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Comparative epigenomic and transcriptomic analysis of *Populus* roots under excess Zn

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ABSTRACT

Epigenetic modifications of chromatin structure are extremely important in mediating stress responses in plants. Epigenetic modifications are especially important in perennial species such as trees, where they contribute to phenotypic plasticity and adaptation to unfavourable environments. *Populus* is a model for physiological studies of trees, and is suitable for phytoremediation of Zn-contaminated soils. Currently, epigenetic modifications involved in Zn stress response are poorly characterized for *Populus*. Here, we compared changes in epigenetic modifications under excess Zn using chromatin immunoprecipitation sequencing (Chip-Seq) for two histone modifications associated with highly expressed genes (H3K4me3) and repressed genes (H3K27me3) in roots of *Populus* × *canadensis* I-214. Chip-Seq data were integrated with RNA-Seq transcript abundance data to examine how epigenetic modifications affect gene expression. These analyses showed that genes with a H3K4me3 modification are generally high-expressed, while genes with a H3K27me3 modification on the 5′-UTR are mainly low-expressed. H3K4me3 modifications in roots under excess Zn condition were enriched in genes involved in carbon (C) catabolism, nitrogen (N) metabolism, and in regulation of sub-cellular vesicular trafficking. These results are consistent with Zn redistribution at a sub-cellular level to buffer Zn-induced nutrient imbalance and osmotic stress in Zn-stressed roots. In contrast H3K27me3 modifications were enriched primarily in genes involved in photosynthetic processes. Together our results provide a useful resource for understanding epigenetic modifications in response to excess Zn in *Populus* roots, and constitute a starting point for the identification of epigenetic markers and improving phytoremediation potential in this species.

1. Introduction

Plants exposed to biotic or abiotic stresses activate several mechanisms to minimize the effects of stress, including modification of gene expression and protein synthesis, and alteration of metabolic pathways and physiological processes (Grativol et al., 2012). In the last ten years it has become clear that dynamic changes in chromatin structure, as well as the bio-genesis of small RNAs, contribute to transcriptional and post-transcriptional regulation of gene expression leading to stress responses (Borsani et al., 2005; Madlung and Comai, 2004). In plants and other organisms the state of chromatin can be modified rapidly and reversibly through the co-operation of different mechanisms including DNA cytosine methylation, post-translational modification of the N-terminal histone tails, or smallRNA-mediated mechanisms, which guide chromatin condensation (Grativol et al., 2012). These modifications, which alter DNA activity without changing its basic nucleotide structure, are referred as epigenetic changes (Madlung and Comai, 2004) and, although reversible, could be heritable both in mitotic and meiotic cell divisions (Grativol et al., 2012).

With the development of Next Generation Sequencing (NGS) technologies several novel approaches have been developed for analysis of whole-genome epigenetic modifications (Metzker, 2010). In particular for the profiling of whole genome histone modifications, chromatin immunoprecipitation (ChIP) has been coupled with deep-sequencing. This approach, named ChIP-seq, allows the identification, with base pair resolution, of the genomic location of a DNA-binding protein of interest (Park, 2009).

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However, understanding histone modifications and their relationship in the regulation of gene expression could be complicated by the high complexity and diversity of histone post-translation modifications and variants that contribute to determining distinct chromatin structures and DNA accessibility (Berger, 2007).

Comprehensive analysis of 12 epigenetic modifications in Arabidopsis identified four main chromatin states relative to active genes, repressed genes, silent repeat elements and intergenic regions (Roudier et al., 2011). This study identify also the histone H3K4 tri-methylation (H3K4me3) as a marker for high expressed genes, instead H3K27 tri-methylation (H3K27me3) for low expressed genes. These findings allowed a more straightforward integration of gene expression and ChIP-seq data, advancing the knowledge of the relationship between epigenetic modifications and gene expression in Arabidopsis and other plants species.

On the other hand few ChIP-seq experiments has been performed on plants exposed to stress conditions, even though this approach could be extremely useful for understanding plant adaptation to unfavourable conditions. To date, the few studies examining plant histone profiling in response to abiotic stress were the detection of H3K4me3 modifications in Arabidopsis and rice under drought stress. Both these studies confirmed the role of H3K4me3 as marker for high expressed genes, even though the inter-relationship between histone modification and differential expression seemed to be more complex than previously thought (Dijk et al., 2010; Zong et al., 2013).

Epigenetic modifications have been proposed to be fundamental in long living plants with complex life cycles such as trees, and they probably contribute to phenotypic variations and plasticity of these plants, but also in adaptation to different environments. Moreover, exposure to stress conditions could create epigenetic heritable traits that can be transmitted to the progeny as 'memory', giving adaptive advantages in the colonization of different environments (Bräutigam et al., 2013).

Among trees the genus Populus has been widely accepted as the model plant for studying tree-specific traits such as secondary growth, perennial growth and specialized adaptation to environmental changes (Jansson and Douglas, 2007). Populus trichocarpa was the first woody species with a sequenced genome (Tuskan et al., 2006) and several high-throughput molecular approaches have been developed for studying tree-specific physiological processes (Wullschleger et al., 2013). However, few studies have addressed the role of epigenetic modification in stress response and phenotypic variability in poplar, and none using ChIP-seq technology. In particular, epigenetic re-programming has been associated with differences in biomass production and drought response in several poplar clones (Gourcilleau et al., 2010), and seems also to play a major role in the regulation of transcription during stress response (Raj et al., 2011).

Populus, being a high-biomass yielding and fast growing tree, has been also used in phytoremediation programs of heavy metal polluted soils, and for the study of plant response to heavy metals (Ali et al., 2013; Sebastiani et al., 2014, 2004). The response to excess Zn, an essential micronutrient that could become toxic at high concentrations (Broadley et al., 2007), has been extensively studied in the hybrid poplar *Populus x canadensis* I-214 clone at physiological, anatomical and molecular level in different organs (Ariani et al., 2015; Di Baccio et al., 2011, 2009, 2003; Romeo et al., 2014a,b). However, little is known about epigenetic re-programming in response to excess Zn in root tissues.

Here, we reports the first ChIP-seq profiling of *Populus* × *canadensis* I-214 clone in response to excess Zn using two different antibodies recognizing histone modifications that have been previously showed to identify high expressed genes (H3K4me3) and low expressed genes (H3K4me3) in *Arabidopsis* (Roudier et al., 2011). We show how the genome-wide histone distribution changes in response to excess Zn and, in turn, gene expression. We also developed an interactive web application where researchers can explore and download the datasets produced in the current study.

2. Materials and methods

2.1. Plant materials, growth parameters and Zn content analysis

Populus × *canadensis* I-214 clone was used for all the experiments in this study. Woody cuttings were grown in plastic pots containing Agrileica clay (Laterlite, Milano, Italy) in a controlled climate chamber under 16/8 h light/dark condition at 23/18 °C day/night temperature and 70% relative humidity. Plant growth and Zn screening were performed in a hydroponic system using the following Zn treatments: (i) basic Hoagland's solution containing 1 μM Zn (corresponding to 0.065 ppm, i.e., the control); (ii) basic Hoagland's solution containing 1 mM Zn (65 ppm) as previously described in Romeo et al. (2014a). For each plant all organs/parts were sampled separately and analyzed. The total concentration of Zn was determined after digestion in concentrated nitric acid (HNO_3) by atomic absorption spectrophotometry (model 373; PerkinElmer, Norwalk, CT, USA).

For ChIP-seq and RNAseq analysis, roots of 1 μM Zn and 1 mM Zn treated plants were sampled and immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Chromatin immunoprecipitation, ChIP-seq and RNA-seq library preparation

Chromatin cross-linking was performed on frozen and ground roots as described by (Luo et al., 2012) with minor modifications. Briefly, 1 g of ground samples was incubated in 10 ml of nuclear isolation buffer (10 mM Hepes pH = 7.6, 1 M Sucrose, 5 mM KCl and 5 mM $MgCl_2$) supplemented with 1% formaldehyde, 0.1% β-mercaptoethanol, 0.6% Triton X-100 and 0.4 mM PMSF for 10 min in ice and briefly vortexed every 2–3 min. The cross-linking reaction was quenched by adding glycine at a final concentration of 0.13 M and incubating the solution for 5 min on ice with gentle shaking. The solution containing the nuclei and cellular debris was filtered through one layer of Miracloth (Calbiochem) and nuclei were collected by centrifugation at 3000*g* for 10 min at 4 °C and the pellet was suspended in 300 μl of nuclear isolation buffer. After adding 500 μl of cold nuclear separation buffer (10 mM Hepes pH = 7.6, 1 M Sucrose, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA pH = 8.0 and 15% Percoll) to the resuspended nuclei, the nuclei were centrifuged at 3000*g* × 5 min at 4 °C. The nuclei were lysed with 600 μl nuclear lysis buffer (1.5 mM Tris-HCl pH = 7.5, 1% Triton X-100, 150 mM NaCl and 1 mM EDTA) by vortexing and the chromatin was sonicated 20 times with 10 s pulse for obtaining chromatin fragment in the range 100–500 bp. The fragmented chromatin was then immuno-precipitated with ChIP-IT kit (Active Motif, CA, USA) using an antibody against H3K4me3 (Millipore, 07-473) and H3K27me3 (Millipore, 07-449) overnight at 4 °C following the manufacturer's instruction. A control sample was similarly processed without adding antibody in the immunoprecipitation reaction. After immunoprecipitation DNA was de-cross-linked and purified with Agencourt AMPure XP beads (Beckman Coulter, CA, USA) and used for ChIP-Seq library preparation.

For each Zn treatment two libraries from two different biological replicates, and one input library from a non-ChIP'd sample, were prepared. Chip-sequencing libraries were prepared with Illumina ChIP-Seq DNA sample prep kit (Illumina, CA, USA) according to manufacturer's instruction. Multiplexed DNA libraries were loaded in a single flow cell and sequenced at 50 bp single-end set-up with Illumina HiSeq2000 platform at the QB3 Genome

Sequencing Laboratory at the University of California, Berkeley, CA, USA.

RNA sequencing libraries were prepared from total RNA using Illumina TruSeq RNA Sample prep kit (Illumina, CA, USA) according to manufacturer's instruction. For each Zn treatments two libraries, from the same biological replicates used for the Chip-seq, were prepared. Multiplexed libraries were loaded in a single flow cell and sequenced at 50 bp single-end set-up with Illumina HiSeq2000 platform at the DNA technology core in the UC Davis genome center. Raw sequencing reads have been deposited in the NCBI Sequence Reads Archive (http://www.ncbi.nlm.nih.gov/sra) under the accession SRP071234, Bioproject PRJNA313970.

2.3. Sequencing data analysis

All the sequencing data analyses were performed in the Atmosphere cloud-based platform of the iPlant cyberinfrastructure (Goff et al., 2011). Sequencing reads quality were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and raw reads were processed using fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Sequencing reads of ChIP-sequencing libraries were trimmed to 35 bp, based on fastQC quality plot, and aligned with SOAP1 (Li et al., 2008) to the *Populus trichocarpa* v3 genome sequence (www.phytozome.net). The alignment parameters were as follows: a maximum of 5 mismatch per reads (-v 5), a maximum gap length of 2 (-g 2), and an iterative 5 bp trimming and re-alignment in the 3′ end of the reads if no hits were found (-c 55). After the alignment only uniquely mapped reads were considered for downstream analysis. Peak calling was performed using BayesPeak package implemented in R language (Spyrou et al., 2009) using control, non immuno-precipitated, DNA as input and only peaks with a posterior probability ≥ 0.95 were considered for further analysis. The distance between the identified peaks and the nearest annotated feature of the genome were evaluated with BEDtools (Quinlan and Hall, 2010). The genes with a peak in the gene sequence (exon/intron/UTRs) and/or in 1 kb flanking regions, and identified in both biological replicates have been considered for further comparison with RNA-seq data in the same clone. The same analysis parameters (i.e. the presence of peak in the 1 kb up-stream region in both biological replicates) were applied for genes with an up-stream H3K27me3 peak.

FastQC analysis of raw RNA-Seq reads showed a constantly high quality over all the sequence, thus the raw reads were directly aligned to the *Populus trichocarpa* v3 genome sequence (www.phytozome.net) using TopHat2 (Kim et al., 2013) with a maximum of 5 mismatches, a maximum gap length of 3 and a maximum of 1 mismatch in the anchor region of a spliced alignment. Each transcript was assigned a read coverage by counting the number of reads overlapping the gene sequence. Only genes with a minimum of 10 reads mapping to them in both biological replicates were considered as expressed in further analyses. The expression datasets were then analyzed separately for each condition. The expression levels were assigned to 3 different classes according to a computed z-score based on reads count (similar as those described in Malone et al., 2011). Briefly, for each single library the raw read counts were $\log_{10}$ transformed, then the mean of the $\log_{10}$ transformed reads count in each library were subtracted from each value and divided by the standard deviation of the $\log_{10}$ read counts. The resulting z-score distribution has a mean of 0 and a standard deviation of 1. The genes were then divided in the subsequent classes: (i) high expressed genes, z-score > 0.5; (ii) medium expressed genes, $-0.5 <$ z-score ≤ 0.5; (iii) low expressed genes, z-score ≤ 0.5. Each expression class had a similar percentage of genes in each expression dataset (data not shown). The genes identified by the ChIP-seq experiment were then compared to these expression classes using a hyper-geometric test, implemented in R, to identify over-representation of genes identified by one of the two histone marks within one of the defined expression classes.

2.4. Gene ontology enrichment analysis

Gene Ontology (GO) enrichment were performed on the genes identified as containing a chromatin mark by ChIP-seq and significant over-represented in particular expression classes (as determined by hyper-geometric test) in both expression datasets. For this analysis Fisher's exact Test was used within Blast2go (Conesa et al., 2005) and only GO terms with a false discovery rate p-value (FDR) < 0.05 were used for data interpretation.

As a background the GO mapping on *P. trichocarpa* genome, available on AgriGO (http://bioinfo.cau.edu.cn/agriGO/), was used. Since the GO analysis utilizes an older version of the *Populus* genome (v2.2) the older IDs were converted to v3 using the synonyms file available on Phytozome (www.phytozome.net). In addition, the genes identified by the ChIP-sequencing experiments that were not already annotated in the background file were manually annotated with blast2go (Conesa et al., 2005) and included in the background file for GO enrichment analysis. For H3K4me3 modified genes GO enrichment was determined for genes belonging to the high and middle expression group, while for the 5′-UTR H3K27me3 modified genes the analysis were performed on genes belonging to low expression group. For interpretation of epigenetic changes induced by Zn in *Populus* roots, enriched GO terms presents only in treatment condition in the same expression class were taken into account. These GO terms were summarized and visualized using REVIGO (Supek et al., 2011).

2.5. Development of web application

The web application of the data produced in this study was created with the shiny package of R (http://shiny.rstudio.com/). The application was developed for allowing researchers to interactively explore and download the data produced in the current study. For this purpose the genes identified by ChIP-Seq analysis were divided into three categories: H3K4me3, H3K27me3 and H3k27me3-upstream (i.e. the genes with a H3K27me3 peak in the 5′-UTR). For each ChIP-Seq category the users can visualize the genes identified in control and/or treatment conditions, and filter these genes by expression classes. Annotation of the different genes are included in the application output.

2.6. Statistical analysis

All the statistical analyses were performed in the R statistical environment (www.r-project.org). Chi-square test were performed with 1000 replicates of simulated p-values using Monte Carlo chain.

3. Results

3.1. Growth parameters and mineral analysis

The plants used in this experiment were from a screening of Zn response of different *P*opulus genotypes, and were also used in a proteomic study of roots of *P.* × *canadensis* I-214 clone in response to excess Zn (Romeo et al., 2014a,b). Zn stress caused an increase of dry biomass and branching in roots of *P.* × *canadensis* I-214 clone, and an almost 10-fold increase of Zn accumulation in this organ (Table 1).

3.2. Analysis of aligned reads and peak calling in ChIP-seq

In initial experiments here we used ChIP-sequencing (ChIP-seq) to assay the frequency of two histone marks associated with genes

Table 1
Summary of phenotypic parameters and Zn accumulation in roots of *P.* × *euramericana* I-214 clone used in this study. Mean and standard deviations are shown. T-test *p* values are shown. DW: dry weight. Data from Romeo et al. (2014a,b).

	Zn		
	1 μM	1 mM	t-test
Roots DW (g)	1.9 ± 0.34	2.8 ± 0.25	*
Roots Zn (mg Kg^{-1})	312.4 ± 107.0	3029.7 ± 544.4	***
Roots branching (branch/cm)	2.6 ± 0.58	4.5 ± 0.67	*

in *Populus* root treated with Zn, versus controls (Methods). Since the two histone marks assayed here, H3K27me3 and H3K4me3, should be associated primarily with gene sequences, the number of reads in each replicate of ChIPed DNA aligning to gene sequences and 1 kb flanking regions was computed. These results were compared to the number of reads for un-ChIP'd total DNA control "input" using Chi-square test with 1000 Monte Carlo simulation of p-value to identify ChIP-seq peaks. A significant ($P < 0.01$) higher percentage (~1–6%) of reads aligned to gene sequences compared to input DNA, except for one treatment replicate for H3K27me3 (Supplementary data S1).

In the H3K4me3 ChIP'd DNA the two biological replicates of the control samples identify a total of 18038 peaks, with 5665 peaks falling in gene sequences or in the flanking 1 kb region. A total of 5120 genes were identified by the two replicates, with 775 genes identified by both replicates. The genes identified by both replicates in the control H3K4me3 ChIP'd DNA, with their relative expression classes, are shown in (Supplementary data S2). The maximum peak width for the two biological replicates were 3551 and 2451 bp. The median peak width was 301 bp for all replicates, with a standard deviation of 216 and 197 bp (Supplementary data S3). In the H3K4me3 ChIPed DNA the two biological replicates of the treated samples identified a total of 24440 peaks, with 9355 peaks falling in gene sequences or in the flanking 1 kb region. A total of 6935 genes were identified by both replicates, with 1676 genes identified by both biological replicates. The genes identified by both replicates in the treated H3K4me3 ChIPed DNA, with their relative expression class, are shown in Supplemental Data S2. The maximum peak width for the two biological replicates were 4101 and 4301 bp. The median peak width was 301 bp for the replicates, with a standard deviation of 206 and 197 bp (Supplementary data S3).

In the H3K27me3 ChIP'd DNA the two biological replicates of the control samples identify a total of 14014 peaks, with 4305 peaks falling in gene sequences or in the flanking 1 kb region. There were 3422 genes identified by the two replicates, with 586 genes identified by both replicates. The genes identified by both replicates in the control H3K27me3 ChIP'd DNA, with their relative expression class, are shown in Supplemental data S2. The maximum peak width for each biological replicates were 2951 and 5501 bp. The median peak width was 301 bp for each replicates, with a standard deviation of 206 and 256 bp (Supplementary data S3).

In the H3K27me3 ChIPed DNA the two biological replicates of the treated samples identify a total of 13116 peaks, with 3630 peaks falling in gene sequences or in the flanking 1 kb region. The genes identified by the two replicates are 2929, with 418 genes identified by both biological replicates. The genes identified by both replicates in the treated H3K27 me3 ChIPed DNA, with their relative expression class, are shown in Supplemental Data S2. The maximum peak width for each biological replicates were 2051 and 5601 bp. The median peak width was 301 bp for each replicates, with a standard deviation of 184 and 273 bp (Supplementary data S3).

Since Malone et al. (2011) showed a correlation between a low gene expression and an up-stream H3K27me3 peaks the same analysis were performed considering only the 5′ up-stream sequence of each gene. This analysis identified a total of 999

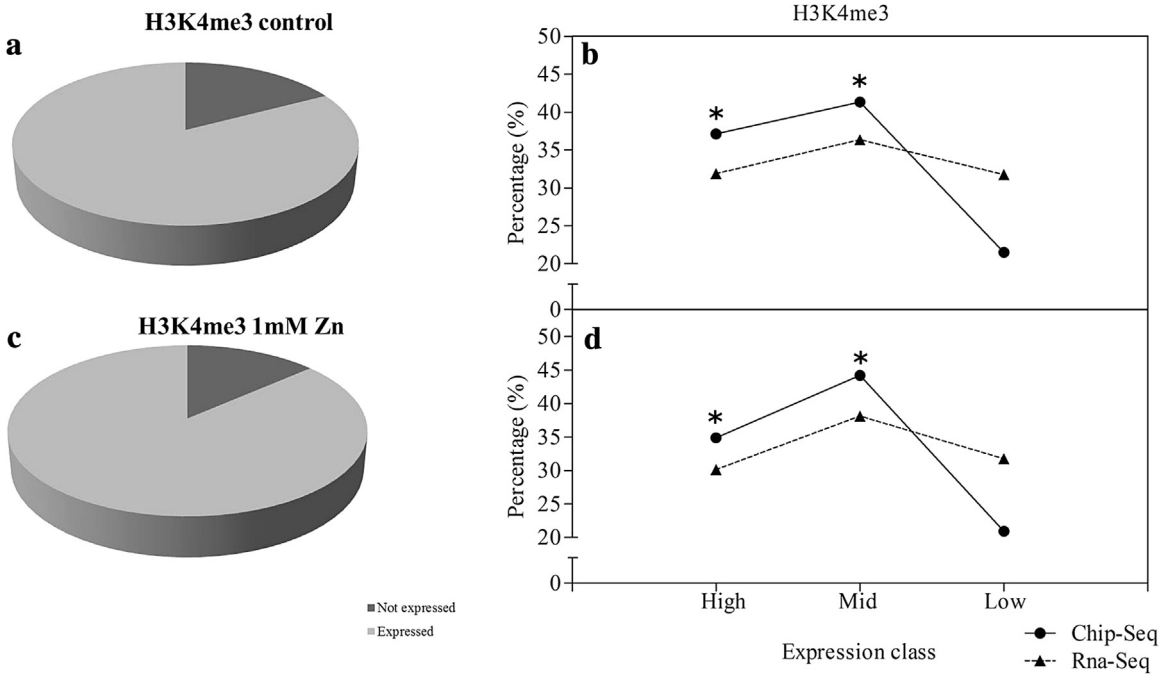

Fig. 1. Comparison of H3K4me3 marked genes with expression level. (a–b) Control condition. a) Percentage of expressed versus not expressed genes among the H3K4me3 marked genes. b) Comparison of the percentage of genes in each expression class between the RNA-seq and H3K4me3 ChIP-seq experiment. (c–d) Zn treatment condition. c) Percentage of expressed versus not expressed genes among the H3K4me3 marked genes. d) Comparison of the percentage of genes in each expression class between the RNA-seq and H3K4me3 ChIP experiment. * represents significant enrichment based on hyper-geometric test. Legend are shown at the bottom of the image and are grouped for (a–c) and (b–d) panels.

peaks with 133 genes identified in control sample; instead treatment sample identify 839 peaks, with 102 genes having a peak in up-stream sequence. The genes with an up-stream H3K27me3 peak, with their relative expression class, are shown in Supplementary data S2.

3.3. Comparison between RNA-seq and ChIP-seq

The ChIP-sequencing results were compared with RNA-Seq expression levels in the different Zn treatments to evaluate how epigenetic modifications influence gene expression in *Populus* roots. Expression levels were discretized in three groups as described in the Materials and Methods. RNAseq data showed the expression of 28910 genes in control condition in root tissue, of which 9215 (32%) were in the high, 10510 (36%) in the middle and 9185 (32%) in the low expression group. In excess Zn condition 26168 genes were expressed based on RNAseq analysis, of which 7887 (30%) are in the high, 9972 (38%) in the middle and 8309 (32%) in the low expression group.

By considering all the genes H3K4me3 modified in control condition (775), 134 (17%) of the ChIPed genes were not expressed in the RNA-seq in the same condition (Fig. 1a). Among the expressed and H3K4me3 modified genes 37%, 41% and 22% were in the high, middle and low expression group, respectively (Fig. 1b). Hyper-geometric test highlighted a significant relationship between a high ($P = 0.002$) and middle ($P = 0.004$) expression, and a H3K4me3 peak in genes or 1 kb flanking sequence.

In excess Zn conditions 228 (13,6%) out of 1676 genes identified as H3K4me3 ChIPed were not expressed (Fig. 1c). Among the expressed, and H3K4me3 modified genes, 35%, 44% and 21% were in the high, middle and low expression group, respectively (Fig. 1d). Hyper-geometric test on the expressed genes showed a significant relationship between a high ($P < 0.001$) and middle ($P < 0.001$) expression and a H3K4me3 peak in a gene. These results suggest that H3K4me3 marked mainly active genes, without a dependence of the position of the peaks.

Among all the genes with a H3K27me3 peak in control condition (586) only 149 (25.5%) of the ChIPed genes identified were not expressed. Among the expressed genes 33.6%, 41.4% and 25% of the ChIPed genes were in the high, middle and low expression group, respectively. Hyper-geometric test showed a significant correlation ($P = 0.01$) between a middle expression and a H3K27me3 peak (data not shown). Among the genes H3K27me3 ChIPed in excess Zn condition (418) only 105 (25%) of the ChIPed genes were not expressed. Among the expressed genes 31.3%, 40% and 28.7% of the expressed and ChIPed genes were in the high, middle and low expression group, respectively. Hyper-geometric test did not show any significant relationship between the expression levels and H3K27me3 peak (data not shown). These findings suggest a possible position-dependent effect of H3K27me3 peaks on gene expression, as described in Malone et al. (2011).

By analyzing only the genes with a H3K27me3 peak in their up-stream sequence in control condition (133), 53% of these genes (71) were not expressed (Fig. 2a), and between them 24% were in the high, 29% in the middle, and 47% in the low expression group (Fig. 2b). Hyper-geometric test highlighted a significant relationship between an up-stream H3K27me3 modification and transcriptional repression ($P < 0.001$), when compared with all the annotated genes of *P. trichocarpa,* or low expression ($P = 0.005$), when compared with all the expressed genes.

In the H3K27me3-ChIPed DNA in the treated samples we identified 102 genes, with 43% (44) of them not expressed (Fig. 2c). Among the 58 expressed genes 24% were in the high, 17% in the middle, and 59% in the low expression group (Fig. 2d). Hyper-geometric test highlight a significant relation between an up-stream H3K27me3 modification and a low expression ($P < 0.001$).

The genes identified by ChIP-Seq and RNA-Seq analysis in the different Zn treatments, as well as their expression classes and

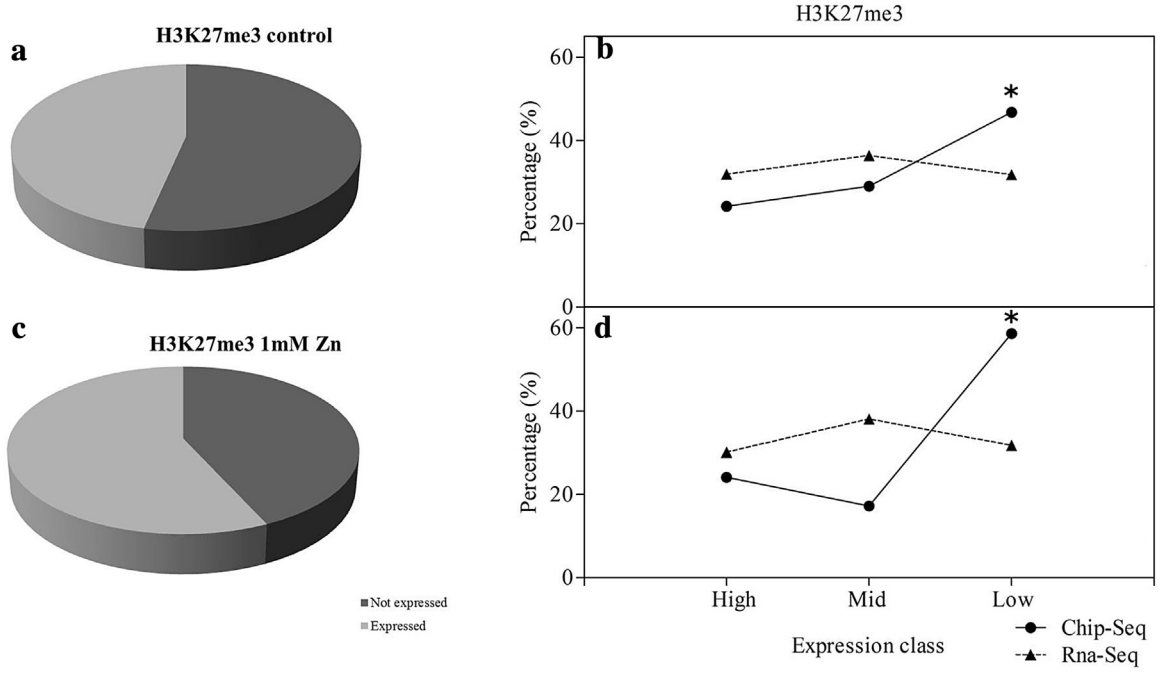

Fig. 2. Comparison of H3K27me3 up-stream marked genes with expression level. (a–b) Control condition. a) Percentage of expressed versus non expressed genes among the H3K27me3 marked genes. b) Comparison of the percentage of genes in each expression class between the RNA-seq and H3K27me3 ChIP-seq experiment. (c–d) Zn treatment condition. c) Percentage of expressed versus non expressed genes among the H3K27me3 marked genes. d) Comparison of the percentage of genes in each expression class between the RNA-seq and H3K27me3 ChIP-seq experiment. * represents significant enrichment based on hyper-geometric test. Legend are shown at the bottom of the image and are grouped for (a–c) and (b–d) panels.

annotations, are freely available for interactive data exploration and download at https://github.com/aariani/PoplarRootZn-dbSearch.

3.4. Gene ontology enrichment analysis

For identifying which biological patterns were modified epigenetically in response to excess Zn we performed Gene Ontology (GO) enrichment analysis on the genes identified as ChIPed by the different histone modifications, and significantly over-represented in a particular expression class (as determined by hyper-geometric test) in both expression datasets.

In the H3K4me3 dataset, GO enrichment analysis of the genes within the high expression group identified three GO enriched in control condition, and 26 GO in treatment condition (Table 2, Supplemental data S4). Instead, GO enrichment analysis of the genes of the middle expression group identified 56 GO enriched in treatment condition, without any enriched GO term in control condition (Table 2, Supplementary data S4). In total the GO terms identified specifically in the Zn treated condition were 26 for the high expressed genes, and 56 for the middle expressed genes.

REVIGO analysis of the GO specifically enriched in treatment condition for high expressed genes identified eight main clusters for Biological processes, four for Cellular component and two for Molecular function ontologies. The most representative clusters were cellular amino acid biosynthesis and protein transport for Biological processes, cell part and nucleus for Cellular component, and binding for Molecular function (Fig. 3). The genes identified in the most representative clusters, together with their annotations, are shown in Supplementary data S5.

The same GO analysis showed enrichment in the Zn treatment for middle expressed genes for six main clusters for Biological Processes, four for Cellular Component and eight for Molecular Function ontologies (Fig. 4). The most representative clusters were carbohydrate catabolism for Biological processes, cell part for Cellular component, and carbohydrate derivative binding for Molecular function. The genes identified in the most representative clusters, together with their annotations, are shown in Supplementary data S6.

In the H3K27me3 dataset, GO enrichment analysis of the genes with an up-stream peak and within the low expression group identified eight enriched GO in control condition, and 65 GO in the Zn treatment condition (Table 2, Supplemental data S4). In total the GO terms identified specifically in treatment conditions were 57. REVIGO analysis of this GO terms identified seven main clusters for Biological processes, five for Cellular component and one for Molecular function ontologies (Fig. 5). The most representative clusters were nucleobase metabolism and photosynthesis for Biological process, chloroplast part for Cellular component, and RNA polymerase activity for Molecular function. The genes identified in the most representative clusters, together with their annotations, are shown in Supplementary data S7.

4. Discussion

Dynamic modifications of chromatin states, and their effects on gene expression, are extremely important in multicellular organisms for a proper regulation of cell cycle, cell identity, and developmental processes (Cantone and Fisher, 2013; Lafos et al., 2011). These reversible modifications have been linked to regulation of biotic and abiotic stress response in plants (Grativol et al., 2012; Madlung and Comai, 2004). In addition, epigenetic variations could be crucial in long-living species like trees, by contributing to phenotypic plasticity and adaptive capacity of these plants (Bräutigam et al., 2013). Regarding *Populus*, epigenetic modifications have been related to differences in biomass production and drought response, probably by regulating transcriptome in response to stress (Gourcilleau et al., 2010; Raj et al., 2011).

In order to characterize chromatin modifications in response to excess Zn in poplar roots (*P.* × *canadensis* I-214 clone), we performed a ChIP-seq analysis using two different histone modification antibodies. These two histone modifications should identify high expressed genes (H3K4me3) and low expressed genes (H3K27me3), according to previous studies in *Arabidopsis* and rice (Dijk et al., 2010; Malone et al., 2011; Roudier et al., 2011; Zong et al., 2013). Since differential expression analysis with RNA-seq in roots of the same clone (with the same experimental design) were described previously (Ariani et al., 2015), we focused mainly on the genes and processes identified by ChIP-seq analysis in response to Zn excess. We integrated the ChIP-Seq results with RNA-seq expression analysis, to understand how chromatin modifications influence gene expression in these trees.

In a previous integrated analysis of 12 histone modifications, Roudier et al. (2011) identified H3K4me3 and H3K27me3 as markers for gene sequences in *Arabidopsis*. In our study, the comparison between the percentage of reads uniquely mapped to gene sequences (or the 1 kb flanking regions) in the ChIPed and input DNA showed a significant increase in ChIPed DNA for all the libraries except for one replicate of H3K27me3 histone in treatment condition. Even though the enrichment towards gene sequences was minimal, the cross-species reads mapping used in this study results in lower mapping efficiencies in *Populus* outside of coding regions (Liu et al., 2014). Regardless, these results suggest that H3K4me3 and H3K27me3 histone modifications mainly tag gene sequences in *Populus*.

The two histone modifications analyzed in this study were related to high or low expressed genes, according to current knowledge. Thus we integrated the genes identified by ChIP sequencing with expression analysis based on RNA-seq. For H3K4me3 the majority of genes identified by ChIP-seq were expressed in both control and Zn excess conditions. In particular, hyper-geometric test highlighted the enrichment of ChIPed genes for high and middle expression classes. These results suggest a conserved role for H3K4me3 histone modification on gene

Table 2
Summary of the number of enriched Gene Ontologies identified by the different ChIP-Seq datasets divided by expression classes.

		Gene Ontologies			
Sample	Expression group	Molecular Function	Biological Process	Cellular Component	Total
H3K4me3 Control	High	2	1	0	3
H3K4me3 Treat	High	2	16	8	26
H3K4me3 Control	Middle	0	0	0	0
H3K4me3 Treat	Middle	30	16	10	56
H3K27me3 Control	Low	0	0	8	8
H3K27me3 Treat	Low	2	19	44	65

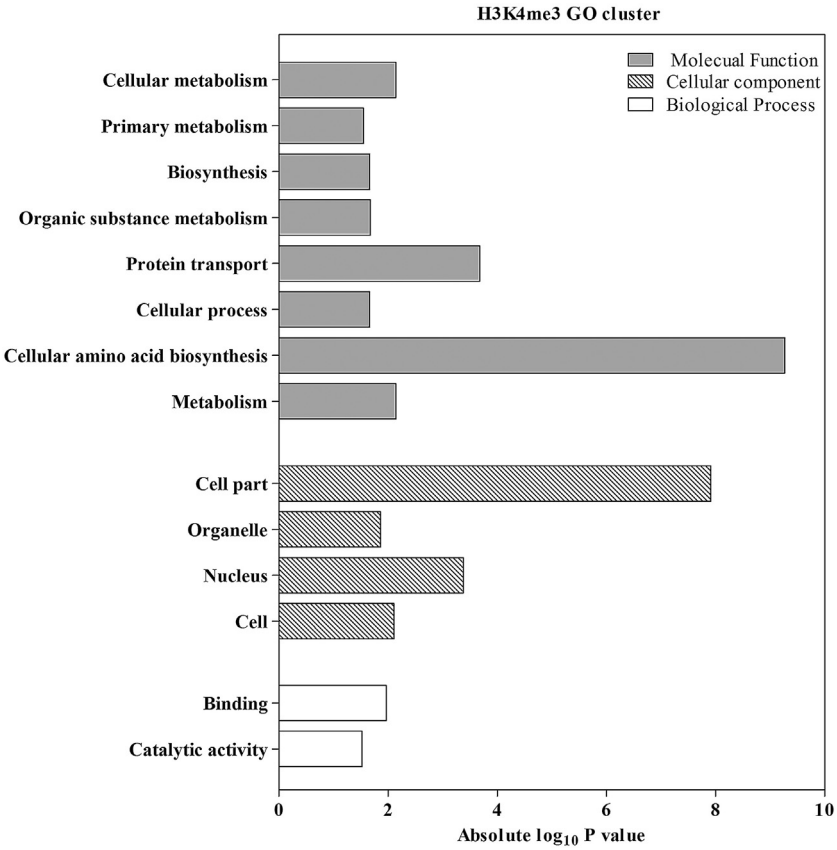

Fig. 3. Enriched Gene Ontology clusters of the H3K4me3 modified genes belonging to the high expression group identified under 1 mM Zn treatment conditions. The identified clusters are colored by Gene Ontology group. The $\log_{10}$ p values of the significance of enrichment are shown as barplots.

expression across different plant species, included *Populus*. For the H3K27me3 histone modification the presence of a peak in the up-stream sequence of a gene could determine its low expression or repression. The low expression was significant for both treatment conditions, while the repression was significant only in control condition. These results are in agreement with previous studies in *Arabidopsis* and rice (Malone et al., 2011; Roudier et al., 2011; Zhang et al., 2007; Zong et al., 2013), suggesting a conserved function for this histone modification across different plant species. The lack of significant association between gene repression and a H3K27me3 up-stream peak in excess Zn condition could be caused by tissue-specific variations for this epigenetic modification (Lafos et al., 2011), probably triggered by the stress condition applied in the experiment.

REVIGO analysis of the genes H3K4me3 modified within the middle and high expression groups identified cellular amino acid biosynthesis, protein transport, and carbohydrate catabolism as the most representative clusters of the enriched gene ontologies. These results could suggest that Zn stress causes an imbalance of nitrogen (N) and carbon (C) metabolisms in poplar roots. The regulation of the metabolism of this two elements is extremely important in plants for growth and developmental processes, and in response to several stresses (Zheng, 2009). An imbalance in the distribution and assimilation of these nutrients was reported in *P.* × *canadensis* I-214 clone in response to excess Zn. In particular, Zn stress caused a reduction of C assimilation (Di Baccio et al., 2009), and an increase of N content in roots (Romeo et al., 2014a). In addition, analysis of leaves transcriptome and roots proteome identified differential expression or variation in protein abundance for genes involved in both N and C metabolism as well (Di Baccio et al., 2011; Romeo et al., 2014b).

Among the genes related to cellular amino acid biosynthesis there were a glutamine synthase (Potri.007G069600) homolog of *Arabidopsis GSR1* (AT5G37600), and a glutamate synthase (Potri.006G038400) homolog of *Arabidopsis GLS1* (AT5G04140). Glutamine synthase (GS) and glutamate synthase (GLR) are essential enzymes in nitrogen assimilation and in amino acid metabolism (Brugière et al., 1999; Forde and Lea, 2007; Masclaux-Daubresse et al., 2006). In particular, GS is involved in ammonia accumulation by condensing this molecule with glutamate, while GLR convert glutamine in 2 molecules of glutamate (Masclaux-Daubresse et al., 2006).

These two enzymes, and their products, have been related to different abiotic stress in several plants. As example, proteomic studies in lettuce plants exposed to Zn or salt stress showed a significant increase of GS abundance (Lucini and Bernardo, 2015), while RNAi inhibition of this gene in transgenic tobacco plants caused a reduction of total prolines (Brugière et al., 1999), a proteinogenic amino acid accumulated in response to heavy metal stress in plants (Sharma and Dietz, 2006). Glutamate could be a precursor of proline (Forde and Lea, 2007), but it could function also as a signaling molecule in regulating root architecture (Forde, 2014; Forde et al., 2013). In particular, application of external glutamate caused a reduction in primary roots growth and an increase in root branching in *Arabidopsis* (Walch-Liu et al., 2006). Such modifications of root architecture in response to heavy metal stress seem to be conserved in plants (Potters et al., 2007), and were shown also in previous studies in *P.* × *canadensis* I-214 clone exposed to excess Zn (Romeo et al., 2014a,b). Thus, we could suggest that regulation of these two enzymes could be crucial in physiological adaptation to toxic concentration of Zn, in particular by modifying roots architecture.

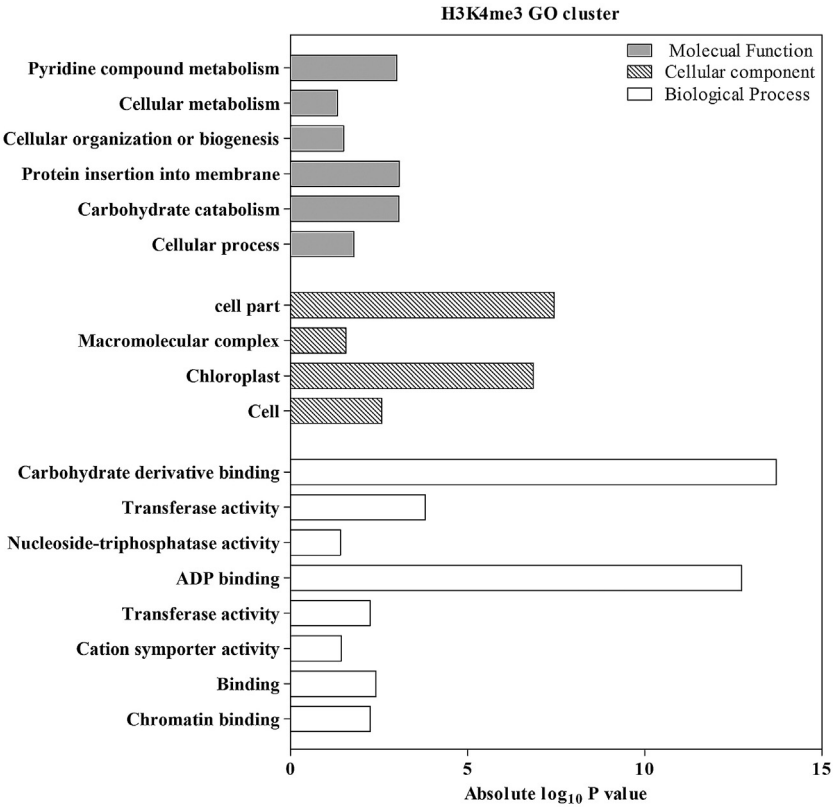

Fig. 4. Enriched Gene Ontology clusters of the H3K4me3 modified genes belonging to the middle expression group identified under 1 mM Zn treatment conditions. The identified clusters are colored by Gene Ontology group. The $\log_{10}$ p values of the significance of enrichment are shown as barplots.

In the same ontolology cluster there were several transcription factor-like (TF-like) genes, such as basic helix-loop-helix (bHLH), homeobox domain (HB), auxin responsive factor (ARF), *RAP2.4*, and basic-leucine zipper (bZIP) transcription factor. Of the two bHLH transcription factors identified, one (Potri.001G416600) is homologous of *Arabidopsis bHLH105* (AT5G54680). This transcription factor is involved in regulation of metal-ion mediated auxin sensing and metal homeostasis, but also in iron (Fe) deficiency response in *Arabidopsis* (Long et al., 2010; Rampey et al., 2006). The interaction between Fe and Zn homeostasis has been widely demonstrated in *Arabidopsis* (Sinclair and Krämer, 2012; Yang et al., 2010), and was suggested also by a previous transcriptome analysis in response to excess Zn in *Populus* (Ariani et al., 2015). In particular, characterization of *Arabidopsis* mutants for *bHLH105* showed the role of this gene in regulating lateral roots elongation and metal homeostasis through vacuolar membrane transporters (Rampey et al., 2006). We suggest that this *Populus* homologous of *bHLH105* (Potri.001G416600) could be involved in excess Zn response by regulating simultaneously vacuolar Zn sequestration and lateral roots formation. This possible double-function makes this transcription factor an interesting candidate for further studies, and also for possibly engineering metal stress response in poplar species.

Auxin is a key plant hormone involved in the regulation of plant growth, morphology, and lateral root formation by integrating endogenous and environmental signals (Lavenus et al., 2013). Variations in auxin homeostasis and distribution are triggered by several environmental stresses, including heavy metals (Wang et al., 2015; Yuan et al., 2013). The identification of three Auxin Response Factor (ARF) genes by ChIP-Seq analysis (Potri.009G011800, Potri.011G091900, and Potri.015G105300) could suggest a similar regulation of auxin homeostasis in *Populus* roots in response to excess Zn.

Between the genes identified by REVIGO as related to protein transport there were several genes involved in vesicular intracellular trafficking, like COPII-mediated transport, vacuole vesicular sorting, and regulation of Multivesicular Bodies (MVBs). The *Arabidopsis* homologous of some of the genes identified are involved in the Endosomal Sorting Complex Required for Transport (ESCRT), like Potri.001G034200 (homologous of *Arabidopsis ELC*), Potri.004G215500 (homologous of *VPS24*), and Potri.018G065100 (homologous of *VPS2*) (Katsiarimpa et al., 2013, 2011; Spitzer et al., 2006; Winter and Hauser, 2006). The ESCRT complex regulates protein sorting, cargo assembly and vesicles formation in the MVBs, and acts as a sub-cellular checkpoint between protein degradation, recycling and secretion (Winter and Hauser, 2006). Other two genes identified (Potri.001G307400, Potri.019G003100) are homologous of *Sec23/Sec24* protein-transport genes of *Arabidopsis*, a class of protein involved in COPII vesicles coat formation (De Craene et al., 2014). COPII is a protein complex specialized in transport between secreting organelles, in particular between the endoplasmic reticulum (ER) and the Golgi apparatus (Marti et al., 2010). These results suggest a finely regulation of sub-cellular localization and vesicles sorting in response to excess Zn, as observed in other plants in response to different environmental stresses (Boursiac et al., 2008; Chevalier and Chaumont, 2015; Levine, 2002; Takáč et al., 2013). Indeed, due to the sessile nature of plants, modifications of sub-cellular trafficking and protein localization/recycling could be the primary and most rapid adaptation mechanism for coping with a challenging environment (Levine, 2002). The genes identified could be also involved in the higher Zn accumulation in roots of I-214 clone, as observed by previous studies (Ariani et al., 2015; Romeo et al., 2014a,b).

Among the genes identified by REVIGO as related to carbohydrate catabolism there were two 6-phosphogluconate dehydrogenase family protein (6PGD) (Potri.001G211500 and

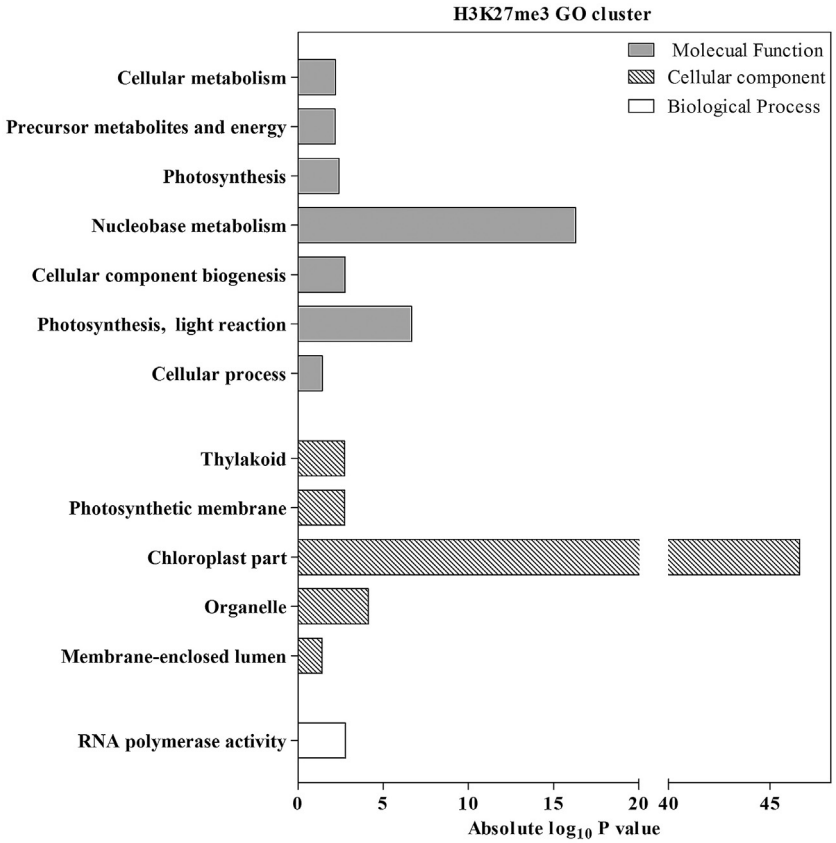

Fig. 5. Enriched Gene Ontology clusters of the H3K27me3 modified genes belonging to the low expression group identified under 1 mM Zn treatment conditions. The identified clusters are colored by Gene Ontology group. The $\log_{10}$ p values of the significance of enrichment are shown as barplots.

Potri.018G088000), two β-amylases (Potri.001G087600 and Potri.008G204200), one Pyruvate kinase (PK) (Potri.008G159700), and a Ribose 5-phosphate isomerase (RPI) (Potri.013G039400). 6-PGD and RPI belong to the oxidative pentose phosphate pathway (oxPPP), a central metabolic pathway essential in response to environmental stresses (Kruger and von Schaewen, 2003). This pathway is divided in two phases: the oxidative phase, where 6PGD is involved, and the non-oxidative phase, where RPI belongs. The oxidative phase is a major source of reducing NADPH for biosynthetic processes such as fatty acid synthesis and nitrogen assimilation (Neuhaus and Emes, 2000), and is essential for maintaining the cellular redox potential necessary for redox scavenging (Juhnke et al., 1996). Instead, the non-oxidative phase is a source of carbon skeletons for the synthesis of nucleotide, aromatic amino acid, phenylpropanoids and their derivatives (Herrmann and Weaver, 1999).

In yeast, mutation of 6PGD caused a higher sensitivity towards hydrogen peroxide in comparison to wild type (Juhnke et al., 1996), suggesting the essential role of this enzyme in maintaining the redox state of the cell. The identification of 6PGD as epigenetically modified during Zn stress could suggest a similar role in *Populus*, probably as a result of the oxidative bursts triggered by this metal. This result is in agreement also with previous transcriptomic and proteomic studies on this clone in response to excess Zn (Ariani et al., 2015; Romeo et al., 2014a,b). *Arabidopsis* mutants for RPI showed a pleiotropic effect on plant growth and development, with an increased cell death under above-normal temperature, probably as a results of an impaired oxPPP pathway and a reduced starch accumulation (Xiong et al., 2009). In addition, due to the positive effects of aromatic amino acid and phenylpropanoids in response to heavy metal (HM) stress in plants (Chen et al., 2014; Izbiańska et al., 2014), we suggest that the poplar homologous for RPI could be involved in response to excess Zn in poplar roots.

β-amylases are involved in starch break-down and maltose production in plants, and have been related to several abiotic stresses like osmotic, salt, cold, drought and heat stresses (Kaplan and Guy, 2004; Krasensky and Jonak, 2012; Prasch et al., 2015). The maltose accumulation attributed to these enzymes could function as osmolyte to maintain cellular turgor, and protect membrane and proteins from stress damage (Kaplan and Guy, 2004; Krasensky and Jonak, 2012). Since high Zn concentration could determine an osmotic-like stress in plants (Lucini and Bernardo, 2015), we suggest that epigenetic modifications of these two β-amylases could be a side-effect of Zn induced osmotic stress in poplar roots, and that these two enzymes probably have a protective role toward different abiotic stresses as well.

Pyruvate kinase (PK) catalyzes the conversion of phosphoenol-pyruvate and ADP to pyruvate and ATP, which is used in numerous metabolic pathways (Baud et al., 2007). Different studies showed that PKs are differentially regulated in response to HM stresses like Cr, Cd and Zn in several plants (Hossain and Komatsu, 2013; Lucini and Bernardo, 2015). Overall, epigenetic modifications of genes involved in carbon catabolism could suggests a re-routing of energy and carbon atoms to counteract excess Zn. This mechanism was observed in several plants using proteomic analysis, and it is supposed to be a physiological adaptation toward a reduced photosynthetic activity in leaves (Hossain and Komatsu, 2013). These results suggest a similar mechanism in *Populus* roots exposed to Zn excess. Previous high-throughput analysis of the same poplar clone under the same experimental condition clearly supported this hypothesis. Indeed, microarray analysis on leaves of I-214 clone showed a down-regulation of photosynthetic

machinery (Di Baccio et al., 2011), while the root proteome showed differential regulation of genes involved in carbon metabolism (Romeo et al., 2014b).

REVIGO analysis of the genes with an up-stream H3K27me3 modification and belonging to the low expression group identified nucleobase metabolism and photosynthesis as the most representative clusters of the enriched gene ontologies. The repression of genes involved in photosynthesis and light is expected in roots, even though photosynthetic activity was detected in aquatic adventitious roots in different plant species (Rich et al., 2012, 2008). Since we analyzed hydroponically-grown adventitious roots, a similar mechanisms could be expected in our experimental conditions. Among the genes identified by REVIGO and related to nucleobase metabolism there were three nuclear genes related to photosynthesis, like a ribulose-bisphosphate carboxylase (Potri. T005800), an ATP synthase (Potri.019G028500), and a NADH-Ubiquinone/plastoquinone (complex I) protein (Potri.013G138800). In particular, the last gene was related to variations in net photosynthesis in a bi-parental mapping population in *Populus* (Wang et al., 2014). The identification of genes involved in photosynthesis as repressed in adventitious roots of *Populus* could suggests the reliability of the data produced in our study and downstream analysis.

5. Conclusions

Populus constitute an interesting model for studying heavy metal stress response in plants, and also for possible phytoremediation applications in polluted soils. Previous studies of *P.* × *canadensis* I-214 clone in response to excess Zn highlighted the complexity of the response to high concentration of this metal. Indeed a wide array of physiological, molecular, biochemical, and anatomical modifications are activated in response to excess Zn treatment. In the current study, the application of ChIP-Seq and RNA-Seq in Zn-stressed roots of *P.* × *canadensis* I-214 confirmed the conserved role of these two histone modifications in regulating gene expression in plants, and highlighted several molecular mechanisms regulated in response to excess Zn in this species. The mechanisms identified as differentially regulated in response to excess Zn largely confirm the physiological and biochemical effects of heavy metal stress in this species. In particular, Zn stress caused a differential regulation of genes involved in Carbon and Nitrogen metabolism, and activated several genes related to sub-cellular vesicular trafficking, probably for finely redistribute excess Zn in different root compartments, and for rapid membrane turn-over. The genes related to N metabolism could be involved both to increased ammonia accumulation, but also to molecular signaling for modifying roots architecture in response to Zn stress. The genes related in carbon catabolism could be involved in regulating cellular redox state, and in the synthesis of stress-responsive molecules. In addition, they could buffer Zn-induced osmotic stress in roots, and the impaired photosynthetic machinery in leaves. These results provide a useful resource for understanding epigenetic modifications in response to excess Zn in *Populus* roots, and constitute a starting points for the identification of epigenetic markers for improving phytoremediation potential of this species.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.08.005.

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