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RESEARCH ARTICLE

Heavy metals modulate DNA compaction and methylation at CpG sites in the metal hyperaccumulator *Arabidopsis halleri*

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Abstract

Excess heavy metals affect plant physiology by inducing stress symptoms, however several species have evolved the ability to hyperaccumulate metals in above-ground tissues without phytotoxic effects. In this study we assume that at subcellular level, different strategies were adopted by hyperaccumulator *versus* the non-accumulator plant species to face the excess of heavy metals. At this purpose the comet assay was used to investigate the nucleoid structure modifications occurring in response to Zn and Cd treatments in the I16 and PL22 populations of the hyperaccumulator *Arabidopsis halleri versus* the nonaccumulator species *Arabidopsis thaliana*. Methy-sens comet assay and RT-qPCR were also performed to associate metal induced variations in nucleoids with possible epigenetic modifications. The comet assay showed that Zn induced a mild but non significant reduction in the tail moment in *A. thaliana* and in both I16 and PL22. Cd treatment induced an increase in DNA migration in nuclei of *A. thaliana*, whereas no differences in DNA migration was observed for I16, and a significant increase in nucleoid condensation was found in PL22 Cd treated samples. This last population showed higher CpG DNA methylation upon Cd treatment than in control conditions, and an up-regulation of genes involved in symmetric methylation and histone deacetylation. Our data support the hypothesis of a possible role of epigenetic modifications in the hyperaccumulation trait to cope with the high Cd shoot concentrations. In addition, the differences observed between PL22 and I16 could reinforce previous suggestions of divergent strategies for metals detoxification developing in the two metallicolous populations.

KEYWORDS
cadmium, DNA integrity, epigenotoxicology, metal hyperaccumulators, methy-sens comet assay, zinc

1 | INTRODUCTION

Excess heavy metals provoke in eukaryotic cells several stresses, such as the induction of DNA damage (i.e., double strand breaks), activation of reactive oxygen species, and inhibition of DNA repair mechanisms (Morales *et al*., 2016; Cortés-Eslava *et al*., 2018; Jaskulak *et al*., 2019). Recently for some heavy metals a role in inducing also epigenetic modifications, as DNA methylation, histone modifications, and modulation of the synthesis of miRNAs has been assessed (Genchi *et al*., 2020).

In this context, among genotoxicity tests to evaluate cells/organisms DNA damages due to heavy metals, the comet assay is a

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powerful approach (Dhawan *et al*., 2009). This assay is able to detect DNA damage even at low concentrations of contaminants, and allows the identification of DNA modifications that alter the molecule's supercoiling, since loops move toward the anode during electrophoresis (Dhawan *et al*., 2009). Several modified versions of the basic alkaline comet assay have been developed to detect specific DNA damage in human cells (Collins *et al*., 2008; Azqueta *et al*., 2014) and/or structural organization of chromatin (Shaposhnikov *et al*., 2015). A recent improvement of the alkaline comet assay, that is the methy-sens comet assay, allows also for detecting global methylation modifications in single cells (Perotti *et al*., 2015) through enzymes, namely *Msp*I and *Hpa*II, that recognize all CpG sites and nonmethylated CpG sites, respectively.

Plants are a good model to evaluate the effects of heavy metal excess, especially considering their possible exposition to environment contaminated by these metals and the existence of plant species able to hyperaccumulate and hypertolerate excess of heavy metals. Nevertheless, few studies report the use of the comet assay to assess plant DNA damage (Seth *et al*., 2008; Pizzaia *et al*., 2019; Rodriguez *et al*., 2019).

Among heavy metals, zinc (Zn) and cadmium (Cd) are naturally occurring in the environment but their presence is often increased because of the anthropogenic activity such as agricultural practices and industrial processes (Balafrej *et al*., 2020; Genchi *et al*., 2020).

Zinc is an essential microelement for plants, but it becomes phytotoxic at high concentrations causing plant growth inhibition, reduction in chlorophyll synthesis, and reduced absorption of macro nutrients, which lead to reduced yields (Khan and Khan 2014; Sturikova *et al*., 2018; Balafrej *et al*., 2020). Genotoxicity of Zn in plants has also been demonstrated (Subhadra and Panda 1994).

Cadmium is considered one of the most dangerous contaminants in soils because of its high solubility in water, non biodegradability and high genotoxicity (Genchi *et al*., 2020). The potential entry of Cd into the food chain following its accumulation in vegetable crops grown in contaminated soils represents a serious threat to human health (Genchi *et al*., 2020). Numerous experimental studies have demonstrated the genotoxicity of Cd salts in plant species (Fojtová and Kovařik 2000; Seoane and Dulout 2001). Furthermore, in animals and humans, Cd has shown to induce epigenetic modifications, as DNA methylation, histone modifications, and modulation of the synthesis of miRNAs. In particular, DNA methylation levels seem to depend on the time of exposure to Cd (Genchi *et al*., 2020).

A limited number of plant species (∼500) known as metal hyperaccumulators have developed a peculiar ability of accumulate enormous amounts of heavy metals or metalloids in their shoots without showing any toxicity symptom (van der Ent *et al*., 2013). Thus, the hyperaccumulator trait and the associated hypertolerance have allowed some species to colonize hostile environments such as metalliferous soils (and more recently, heavy-metal polluted sites) through metal uptake and hyperaccumulation rather than metal exclusion. In particular, Zn hyperaccumulators are able to accumulate up to 1% of the shoot dry biomass of Zn in their aerial parts (Broadley *et al*., 2007), while plant species considered Cd hyperaccumulators can accumulate over 0.01% Cd in shoot dry biomass (Verbruggen *et al*., 2013a). The comprehension of this phenomenon will shed light on the evolution of this trait and on the adaptation of these plants to extreme environments, and a recent literature is indeed pointing to this direction (Manara *et al*., 2020).

Interestingly, for some species as *Noccaea caerulescens* and *Arabidopsis halleri* different populations are present which showed metal tolerance and accumulation traits specific to the metal concentration in the soil of origin (Escarrè *et al*., 2000; Corso *et al*., 2018). Indeed, both *N. caerulescens* and *A. halleri* are considered as model species when studying the molecular and genetic basis underlying hyperaccumulation as well as plant metal homeostasis due to their phylogenetic proximity to *Arabidopsis thaliana* in which genetic maps, genome sequence, and commercial microarrays are available (Verbruggen *et al*., 2013b; Reeves *et al*., 2017).

Besides this, not much is known about the DNA structure and the epigenetic modifications occurring in hyperaccumulator species due to heavy metals. In a recent study, alkaline comet assay was applied to leaves of the Ni hyperaccumulator *N. caerulescens* grown in a Ni-rich culture substrate, and it showed that leaf cells presented more condensed nucleoids, associated with hyper-methylation of DNA and up-regulation of the genes involved in DNA and histone epigenetic alterations (Gullì *et al*., 2018). In this research we utilized a similar approach to investigate for the first time possible variation in nucleoid structure occurring in two *A. halleri* populations in response to the heavy metals Cd and Zn with the aim to correlate those data with variation in the expression of genes connected to epigenetic marks. In particular, two populations with contrasting Zn and Cd accumulation profiles (i.e., I16 and PL22; Meyer *et al*., 2015; Corso *et al*., 2018) were chosen to assess the impact of Zn and Cd exposure on: (i) the compaction and relaxation of leaf cell nucleoids; (ii) the CpG DNA methylation percentage, and (iii) the modulation of genes linked to epigenetic modifications. The same analyses were performed on the phylogenetically related species *A. thaliana* which is a nonaccumulator.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

A. halleri (L.) O'Kane & Al-Shehbaz population I16 (Val del Riso, northern Italy) and population PL22 (Bukowno, south of Poland) were propagated *in vitro*. Seeds from I16 population were harvested by the authors (45°51′34.40 N 9°52′34.94E) whereas PL22 population was kindly provided by (Corso *et al*., 2018). Seeds were germinated *in vitro* on standard agarised MS medium (Murashige and Skoog 1962) supplemented with 10 g L^{-1} sucrose. In these conditions, seedlings of *A. halleri* are readily growing and can be multiplied by micropropagation of cuttings obtained on the main stem. Rooted plants were maintained *in vitro* with a 16 hr light/8 hr dark photoperiod (120 μmol m^{-2} s^{-1} photosynthetic photon flux, 75% relative humidity (RH)) and 22°C. Developed plants (2-week old after the cutting) consisted in a vegetative rosette apparatus without inflorescence stem,

with totally developed primary and secondary roots, roughly ranging from 3 to 4 cm in length. At this timepoint, plants were transferred into hydroponic culture in 3 L polyethylene vessels containing 1X Hoagland solution (Hoagland and Arnon 1950) and moved to a growth chamber under controlled conditions (22°C; 16 hr/8 hr light/dark; 120 μmol m^{-2} s^{-1} photosynthetic photon flux, 75% RH). The hydroponic solution was substituted every week and kept oxygenated by constant air bubbling. After 1 week of acclimation, six plants per population were treated with 100 μM $ZnSO_4$ or with 50 μM $CdSO_4$ for 2 weeks. Control plants were grown in 1X Hoagland solution without addition of Cd or extra-addition of Zn. The experiment was performed in duplicate, to have two biological replication of both control and metal treatments.

A. thaliana Columbia_0 seeds were sterilized following standard protocols and placed at 4°C (dark) for 3 days to synchronize germination. The seeds were plated on agarised MS medium (Murashige and Skoog 1962) and left to germinate and grow in the growth chamber (22°C; 16 hr/8 hr light/dark; 120 μmol m^{-2} s^{-1} photosynthetic photon flux, 75% RH). One-week-old seedlings were transferred to 3 L polyethylene pots (6 seedlings per pot) filled with 1X Hoagland solution as described above. The nutrient solution was replaced every week and kept oxygenated by constant air bubbling. After 1 week of acclimation, six plants were treated with 5 μM $ZnSO_4$ for 7 days, or with 5 μM $CdSO_4$ for 5 days. Also in this case, six plants were maintained in 1X Hoagland solution (control conditions), without addition of Cd or extra-addition of Zn. The experiments were replicated, obtaining two biological replicates for each condition tested.

The metal concentrations applied in the growth solution for both *A. halleri* and *A. thaliana* were reported not to be toxic and allow the induction of metal response in plants without killing them (Fasani *et al*., 2017). At the end of the experiments, that is, after the above mentioned days of treatment in the heavy metal contaminated solution, three leaves of the same size from each treated and untreated plant, both *A. halleri* and *A. thaliana*, were sampled to perform the alkaline comet assay (Tice *et al*., 2000) and the methy-sens comet assay. Other leaves were sampled and stored at −80°C for RNA extractions, or oven-dried at 70°C for 3 days for metal quantification.

2.2 | Chemical analyses

About 0.1 g oven-dried samples, weighed in quartz vessels and rehydrated with 800 μl of ultrapure deionized water (0.05 μS cm^{-1}, Purelab® Ultra ELGA, High Wycombe, UK), were mineralized in duplicate with 2 ml of ultrapure inverted aqua regia (HNO_3:HCl:H_2O, 3:1:1 v/v/v) by means of UltraWAVE (UltraWAVE Milestone, Sorisole, Italy), as described in (Gullì *et al*., 2018).

The analytic quantification of Cd (214.439 nm, IDL = 0.001 mg L^{-1}) and Zn (213.857 nm, IDL = 0.009 mg L^{-1}) in the suitable diluted mineralized samples was carried out with a ICP-OES spectrometer (Vista-MPX, Varian, Agilent Technologies, Santa Clara, CA) by employing two 7-point external calibration curves (0.005–50 mg L^{-1} for Cd and 0.1–1.6 mg L^{-1} for Zn). The calibration standards were derived from 1,000 mg L^{-1} mono-element solutions of Cd and Zn (TraceCERT® Fluka Analytical, Sigma-Aldrich, St. Louis, MO) in 1% (v/v) ultrapure HNO_3 (67–69% m/v, Chem-Lab NV, Pico-Pure Plus, Zedelgem, Belgium).

2.3 | Alkaline comet assay

Extraction of the plant cell nuclei, preparation of the slides and electrophoresis for the alkaline comet assay (pH >13) were performed according to Restivo *et al*. (2002), with minor modifications. In particular, cut leaves were sprinkled with 200 μl of 0.7% low melting agarose (LMA) and then nuclei suspension was transferred to a slide. For scoring, the slides were observed through a fluorescence microscope (Leica DMLS, Leica Microsystems, Wetzlar, Germany) (excitation filter: BP 515–560 nm; barrier filter: LP 580 nm), after staining with 75 μl ethidium bromide (10 μg ml^{-1}). The comet images were captured by means of a monochromatic camera (Pulnix PE-2020P, Pulnix, Alzenau, Germany) and examined via an automatic image analysis system (Comet assay IV; Perceptive Instruments Ltd., Bury St. Edmunds, UK). Two slides were analyzed for every tested condition, and 50 random selected nucleoids were collected per slide. The tail moment was chosen as parameter to evaluate nucleoid structure modifications since it takes into consideration the migration of the genetic material as well as the relative amount of DNA in the tail, and it is useful to describe heterogeneity in a cell population. For each of the two biological replicates, the assay was performed using three leaves (treated or untreated) to prepare three independent technical replicates.

2.4 | Methy-sens comet assay

The plant cell nuclei obtained and transferred onto glass slides as described above were subjected to an enzymatic restriction reaction, using FastDigest *Msp*I and *Hpa*II enzymes (Thermo Fisher Scientific). These two isoschizomer endonucleases recognize the same restriction site, but *Hpa*II can cut into the restriction site in presence of methylation. The test was performed as described by (Perotti *et al*., 2015), with minor modifications. After their preparation, the slides were dipped into PBS for 10 min at RT, placed horizontally and sprinkled with 100 μl enzymatic solution, containing either *Msp*I or *Hpa*II diluted in FastDigest Buffer (0.5 μl of Enzyme, 10 μl of FastDigest Buffer and 89.5 μl of double distilled water). A microscope coverslip was moved onto the nuclei suspension, and the slides were incubated at 37°C for 10 min. A 100 μl FastDigest buffer was used as negative control. The enzymatic activity was stopped by transferring the slides into the electrophoretic chamber, filled with the electrophoretic alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH ≥13). After 30-min electrophoresis (0.66 V/cm, 300 mA), the slides were neutralized, fixed, and stained as described above. After fluorescence microscope observation, the tail moment was chosen as reference parameter. The fragmentation observed after *Msp*I treatment represents the positive control. This restriction enzyme digests DNA at all the CpG sites, allowing us

to observe the total fragmentation obtainable in this reaction condition. The fragmentation induced through the digestion with *Hpa*II enzyme is inversely proportional to the DNA methylation status. For each of the two biological replicates, the assay was performed using three leaves (treated or untreated) to prepare three independent technical replicates.

2.5 | RNA isolation and RT-qPCR

The total RNA was obtained through extraction from 0.5 g leaf tissues by means of the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany); the sample quality and purity were analyzed by means of agarose gel electrophoresis and the A_{260}/A_{280} determination at spectrophotometer. All the samples showed appropriate values.

The target genes were selected within the *A. halleri* transcriptome shotgun assembly (TSA) on the basis of the homology with *A. thaliana* gene sequences available in GenBank; if possible, the same primer pairs were identified for both species on conserved regions. The gene specific primer pairs were designed through the online tool Primer3 (http://primer3.ut.ee), and purchased from BMR Genomics (Padua, Italy). Each primer pair was tested for efficiency and specificity. The efficiency was established with the standard curve method (data not shown), while the specificity was verified by sequencing the fragments obtained with the amplification of *A. halleri* and *A. thaliana* cDNAs. The list of primers and their target genes are shown in Table 1. The RNAs extracted from the leaves of *A. halleri* and of *A. thaliana* were analyzed by means of Quantitative Reverse Transcriptase-PCR (RT-qPCR) as previously described (Gullì *et al*., 2018). The data were analyzed with the $2^{-\Delta\Delta Ct}$ method, using *tubulin* as housekeeping gene and the control samples (without Zn or Cd) as calibrators (Livak and Schmittgen 2001). The RT-qPCR data are presented as the mean values calculated from three technical replicates from two separate biological experiments.

2.6 | Statistical analyses

Statistical analyses were carried out by means of SPSS 21.0.0 software for Windows (© Copyright IBM Corporation 1989, 2012), while Zn and Cd contents, gene expression variation, and comet assay results were evaluated by means of Student's *t* test.

3 | RESULTS

3.1 | Zn and Cd content in leaf tissues

As for Zn, *A. halleri* PL22 and I16 populations were grown in hydroponic solution in control (0.7 μM Zn), or Zn treatment (100 μM Zn) conditions for 2 weeks. I16 displayed higher amounts of Zn than PL22, in high-Zn conditions. The leaves of PL22 showed an average Zn content equal to 1,140 and 1,683 μg g^{-1} of tissue dry weight (DW) in control and treated samples, respectively, while leaves of I16 accumulated an average Zn content equal to 1,070 μg g^{-1} DW in control plants while the average Zn content in treated plant reached 10,535 μg g^{-1} DW. *A. thaliana*, the non-metal accumulator species grown in hydroponic solution in control (0.7 μM Zn) or sub-toxic treatment (5 μM Zn) conditions for 1 week, showed markedly lower Zn accumulation than the two *A. halleri* populations, with an average Zn content equal to 84 and 115 μg g^{-1} DW, respectively (Table 2). No visible symptoms of toxicity were noticed in any plants in all the conditions tested.

TABLE 1 Genes analyzed in this study and primers utilized for RT-qPCR

Gene name	Accession number (At)	Accession number (Ah)	Function	Primer sequences 5′-3′	Amplicon length (bp)
DRM2	AT5G14620	GFUL01023011	DNA (cytosine-5) methyltransferase DRM2	F-CAGATGGGCTTTTCAGACGAG R-GAAGCTCCGAAACGATTGCT	152
HDA8	AT1G08460	GFUL01018416	Histone deacetylase 8	F-TATTGGAGAAGCACCCGGAG R-GAAGCTCCGAAACGATTGCT	123
IBM1	AT3G07610	GFUL01019866	Histone H3mK9 demethylation activity, RNA directed DNA methylation	F-TGCCGAACAAGATCTCAAGG R-TGTCCCAAAGAGCTCCATCA	125
MET1 (At)	AT5G49160		DNA (cytosine-5)-methyltransferase 1	F-CACAACGGTTGGAAGGGACT R-CTGCAAACTCGTAGCTATCCG	108
MET1 (Ah)		GFUL01025078	DNA (cytosine-5)-methyltransferase 1	F-AGCCTCTCTATCGACAAGCC R-CCACAAAATAGATGGCCGGG	181
KYP	AT5G13960	GFUL01012674	Histone-lysine *N*-methyltransferase, (H3 lysine-9 specific)	F-CTCTGCAAAGAAGGGTTGGG R-ACCTTGCATTGTCTGTTGGC	160
VIM1	AT1G57820	GFUL01031384	Methylcytosine-binding protein	F-CTCAGTGGCAACAAAAGGAC R-TTGTAGACCTGACAACTCG	102
TUB	NM_100360	GFUL010929527	Tubulin alpha-4 chain	F-TAAAGACGTGAACGCAGCTGTT R-TGAATCCAGTAGGACACCAGT	80

Note: The following information is given for each primer pair: target gene name, accession number of *Arabidopsis thaliana* (At) or *Arabidopsis halleri* (Ah), protein function, primer sequence, and amplicon length (bp).

TABLE 2 Zn and Cd content in leaves in *Arabidopsis halleri* and *Arabidopsis thaliana*

Species	Growth condition	Sample	Zn μg g^{-1} DW (mean ± *SD*)	Growth condition	Sample	Cd μg g^{-1} DW (mean ± *SD*)
A. thaliana	0.7 μM Zn	At	84 ± 1	−Cd	At	n.d.
	5 μM Zn	At +Zn	115 ± 1**	5 μM Cd	At +Cd	332 ± 2
A. halleri I16	0.7 μM Zn	Ah I16	1,070 ± 23	−Cd	Ah I16	n.d.
	100 μM Zn	Ah I16 +Zn	10,535 ± 66***	50 μM Cd	Ah I16 +Cd (2)	1,604 ± 8
A. halleri PL22	0.7 μM Zn	Ah PL22	1,140 ± 17	−Cd	Ah PL22	n.d.
	100 μM Zn	Ah PL22 +Zn	1,683 ± 13**	50 μM Cd	Ah PL22 +Cd (2)	2,149 ± 11

Note: Asterisks correspond to statistically different values (Student's *t* test, $^{**}p \leq .01$; $^{***}p \leq .001$).
Abbreviation: n.d., not detectable.

Regarding Cd, PL22 and I16 were grown in hydroponic solution in control (absence of Cd) or treatment (50 μM Cd) conditions for 2 weeks. PL22 displayed higher Cd accumulation in shoots than I16, showing an average Cd content of 2,149 μg g^{-1} and 1,604 μg g^{-1} DW, respectively (Table 1). *A. thaliana* grown in hydroponic solution in control (absence of Cd) or treatment (5 μM Cd) conditions for 5 days showed markedly lower Cd accumulation than the hyperaccumulator *A. halleri*, with an average Cd content of 332 μg g^{-1} DW in the leaves (Table 2). While no visible symptoms of chlorosis or toxicity were observed in PL22 and I16 plants, *A. thaliana* treated with Cd showed initial symptoms of chlorosis in the leaves after 5 days' treatment.

3.2 | Zn effects on nucleoids

The alterations in the structure of the DNA molecule of leaf cell nuclei of *A. thaliana* and *A. halleri* (I16 and PL22) plants grown in a Zn-rich environment were analyzed by means of the alkaline comet assay (Figure 1). The integrity/relaxation of the DNA from free nucleoids was analyzed using the tail moment parameter, which takes into consideration both the length of the electrophoretic migration and the percentage of DNA migrated into the comet's tail. Zn treatment induces a mild reduction in the tail moment in all the species tested: *A. thaliana* (Figure 1a–c), I16 (Figure 1d–f), and PL22 populations (Figure 1g–i). In particular, the DNA nucleoids derived from plants grown in the presence of 100 μM $ZnSO_4$ appear a little more condensed than the ones derived from plants grown with less $ZnSO_4$ (0.7 μM $ZnSO_4$). In general, Zn induces no significant variation in DNA structure, in both the nontolerant and the hyperaccumulator species.

3.3 | Cd effects on nucleoids

A. thaliana and *A. halleri* (I16 and PL22) plants, untreated controls, and 5 μM- and 50 μM- $CdSO_4$ treated respectively, were also subjected to leaf nuclei extraction, and the integrity of the DNA molecules was analyzed by means of the alkaline comet assay. The data reported in Figure 2 show the contrasting behavior of the nontolerant and the hyperaccumulator species following the Cd treatment. An increase in

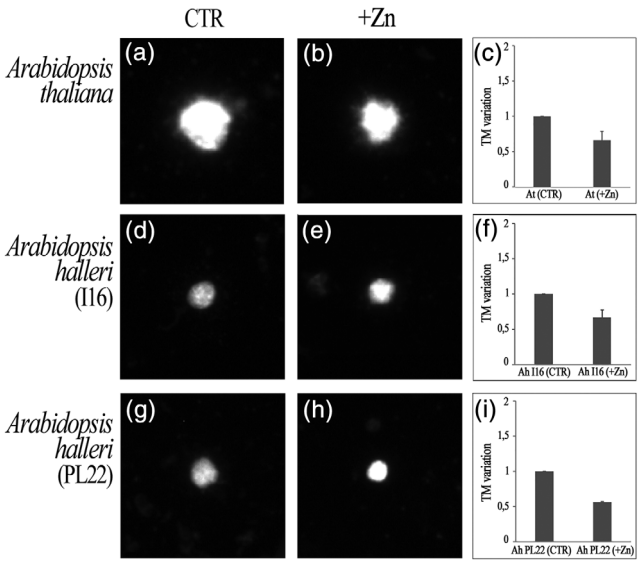

FIGURE 1 Alkaline comet assay on *A. thaliana* and *A. halleri* leaf nuclei (I16, PL22). (a,d,g). Comet images of nucleoids extracted from leaves of untreated *A. thaliana* and *A. halleri* (I16, PL22; (b,e,h) comet images of nucleoids extracted from leaves of 5 μM Zn-treated *A. thaliana* and 100 μM Zn *A. halleri* (I16, PL22); (c,f,i) average mean variation (±*SE*) of Tail Moment (TM) (50 nuclei/duplicate slide). CTR, plant grown in standard hydroponic solution; +Zn, plant grown in the presence of 5 μM for *A. thaliana*, or 100 μM $ZnSO_4$ for I16 and PL22; Ah, *Arabidopsis halleri*; At, *Arabidopsis thaliana*. Images were captured at 200×

DNA migration was observed in *A. thaliana* after Cd treatment, in comparison with the untreated control (Figure 2a–c). Cd-treated *A. halleri* populations showed major condensation of the DNA molecules (Figure 2d–i). This datum appears to be significant for PL22 only ($p < .01$) (Figure 2i).

3.4 | Zn and Cd effects on GpG DNA methylation

The methy-sens comet assay was performed on I16 and PL22 treated with Zn and Cd to investigate the possible role of CpG methylation on nucleus condensation.

As far as Zn treated samples is concerned, a different behavior was observed after methy-sens comet assay, in the two *A. halleri* populations. I16, showed an increase of methylation percentage while PL22 showed an opposite trend due to Zn treatment (Figure S1).

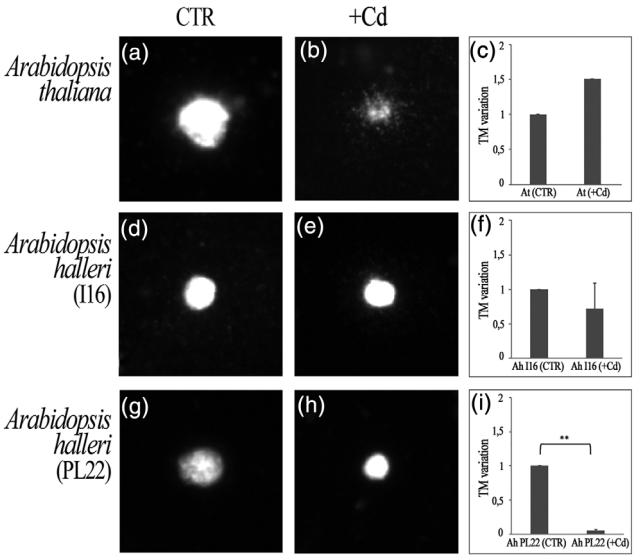

FIGURE 2 Alkaline comet assay on *A. thaliana* and *A. halleri* leaf nuclei (I16, PL22). (a,d,g). Comet images of nucleoids extracted from leaves of untreated *A. thaliana* and *A. halleri* (I16, PL22); (b,d,f) comet images of nucleoids extracted from leaves of 5 μM Cd-treated *A. thaliana* and 50 μM Cd-treated *A. halleri* (I16, PL22); (c,f,i) average mean variation (±*SE*) of Tail Moment (TM) (50 nuclei/duplicate slide). Significantly different values are marked with asterisks, (**p <.01). CTR, plant grown in standard hydroponic solution; +Cd = plant grown in the presence of 5 μM for *A. thaliana* or 50 μM $CdSO_4$ for I16 and PL22; Ah, *Arabidopsis halleri*; At, *Arabidopsis thaliana*. Images were captured at 200×

As far as Cd is concerned a decrease in the methylation percentage was observed in I16 (Figure 3a). On the contrary, an important increase in methylation was detected in PL22 (Figure 3b–d). This data may be related to the significant condensation of the DNA molecule observed with the comet assay (Figure 2i).

DNA modifications induced by $ZnSO_4$ and $CdSO_4$ treatment, could include epigenetic modifications, as variations in the amount of methylated DNA.

3.5 | Gene expression analysis

The expression of genes involved in epigenetic modifications was evaluated in all samples of *A. halleri* and *A. thaliana* grown in control condition and in the presence of Zn or Cd. In particular, the target genes involved in chromatin modification were: *MET1*, involved in CpG DNA methylation (Chan *et al*., 2005); *DRM2*, involved in RNA-dependent DNA methylation (Chan *et al*., 2005); and *VIM1*, a methyl-cytosine-binding protein (Furner and Matzke 2011). We also selected a group of genes involved in histone modifications, namely: *KYP*, a histone H3 lysine 9 methyltransferase; *IBM1*, a histone H3 lysine 9 demethylases; and *HDA8*, a histone deacetylase (Pandey *et al*., 2002) (Table 1).

In plants treated with Zn, the selected genes showed moderate variations in response to the metal treatment (Figure 4a). However, most of the genes showed a different behavior in *A. halleri* I16 respect to PL22. *MET1* and *IBM1* were down-regulated in both I16 and *A. thaliana* differently from PL22 in which they showed no variation in response to Zn (p <.05). *DRM2* was down-regulated in *A. halleri* I16 and up-regulated in PL22 and *A. thaliana* (p <.01). *VIM1* was down-regulated in *A. thaliana* differently from I16 (p <.05). *HDA8* was down-regulated in both I16 and *A. thaliana*, while in PL22 it was

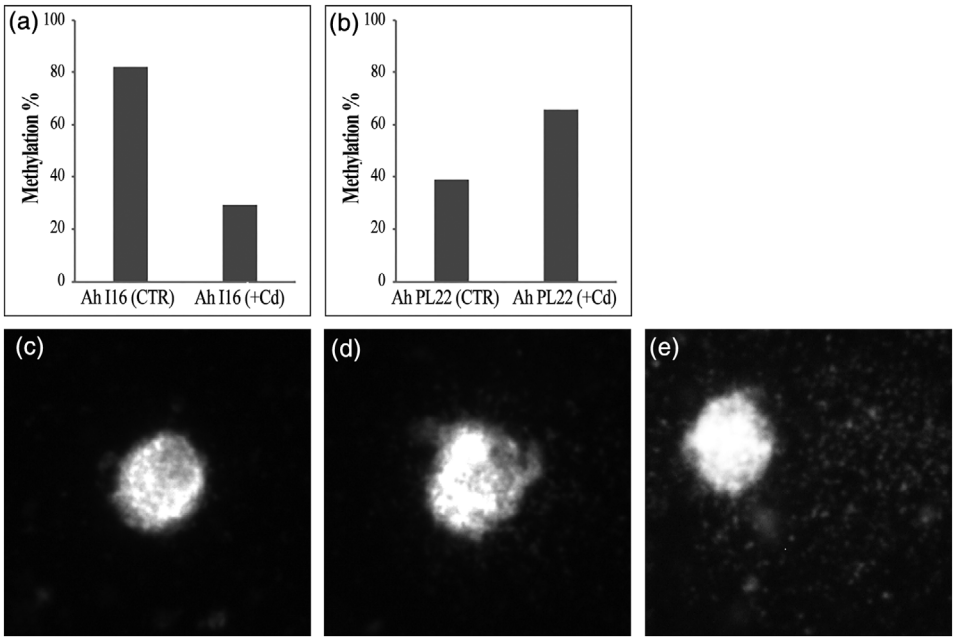

FIGURE 3 Methy-sens comet assay on nucleoids from leaves of *A. halleri* I16 (a) or PL22 (b) untreated or treated for 2 weeks with 50 μM $CdSO_4$. (a,b) Percentage of methylation after $CdSO_4$ treatment assessed through enzymatic digestions (100 − (TI%[*Hpa*II]/TI% [*Msp*I] * 100)); *A. halleri* PL22 treated with 50 μM $CdSO_4$: examples of the undigested (c), digested with *Hpa*II (d), or *Msp*I (e) nucleoids. CTR, plant grown in standard hydroponic solution; +Cd, plant grown in the presence of 50 μM $CdSO_4$; Ah, *Arabidopsis halleri*. Images were captured at 400×

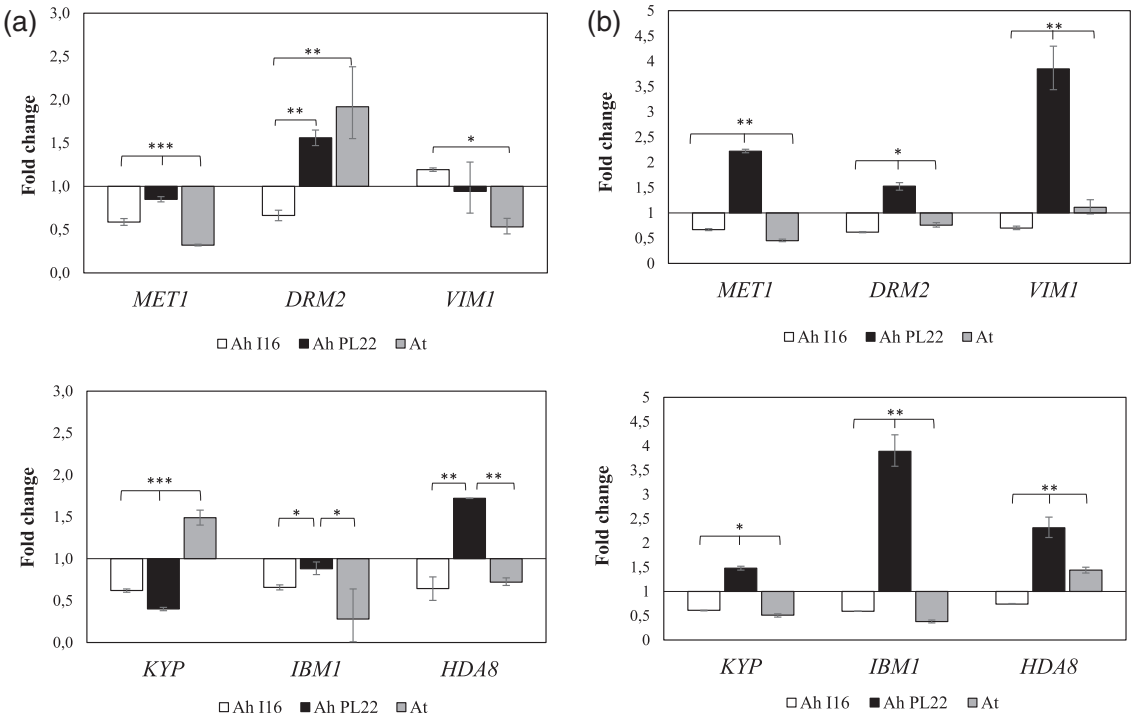

FIGURE 4 Expression analysis of genes involved in DNA and histone alterations in *A. thaliana*, *A. halleri* I16 and PL22 populations. Each gene's relative expression (Fold Change) was measured in: (a) leaves of *A. thaliana* treated with 5 μM Zn for 7 days, and in leaves of *A. halleri* populations I16 and PL22 treated with 100 μM Zn for 2 weeks; (b) leaves of *A. thaliana* treated with 5 μM Cd for 5 days, and in leaves of *A. halleri* populations I16 and PL22 treated with 50 μM Cd for 2 weeks. Average mean values (±*SD*) were derived from two biological replicates. All values are normalized on the corresponding values from control samples taken from *A. thaliana* and *A. halleri* I16 and PL22 plants grown without metals. Asterisks correspond to statistically different values (Student's *t* test, **p* ≤.05, ***p* ≤.01, ****p* ≤.001). Ah, *Arabidopsis halleri*; At, *Arabidopsis thaliana*

up-regulated (p <.01). The only exception is *KYP* that was down-regulated in both *A. halleri* populations, and up-regulated in *A. thaliana* (p >.001) (Figure 4a).

In plants treated with Cd, all the tested genes were up-regulated in PL22. In particular, the expression of *MET1* (FC 2.22), *VIM1* (FC 3.85), *IBM1* (FC 3.89), and *HDA8* (FC 2.31) was significantly different from I16 and *A. thaliana* (p <.01) (Figure 4b). The variation in the expression of *DRM2* and *KYP* (FC 1.5) is lower than that observed for the other genes, but significantly different in PL22 compared to I16 and *A. thaliana* (p <.05).

4 | DISCUSSION

In recent decades there has been increasing evidence of the important role of epigenetic modifications in the adaptation of plants to environmental stressful conditions (Kinoshita and Seki 2014; Lamke and Baurle 2017). Moreover, little information has been reported to show how Zn- and Cd-induced DNA and protein methylation regulate Zn and Cd accumulation and tolerance in plants: Fan *et al*. (2020) showed that DNA demethylation enhances plant tolerance to Cd toxicity in *A. thaliana*, while Serre *et al*. (2020) pointed out differences in the Lys methylation of nonhistone proteins in two Cd-sensitive species, *A. thaliana* and *A. lyrata*, and in three populations of *A. halleri* AU, I16 and PL22 with contrasting Cd accumulation and tolerance.

We adopted the comet assay in a previous work to compare the toxicity effects of Ni on leaf cell nuclei in the Ni hyperaccumulator *N. caerulescens* and the nontolerant *A. thaliana*. We observed that upon Ni treatment the hyperaccumulator species showed a more compact nuclear structure, in comparison with the nontolerant species, which suggested the existence of a defense mechanism preventing Ni-induced DNA damage in the hyperaccumulator species (Gullì *et al*., 2018).

In this work we applied a similar procedure to compare I16 and PL22 metallicolous populations of *A. halleri* belonging to distant genetic units in Europe. The methy-sens comet assay was performed for the first time on plant tissues too, in order to correlate differences in nucleoid structure with possible variations in DNA methylation in I16 and PL22 subjected to metal stress. The data obtained were supported by the expression analyses performed on some genes involved in DNA and histone modifications.

Data regarding the higher Zn accumulation in leaf tissues of I16 compared to PL22 (Table 2) were in accordance with previous works which highlighted the contrasting Zn accumulation of the two populations (Corso *et al*., 2018; Schvartzman *et al*., 2018). Differences between I16 and PL22 were also found in gene expression following Zn treatment, suggesting that these populations developed distinct mechanisms to adapt themselves to high Zn in soils (Meyer *et al*., 2010; Schvartzman *et al*., 2018). In our work we showed that Zn treatment induced a mild condensation of nucleoids

and variations in the amount of methylated DNA in both I16 and PL22. Even though no significant difference in the expression of epigenetic marks was observed in all the plants exposed to Zn treatment, in I16 it was observed, through the methy-sens comet assay, an increase in CpG methylation between nuclei from treated and nontreated plants, concomitantly PL22 nuclei showed a decrease in methylation after Zn treatment. This observation reinforces the results previously reported (Schvartzman *et al*., 2018) that the PL22 shows greater sensitivity to modifications in the external Zn concentration respect to I16. Schvartzman *et al*. (2018) reported that at high Zn concentration PL22 displays iron deficiency in root and shoots with an upregulation of various transcription factors linked to Fe deficiency response and in oxidative cell damage protection, compared to I16 population.

Data regarding Cd content in the leaves of the two *A. halleri* populations are in accordance with previous studies showing different behaviors of PL22 and I16: both populations are hypertolerant to Cd but only PL22 hyperaccumulate Cd both *in situ* and when grown in hydroponic condition (Meyer *et al*., 2015; Corso *et al*., 2018). Nucleoids of PL22 plants were significantly more condensed upon Cd treatment while nucleoids of I16 were similar in both Cd-treated and nontreated samples (Figure 2). An opposite behavior was observed in the nonmetal tolerant *A. thaliana*, in which serious damage to nuclei occurred when Cd penetrated the leaves and reached the limit of toxicity for a nonaccumulator species (Wang *et al*., 2016; Cui *et al*., 2017). Interestingly, also in this case, the nucleoids of the two metallicolous populations showed contrasting DNA methylation proportion following Cd treatment: PL22 showed higher global CpG DNA methylation upon Cd treatment than in control conditions, while I16 showed the opposite trend, as assessed by the methy-sens comet (Figure 3). These data are in accordance with the expression analyses performed on genes linked to DNA methylation and histone alteration processes in the two populations (Figure 4). *MET1*, which is a DNA methyltransferase which catalyzes methylation at CpG dinucleotides and is responsible for maintaining a pattern of symmetric methylation during DNA replication (Kankel *et al*., 2003), showed a two-fold increase upon Cd treatment in PL22. No variation instead was found in either I16 or *A. thaliana*. This datum is reinforced by the four-fold increase of *VIM* expression, encoding the methyl cytosine binding protein, a prerequisite for CpG DNA methylation in the centromeric regions of PL22 Cd-treated plants (Woo *et al*., 2008). Besides CpG methylation, the nucleoid condensation which occurred in PL22 only upon Cd treatment could also be ascribed to variations in the expression of genes coding for enzymes linked to histone modifications. Indeed, a two-fold increase in *HDA8* gene transcript was observed in PL22 Cd-treated plants. This gene encodes for a histone deacetylase related to the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3, and H4), acting in concert with *MET1* in inducing chromatin condensation and remodeling (Pandey *et al*., 2002).

DRM2 and *KYP* were also up-regulated in PL22 exposed to Cd, although not at significant levels. *DRM2* is linked to DNA methylation processes in the nonsymmetric CHH context, which is targeted by small RNAs (Cao and Jacobsen 2002). *KYP* catalyzes methylation of H3K9me2 and induces chromomethylase 3 (*CMT3*) to perform methylation of CHG trinucleotides in transposons (Saze *et al*., 2008; Inagaki *et al*., 2010). Besides the limited induction of these genes in response to Cd treatment in PL22, which could denote few movements in transposon and smRNA, nonsymmetric methylation might also occur in transcribed genes upon abiotic stress in plants (Furner and Matzke 2011). Indeed, we found a four-fold increase in the expression level of gene coding for histone demethylase *IBM1* in PL22 Cd-treated samples. This enzyme has a dual function: on the one hand it is involved in removing the H3K9me2 modifications enacted by *CMT3-KYP*, thereby protecting the transcribed genes from silencing; on the other hand, it has a role in transposon silencing (Saze *et al*., 2008; Fan *et al*., 2012). Considering the data from the methy-sens comet assay and the expression analysis on genes linked to CpG DNA methylation and histone alterations, we can suppose that PL22 responds to high Cd concentration in shoot tissues by condensing the structure of its nuclear DNA. In a recent work it was demonstrated that the total ascorbic acid content, and the ratio of reduced and oxidized glutathione, both of which indicate the cellular redox state and antioxidant capacity, were significantly higher in shoots in PL22 than in I16, thereby indicating an important role as antioxidant defense for PL22 in the adaptation to metallicolous sites (Corso *et al*., 2018). Besides the high antioxidant capacity in PL22, we observed an enhanced nucleoid condensation in PL22 after Cd treatment, which could limit the possible negative action of reactive oxygen species and Cd itself on DNA, and allow better adaptation of this population to Cd-contaminated soils.

In conclusion, the analysis of genetic and epigenetic alterations could be a good approach to assess the toxicity of environmental pollutants to plants and animal organisms. We have demonstrated the applicability of the comet assay and the methy-sens comet assay in this field, in particular to evaluate differences between metal-tolerant and hyperaccumulator and nontolerant species. Our data suggest a possible defense role of epigenetic modifications in tolerant and hyperaccumulator species under stress conditions, such as heavy metal accumulation. Finally, the differences between PL22 and I16 observed in nuclear structure and in the expression of genes coding for DNA methylation and histone modifications, after metals' treatment, could reinforce previous suggestions of divergent strategies for metals detoxification developing in PL22 and I16 metallicolous populations.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Giovanna Visioli, Mariolina Gullì, and Annamaria Buschini conceived and designed the experiments. Serena Galati, Giovanna Visioli and Gianluigi Giannelli carried out the experiments. Rosaria Fragni performed chemical analyses. Serena Galati, Giovanna Visioli, Mariolina Gullì, and Annamaria Buschini analyzed the data. Giovanna Visioli, Mariolina Gullì, and Annamaria Buschini contributed reagents/materials/analytical tools. Giovanna Visioli wrote the first draft of the article. Antonella Furini and Giovanni DalCorso contributed by improving the *Introduction* and *Discussion* sections. All authors contributed to interpreting and discussing the results, and read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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