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# Epigenetic changes induced by chronic and acute chromium stress treatments in *Arabidopsis thaliana* identified by the MSAP-Seq

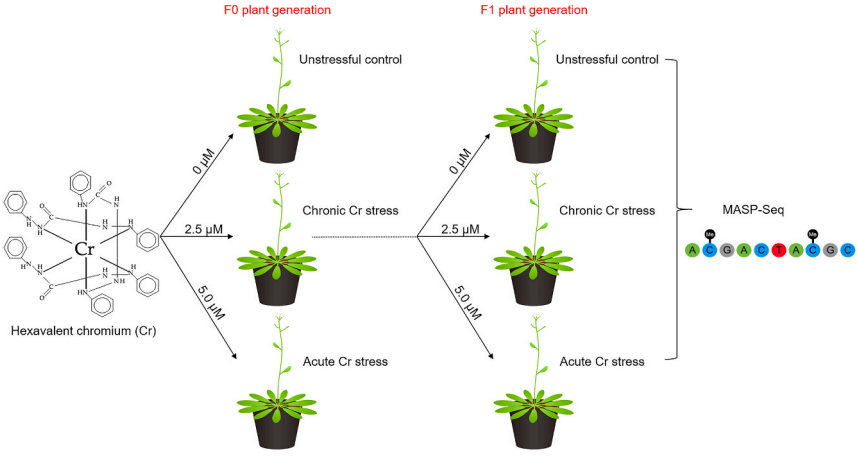
Francesco Guarino [a], Angela Cicatelli [a], Werther Guidi Nissim [c], Ilaria Colzi [b], Cristina Gonnelli [b], Marcos Fernando Basso [b], Chiara Vergata [b], Felice Contaldi [b], Federico Martinelli [b,*], Stefano Castiglione [a]

[a] Department of Chemical and Biology "A. Zambelli", University of Salerno, 84084, Fisciano, Salerno, Italy
[b] Department of Biology, University of Florence, Sesto Fiorentino, 50019, Florence, Italy
[c] Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126, Milan, Italy

## HIGHLIGHTS

- Epigenetic status modulated by chronic and acute chromium stress in *Arabidopsis thaliana*.
- Cytosine methylation status was identified by MSAP-Seq.
- Methylated or unmethylated genes in Arabidopsis plants kept under chronic and acute Cr stress conditions.
- Chronic Cr stress affected the gene methylation involved in photorespiration, transcription, and translation, and posttranslational.
- Acute Cr stress affected the gene methylation involved in cell cycle, translation, cytochrome, defense response, and photorespiration.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Chromium (Cr) is an highly toxic metal to plants and causes severe damage to their growth, development, and reproduction. Plant exposure to chronic and acute Cr stress treatments results in significant changes at short time in the gene expression profile and at long time in the genomic DNA methylation profile at a transgenerational level and, consequently, in gene expression. These epigenetic modifications and their implications imposed by the Cr stress are not yet completely known in plants. Herein, were identified the epigenetic changes induced by chronic and acute Cr stress treatments in *Arabidopsis thaliana* plants using Methylation Sensitive Amplification Polymorphism coupled with next-generation sequencing (MSAP-Seq). First-generation Arabidopsis plants (termed F0 plants) kept under hoagland solution were subjected to Cr stress treatments. For chronic Cr stress, plants were treated through hoagland solution with 2.5 μM Cr during the entire cultivation period until seed

* Corresponding author. Department of Biology, University of Florence, Via Madonna del Piano, 6, Sesto Fiorentino, Florence, 50019, Italy.
*E-mail address:* federico.martinelli@unifi.it (F. Martinelli).

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harvest. Meanwhile, for acute Cr stress, plants were treated with 5 μM Cr during the first three weeks and returned to unstressful control condition until seed harvest. Seeds from F0 plants were sown and F1 plants were re-submitted to the same Cr stress treatments. The seed germination rate was evaluated from F-2 seeds harvested of F1 plants kept under different Cr stress treatments (0, 10, 20, and 40 μM) compared to the unstressful control condition. These data showed significant changes in the germination rate of F-2 seeds originating from stressed F1 plants compared to F-2 seeds harvested from unstressful control plants. Given this data, F1 plants kept under these chronic and acute Cr stress treatments and unstressful control condition were evaluated for the trans-generational epigenetic modifications using MSAP-Seq. The MSAP-Seq data showed that several genes were modified in their methylation status as a consequence of chronic and acute Cr stress treatment to maintain plant defenses activated. In particular, RNA processing, protein translation, photorespiration, energy production, transmembrane transport, DNA transcription, plant development, and plant resilience were the major biological processes modulated by epigenetic mechanisms identified in F1 plants kept under chronic and acute Cr stress. Therefore, collective data suggested that Arabidopsis plants kept under Cr stress regulate their epigenetic status over generations based on DNA methylation to modulate defense and resilience mechanisms.

## 1. Introduction

Land plants, being immobile organisms, cannot escape biotic and abiotic stress without consequent negative impacts on their physiological status (Saud et al., 2022). Biotic and abiotic stress events can occur in an acute and chronic manner (Ali et al., 2023). Molecular mechanisms, underlying stress stimuli transduction, and modulating gene regulatory networks linked to tolerance/resistance/susceptibility to environmental stresses have been extensively investigated (Tosetti et al., 2010). Likewise, the acute responses to different abiotic stresses, in general, are well-studied (Colzi et al., 2023; Mishra et al., 2023). However, stresses are often chronic or recurring in open field conditions, and the understanding of the molecular mechanisms behind the plant response to this type of stress is not yet fully understood. Adaptation and resilience mechanisms allow plants to survive and generate progenies even during chronic stress, in part thanks to epigenetic memory mechanisms (Lämke and Bäurle, 2017).

Heavy metal pollution is one of the most dangerous environmental hazards for all living organisms on a global scale (Bertucci et al., 2018; Abou-Elwafa et al., 2019; Han et al., 2023). In particular, chromium (Cr) is one of the most abundant elements on Earth, which is used in several industrial activities and products such as leather tanning, printing, and dyeing processes (Lunk, 2015). Cr uptake and accumulation in agricultural products expose humans to several health risks (Zayed and Terry, 2003; Xu et al., 2023). In plants, hexavalent (VI) Cr is actively taken up by unspecific carriers of other essential ions (*i.e.*, sulfate or iron), whereas trivalent (III) Cr is taken up passively by cation exchange (Shanker et al., 2005; Sharma et al., 2020). Plants can withstand low environmental Cr concentrations but high amounts of both III and VI forms limit plant growth and development (Sharma et al., 2020; Yu et al., 2023). In fact, Cr inhibits seed germination, seedling growth, plant development, and plant reproduction as a result mainly of the drastic oxidative damage caused to cells (Panda and Choudhury, 2005; Colzi et al., 2023). Previous studies highlighted that both chronic and acute exposure to Cr generally inhibits Arabidopsis plant growth due to impaired photosynthesis, oxidative stress, and alteration of nutrient and water uptake (Patra et al., 2019; Christou et al., 2020; Colzi et al., 2023; Abdullah et al., 2024). Arabidopsis plants kept for 24 h under 200 μM Cr stress treatment showed down-regulation of several genes involved in redox, secondary metabolism, and energy metabolism processes, while genes related to stress defense response, photosynthesis, sulfur metabolism, indole-3-acetic acid, and carotenoids were up-regulated (Liu et al., 2020a,b). A transgenerational priming effect in Arabidopsis plants kept under 2.5 and 5 μM Cr stress treatments was demonstrated by the higher tolerance to the metal of F1 plants from parental plants kept under both chronic or acute Cr stresses compared with F1 plants from parents grown under unstressful control condition (Colzi et al., 2023).

It is widely known that DNA methylation is an epigenetic mechanism that modulates gene expression, transposon mobility, genome integrity, and chromatin structure and condensation in the nucleus (Mladenov et al., 2021). In green plants, DNA methylation typically occurs in three contexts of DNA sequence: CG, CHG, and CHH, where H is A, C, or T (Guarino et al., 2020; Guarino et al., 2022). The whole methylomes of several plant species have been already characterized, even those that have a genome with a high percentage of repetitive elements (Johannes and Schmitz, 2019). Plants have a unique memory of stress cues through mitotically inheritable epigenetic regulation that might be transmitted to the next generation (Gallusci et al., 2023). Heritable epimutations can contribute strongly to heritable phenotypic variation, such as plant tolerance to biotic stresses (Guarino et al., 2022). Several phenotypic changes due to epigenetic modifications have been documented, such as an increase in somatic homologous recombination (Molinier et al., 2006), reactivation of the ONSEN retrotransposon (Nozawa et al., 2022), and abscisic acid (ABA) insensitivity driven by chromatin-remodeling genes identified as *DDM1* and *MOM1* (Iwasaki, 2015). Methylation Sensitive Amplification Polymorphism (MSAP) is one of the most commonly used genome-wide and untargeted approaches for determining DNA methylation changes in plant genomes (Aina et al., 2004). The MSAP method is based on different sensitivities of two isoschizomer enzymes to DNA methylation. An improved version of this method is named MSAP coupled to next-generation sequencing (MSAP-Seq) (Guarino et al., 2020). The MSAP-Seq consists of replacing the conventional separation of amplicons on polyacrylamide gels with direct and high-throughput DNA sequencing (Guarino et al., 2020). Therefore, the combination of high-throughput sequencing with the MSAP protocol allows the DNA methylation analysis in hundreds of thousands of sites across the plant genome. The MSAP-Seq method may analyze up to three-quarters of all genes, even in large plant genomes, and almost all genes present in Arabidopsis (Chwialkowska et al., 2017; Guarino et al., 2019, 2020). In addition, this technique is cost-effective, and its high-multiplexing capability makes it highly affordable (Chwialkowska et al., 2017). Therefore, knowing the epigenetic changes and the plant's defense mechanisms to the Cr phytotoxicity allows us to better understand the plant defense response and to develop biotechnological tools to obtain crops with improved resilience to this stress (Basso et al., 2019, 2020).

In this study, the transgenerational epigenetic changes induced by chronic and acute Cr stress treatments in Arabidopsis plants were exploited using the MSAP-Seq approach. First generation plants (F0 plants) were submitted to chronic and acute Cr treatments. Then, F1 plants originating from F0 plants were re-submitted to the same chronic and acute Cr treatments, and genome DNA was purified and analyzed by MSAP-Seq to determine the epigenetic changes caused by Cr stress after a successive treatment over two plant generations. Therefore, the epigenetic modifications imposed by Cr stress treatment in Arabidopsis plants were successfully identified and their effect on plant resilience was addressed and discussed.

## 2. Materials and methods

### 2.1. Plant material, seed germination, and experimental design

Arabidopsis ecotype Col-0 (Columbia) seeds were sown in Peat Jiffy discs and, subsequently, seedlings were transplanted and cultivated in commercial substrate for 15 days under growth room conditions. Then, adult plants were transferred to a hydroponic culture system containing 500 mL hoagland solution (Hoagland and Arnon, 1938) per container/per plant and kept for eight days for previous acclimatization. After, first-generation Arabidopsis plants (termed F0 plants) were submitted to chronic and acute Cr treatments. For chronic stress treatment, F0 plants were treated through hoagland solution with 2.5 μM Cr during the entire cultivation period until seed harvest. For acute stress treatment, F0 plants were treated with 5 μM Cr during the first three weeks and returned to unstressful conditions (Cr-free hoagland solution) until seed harvest. These two Cr concentrations used here were chosen based on the previous study in which the maximal effective concentration of Cr ($EC_{50}$) determined in Arabidopsis plants was 1.32 ± 0.18 μM (Colzi et al., 2023). The hoagland solution was changed once every eight days. F-1 seeds originating from F0 plants were sown and re-submitted to the same Cr stress treatments undertaken for F0 plants. The seed germination rate was carried out using 90 × 15 mm Petri dishes on filter paper soaked with sterile water with F-2 seeds harvested from F1 plants ($n = 3$ plates × 20 seeds per treatment) under different Cr stress treatments (0, 10, 20, and 40 μM hexavalent Cr, $K_2Cr_2O_7$) compared to the unstressful control condition. Petri plates were maintained under a 16/8 (light/-dark) hours photoperiod at 22 (dark) to 25 °C (daylight), 60% humidity, and 120 μmol $m^{-2}$ $s^{-1}$ light. The seed germination rate over time was evaluated on the third and fourth days after seed sowing. The daily germination rate was calculated based on the formula proposed by Ellis and Roberts (1980). Data were evaluated for statistical significance by two-way ANOVA followed by the HSD-Tukey test for *post-hoc* comparisons, using GraphPad Prism 7 (GraphPad 173 Software, San Diego, CA).

### 2.2. Plant sampling for epigenetic analyses, DNA extraction, and DNA fragmentation

F1 plants submitted to the chronic and acute Cr treatments and unstressful control condition were evaluated for transgenerational epigenetic modifications by the MSAP-Seq approach. Arabidopsis leaves were harvested at two time points: T1, one week after starting the Cr stress treatments, and T2: three weeks after starting the Cr stress treatments. Total genomic DNA was extracted from 100 mg of fresh material using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Purified DNA was then processed using the MSAP-Seq standard protocol, slightly modified from that employed by Guarino et al. (2020). Briefly, the isoschizomer restriction enzymes *Hpa*II and *Msp*I (with different levels of sensitivity to DNA methylation) and *Mse*I (a methylation-insensitive restriction enzyme that is specifically used as a "frequent cutter") were used to fragment the plant genome. In particular, *Hpa*II or *Msp*I enzymes can digest DNA when one of these conditions are met: (i) double-strand methylation of the inner cytosine (*Msp*I: digestion, *Hpa*II: no digestion); (ii) hemi-methylation of the outer cytosine (*Msp*I: digestion, *Hpa*II: no digestion); (iii) hemi-methylation of the inner and outer cytosine (*Msp*I: no digestion, *Hpa*II: digestion); and (iv) no methylation of the target sequence (*Msp*I: digestion, *Hpa*II: digestion). The MSAP-Seq standard protocol was modified replacing the rare cutter *Eco*RI restriction enzyme, commonly used in the MSAP protocol, by the *Mse*I enzyme, to reduce the size of the DNA fragments and then proceed with the DNA sequencing (Guarino et al., 2020). The digested DNA fragments were purified and ligated to primer adapters to perform a single PCR amplification (pre-selective) step, that was not followed by a second PCR (selective step) amplification, which is normally performed to reduce the number of MSAP amplicons.

### 2.3. Library preparation and next-generation sequencing

The purified amplicons obtained at the end of the selective PCR were complexed in libraries and sequenced using the Illumina MISEQ (Illumina, San Diego, CA, USA) by Genomix4Life facility (Baronissi, SA, Italy). The sequence quality was verified with the UNIX-based package FastQC-0.10.1 (Andrews, 2010). Raw reads were processed by the CLC suite (CLC/Qiagen, Aarhus, Denmark) to filter, to remove the unused adapters, and to trim the sequences. Then, the MSAP-Seq protocol was performed as described by Guarino et al. (2020). Briefly, a *de novo* assembly and pooling of all the reads relative to each biological sample/library, for both *Mse*I-*Msp*I and *Mse*I-*Hpa*II fragments, was performed generating a list of contigs. Then, the obtained DNA fragments from each *Mse*I-*Msp*I and *Mse*I-*Hpa*II double digestion were mapped using these contig lists. The DNA sequences with less than two read counts and shorter than 200 bp were discarded. Contigs of each biological sample/library and for both *Mse*I-*Msp*I and *Mse*I-*Hpa*II fragments were analyzed for similarity by BLASTN using the Arabidopsis TAIR10 genome as a reference to identify genes affected or unaffected by DNA methylation.

The two MSAP profiles were separately considered to highlight the Cr stress effects on the DNA methylation status, revealed by *Mse*I-*Msp*I or *Mse*I-*Hpa*II, following a previously described protocol (Guarino et al., 2015, 2019). Therefore, in this first step of the analysis were considered separately the gene datasets identified by the *Mse*I-*Msp*I or *Mse*I-*Hpa*II digestions. Subsequently, to elaborate the data according to the MSAP protocol, consisting of a comparison of both MSAP profiles (*Mse*I-*Msp*I and *Mse*I-*Hpa*II) of the same biological sample, Venn diagrams for the gene datasets of each experimental group were carried out. Based on the presence or absence of the genes in one category or both categories (*Mse*I-*Msp*I and/or *Mse*I-*Hpa*II), the DNA methylation status of the identified genes was estimated as follows: (i) double-strand methylation of the inner cytosine or hemi-methylation of inner cytosine (*Msp*I: 1, *Hpa*II: 0 = M1-H0); (ii) hemi-methylation CHG-sites of the inner and outer cytosine (*Msp*I: 0, *Hpa*II: 1 = M0-H1); (iii) uninformative state caused either by different types of methylation or due to restriction site polymorphism (*Msp*I: 0, *Hpa*II: 0 = M0-H0); and (iv) no methylation of the target sequence (*Msp*I: 1, *Hpa*II: 1 = M1-H1) (Fig. S1) (Guarino et al., 2019, 2020).

### 2.4. Functional analysis of differentially methylated genes

Venn diagrams were elaborated by counting the number of genes involved based on common or unique methylations between unstressful control condition and Cr stress treatments at the T1 and T2 time points. Enrichment analysis from Gene Ontology (GO) categories including their pathway for biological processes was performed by ShinyGO software using the Benjamini-Hochberg method (Ge et al., 2020). Processes with a false discovery rate and $p$-value $<0.05$ were considered as significant, and only categories with a minimum of 20 overlapping genes were selected. The protein-protein interaction and gene co-expression networks among differentially methylated genes identified in Arabidopsis plants kept under unstressful control condition or identified in Arabidopsis plants kept under 2.5 μM and 5.0 μM Cr stress treatments at T1 and T2 time points were provided by the STRING (Szklarczyk et al., 2019) using TAIR10 dataset as a reference.

## 3. Results and discussions

### 3.1. Cr stress, plant generation advancement, and seed germination tests

Growth of F0 to F1 plants and the harvesting of F-1 and F-2 seeds were successfully conducted according to technical planning (Fig. 1). F-2 seed germination rate carried out under Cr stressful treatments and unstressful control condition was successfully determined 3 and 4 days after sowing. A significant decrease in seed germination rate under

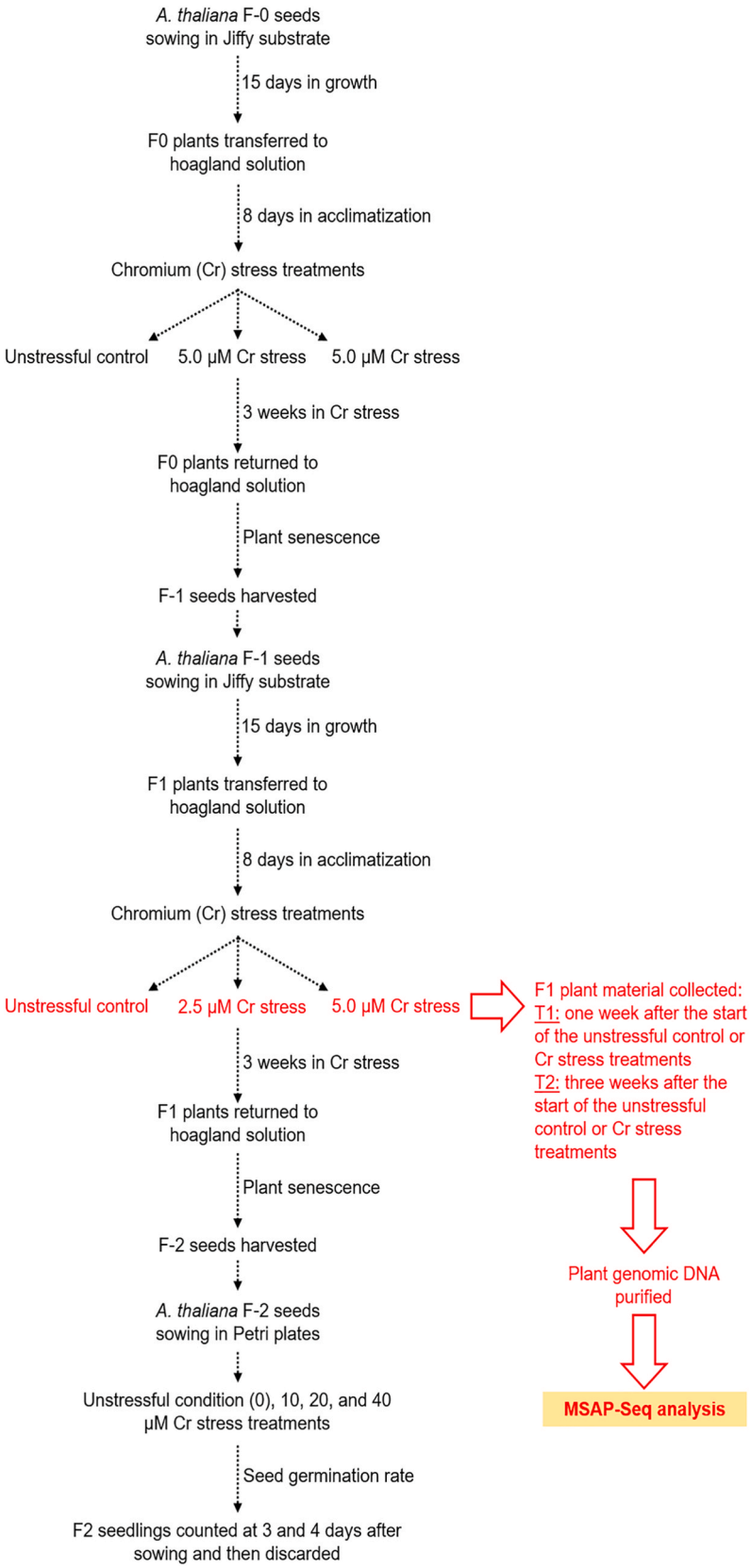


**Fig. 1.** Experimental design of chromium (Cr) stress treatments in Arabidopsis plants. F0 and F1 plants were subject to unstressful control (0), 2.5 µM Cr, and 5 µM Cr stress treatments. F1 plants were sampled and evaluated by the MSAP-Seq approach. F-2 seeds originating from F1 plants were screened to evaluate the seed germination rate under unstressful control condition and Cr stress treatments.

higher Cr concentrations was observed for F-2 seeds originating from F1 plants kept under unstressful control condition (Fig. 2a and b). In contrast, F-2 seeds originating F1 plants kept under chronic and acute Cr stress treatments showed similar seed germination rates both in the absence and presence of Cr, even at higher concentrations such as 20 and 40 μM Cr. Therefore, these data revealed that Arabidopsis plants kept under chronic and acute Cr stress treatments present better physiological performance and resilience over generations to overcome these adverse phytotoxicity conditions. Supported by these data, the epigenetic changes associated with this improved phenotype were further explored.

The excessive Cr in the soil is allocated to plant organs and causes adverse effects on plant growth, development, nutrient absorption, photosynthesis, and reproduction (Saud et al., 2022). It is already been reported that the exposure of plants to Cr stress treatments can result in changes in a short time in the gene expression profile and at medium to a long time in the genomic DNA methylation status at a transgenerational level and, consequently, in the gene expression profile (Cong et al., 2019; Tang et al., 2022; Colzi et al., 2023; Sun et al., 2023). Our previous data revealed that exposing Arabidopsis plants to chronic and acute Cr stress treatments during two generations (F0 and F1) conditioned the subsequent F2 plants to be more tolerant to this same stress. Given this, this present study aimed to investigate by using the MSAP-Seq approach the epigenetic changes in these F1 plants kept under chronic and acute Cr stress treatments compared with unstressful control condition.

### 3.2. *Epigenetic analyses of Arabidopsis plants kept under Cr stress treatments by MSAP-Seq*

By using the methylome data, we aimed to reveal key genes and major biological processes involved in plant defense response and resilience against Cr stress. In particular, the MSAP-Seq can effectively estimate the methylation status of the cytosine within the CpG islands of plant genomes (Guarino et al., 2020). In Arabidopsis, most of the CpG islands are known to be associated with protein-coding genes, implying that these clusters are useful landmarks for identifying functional genes in plants with small genomes (Ashikawa, 2001). The two isoschizomer restriction enzymes, *Msp*I and *Hpa*II, which recognize the C^CGG sites but are differentially blocked by CpG methylation, can digest the genomic DNA based on the methylation status of the inner and/or outer cytosines (hemi-methylation or double-strand methylation) and they are, therefore, commonly exploited for the estimation of DNA methylation profile. Conventionally, the estimation of DNA methylation status through MSAP-Seq is performed by comparing the two profiles obtained independently through *Mse*I-*Msp*I and *Mse*I-*Hpa*II enzymes. In the first analysis step of this study, were separately considered the datasets of genes identified after *Mse*I-*Msp*I or *Mse*I-*Hpa*II digestions. Therefore, in this first step, the comparisons (intra digestions) aimed at assaying the effects of the unstressful control (0) condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T1 and T2 time points on the DNA methylation status. Successively, the canonical comparisons between gene datasets generated by *Mse*I-*Msp*I and *Mse*I-*Hpa*II enzyme digestions, from each sample group, allowed us to identify key genes with different DNA methylation status exclusively associated with defense responses to chronic and acute Cr stress.

#### 3.2.1. *Differentially methylated genes identified by MseI-MspI enzyme digestion*

The gene datasets identified after the *Mse*I-*Msp*I enzyme digestion revealed 38, 18, and 25 genes, respectively, in Arabidopsis plants kept under unstressful control condition, 2.5 μM, and 5.0 μM Cr stress treatments at the T1 (F1 plants one week after starting the unstressful control condition or Cr stress treatments) time point (Table S1). The pairwise comparison between the unstressful control condition *versus* 2.5 μM Cr stress treatment revealed that 18 genes were shared between the two groups, indicating that the methylation profile of these genes was not affected by the Cr stress treatment. In contrast, 20 genes were specific for unstressful control condition and only one gene for the 2.5 μM Cr stress treatment (Table S2). The comparison between the unstressful control condition *versus* 5.0 μM Cr stress treatment highlighted 21 methylated genes shared between the two groups, while 17 genes were present in the unstressful control condition and only five genes in the 5.0 μM Cr stress treatment. These data indicated that Cr stress treatment strongly modified the DNA methylation profile detectable by *Mse*I-*Msp*I enzyme digestion. This pairwise comparison also showed that no genes were affected specifically by the 2.5 μM Cr stress treatment while four genes were affected specifically by the 5.0 μM Cr stress treatment. In addition, only one gene maintained the same DNA methylation status for both Cr stress treatments (Table S2).

In contrast, the gene datasets identified after the *Mse*I-*Msp*I enzyme digestion revealed 36, 40, and 50 genes, respectively, in Arabidopsis plants kept under unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T2 (F1 plants three weeks after starting the unstressful control condition or Cr stress treatments) time point (Table S1). The pairwise comparison between the unstressful control condition *versus* 2.5 μM Cr stress treatment demonstrated that 22 genes were shared, while 14 genes were present only in the unstressful control condition and 18 genes were present only in the 2.5 μM Cr stress

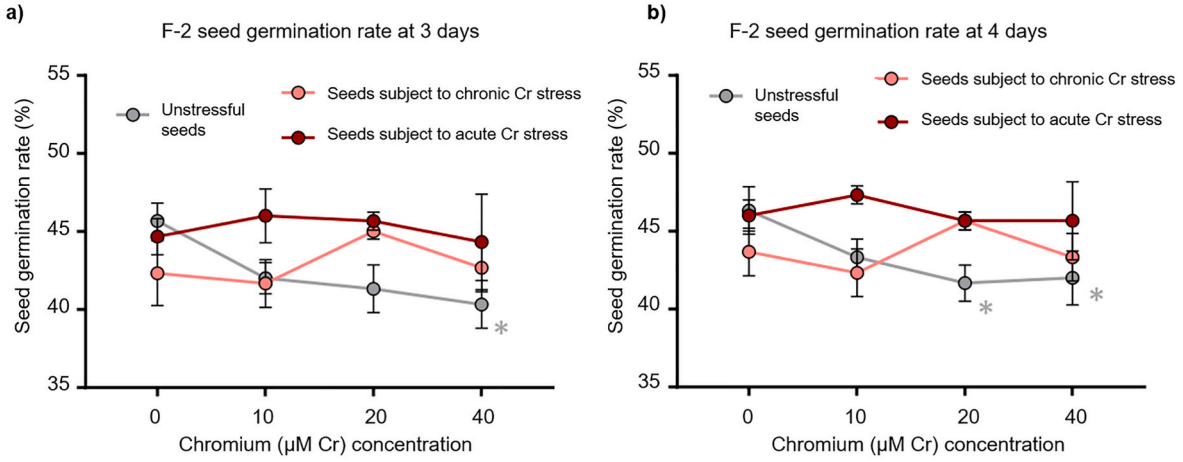


**Fig. 2.** Seed germination rate of Arabidopsis plants kept under unstressful control condition and different chromium (Cr) stress treatments. F2 seeds originating from F1 plants (F-2) were sowed in Petri plates under unstressful control (0), 10, 20, and 40 μM Cr stress treatments. The seed germination rate was determined at (**a**) 3 and (**b**) 4 days after sowing. Asterisks indicate significant statistical differences by the HSD-Tukey test.

treatment, showing that DNA methylation status related to *Mse*I-*Msp*I enzyme digestion, was significantly modified by 2.5 μM Cr stress treatment (Table S3). The pairwise comparison between the unstressful control condition *versus* 5.0 μM Cr stress treatment showed that 24 genes were not affected by the Cr stress treatment, while 12 genes were present only in the unstressful control condition and 26 genes were present only in the 5.0 μM Cr stress treatment (Table S3). This pairwise comparison also showed that 14 genes were affected specifically by the 2.5 μM Cr stress treatment while 22 genes were affected specifically by the 5.0 μM Cr stress treatment. In addition, only four genes maintained the same DNA methylation status for both Cr stress treatments (Table S3).

#### 3.2.2. Differentially methylated gene datasets generated by MseI-HpaII enzyme digestion

The gene datasets identified after *Mse*I-*Hpa*II enzyme digestion included 41, 35, and 31 genes, respectively, in Arabidopsis plants kept under unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T1 time point (Table S4). The pairwise comparison between the unstressful control condition *versus* 2.5 μM Cr stress treatment showed that 21 genes were shared, while 20 genes were present only in the unstressful control condition and 14 genes were present only in the 2.5 μM Cr stress treatment (Table S5). This data suggests that Cr stress treatment at the T1 time point caused changes in the DNA methylation status. The comparison between the unstressful control condition *versus* the 5.0 μM Cr stress treatment highlighted 20 genes shared between the two groups, while 21 genes were present in the unstressful control condition and 11 genes in the 5.0 μM Cr stress treatment (Table S5). This pairwise comparison also showed that 11 genes were affected specifically by the 2.5 μM Cr stress treatment while eight genes were affected specifically by the 5.0 μM Cr stress treatment (Table S5). In addition, three genes maintained the same DNA methylation status for both Cr stress treatments.

In contrast, the gene datasets identified after *Mse*I-*Hpa*II enzyme digestion revealed 44, 28, and 62 genes, respectively, in Arabidopsis plants kept under unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T2 time point (Table S4). The pairwise comparison between the unstressful control condition *versus* 2.5 μM Cr stress treatment demonstrated that 12 genes were shared, while 32 genes were present only in the unstressful control condition and 16 genes were present only in the 2.5 μM Cr stress treatment, showing that DNA methylation status related to *Mse*I-*Hpa*II enzyme digestion was also modified by 2.5 μM Cr stress treatment (Table S6). The comparison between the unstressful control condition *versus* the 5.0 μM Cr stress treatment highlighted that 15 genes were shared between the two groups, while 29 genes were present in the unstressful control condition and 47 genes in the 5.0 μM Cr stress treatment (Table S6). This pairwise comparison also showed that 10 genes were affected specifically by the 2.5 μM Cr stress treatment while 41 genes were affected specifically by the 5.0 μM Cr stress treatment (Table S6). In addition, six genes maintained the same DNA methylation status for both Cr stress treatments.

#### 3.2.3. Comparison intra MseI-MspI or MseI-HpaII enzyme digestion profile and methylation

By considering the comparison between unstressful control condition *versus* 2.5 μM Cr stress treatment at the T1 time point, the independent analysis showed that 67.9 and 72.4% of the identified genes by *Mse*I-*Msp*I or *Mse*I-*Hpa*II enzyme digestions, respectively, had their DNA methylation status altered by chronic Cr stress treatment. Meanwhile, at the T2 time point, the comparison between unstressful control condition *versus* 2.5 μM Cr stress treatment showed that 71.1 and 83.4% of the identified genes by *Mse*I-*Msp*I or *Mse*I-*Hpa*II enzyme digestions, respectively, had their DNA methylation status also altered by chronic Cr stress treatment. Similarly, considering the comparison between unstressful control condition *versus* 5.0 μM Cr stress treatment at the T1 time point, the analysis showed that 66.7 and 72.3% of the identified genes by *Mse*I-*Msp*I or *Mse*I-*Hpa*II digestions, respectively, had their DNA methylation status also altered by acute Cr stress treatment. Meanwhile, at the T2 time point, the comparison between unstressful control condition *versus* 5.0 μM Cr stress treatment showed that 72.1 and 85.9% of the identified genes by *Mse*I-*Msp*I or *Mse*I-*Hpa*II enzyme digestions, respectively, also had their DNA methylation status altered by acute Cr stress treatment.

#### 3.2.4. Comparison of gene methylation status at the T1 and T2 time points: effects of the exposure time

In the unstressful control condition was observed that 22 genes identified by *Mse*I-*Msp*I enzyme digestion were shared between the T1 and T2 time points, indicating the same methylation status, while 30 genes showed a modified methylation status at the two different time points (16 and 14 genes at T1 and T2, respectively; Table S7). In the same way, comparing the 2.5 μM Cr-related group was observed that 18 genes were shared, indicating an altered DNA methylation status but not related to time points, while 23 genes showed a modified methylation status at the two different time points (1 and 22 genes at T1 and T2, respectively; Table S7). Similarly, comparing the 5.0 μM Cr-related group was observed that 21 genes were shared between T1 and T2, indicating an altered methylation status but not related to time points, while 30 genes showed a modified methylation status at the two different time points (five and 25 genes at T1 and T2, respectively; Table S7).

In the unstressful control condition was observed that 19 genes identified by *Mse*I-*Hpa*II enzyme digestion were shared at T1 and T2, indicating the same methylation status, while 46 genes showed a modified methylation status at the two different time points (22 and 24 genes at T1 and T2, respectively; Table S8). In the same way, comparing the 2.5 μM Cr-related group was observed that 19 genes were shared, indicating an altered DNA methylation status but not related to time points, while 25 genes showed a modified methylation status at the two different time points (16 and nine genes at T1 and T2, respectively; Table S8). Similarly, comparing the 5.0 μM Cr-related group was observed that 18 genes were shared, indicating the same DNA methylation status, while 55 genes showed a modified methylation status at the two different time points (12 and 43 genes at T1 and T2, respectively; Table S8).

### 3.3. DNA methylation status and biological implications for Arabidopsis plants

The comparison between gene datasets identified by *Mse*I-*Msp*I and *Mse*I-*Hpa*II enzyme digestions from each sample group of the unstressful control condition at the T1 time point revealed that 68.3% of the identified genes were methylated (Table 1). The most frequent methylation event was related to the hemi-methylation of the inner and outer cytosine (36.6%), while the remaining 31.6% were associated with double-strand methylation of inner cytosine or hemi-methylation of inner/outer cytosine (Table 1). In contrast, from each sample group of the 2.5 μM Cr and 5.0 μM Cr stress treatments were observed that 57.8 and 41.6% of the identified genes were methylated, respectively (Table 1). The percentage related to the double-strand methylation of inner cytosine or hemi-methylation of inner/outer cytosine strongly decreased up to 7.8% and 13.8%, respectively. Meanwhile, hemi-methylation of the inner and outer cytosine was represented by 50 and 27.7%, respectively. The DNA methylation status most affected by 2.5 μM Cr and 5.0 μM Cr stress treatments at the T1 time point was the double-strand methylation of the inner cytosine or hemi-methylation of the inner/outer cytosine, which strongly decreased 92.1% and 86.1%, respectively (Table 1). These collective data showed that the two Cr stress treatments at the T1 time point induced a clear double-strand demethylation of inner cytosine or hemi-demethylation of inner/outer cytosine of several target genes (Table S9). Furthermore, in the case of the 5.0 μM Cr-related group, the hemi-methylation of inner and outer cytosine was also reduced by 54.5% compared with the unstressful control condition.

**Table 1**
Number of genes identified in Arabidopsis plants kept under chromium (Cr) stress treatments and unstressful control condition at the T1 (F1 plants after 1 week) and T2 (F1 plants after 3 weeks) time points characterized by the three different DNA methylation status detectable using the MSAP-Seq approach (*Mse*I-*Msp*I and *Mse*I-*Hpa*II enzyme digestions). T1 corresponds to F1 plants one week after starting the unstressful control condition or Cr stress treatments; T2 corresponds to F1 plants three weeks after starting the unstressful control condition or Cr stress treatments.

| T1 time point | Unstressful | 2.5 μM Cr | 5.0 μM Cr |
|---|---|---|---|
| No methylation | 19 | 16 | 21 |
| Double-strand methylation of inner cytosine or hemi-methylation of inner/outer cytosine | 19 | 3 | 5 |
| Hemi-methylation of the inner and outer cytosine | 22 | 19 | 10 |
| **T2 time point** | **Unstressful** | **2.5 μM Cr** | **5.0 μM Cr** |
| No methylation | 36 | 18 | 25 |
| Double-strand methylation of inner cytosine or hemi-methylation of inner/outer cytosine | 0 | 22 | 25 |
| Hemi-methylation of the inner and outer cytosine | 7 | 10 | 37 |

In the case of the unstressful control condition at the T2 time point, 83.7% of the identified genes were not methylated (Table 1). The remaining 16.3% genes were characterized by the hemi-methylation of inner and outer cytosine (Table 1). The DNA methylation status most affected by 2.5 μM Cr- and 5.0 μM Cr-related groups revealed that the percentages of methylated genes were 64% and 71.2%, respectively. In contrast, comparing the unstressful control condition with 2.5 μM Cr stress treatment, the percentage of unmethylated genes was strongly reduced from 36 to 83.7%, respectively. Meanwhile, the double-strand methylation of the inner cytosine or hemi-methylation of the inner/outer cytosine, not represented in the unstressful control condition, was associated with 44% in the case of the 2.5 μM Cr-related group (Table 1). The percentages of the genes characterized by the hemi-methylation of the inner and outer cytosine were similar in the unstressful control condition and 2.5 μM Cr stress treatment, being of 16.2 and 20%, respectively. In the 5.0 μM Cr stress treatment, was observed a higher number of methylated genes compared with the unstressful control condition and 2.5 μM Cr stress treatment. In particular, the most represented methylation status was the hemi-methylation of the inner and outer cytosine (42.5%), while the double-strand methylation of the inner cytosine or hemi-methylation of the inner/outer cytosine was associated with 28.7% of the identified genes (Table 1). These collective results showed that at the T2 time point, both Cr stress treatments induced a clear double-strand methylation of the inner cytosine or hemi-methylation of the inner/outer cytosine of several genes (Table S9).

The number of genes for each methylation status identified in the unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments were compared for the T1 and T2 time point groups. In the stressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T1 time point were identified 19, three, and five genes, respectively, with double-strand methylation of inner cytosine or hemi-methylation of inner cytosine, and 22, 19, 10 genes, respectively, with hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine). In addition, 19, 16, and 21 genes were identified as unmethylated, respectively, in the unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress

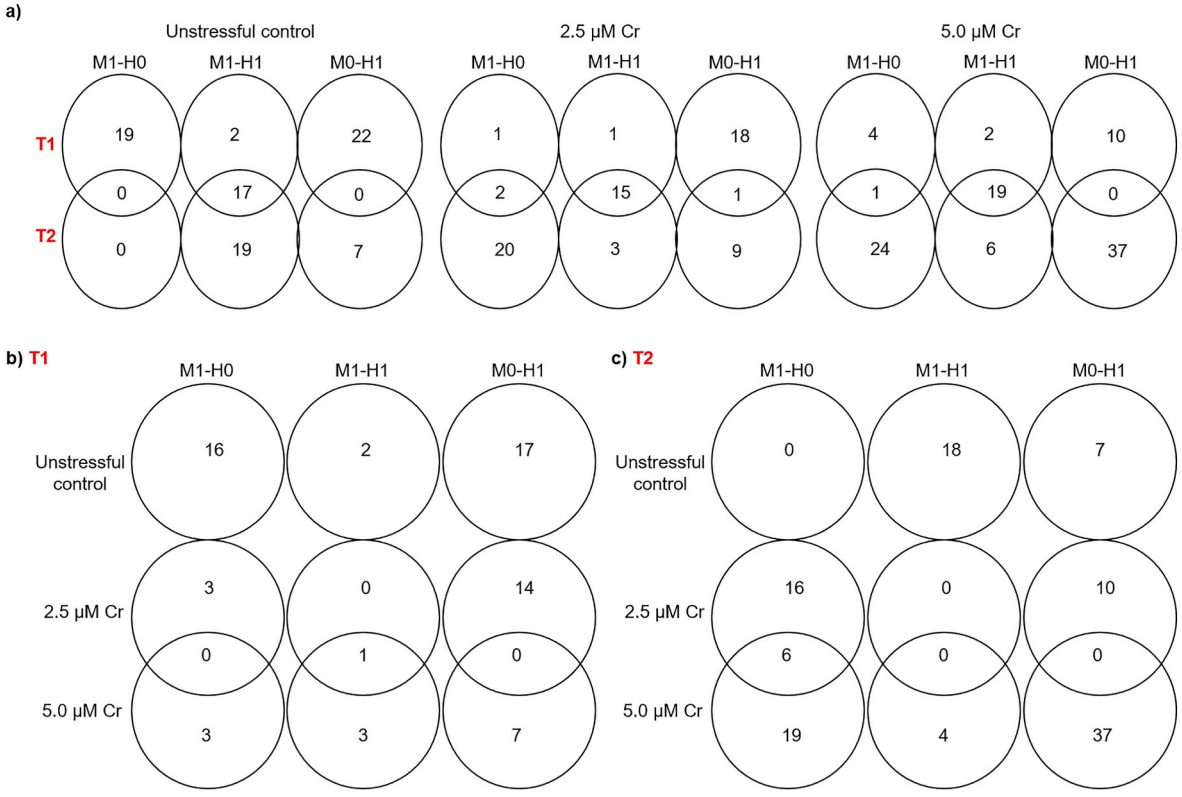


**Fig. 3.** Venn diagrams representing the number of genes for each methylation status identified in the unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T1 (F1 plants after 1 week) and T2 (F1 plants after 3 weeks) time point groups. Methylation profile of genes identified at **(a)** T1 and T2 time points (Table S9). Differentially methylated genes exclusively identified in Arabidopsis plants kept under unstressful control condition or exclusively identified in plants kept under 2.5 μM and 5.0 μM chromium stress treatments at the **(b)** T1 (Table 2) and **(c)** T2 (Table 3) time points. MSAP-Seq abbreviations: M1-H0 status corresponds to genes with double-strand methylation of inner cytosine or hemi-methylation of inner cytosine (*Msp*I: 1, *Hpa*II: 0); M1-H1 status corresponds to unmethylated genes (*Msp*I: 1, *Hpa*II: 1); and M0-H1 status corresponds to gens with hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine) (*Msp*I: 0, *Hpa*II: 1).

treatments at the T1 time point (Fig. 3a). Similarly, in the stressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T2 time point were identified 0, 22, and 25 genes, respectively, with double-strand methylation of inner cytosine or hemi-methylation of inner cytosine, and seven, 10, and 37 genes, respectively, with hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine). In addition, 36, 18, and 25 genes were identified as unmethylated, respectively, in the unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments at the T2 time point (Fig. 3a). The GO analysis showed that for both unstressful control condition, 2.5 μM Cr, and 5.0 μM Cr stress treatments, the gene set with unmethylated status at the T1 and T2 time points enriched the photosynthesis, DNA transcription, and energy production processes, while the gene sets with different methylated status enriched the cell proliferation, defense response, DNA methylation, senescence, energy production, transport, and photosynthesis processes (Tables S10 and S11).

In contrast, considering the differentially methylated genes exclusively identified in Arabidopsis plants kept under unstressful control condition or exclusively identified in Arabidopsis plants kept under 2.5 μM and 5.0 μM Cr stress treatments at the T1 and T2 time points were revealed the gene sets directly modulated by epigenetic mechanisms in consequence of chronic and acute Cr stress treatments (Tables 2 and 3). Comparatively, in unstressful control condition was identified two and 18 unmethylated genes at the T1 and T2 time points, respectively, while up to four unmethylated genes were identified exclusively in each Cr stress treatment (Fig. 3b and c). Meanwhile, in unstressful control condition was identified 16 double-strand methylated genes at the T1 and T2 time points, while up to 19 double-strand methylated genes were identified exclusively in each Cr stress treatment (Fig. 3b and c). In sequence, in the unstressful control condition was identified 17 and seven hemi-methylated genes at the T1 and T2 time points, respectively, while up to 14 and 37 hemi-methylated genes were identified exclusively in the 2.5 μM and 5.0 μM Cr stress treatments, respectively (Fig. 3b and c).

Therefore, a greater number of genes with methylation status of the double-strand methylation of inner cytosine or hemi-methylation of inner cytosine type was observed in unstressful control at the T1 time point, while at the T2 time point, this type of epigenetic change was observed in greater number in genes of Arabidopsis plants kept under both Cr stress treatments. In contrast, a greater number of genes with the epigenetic status of the hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine) was observed in Arabidopsis plants kept under Cr stress treatments at the T2 time point. Therefore, these data revealed the significant changes in the gene methylation profile as a result of chronic and acute Cr stress treatments, as well as a result of the sampling period of F1 plants at the T1 and T2 time points. Supported by seed germination rate data, this profile of epigenetic modification in several genes may be in line with the improved resilience of Arabidopsis plants kept under chronic and acute Cr stress treatments over two generations. Similar results to those obtained in the present study, Labra et al. (2004) showed that *Brassica napus* plants kept under Cr stress have significant epigenetic changes and that the amount of these genomic

**Table 2**
Functional annotation of differentially methylated genes identified in Arabidopsis plants kept under unstressful control condition or identified in plants kept under 2.5 μM and 5.0 μM chromium (Cr) stress treatment at the T1 (F1 plants after 1 week) time point. M1-H1 status corresponds to unmethylated genes; M1-H0 status corresponds to genes with double-strand methylation of inner cytosine or hemi-methylation of inner cytosine; and M0-H1 status corresponds to gens with hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine).

| Treatment | M1-H1 | Function | M1-H0 | Function | M0-H1 | Function |
|---|---|---|---|---|---|---|
| Unstressful | Atmg01200 | ATPase | Atmg00090 | translation US3M | At2g07725 | RPL5 translation |
| | At2g07656 | RNase H | At1g02270 | defense response | At3g14330 | CREF3 deaminase |
| | | | At4g20953 | hypothetical | At5g64830 | pre-rRNA/PDCD2 |
| | | | At1g15430 | hypothetical | Atmg00180 | CCB452 cytochrome |
| | | | At2g07708 | hypothetical | Atmg01170 | ATPase |
| | | | At1g10680 | ABCB10 | Atmg00300 | GAG integrase |
| | | | Atmg00900 | ABCI3 | At3g22600 | LTPG5 seed storage |
| | | | At1g25540 | MED25 | At1g53440 | LRR kinase |
| | | | At2g07728 | hypothetical | At3g49840 | DUF2215 transmembrane |
| | | | At1g73850 | DNA ligase | Atmg00560 | RPL2 translation |
| | | | Atmg01320 | NAD2B | At2g07626 | hypothetical |
| | | | At5g60920 | COBRA | At5g41315 | BHLH transcription |
| | | | Atmg00580 | NAD4 | At3g57430 | OTPR82/deaminase |
| | | | At2g05790 | GHL17 | At1g02880 | TPK1 pyrophosphokinase |
| | | | Atcg01280 | ATPase | At3g07180 | GPI transamidase |
| | | | At2g44880 | ABA/AHG11 | At3g12915 | RPS5 translation |
| | | | | | At2g43480 | peroxidase |
| 2.5 μM Cr | Atcg01280 | ATPase | Atcg00680 | photosystem II | Atmg01200 | ATPase |
| | | | At2g07631 | mitochondrial | At2g07681 | ABCI4 cytochrome |
| | | | At2g07667 | mitochondrial | At1g02270 | Ca-binding phosphatase |
| | | | | | At2g07706 | hypothetical |
| | | | | | At3g04410 | NAC4 transcription |
| | | | | | At2g07777 | ATP synthase |
| | | | | | At5g15950 | AdoMetDC |
| | | | | | Atmg00510 | NDUFS2 |
| | | | | | Atmg01280 | cytochrome C oxidase |
| | | | | | At3g13400 | SKS13 Cu-oxidase |
| | | | | | At5g10360 | RPS6A translation |
| | | | | | Atcg00730 | cytochrome *b6/f* |
| | | | | | Atmg01320 | NADPH dehydrogenase |
| | | | | | At3g11430 | suberin/cutin |
| 5.0 μM Cr | At2g07631 | hypothetical | At1g15150 | MATE efflux | At1g06220 | EFTUD2 elongation factor |
| | At2g07815 | cytochrome C | At2g07626 | hypothetical | At4g13630 | MYOB13 zein-binding |
| | Atmg01320 | NAD2/NADH | At4g09810 | UDP-RHA | At2g30440 | PLSP2B signal peptidase |
| | Atcg01280 | ATPase | | | At3g43960 | cysteine protease |
| | | | | | At3g10985 | WUN/SAG20 |
| | | | | | At2g07728 | hypothetical |
| | | | | | At2g27990 | BLH8 homeobox |

**Table 3**
Functional annotation of differentially methylated genes identified in Arabidopsis plants kept under unstressful control condition or identified in plants kept under 2.5 μM and 5.0 μM chromium (Cr) stress treatments at the T2 (F1 plants after 3 weeks) time point. M1-H1 status corresponds to unmethylated genes; M1-H0 status corresponds to genes with double-strand methylation of inner cytosine or hemi-methylation of inner cytosine; and M0-H1 status corresponds to gens with hemi-methylated CHG-sites (hemi-methylation of inner and outer cytosine).

| Treatment | M1-H1 | Function | M1-H0 | Function | M0-H1 | Function |
|---|---|---|---|---|---|---|
| Unstressful | At4g09980 | EMB1691 | – | – | At4g25870 | GlcNAc |
| | At2g20370 | glycosyltransferase | | | At4g18240 | starch synthase 4 |
| | Atcg00680 | photosystem II | | | At4g26490 | LEA |
| | At1g20480 | 4CL-related | | | At2g32460 | MYB101 |
| | At1g15430 | DUF1644 Znf_RING | | | At5g05750 | DnaJ |
| | At2g07719 | lipoprotein | | | At4g13610 | MET3 transferase |
| | At2g40400 | heme-binding | | | At2g35310 | REM23/B3 domain |
| | At5g12280 | ubiquitin | | | | |
| | At4g05612 | hypothetical | | | | |
| | At3g10985 | WUN/SAG20 | | | | |
| | At3g63370 | OTP86 deaminase | | | | |
| | At3g10660 | CPK2 kinase | | | | |
| | At2g01008 | MEE embryonary | | | | |
| | At5g45260 | WRKY52/RRS1 | | | | |
| | At1g01010 | NAC001 transcription | | | | |
| | Atmg00580 | NADH4 | | | | |
| | At2g19640 | ASHR2/SET domain | | | | |
| | At1g47290 | NSDHL/ERG26 | | | | |
| 2.5 μM Cr | – | | Atcg00680 | photosystem II | At4g09310 | SPRY RNA-binding |
| | | | At1g21710 | DNA lyase | At4g35870 | ERD4 membrane |
| | | | At5g66160 | RMR1 receptor | At1g59780 | disease response |
| | | | Atmg00180 | cytochrome CCB | At1g30480 | DRT111 spliceosome |
| | | | At1g02550 | PMEI inhibitor | At5g45380 | solute transporter |
| | | | At2g07667 | hypothetical | At1g10180 | EXO84 exocyst |
| | | | At1g50580 | xylosyltransferase | At3g61040 | CYP76C cytochrome |
| | | | At1g14640 | SWAP/splicing | At2g07806 | hypothetical |
| | | | At4g37650 | GRAS/transcription | Atmg00670 | NADH membrane |
| | | | At4g05612 | hypothetical | At2g26980 | CIPK3 kinase |
| | | | At1g15150 | MATE efflux | | |
| | | | At3g10985 | WUN/SAG20 | | |
| | | | At1g14970 | fucosyltransferase | | |
| | | | At2g18330 | ATPase | | |
| | | | At5g25890 | IAA28/auxin | | |
| | | | At2g01008 | embryonary MEE | | |
| | | | At2g07795 | transmembrane | | |
| | | | Atmg00513 | ND5/NADH | | |
| | | | At1g32490 | EMB2733 splicing | | |
| | | | At4g20575 | kinase receptor | | |
| | | | At2g12170 | hypothetical | | |
| | | | Atmg00070 | NADH9 ubiquinone | | |
| 5.0 μM Cr | At2g07681 | ABCI4 cytochrome | Atmg01200 | F-type H + -transport | At5g26150 | kinase receptor |
| | At2g07718 | heme exporter/NADH6 | At5g02820 | BIN5/cell division | At2g44350 | citrate synthase |
| | At2g07777 | ATP synthase 9 | At1g11420 | DUF724 protein | At3g50590 | WD40-1 |
| | Atmg00060 | NADH5 ubiquinone | At5g62910 | RING/U transcription | At5g13220 | JAZ10/jasmonate |
| | | | At1g21710 | DNA lyase | At1g26650 | SOS1 |
| | | | Atmg01120 | NADH1 ubiquinone | At3g01690 | SMP1 β-hydrolase |
| | | | At1g11270 | F-box ubiquitin | At2g07724 | hypothetical |
| | | | At4g05612 | hypothetical | At2g19860 | HXK2 hexokinase |
| | | | At1g11660 | HSP70-16 | Atmg00160 | cytochrome C2 |
| | | | At2g07806 | hypothetical | At5g42810 | IPK1 kinase |
| | | | At2g33080 | RLP28 receptor | At1g50250 | FTSH1 protease |
| | | | At5g25890 | IAA28/auxin | At5g10180 | AST68 S-transporter |
| | | | At4g14940 | putrescine oxidase | At1g22770 | GIGANTEA |
| | | | At2g07749 | RdR polymerase | At4g00230 | subtilase protease |
| | | | Atmg00516 | NAD1/NADH1 | At2g07656 | RNase H |
| | | | At1g50820 | kinase receptor | At5g60920 | COBRA |
| | | | At4g39960 | DnaJ5 | At1g49240 | actin 8 |
| | | | At2g38550 | FAX2 membrane | At1g21150 | mTERF transcription |
| | | | At2g07661 | hypothetical | At3g24850 | B3 domain |
| | | | At4g31080 | LNPA membrane | At5g56870 | β-galactosidase 4 |
| | | | At4g37650 | SGR7 transcription | At3g19515 | apoptosis inhibitor |
| | | | At3g10985 | WUN/SAG20 | At5g43560 | TRAF1A receptor |
| | | | At4g32030 | RNase H/RuvC | At2g48120 | RNA maturation |
| | | | At2g07795 | transmembrane | At4g24510 | CER2 a-transferase |
| | | | At2g19640 | ASHR2/SET domain | At5g39940 | FAD/NAD reductase |
| | | | | | At5g13630 | Mg-chelatase |
| | | | | | At2g33580 | LYK5 receptor |
| | | | | | Atmg01330 | NADH reductase |
| | | | | | At5g66100 | LAM leaf senescence |
| | | | | | At4g15370 | baruol synthase |

(*continued on next page*)

**Table 3** (*continued*)

| Treatment | M1-H1 | Function | M1-H0 | Function | M0-H1 | Function |
|---|---|---|---|---|---|---|
| | | | | | At3g15030 | TCP10 transcription |
| | | | | | At3g53320 | GPT2 microtubule |
| | | | | | At2g40650 | pre-mRNA-splicing |
| | | | | | At2g45300 | EPSP synthase |
| | | | | | At2g43960 | SWAP spliceosome |
| | | | | | At1g07320 | EMB2784 ribosomal |
| | | | | | At3g44310 | IAN nitrilase |

changes has a positive correlation with the dosage of Cr used. In addition, Aina et al. (2004) also revealed the occurrence of major changes in the methylation profile of the genome of *Trifolium repens* and *Cannabis sativa* plants kept under Cr, nickel, and cadmium stress treatments, and that these DNA methylation changes are mainly related to hypomethylation events of specific genomic regions. In accordance, Ferrari et al. (2020) showed that both hyper- and hypomethylation status of specific genome regions are important to improve algae tolerance to Cr stress. In fact, several plant species kept under different abiotic stresses, such as heavy metals and high salinity, develop notable epigenetic changes to improve their resilience to these severe adverse conditions (Aina et al., 2004; Guangyuan et al., 2007; Guarino et al., 2015; Guarino et al., 2019).

In the gene set exclusively identified in unstressful control condition (unmethylated and methylated), the protein-protein interaction and gene co-expression network analysis revealed four major functional linkage groups: (i) mitochondrial respiratory chain and photosystem II, (ii) protein posttranslational modification at the T1 time point (Fig. 4a), (iii) photosystem II reaction center, and (iv) mitochondrial respiratory chain at the T2 time point (Fig. 4d). In contrast, in the gene set exclusively identified in 2.5 μM Cr stress treatment, the protein-protein interaction and gene co-expression network analysis revealed also four major functional linkage groups: (i) mitochondrial respiratory chain and photosystem II, (ii) ribosomal, cell growth and proliferation at the T1 time point (Fig. 4b), (iii) photosystem II reaction center, and (iv) mitochondrial respiratory chain at the T2 time point (Fig. 4e). Meanwhile, in the gene set exclusively identified in 5.0 μM Cr stress treatment, the protein-protein interaction and gene co-expression network analysis revealed five major functional linkage groups: (i) spliceosome and cell cycle at the T1 time point (Fig. 4c), (ii) chloroplast protein translation, (iii) cuticular wax and suberin biosynthesis, (iv) cytochrome, and (iv) photorespiration at the T2 time point (Fig. 4f). These collective data showed that Arabidopsis plants kept under chronic and acute Cr stress treatments undergo transgenerational significant epigenetic changes to regulate target genes associated with plant resilience, mainly involved in major processes such as photorespiration, splicing, post-translational modification, and plant defense response. Furthermore, it was also observed in these Arabidopsis plants that epigenetic modulation is slightly different between chronic and acute stress treatments, with modifications at the spliceosome and cell cycle level being mainly observed in Arabidopsis plants kept under acute Cr stress treatment.

Therefore, in this study, was observed that chronic Cr stress treatment affected the DNA methylation status of several genes involved mainly in the mitochondrial respiratory chain, photosystem II, ribosomal, cell growth and proliferation, and protein posttranslational modification (Fig. 4a to f; File S1). Meanwhile, Arabidopsis plants kept under acute Cr stress treatment showed affected DNA methylation status in various genes involved mainly in the spliceosome, cell cycle, RNA processing, chloroplast protein translation, DNA transcription, cytochrome, defense response, and photorespiration. Similarly, Tang et al. (2022) and Tang et al. (2023) showed that antioxidant and oxidoreductase genes were differentially methylated in kenaf (*Hibiscus cannabinus*) seedlings kept under extreme doses of Cr and it was revealed that these enzymes are important in plant defense response to Cr stress. Comparatively, Liu et al. (2020a,b) revealed that Arabidopsis plants kept under 200 μM Cr stress treatment showed a down-regulation of genes involved in redox, secondary metabolism, and energy metabolism processes, while up-regulation of genes related to stress defense response, photosynthesis, sulfur metabolism, indole-3-acetic acid, and carotenoids was also observed. Similarly, Colzi et al. (2023) showed a transgenerational priming effect conditioned by chronic and acute Cr stress treatments in F1 plants of Arabidopsis, highlighting the up-regulation mainly of genes involved in iron starvation, plant defense response, and cell redox homeostasis.

In addition to these biochemical processes modulated by Cr stress treatment, genes involved in specific functions were observed and in particular discussed. Thus, *AtMED25* (At1g25540), *AtABCB10* (At1g10680), *AtABCI3* (Atmg00900), *AtCOBRA* (At5g60920), *AtGHL17* (AT2G05790), *AtABA/AHG11* (At2g44880), *AtNAD2B* (Atmg01320), and *AtNAD4* (Atmg00580), *AtRPL5* (At2g07725), *AtRPL2* (Atmg00560), *AtRPS5* (At3g12915), *AtCREF3* (At3g14330), *AtOTPR84* (At3g57430), *AtLRR* (At1g53440), and *AtTPK1* (At1g02880) are some of the main genes identified as methylated specifically in Arabidopsis plants kept under unstressful control condition (but not detected in the Arabidopsis plants kept under Cr stress treatments) at the T1 time point. GO category analysis showed that this gene set was enriched for photorespiration, nucleotide metabolism, and metabolic processes (File S1). Functionally, the AtMED25 protein acts in the phytochrome and flowering time regulation by modulating the alternative splicing of *JASMONATE ZIM-DOMAIN* genes through recruiting the PRP39a and PRP40a splicing factors (Wu et al., 2020). Meanwhile, AtABCB10 and AtABCI3 proteins act in ATP hydrolysis and transmembrane transport, and are important to cell integrity and to improve tolerance plant to Cr stress (Gräfe and Schmitt, 2021; Nie et al., 2021a, 2021b; Li et al., 2022). In the meantime, AtCOBRA proteins are primarily localized in the plasmatic membrane of the longitudinal sides of root cells and are necessary for oriented cell expansion and to regulate the cellulose deposition (Yang et al., 2019). The AtGHL17 proteins act by hydrolyzing O-glycosyl compounds in complex sugars (Gaudioso-Pedraza and Benitez-Alfonso, 2014). The AtABA/AHG11 are pentatricopeptide repeat-containing proteins that act in RNA editing and ABA hypersensitive for seed germination (Murayama et al., 2012). Likewise, AtNAD2B and AtNAD4 are part of the mitochondrial respiratory chain complex I acting as mitochondrial NADPH dehydrogenase (Marchetti et al., 2020). In addition, AtNAD2B is recognized to have evolved as a component of the collection of innate immunity molecules in Arabidopsis plants. Its engagement with other significant proteins, coupled with diverse mechanisms, provides effectiveness against several biotic and abiotic stresses (Dracatos et al., 2016). On the other side, AtRLP5, AtRPL2, and AtRPS5 ribosomal proteins act in the regulation of protein translation (Scarpin et al., 2023), while AtCREF3 and AtOTPR84 deaminases are pentatricopeptide repeat proteins involved in chloroplast cytidine to uridine editing of mRNA molecules (Yagi et al., 2013; Hayes et al., 2015). AtLRR encodes a leucine-rich repeat transmembrane protein kinase (ten Hove et al., 2011) while AtTPK1 encodes a thiamine pyrophosphokinase capable of producing thiamine pyrophosphate from free thiamine (Ajjawi et al., 2007).

Meanwhile, the main genes identified as differentially methylated specifically in Arabidopsis plants kept under Cr stress treatments (but not detected in the Arabidopsis plants kept under unstressful control condition) at the T1 time point were *AtATPase* (Atcg01280 and Atmg01200), *AtABCI4* (At2g07681), *AtRPS6A* (At5g10360),

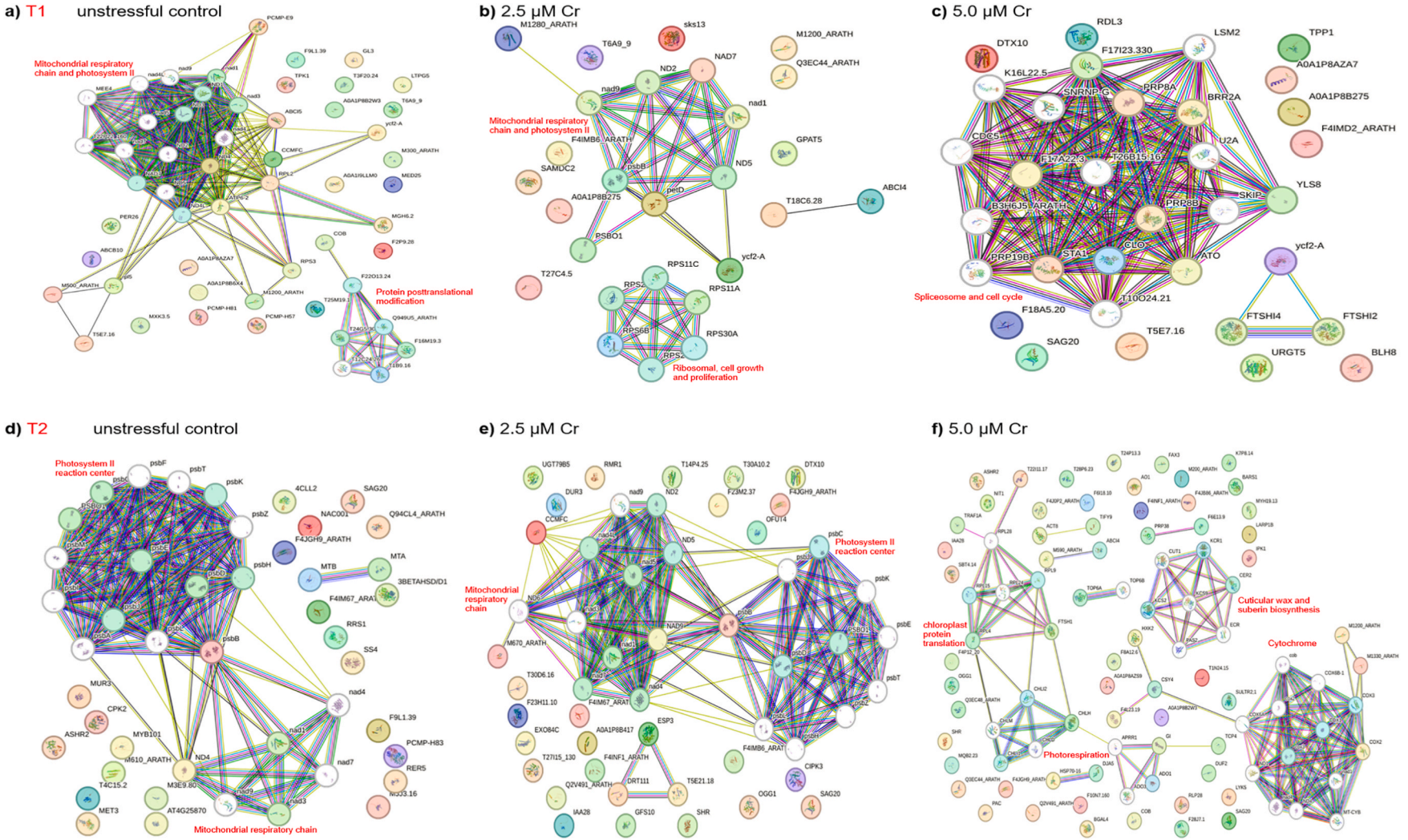


**Fig. 4.** Protein-protein interaction and gene co-expression networks among differentially methylated genes identified in Arabidopsis plants kept under (**a** and **d**) unstressful control condition or identified in Arabidopsis plants kept under (**b** and **e**) 2.5 µM chromium (Cr) and (**c** and **f**) 5.0 µM Cr stress treatments at T1 (F1 plants after 1 week; Table 2) and T2 (F1 plants after 3 weeks; Table 3) time points. In addition to the differentially methylated genes used as input, more nodes were added to current networks. The interaction network provided by the STRING represented the known interactions are shown in light blue lines when from curated databases, and pink lines when experimentally determined; predicted interactions are shown in dark green lines when gene neighborhood, red lines when gene fusions, and dark yellow lines when gene co-occurrence; while other protein-protein associations are shown in light green lines when text-mining, black lines when co-expression, and light blue lines when by protein homology. Colored nodes indicate query proteins and the first shell of interactors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

*AtAdoMetDC* (At5g15950), *AtNAC4* (At3g04410), *AtSKS13* (At3g13400), *AtMATE* (At1g15150), *AtEFTUD2* (At1g06220), *AtMYOB13* (At4g13630), *AtWUN/SAG20* (At3g10985), *AtPLSP2B* (At2g30440), and *AtBLH8* (At2g27990). GO category analysis showed that this gene set was enriched for metabolic processes, photorespiration, and peptide metabolism (File S1). In particular, AtATPases are P-loop containing nucleotide triphosphate hydrolases superfamily proteins involved in the nucleoside-triphosphatase activity and ATP binding (Shalaeva et al., 2018). The AtABCI4 is an ATP-BINDING CASSETTE I4 protein involved in cytochrome C assembly and located in the chloroplast (Gräfe and Schmitt, 2021), while AtRPS6A are proteins located in the cytosolic ribosome and involved in embryo development during seed dormancy and in the regulation of rRNA genes by possible epigenetic changes (Kim et al., 2014). AtAdoMetDC is an adenosylmethionine decarboxylase family protein, essential for polyamine homeostasis and normal plant embryogenesis, growth, and development (Majumdar et al., 2017). In turn, AtNAC4 is a protein of typical No Apical Meristem domain transcriptional regulator superfamily (Seo et al., 2011), while AtSKS13 is a protein involved in the pollen development and pollination/fertilization processes (Ji et al., 2019). Meanwhile, AtMATE is a multidrug and toxin extrusion protein that plays a pivotal role in controlling plant development by affecting phytohormone transport, tip growth, and senescence (Upadhyay et al., 2019). AtEFTUD2 is a ribosomal protein S5/elongation factor G/III/V family protein involved in pre-mRNA splicing and essential for plant reproduction (Pagnussat et al., 2005; Liu et al., 2009). AtMYOB13 is a myosin-binding protein 13 that drives endomembrane trafficking and cytoplasmic streaming in plant cells (Kurth et al., 2017). AtWUN/SAG20 is a senescence-associated protein whose accumulation is induced in response to multiple stresses (Kanojia et al., 2020). In turn, AtPLSP2B encodes a thylakoidal processing peptidase that removes signal sequences from proteins synthesized in the cytoplasm and transported into the thylakoid lumen (Hsu et al., 2011). In the meantime, the AtBLH8 is a BEL1-like homeobox protein that acts together with PNY factors in meristem maintenance by regulating the allocation process during vegetative and reproductive development (Niu and Fu, 2022).

In sequence, the differentially methylated genes at the T2 time point differed greatly from the genes identified in Arabidopsis plants kept under unstressful control condition and Cr stress treatments at the T1 time point. The main genes identified as methylated specifically in Arabidopsis plants kept under unstressful control condition, but not detected in the Arabidopsis plants kept under Cr stress treatments at the T2 time point, were *AtLEA* (At4g26490), *AtMYB101* (At2g32460), *AtDnaJ* (At5g05750), and *AtREM23/B3* (At2g35310). GO category analysis showed that this gene set was enriched for photorespiration and transferase activity (File S1). The AtLEA protein is a late embryogenesis abundant hydroxyproline-rich glycoprotein family member associated with plant resistance against multiple abiotic stresses (Hundertmark and Hincha, 2008). In contrast, AtMYB101 is a transcription factor containing a MYB-like DNA-binding domain that plays an important role in pollen tube reception by controlling the expression of downstream genes (Liang et al., 2013). The AtDnaJ is a DNAJ heat shock N-terminal domain-containing protein that acts as a molecular chaperone in protein folding and plays critical roles in plant growth and development, and defense response to heat stress (Pulido and Leister, 2018). Meanwhile, the AtREM23/B3 protein is a member of the Reproductive Meristem (REM) family and is part of the B3 DNA-binding domain superfamily that acts as a transcription factor (Mantegazza et al., 2014).

In contrast, the main genes identified as unmethylated specifically in Arabidopsis plants kept under unstressful control condition at the T2 time point were *AtEMB1691* (At4g09980), *AtCPK2* (At3g10660), *AtWRKY52/RRS1* (At5g45260), *AtNAC001* (At1g01010), and *AtASHR2/SET* (At2g19640). The AtEMB1691 is the EMBRYO DEFECTIVE 1691 protein that acts as an S-adenosylmethionine-dependent methyltransferase, which is essential for adenosine methylation at specific mRNA sequences and plays a notable role in mRNA stability, processing, translation efficiency, and editing (Tzafrir et al., 2004). AtCPK2 is a calmodulin-domain protein kinase CDPK isoform 2 that acts in the regulation of pollen tube growth (Gutermuth et al., 2013). The AtWRKY52/RRS1 is a disease-resistance protein of the TIR-NBS-LRR class that acts in plant resistance against biotic stresses (Rinerson et al., 2015), while AtNAC001 is a NAC domain-containing protein that acts as a sequence-specific DNA binding transcription factor (Ooka et al., 2003). On the other hand, AtASHR2/SET is an ASH1-related protein 2 that belongs to the class V-like SAM-binding methyltransferase superfamily and acts as a histone-lysine methyltransferase (Ng et al., 2007).

In the meantime, the main genes identified as differentially methylated specifically in Arabidopsis plants kept under Cr stress treatments at the T2 time point were *AtRMR1* (At5g66160), *AtGRAS* (At4g37650), *AtWUN/SAG20* (At3g10985), *AtMATE* (At1g15150), *AtERD4* (At4g35870), *AtBIN5* (At5g02820), *AtCOBRA* (At5g60920), *AtASHR2* (At2g19640), *AtDnaJ5* (At4g39960), *AtSWAP* (At1g14640), *AtRLP28* (At2g33080), *AtIAA28* (At5g25890), and *AtTCP10* (At3g15030), as well as several other important genes that play notable roles mainly in RNA processing and spliceosome (At4g09310, At2g07749, At2g48120, At1g30480, At1g32490, At2g43960, and At2g40650), transmembrane proteins (At2g07795, Atmg00670, At2g38550, and At4g31080), and kinases (At2g26980, At1g50820, At5g26150, At5g42810, At2g19860, At4g20575, and At2g33580). GO category analysis showed that this gene set was enriched for photorespiration, RNA processing, metabolic processes, plant development, and nucleotide binding (File S1). In particular, AtRMR1 is receptor homology-transmembrane-ring H2 domain protein 1 involved in transporting storage proteins within vacuoles (Occhialini et al., 2016). AtGRAS is a transcription factor belonging to the GRAS family involved in the transcriptional regulation of genes that play critical roles in plant growth and development (Cenci and Rouard, 2017). AtERD4 is an early-responsive to dehydration stress protein involved in conferring abiotic stress tolerance and enhancing plant growth (Rai et al., 2016). AtBIN5 is a BRASSINOSTEROID INSENSITIVE 5 protein involved in the brassinosteroids signaling pathway and that plays important roles throughout plant growth and development (Yin et al., 2002). AtDnaJ5 is a molecular chaperone Hsp40/DnaJ family protein involved in the mechanism of iron utilization during chloroplast Fe–S cluster biogenesis (Zhang et al., 2021). The AtSWAP is a Suppressor-of-White-APricot/surp domain-containing protein with RNA binding activity and is involved in RNA processing and spliceosome to promote photomorphogenesis (Wang and Brendel, 2004). AtRLP28 is a receptor-like protein 28 located extracellularly in the transmembrane and involved in signal transduction in defense response to stresses (Wu et al., 2016). In turn, the AtIAA28 is a member of the *IAA/ARF* gene family that encodes an auxin-responsive protein that may be a negative regulator of lateral root formation in response to auxin (Rogg et al., 2001; Overvoorde et al., 2005). Lastly, AtTCP10 is a TCP family transcription factor involved in the heterochronic regulation of leaf differentiation, in plant development, and inhibits the high temperature-induced homeotic conversion of the ovules (Lan et al., 2023; Koyama et al., 2007).

Therefore, these collective data reveal in detail many biological processes differentially modulated through demethylation and methylation of specific transcription units within the genome as a consequence of chronic or acute Cr stress. In addition, these data evidence the epigenetic marks in Arabidopsis plants exposed to Cr stress conditions that modulate from development to reproduction, as well as to improve plant resilience. These findings obtained in this study are in agreement with those obtained in other studies that kept plants under Cr stress and studied the effect at the epigenetic level, such as with the epigenetic modulation of genes encoding metal transporters, DNA transcription, antioxidants, defense response to abiotic stress, RNA processing, and protein post-translational modifications (Aina et al., 2004; Cong et al., 2019; Tang et al., 2022; Colzi et al., 2023; Sun et al., 2023). Tang et al. (2021) and Ding et al. (2014) showed differential methylation of genes encoding antioxidant, MYB, AP2/ERF, bZIP, TPR, F-box, and kinase

receptors and pointed out this mechanism necessary to enhance antioxidant response and lead (Pb) detoxification in radish and maize. Feng et al. (2021) and Miao et al. (2022) showed by epigenetic study that heavy metal stress significantly reduced the DNA methylation at CpG (Cytosine-p-Guanine) and histone H3K9me2 marks in the upstream region of heavy metal responsive genes of rice. In addition, these authors also showed that overexpression of *OsHMP* gene improves rice adaptation to the environment contaminated with cadmium. Likewise, Kashino-Fujii et al. (2018) showed that the multiretrotransposon-like (MRL) sequence insertion in the upstream region of the *HvAACT1* gene is responsible for increasing the expression of this gene while MRL methylation affects its expression and reduces barley tolerance to aluminum toxicity. Lastly, Tang et al. (2022) showed that differential DNA methylation of kenaf plants kept under Cr stress affects several genes, such as ABC transporters, kinase receptors, and F-box proteins. Furthermore, it can also be observed that even exposing Arabidopsis plants to Cr stress for a few generations is enough to detect epigenetic changes, which when accumulated can lead to significant changes ranging from DNA transcription and protein accumulation to transcriptional changes in non-coding RNA transcripts.

## 4. Conclusions

MSAP-Seq allowed us to estimate the methylation status of the cytosine within the CpG islands in genes of Arabidopsis plants kept over two generations (F0 and F1) under unstressful control (0) condition, chronic (2.5 μM Cr), and acute (5.0 μM Cr) stress treatments. These collective data revealed that several key genes were specifically methylated or unmethylated when plants were kept under chronic and acute Cr stress treatments compared with plants kept under unstressful control condition. In addition, the functional profile of genes modified by epigenetic mechanisms in these plants kept under acute Cr stress, despite sharing some genes, has notable differences with plants kept under chronic Cr stress. Several genes with major functional effects were described here as being modulated by epigenetic marks to possibly act on the plant's resilience to Cr stress. This evidence demonstrates that Arabidopsis plants orchestrate epigenetic mechanisms to activate a prompt and long-lasting physiological response to the presence of this heavy metal. In particular, energy production, RNA processing, protein translation, photorespiration, transmembrane transport, DNA transcription, plant development, and plant resilience are highlighted as major processes modulated by epigenetics to improve the Arabidopsis defense to Cr stress. These data provide valuable insights into the adaptive strategies employed by plants kept under Cr stress conditions. Furthermore, these data also suggest that the exposure of Arabidopsis plants to more prolonged Cr stress treatment over generations can lead to cumulative epigenetic changes to the point of significantly altering the expression profile of target genes. Therefore, further studies should clarify how these epigenetic changes remain over generations and their implications for plant resilience.

## Consent for publication or Ethics approval and consent to participate

Not applicable.

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## CRediT authorship contribution statement

**Francesco Guarino:** Investigation. **Angela Cicatelli:** Investigation. **Werther Guidi Nissim:** Investigation. **Ilaria Colzi:** Investigation. **Cristina Gonnelli:** Investigation. **Marcos Fernando Basso:** Formal analysis, Writing – original draft. **Chiara Vergata:** Investigation. **Felice Contaldi:** Investigation. **Federico Martinelli:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Stefano Castiglione:** Conceptualization, Funding acquisition, Project administration, Supervision.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142642.

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