culture media after 1, 3, 6 and 12 h of treatment. All graphics are normalized to its initial concentration.

Pyrimidinedione induces cell death after 72 h of treatment

The effect of Pyr over Chlamydomonas exposed to cold temperatures was followed during 120 h. After 5 h of cold, Pyr cultures growth and Fv/Fm decreased (*Figure 3A, 3B; Supplementary table 3*). Between 24 h and 120 h of cold, Pyr cultures growth continue decreasing and Fv/Fm values were completely blocked. Pyr cultures died 72 h into the cold treatment. Moreover cultures were left to recover for 72 h at 25 ºC. Control cultures completely recovered, but not Pyr cultures, as they had died previously during the cold treatment (*Figure 3C*).

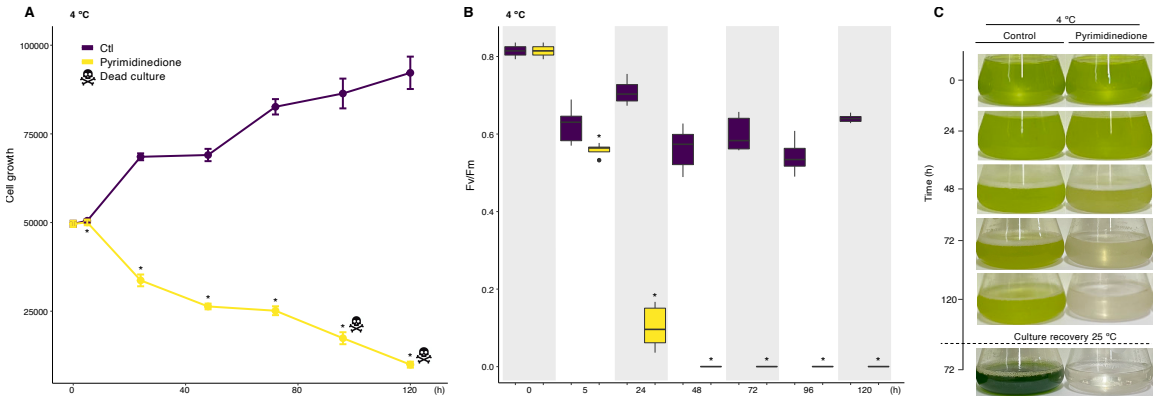


Figure 3. A) Chlamydomonas control and Pyr treatments growth rate (A) and Fv/Fm (B) under 4 ºC and Pyr. C) Aspect of the growth during the time course experiment of 120 h and the recovery of them at 25 ºC during 72 h. Asterisks indicate significant differences compared to controls ($p<0.05$).

Transcriptomic analysis showed up cold acclimation epigenetic components

Transcriptomic approach was performed to explain the changes in gene expression under cold stress in control and pyrimidinedione treated samples. A total of 17,615 genes were confidentially mapped to Chlamydomonas (v6.1) genome and quantified considering search and abundance threshold (*Supplementary table 4*). From these, 16,882 (95.84 %) genes showed significant differences between, at least, two treatments (Kruskal-Wallis, q-value <0.05).

To obtain a comprehensive view of the transcript group categories induced by cold, we classified according to MapMan onthology employing Mercator tool and annotating 4,022 transcripts to functional bins (*Supplementary table 5, Figure 4A*). The exposition to cold short-term period triggered changes in secondary metabolism, cellular respiration, polyamines and cell division comparing to optimal temperature samples. Long-term cold exposition induced changes in cytoskeleton organization, DNA damage response, plant reproduction, cellular respiration, nucleotide metabolism and RNA processing. Regarding to Pyr treated cultures, the rearrangement of transcriptome was profound at the two times sampled (5 and 24 h of treatment). All categories showed differences with their cold treated controls, being the most RNA biosynthesis and processing, external stimuli response, redox homeostasis, nucleotide metabolism, cell respiration and plant reproduction.

Together, the obtained transcripts were classified with Gene Onthology (GO) and the derived enrichment analysis performed with g:Profiler revealed that the obtained transcriptome was enriched in nine categories (*Figure 4B, Supplementary table 6*). 5 h of cold stress treatment

induced the enhancement of ATP binding , binding and RNA binding, and response to freezing while deacreasing protein tyrosine kinase and membrane categories. During time, these categories balanced to control conditions arriving almost at control temperature levels at t 120 h when cultures pretended to acclimate, except RNA binding which remained increased during cold control conditions. This acclimation response derived into a deacreasing of the protein phosphorylation and protein kinase, together with membrane category. Transcripts regarding to pyrimidinedione 5 h of treatment, enhanced their abundance in the categories of ATP binding, response to freezing and membrane, while deacreasing or not arriving to the cold controls levels in the rest of them, as binding and RNA binding. However, pyrimidinedione 24 h treated cultures didn't arrive to the cold treated controls levels in any of the enriched categories, suffering a decrease in all the classified enriched transcripts.

We employed the Principal Component analysis (PC) for observing the transcripts more related to the observed variations (*Figure 4C, Supplementary table 7*). This method adequately distinguishes between the different treatments. Principal component 1 (PC1, 42.31 % of variance) separated the cultures grade of acclimation, being in the same line the control temperature samples and the acclimated cultures at 120 h, followed by 24 h and then 5 h. In the opposite extreme to the control temperature condition samples were located the pyrimidinedione 5 and 24 h treated. Principal component 2 (PC2, 14.78 % of variance) separated the optimal temperature controls to all cold treatments. In the top loadings can be found the yet known necessary elements for Chlamydomonas cold acclimation, as different protein kinases as two *sucrose non-fermenting related kinase* (*SnRK*) members (Cre12.g528000, Cre03.g209505), different *gamma carbonic anhydrases* (Cre06.g293850, Cre09.g415850), the *Phosphatase PP2A* (Cre01.g055420), the *MAPK3* (Cre04.g213904) and different *SNF2-related DNA/RNA helicases* (Cre12.g508150, Cre14.g614400, Cre03.g183350). Moreover, the top-loadings of both components revealed new key elements for cold acclimation, highlighting the importance of the epigenetic machinery at DNA, RNA and histone levels. Different methyltransferases of these three levels are present as the homologue to mammalian *DNA methyltransferase 1-associated protein 1* (Cre03.g144907), the *RNA cap guanine-N2 methyltransferase* (Cre06.g290800) and different *Histone-lysine N-methyltransferases* (Cre16.g664000, Cre02.g089200). Moreover, the key players in gene-silencing pathways guided by small RNAs, *Argonaute 1* and *2* (Cre02.g141050, Cre04.g214250) appeared, revealing the importance of the epigenetic silencing. Moreover, some *histone acetyltransferases* had a role in cold acclimation (i.e.: Cre01.g048800, Cre13.g587150). All of these transcripts deacreased significantly their abundance, or even are absent in the

pyrimidinedione treated cultures, highlighting the necessity of epigenetic machinery for the Chlamydomonas cold acclimation.

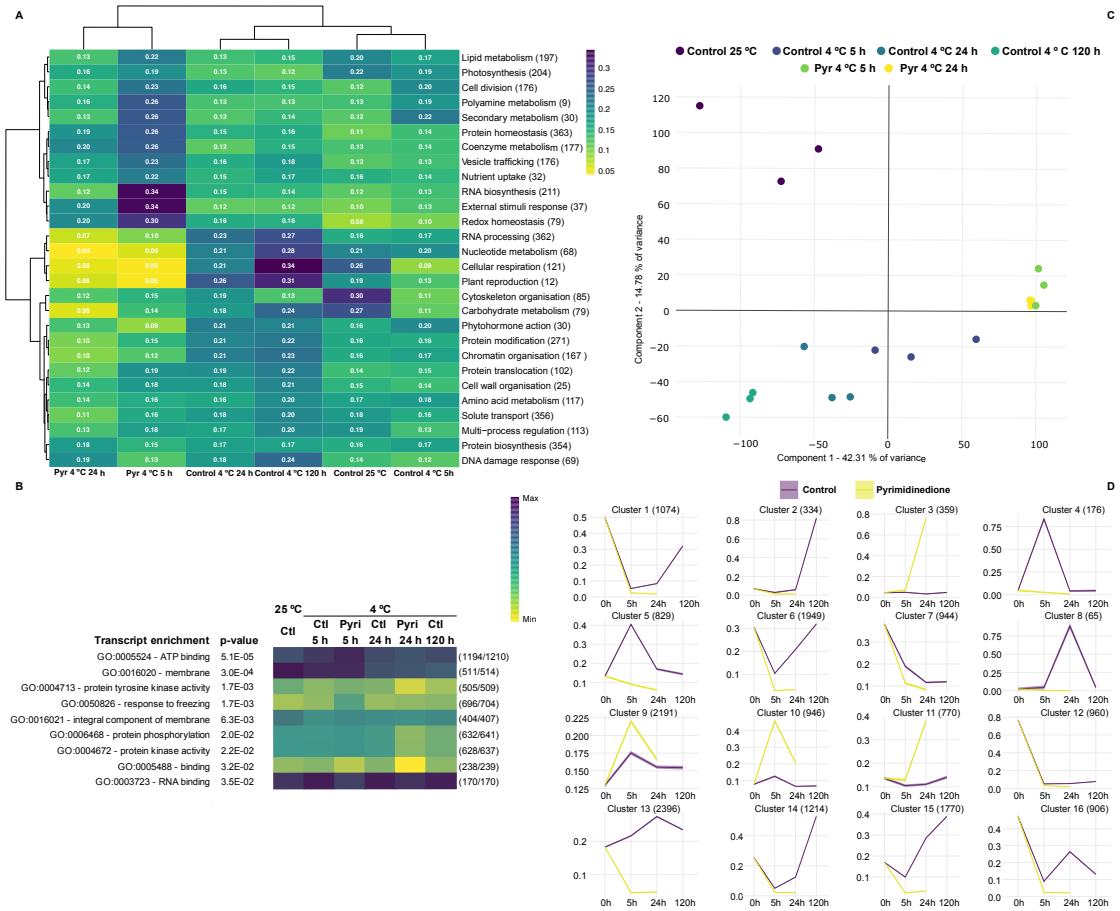


Figure 4. A) Transcripts separated into MapMan categories heatmap. B) Transcripts enrichment aggrupation into Gene Onthology categories. C) Principal component analysis showing components 1 and 2 in horizontal and vertical axes. D) K-means clustering analysis.

Differences in transcript patters were visible in k-means analysis (*Supplementary table 4, Figure 4D*). Control under cold enhanced their abundance in the most of the clades comparing to optimal temperature growth. In addition, 12 of the 16 clusters, transcripts regarding to cold controls level were higher at almost one of the sampled times comparing with pyrimidinedione treated ones. Only in clusters 3, 9, 10 and 11 pyrimidinedione cultures showed enhanced abundance at some sampled time comparing to cold controls.

Effects of pyrimidinedione exposure in 6mA methylation

Pyrimidinedione affects global levels of 6mA during cold stress

Chlamydomonas exposure to pyrimidinedione and cold had an impact in global 6mA content. From DNA sequenced with ONT, we quantified the 6mA content in all samples during time, with a minimum coverage of 8x and a methylation threshold of a 90 % for an A to qualify as methylated (*Figure 5A, Supplementary table 8*). After 5 h of cold exposure, pyrimidinedione treated cells exhibit significantly less 6mA than controls. Conversely, at 24 h, 6mA content did not change. At 120 h, cultures were totally dead, so no data was available to compare with control.

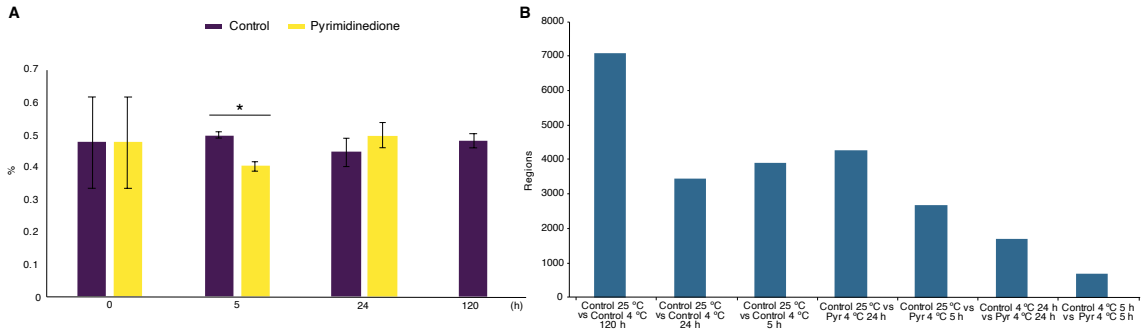


Figure 5. A) Global adenine methylation levels. B) Number of differentially adenine methylation genome regions between samples (q-value<0.05, minimum coverage 8x).

6mA methylation variates between the different genome and gene regions

Adenine methylation levels in genome regions differed between treatments and with time (*Figure 5B, Supplementary table 9*). The greatest number of regions differentially methylated in adenine (q<0.05, minimum coverage 8x) were found between 25 ºC and 4 ºC controls and increased with the time of exposure to cold treatment, being the highest number of differentially methylated regions found after 120 h of cold temperature. Regarding to the pyrimidinedione treated cells, the highest number of differential regions were found compared with 25 ºC controls, while a lower number of them was found when comparing them with their corresponding 4 ºC controls.

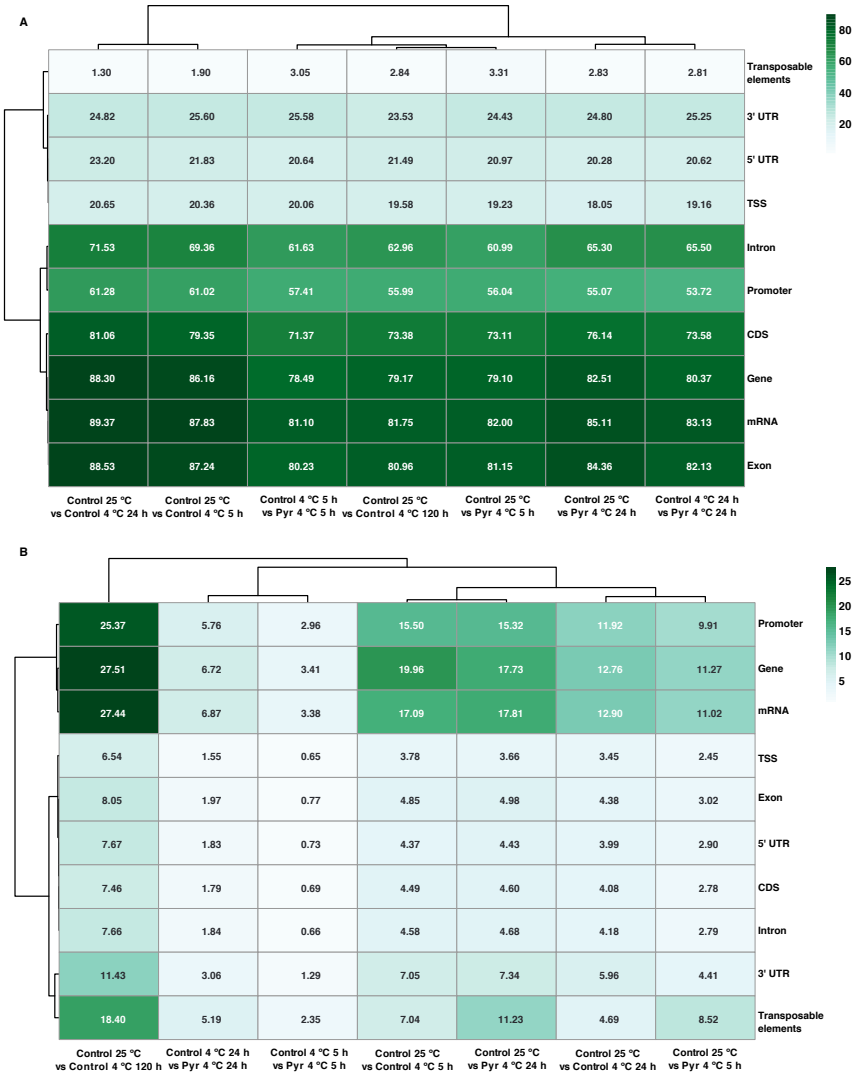


Figure 6. A) Heatmap representing the percentage of total differentially adenine methylated regions that are distributed in the total genome. B) Heatmap representing the percentage of each region differentially methylated explaining the methylations.

The annotation of the differentially methylated regions in each comparison to genomic features and their representation in a heatmap showed a quite uniform distribution between comparisons, each feature representing a similar % of the total differential regions throughout the dataset (*Figure 6A, Supplementary table 10*). A complementary analysis consisted in determining the percentage the differential regions in each comparison represented over the total number of each feature in the genome (*Figure 6B, Supplementary table 10*). Distribution of regions that are methylated in all the genomes representing in a heatmap (*Figure 6A, Supplementary table 10*) showing that between samples the percentage of methylation between samples is maintained. However, pyrimidinedione caused differential methylation between gene regions between

samples. Heatmap representing the differentially methylation between samples (*Figure 6B, Supplementary table 10*) showed the TSS has the greatest number of adenines methylated differences between control at 25 ºC and control 120 h 4 ºC. Moreover, samples of 5 h after pyrimidinedione treatment showed less changes than controls of 5 h 4 ºC when comparing the stress, which would match the global methylation (*Figure 5B, Supplementary table 8*). Not only the TSS presents this difference, but also all gene regions showed. Therefore, not only the temperature treatment impacts on adenine methylation content in the different gene regions but also the pyrimidinedione treatment directly or indirectly has a negative effect on methylation content in all regions in genes.

Differences in adenine methylation are also visible in chromosomes during time and treatments. After 5 h of treatments, thirteen chromosomes are differentially methylated, but in 6mA percentage is higher when comparing controls of 4 ºC to controls of 25 ºC than pyrimidinedione treated versus controls of 4 ºC, reinforcing only the partial inhibitory effect of pyrimidione at applied dosage. In contrast, after 24 h of low temperature, the pyrimidinedione treated cells enhanced their 6mA content, probably due to the neutralization of this drug. In addition, hypomethylation is also taking place under cold. Genes related to the primary metabolism repressed when no methylation is present while methylated second metabolism genes were transcriptionally activated to enhance the survival.

Several examples of genes methylated not only in the surrounding of TSS (*Figure 6B*) but also in the gene body were found in response to low temperatures. The *RNA adenine methyltransferase* in the chromosome 6, related to have a differential adenine methylation (*Figure 7A, Supplementary table 11, Figure 8*), was not adenine methylated during 25 ºC, but several of these genes adenines methylated in response to cold 24 h (*Figure 7A, Supplementary table 11, Figure 8*). In Arabidopsis and bacteria, RNA adenine methyltransferase are related to confer a better resistance to cold stress [36]. When applying pyrimidinedione, 6mA is absent in both TSS surrounds and all gene body. This situation is common to several genes responsible to cold acclimation. For instance, a thermitase of the chromosome 17 (Cre17.g708400), shared the same adenine methylation pattern as the *RNA adenine methyltransferase*. Under cold, this gene methylated in all gene while the effect of pyrimidinedione is represented as no methylation was found in this treatment cells. Thermitases are a family of subtilases (serine proteases). Subtilases are related to confer acclimation to low temperatures in mesophilic organisms [37].

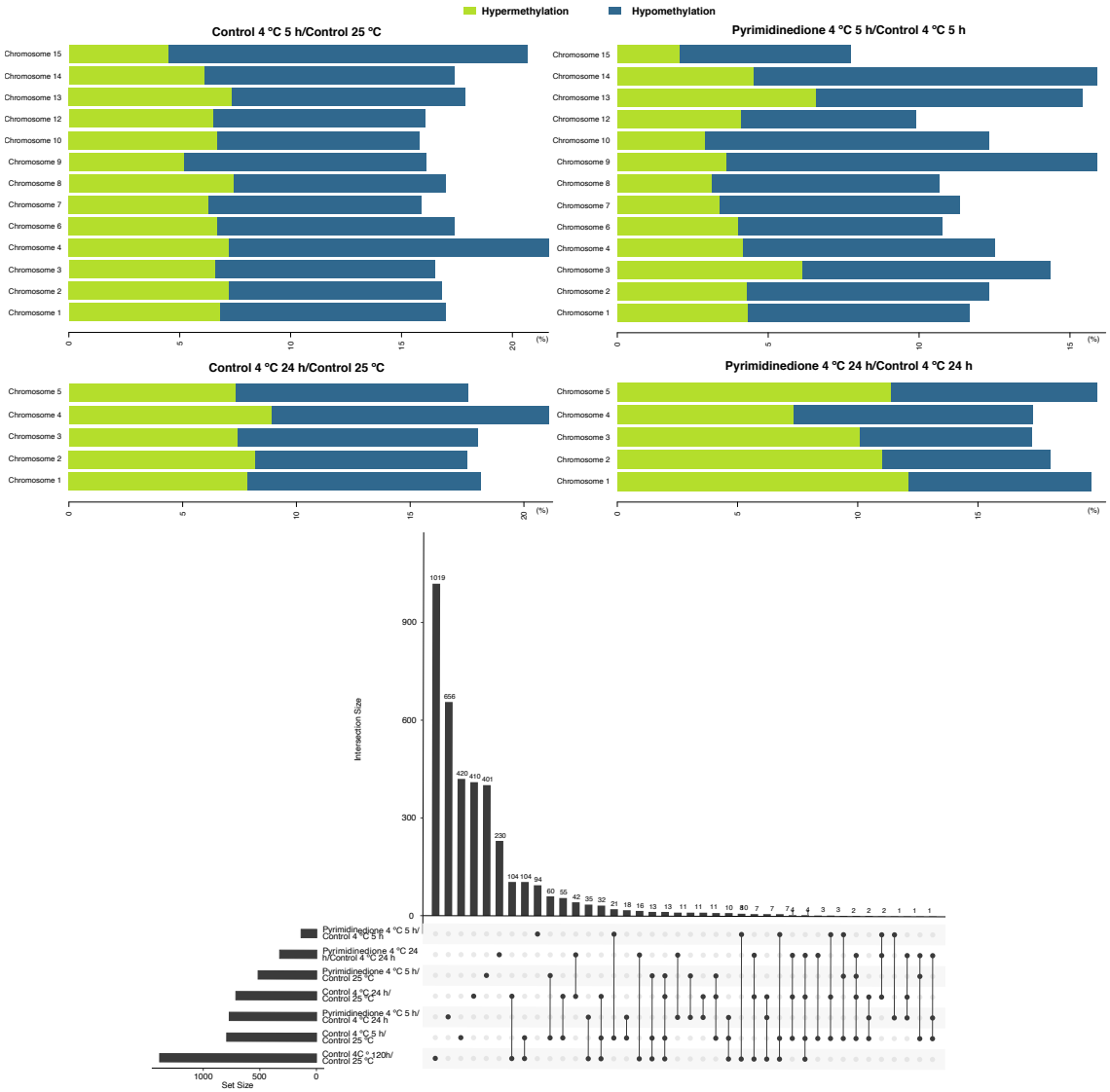


Figure 7. A) Percentage of differential adenine hyper and hypomethylation (greater than 0, q<0.05) per chromosomes and treatments. B) Upset plot regarding to the differential TSS adenine methylation conserved under different treatments.

Regarding to the differential adenine methylation in TSS (*Figure 7B, Supplementary table 12*), an accumulation of this mark in genes has been reported to have a key role in cold stress response. In the first hours of low temperature (5 h), the *DEAD/DEAH box helicase* (Cre06.g289950, *Supplementary table 13*) is methylated under cold, but not under pyrimidinedione 5 h of treatment. This protein accumulated after 48 h of cold stress in Chlamydomonas [1], and is related to be coexpressed with several stress responsive enzymes in this species. The *flagellar protein 6*, related to *protein phosphatase 2 C* for carbon concentration mechanisms occurring during cold stress [1] (Cre01.g030200, *Supplementary table 13*) is also methylated in adenine in TSS during

the first 24 h of cold stress. After 24 h of cold treatment, the *putative triacylglycerol lipase* (Cre03.g195500, *Supplementary table* 13) methylates. These enzymes families are associated to thylakoid membrane remodeling under stress in Chlamydomonas [38]. At t 24 h, genes of related to have a role during cold stress response are methylated in the TSS region, as the *Coproporphyrinogen III oxidase* (Cre02.g085450, *Supplementary table 13*), involved in the biosynthesis pathway of Mg-protoporphyrinogen IX, which activates protective signals for cold stress resistance to Arabidopsis [45]. This gene continues hypermethylated at 120 h of cold stress. Together, also in sampled times, the *non-canonical TET-dioxygenase*, putative 5-methylcytosine-modifying enzyme (Cre02.g081150, *Supplementary table 13*) highly methylates in TSS under cold treatment. This enzyme is related to oxidate 5mC to 5hmC (among others), participating in the demethylation process required to control multiple biological processes [39]. Other stress related enzymes as the *N-acetyltransferase related to GCN5* (Cre13.g583200, *Supplementary table 13*) that catalyzes the polyamine acetylation, participating in for example spermidine and putrescine synthesis in plants [42], was methylated under cold 24 and 120 h.

Adaption-responsive genes (120 h) were also methylated. Among others, two members of the *SnRK* family (*CKIN1L* Cre13.g570250, *CKIN2.14* Cre13.g568050, *Supplementary table 13*) methylated. Both genes are related to take a key role in cold stress resistance in Chlamydomonas [1,5]. These examples are only a few of the several enzymes that are relevant in the response to cold stress and appeared as adenine methylated in the TSS region. Reversely, some genes demethylated at 120 h, following the proteome tencence under cold stress [1], as the *cytochrome C oxydases* (Cre13.g567600, Cre12.g516350, *Supplementary table 13*).

During all cold treatment was methylated the *membrane-associated hydroxyproline-rich glycoprotein 2* (Cre03.g185400, *Supplementary table 13*), which is related to have several roles in plant cell wall, including the abiotic stress response [40]. Different *3', 5'-cyclic nucleotide phosphodiesterase* (Cre12.g499250, Cre14.g617700, Cre02.g119850, Cre01.g015200, *Supplementary table 13*), methylation accumulated also at almost one of the three cold sampled times. This enzyme controls the levels of cyclic nucleotides, related to accumulate during cold treatment in plants [42]. Additionally, sugar related genes appeared to adenine methylated in TSS during cold, as the *Dual-specificity tyrosine regulated protein kinase* (Cre02.g146500, *Supplementary table 13*). Proteins from this family positively correlate with sugar accumulation in Chlamydomonas UV-C stress response [41].

Even the pyrimidinedione presence, at 24 h of cold treatment, a *histone acetyl transferase* (Cre01.g002250, *Supplementary table 13*), methylated at cold control of 5 h. Histone methylation are very important mark in plants cold stress response [46,47]. The channelrhodopsin 2 (Cre02.g085257, *Supplementary table 13*) was also methylated under 24 h of pyrimidinedione treatment together with 24 and 120 h of the control control conditions. This channel act as light-gated ion (Ca^{2+} and H^{+}) channel.

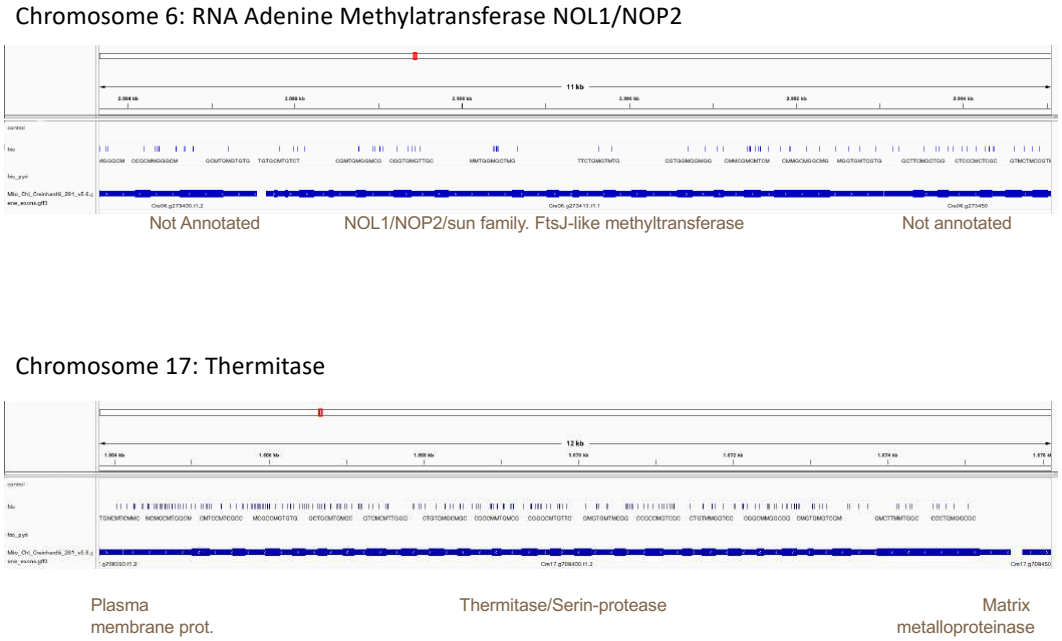


Figure 8. Adenine methylation accumulates in TSS flanks and gene body under cold for transcriptionally activation of the RNA Adenine methyltransferase NOL1/NOP2 and a thermitase.

Correlation of methylation with transcription

For identifying the regions differentially methylated that correlate with the transcriptome, we obtained the GO categories enrichment per treatments (*Supplementary tables 14, 15*). From one hand, under cold stress, all times sampled shared the enriched category of ATP binding, 5 h and 120 h shared also the cyclic nucleotide biosynthetic process, phosphorus-oxygen lyase activity and the transmembrane transport. Also, 24 h and 120 h of cold shared the enrichment of ATP hydrolysis activity. From the other hand, at 5 h appeared intracellular signal transduction, at 24 h protein tyrosine kinase activity, and at 120 h is exclusive the binding one. Some of these categories were also enriched in the transcriptomic analysis. In addition, this correlation revealed the intracellular signal transduction, cyclic nucleotide biosynthetic process, phosphorus-oxygen

lyase activity, transmembrane transport, ATP hydrolysis activity as processes exclusive to cold stress response, that were not described through the transcriptomic analysis.

Regarding to the adenine methylation linked to cold and pyrimidinedione treatments, the number of genes diferentially methylated was lower than in cold controls. Moreover, only at 5 h of Pyr the membrane category appeared to be enriched, while no results were obtained for Pyr 24 h of treatment.

Discussion

Chlamydomonas exposure to cold is mediated through complex mechanisms involving a wide range of players, from the epigenome to the post-translational modifications. In the last years, several publications focused the acclimation stress response in microalgae, exploring genomic, transcriptomic, proteomic and metabolomic responses. However, any of them covers the epigenomic mechanisms. Because of this reason, and taking into account the results from the previous chapter, we characterized and integrated the epi-genomic and the transcriptomic layers from the Chlamydomonas cultures growing not only under cold but also treated with a reducing adenine methylation epi-drug pyrimidinedione.

Despite adenine methylation is not as well studied as the role of cytosine methylation due to its low content in eukaryotes [48], we focused in it as in Chlamydomonas seem to be a transcriptional activation mark while its concentration is almost as 5mC [7]. However, in fly and worm is being studied and in the last two years some research relating cancer and 6mA has been published. Conversely, in plants, 6mA is still poorly understood, and in Chlamydomonas, not only this mark but also the 5mC. Therefore, we used Nanopore sequencing to identify not only 6mA but also 5mC at single base resolution across the Chlamydomonas genome. In addition, we employed the Illumina platform for RNA-seq which allowed from one hand to enhance the knowledge of Chlamydomonas cold stress acclimation and from the other the correlation with the methylation.

To explore 6mA dynamics under cold stress, we employed the epi-drug pyrimidinedione, which has been related to inhibit the DAM together with the CcrM in bacteria [21]. Its effect was never tested in eukaryotes before. In Chlamydomonas, no homology has found for the CcrM, so this drug is supposed to interact with DAM, reduce its function and thus, reduce the *de novo* 6mA. Its influence in growth was tested out by treating Chlamydomonas with this epi-drug. It induced a profound decrease in both growth and Fv/Fm from the first 5 h of treatment. Moreover, eliminating

this drug from the media by sequential washes didn't recover the acclimation capacity. There is clear evidence of the induced decreasing in adenine methylation during the first 5 h. From this, probably the epi-drug would be metabolized/disarmed. Results on the adenine methylation global content, together with the differentially 6mA gene regions and in chromosomes indicated that there is not only a change induced by cold, but also by pyrimidinedione reducing this mark. Thus, cells would start to enhance adenine methylation, but it would be too late to ensure the survival of the population, as cells finally died from 72 h. Therefore, the fist hours of adenine methylation are key in the acclimation response to low temperatures in Chlamydomonas.

Though this drug has this evident effect, total inhibiting the DNA Adenine methyltransferase by knockout would be an approach to considered. However, the decreasing effect on the growth of Chlamydomonas even under optimal conditions when partial inhibition with pyrimidinedione, and considering that is a transcriptional active mark, would lead to think that the total inhibition of this protein would be lethal. Probably, the next experimental option to check the effect of DAM would be to overexpress this enzyme and check if Chlamydomonas increases the stress acclimation.

Not only pyrimidinedione affect the 6mA content, but also the cold, being the most increased content in adenine content at 120 h when comparing with 25 ºC cultures. This correlates with the long-term acclimation response described in Valledor *et al.* (2014) [2014], when major differences as cell size increase are taking place. At short term (5 and 24 h), they described major changes in the central metabolism [1], which correlates with our results. Together from the 5 h of treatment, cold induces a decay on Fv/Fm and growth comparing with controls at 25 ºC, when photosynthetic apparatus starts to be damaged, and the cell ensures the survival by redirecting C to the sugar metabolism.

The Chlamydomonas response to cold acclimation already analyzed proteome related by Valledor *et al.* (2014) [1] was also supported by the transcriptome analisys performed in this chapter. The *SnRK* family, the *carbonic anhydrases*, the *PP2A*, the *SNF2* and the MAPK3 during cold acclimation in this species are key enzymes for the adequate stress response, and all of them are differentially expressed in at least one of the sampled times under cold. Besides, the transcriptomic results contribute to get new insights into the cold stress response knowledge, as revealed the importance of epigenetic processes in genome, transcriptome and histones in Chlamydomonas. These processes are well-described in other model organisms as Arabidopsis [13], but Chlamydomonas epigenetic processes and even more over stress conditions are still to

be described. Some of these epigenetic related genes, as methyltransferases of DNA, RNA and histones, are differentially methylated in adenine under cold and even under Pyr treatment.

6mA enhancement in response to stress has been described in rice, and has been related to contribute to heat and cold enhancement [1]. However cold is one of the major growth arrestment in plants, together with the fact that chilling temperatures control flowering, adenine methylation during this stress has only investigated in rice but in a very shallow investigation, being this research the first time at nucleotide level. Nevertheless, the 6mA increasing levels under cold stress shared the same pattern as in rice response to these temperatures. This fact shows up that probably the adenine methylation role is important not only for cold stress but also for other stress response as reported in bacteria. The last organisms 6mA dynamics is well-studied and gene transcriptional activation by this mark seem to be conserved across the tree life, from prokaryotes to eukaryotes. Thus, studying 6mA in response to stress would contribute to understand the evolution of organisms, and the position of Chlamydomonas in the tree linage makes the organism perfect to research this topic.

Adenine methylation is a transcriptional activation mark that accumulates principally in TSS of genes. Here, we reported several genes related to have a key role in stress response acclimation in land plants and Chlamydomonas. In the first 5 h of low temperatures application, several changes are taking place in the chloroplast for continuing the photosynthesis. Together, cold stress implies in Chlamydomonas the remodeling of membranes. Taking these effects into account, the activation of a *triacylglycerol lipase* at 24 h through 6mA for thylakoid membrane remodeling seem to be key for continuing the electron transport chain in the chloroplast.

Valledor *et al.* (2014) reported the accumulation of MAPK during cold stress [1], very important on Ca^{2+} transduction signals. One of these genes is methylated during all cold treatment, reveling that is function is due to the triggered 6mA in the TSS. Even the Ca^{2+} accumulation under chilling temperatures in Chlamydomonas remains to be determined, some ion channels adenine methylated in TSS, as the *channelrhodopsin 2*, supporting the hypothesis of the Ca^{2+} importance during this stress acclimation. Moreover, the methylated *N-acetyltransferase related to GCN5* contributes to the polyamine biosynthesis [43], and is well-known that in Chlamydomonas, spermidine and putrescine are key secondary metabolite to enhance stress acclimation [44], but their presence under cold stress in Chlamydomonas is not clear [6].

In addition, the cold responsive genes that appeared as key to cold acclimation in Chlamydomonas as the previously mentioned *MAPK*, the *SNF2* and the *SnRK*'s showed an opposite abundance in transcriptome and 6mA methylome when comparing the pyrimidinedione treatment under cold and the corresponding cold controls. This response is also showed in the GO enrichment analysis, when even in 24 h of Pyr treatment no results were found, unveiling one more time the necessity of 6mA for cold response activation.

Even though pyrimidinedione decreases the number of adenines methylated under cold stress, genes responsive to stress are still methylated, as the drug effect is not total. Hence, genes as histones methyltransferases, among other methyltransferases methylated at 24 h of treatment, probably a failed attempt to maintain cell homeostasis.

Not only the cold stress acclimation response is triggered by the TSS adenine methylation. Two key genes in this response were methylated in all gene body at 24 h of chill temperature, while no methylation was found during optimal and pyrimidinedione treatments. Sugars and concretely the accumulation of starch is one of the most characteristic effects of cold stress in Chlamydomonas. The adenine methylation of the *β-galactosidase* and thus, its activation would have a relevant role in starch accumulation, as mutants of a *β-galactosidase* (Cre02.g117200, *Supplementary table 14*) under N deprivation induces the accumulation of this metabolite instead of lipids in this microalga [49]. Stress responsive genes activated thanks to the adenine methylation, were the RNA adenine methyltransferase and a thermitase. RNA methyltransferases have a key role in stress response in plants. Concretely, Arabidopsis increases the transcript of this gene for protecting against cold stress [36]. The thermitases are a family of proteins more characterized to confer stress acclimation in bacteria, but recently, it has been reported that has a role in abiotic stress acclimation in other chlorophyte, *Pedinomonas minor* [50].

Chlamydomonas cold stress response is thus mediated though 6mA, being key to survive the methylation in the first hours of cold of the responsive acclimation genes. Reducing this mark, even in the first 5 hours caused the death of this chlorophyte. Adenine methylation is key to ensure the survival as transcriptionally activates genes that have an important role for chilling temperatures acclimation, as other epigenetics components.

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