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The development dynamics of the maize root transcriptome responsive to heavy metal Pb pollution

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ABSTRACT

Lead (Pb), as a heavy metal element, has become the most important metal pollutant of the environment. With allocating a relatively higher proportion of its biomass in roots, maize could be a potential important model to study the phytoremediation of Pb-contaminated soil. Here we analyzed the maize root transcriptome of inbred lines 9782 under heavy metal lead (Pb) pollution, which was identified as a non-hyperaccumulator for Pb in roots. In the present study, more than 98 millions reads were mapped to define gene structure and detect polymorphism, thereby to qualify transcript abundance along roots development under Pb treatment. A total of 17,707, 17,440, 16,998 and 16,586 genes were identified in maize roots at four developmental stages (0, 12 h, 24 h and 48 h) respectively and 2,825, 2,626, 2161 and 2260 stage-specifically expressed genes were also identified respectively. In addition, based on our RNA-Seq data, transcriptomic changes during maize root development responsive to Pb were investigated. A total of 384 differentially expressed genes (DEGs) ($\log2\text{Ratio} \geq 1$, $\text{FDR} \leq 0.001$) were identified, of which, 36 genes with significant alteration in expression were detected in four developmental stages; 12 DEGs were randomly selected and successful validated by qRT-PCR. Additionally, many transcription factor families might act as the important regulators at different developmental stages, such as bZIP, ERF and GARP et al. These results will expand our understanding of the complex molecular and cellular events in maize root development and provide a foundation for future study on root development in maize under heavy metal pollution and other cereal crops.

1. Introduction

Lead (Pb), as one of the most common pollutants, that has readily accumulated in soils of the environment [26], which would particulate accumulate and contaminate the soil from pipes, lead paint and residual emissions from leaded gasoline that we used [5]. In plants, it is reported that different plant species can uptake, transport and accumulate Pb in different soil type. Especially, Lead (Pb) is absorbed and accumulated with the highest amount in the root tissues than the other plant tissues [15,24].

Maize (*Zea mays* L.) is the most widely grown grain crop throughout the China, with a larger biomass than *Arabidopsis*, rice and most of other crops, which has a greater putative capacity of phytoextracting heavy metals from contaminated soil. Roots, as a important organs of plants, that could accumulate large amounts of lead (Pb), thereby causing a potential health risk by absorbing from polluted soils [8,19]. Considerable efforts have been reported to investigate Pb stress in roots, such as its accumulation, translocation, physiological and metabolic variations, and cell deposition [1,6,8,16]. Therefore, it is an important step towards being able to control the health risk in pollution condition by understanding the mechanism of Pb accumulation in plants roots. In our previous study, we combined the series Pb1000 treatment time at four developmental stages (0, 12 h, 24 h and 48 h), to investigate the

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dynamically transcriptional changes in response to heavy metal Pb stress in Maize revealed by RNAseq.

2. Materials and methods

2.1. Seed sterilization and experiment design

The seeds of maize (*Z. mays*) inbred line 9782 were sown on filter paper saturated with distilled water and incubated at 26 °C in the dark. Seedlings with uniform growth were selected after three days, transplanted into an aerated complete nutrient solution (see Table S1) and grow in growth chamber as follows: kept for 3 days with a photoperiod of 14 h light/10 h dark at 26 °C and a relative humidity of 70%. After that, the maize seedlings were randomly divided into two groups, CK—grown (C1A) seedlings, grown only in half-strength Hoagland solution, Pb1000—grown in CK + Pb1000 (1000 mg L−1 $Pb(NO_3)_2$) for Pb stress.

2.2. Superoxide dismutase (SOD) and peroxidase (POD) enzymatic activity assays

The seedlings for 6 h, 12 h, 24 h, 48 h, 72 h and 96 h were treated with 1000 mg L^{-1} of $Pb(NO_3)_2$ solution and then collected to conduct the SOD and POD enzyme activities screening, mock-treated seedlings were set as control (CK). According to the method described by Beyer and Fridovich (1987) [2] and Kim and Yoo (1996) [11], the SOD activity and Peroxidase activity were determined respectively, we repeated all the measurements for three times with three biological replicates.

2.3. Maize roots collection and RNA isolation

All samples from maize inbred line 9782 roots were cleaned and immediately frozen in liquid nitrogen for further study. Four mRNA libraries were constructed using RNA extracted from the CK- grown and Pb1000-grown maize roots at 12 h, 24 h and 48 h according to the results from POD and SOD assay, respectively. According to the manufacturer's instructions, total RNA samples were prepared as follows in three replicates: equal quantities of RNA isolated from roots were pooled for each stress stage using Trizol Reagent (Invitrogene, Nottingham, UK).

2.4. Calculation of RNA-Seq expression values

From approximately 5 μg of total RNA, mRNA was isolated, fragmented, converted to cDNA, and PCR amplified according to the Illumina RNA-Seq protocol (Illumina, Inc. San Diego, CA). Sequence reads were generated using the Illumina Genome Analyzer II (San Diego, CA) and Illumina HiSeq 2000 (San Diego, CA) at the Beijing Genomics institution (ShenZhen, China). Illumina barcodes were used to multiplex a portion of the samples. Sequence reads generated were between 35 and 101 bp single-end reads. We used the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) to clean reads prior to mapping. We removed the Illumina adapter sequences by the fastx_clipper program, with a minimum sequence length of 20 bp after clipping, in addition, we removed low quality bases from the ends of reads by the fastq_quality_-trimmer, requiring a minimum Phred score of 20 and a minimum length of 20 bp after trimming. RSeQC software were used to quality control of all the data of RNA-seq experiments [25]. Sequence reads for each tissue were mapped to v2 of the B73 reference pseudomolecules (http://ftp.maizesequence.org/) [18] using Bowtie version 0.12.7 [12] and the splice site aware aligner TopHat version 1.2.0 [22]. Fragments per kilobase pair of exon model per million fragments mapped (FPKM) was used to normalize gene expression values expressed and determined using Cufflinks version 0.9.3 [23]. The 5b annotation (http://ftp.maizesequence.org/) was provided as the reference annotation. After constructing the union of the transcripts, cuffmerge overlays the reference annotations and classifies all the assembled transcripts into different categories, further, cuffmerge can integrate reference transcripts into the merged assembly based on the maize reference genome annotation [23]. Cuffdiff is used to calculate the significance of observed changes with high expression in different samples in our research, and data from Cuffdiff analysis were managed, visualized and integrated by CummeRbund [23].

2.5. Confirmation of candidate differential expression genes (DGEs) by performing qRT-PCR

To validate the differential expression genes (DGEs) from RNA-seq, 12 DGEs were randomly selected from 36 common differentially expressed genes in four libraries and then subjected to quantitative real-time PCR analysis. Actin1 (GRMZM2G126010) was set as the endogenous control in this study. Primer5 software was used to design the corresponding primers, which are listed in (Table S2). According to the standard protocol of the ABI7500 system, the amplification programs were performed as follows: 95 °C for 30s; 95 °C for 5s, 60 °C for 30s, 40 cycles, and then generated the melt curves for verification of amplification specificity by a thermal denaturing step. We set all reactions in triplicate, as well as non-template controls. In addition, the threshold cycles (Ct) of each tested genes and the control products of Actin1 gene were averaged for triplicate reactions and normalized the values respectively. The $2^{-\triangle\triangle Ct}$ method were performed for statistical analysis [17].

2.6. Single nucleotide polymorphism diversity analysis

Bowtie version 0.12.7 [12] and TopHat version 1.2.0 [22] were used to map the cleaned RNA-seq reads to maize B73 5b pseudomolecules (http://ftp.maizesequence.org/) [18],with a minimum intron size of 5 bp and a maximum intron size of 60,000 bp respectively. After that, SAMtools version 0.1.7 were used to sort, index, and pileup the mapped unique reads [13] to generate unfiltered pileup files. Run mpileup and bcftools to call SNP and INDELs, Subsequently, we use the annovar software to annotate the SNP and indels.

2.7. GO annotation by parametric analysis of gene set enrichment (PAGE)

The Singular Enrichment Analysis (SEA) of agriGO were used to GO analysis and GO enrichment with Zea mays ssp V5a as reference background [4]. In addition, the P-value is adjusted by Hypergeometric tests with Benjamini and Hochberg FDRs with the default parameters in this study, moreover, based on differential gene expression levels (Log2FC), Parametric Analysis of Gene Set Enrichment (PAGE) [10] was also executed. MapMan [21] was also used to annotate the expression of genes onto metabolic pathways.

3. Results

To confirm the suitable treatment time for RNAseq, the enzymatic activities of POD and SOD were analyzed in the maize roots at a series of time points (6 h—96 h) with 1000 mg L^{-1} concentrations of Pb treatment (Fig. S1). The results showed that the SOD activities increased noticeably within 12 h, 24h and 48h of treatment with Pb, however, it is contrary to POD activities. After 48h, both POD and SOD activities showed a gradually decreasing at a constant level. In this research, four mRNA libraries were constructed using

Table 1
Summary of read numbers based on the RNA-Seq data from Maize root under Pb treatment.

	C1A	M2A	M3A	M4A
Total reads	25,452,458	24,828,108	24,750,366	23,044,164
Mapped reads	19,562,150	19,171,257	19,248,546	17,813,916
	76.86%	77.22%	77.77%	77.30%
Multi-position match	1,866,828	1,761,227	2,268,676	2,028,618
	7.33%	9.19%	9.17%	8.80%
Unique match	17,695,322	17,410,030	16,979,870	15,785,298
	69.53%	68.03%	68.60%	68.50%
Unmapped reads	5,890,308	5,656,851	5,501,820	5,230,248
	23.14%	22.68%	22.23%	22.70%
SNP numbers	27,036	27956	29,100	27,609
	0.138%	0.146%	0.151%	0.155%

RNA extracted from the CK- grown and Pb1000-grown maize roots at 12 h, 24h and 48 h, as well as control, according to the results from POD and SOD assay, respectively.

To investigate the expression patterns of genes in maize roots, in this study, we used Illumina Hiseq 2000 platform for sequencing the RNA extracted from the roots and generating sequence reads, including CK sample (C1A), Pb1000 treat for 12h (M2A), Pb1000 treat for 24h (M3A), Pb1000 treat for 48h (M4A) samples. An overview of the sequencing is outlined in Table 1. A total of 25,452,458 (C1A), 24,828,108 (M2A), 24,750,366 (M3A) and 23,044,164 (M4A) were generated by mapping the sequenced reads to the genome respectively, 19,562,150, 19,171,257, 19,248,546 and 17,813,916 high-quality clean reads (almost 77% of the raw data) were remained in C1A, M2A, M3A and M4A respectively after removal of adaptor sequences, duplication sequences, ambiguous reads and low-quality reads. In the CK sample (C1A) and Pb1000 treat for 12h (M2A), Pb1000 treat for 24 h (M3A), Pb1000 treat for 48h (M4A) samples, 68.0%, 68.6%, and 68.5% of the total reads (72 million) from RNA-seq data were mapped uniquely to the genome respectively, only small proportions were mapped multiply to genome. Using RNA-seq, we were able to identify 27,036, 27,956, 29,100 and 27,609 high confidence SNP polymorphisms in these four different groups respectively (Table 1). Interestingly, most of SNPs distributed in Chr 1 and Chr 5 of maize chromosome (Fig. 1A), in addition, the occurrence of SNPs is higher in exons than others, followed by UTR3 and intron, only few SNPs were checked in upstream and downstream (Fig. 1B). Moreover, we found that most of SNPs were checked in synonymous SNV (11680), comparing with that in non-synonymous SNV (4677), 308 and 263 were found in frameshift deletion and frameshift insertion respectively (Fig. 1C).

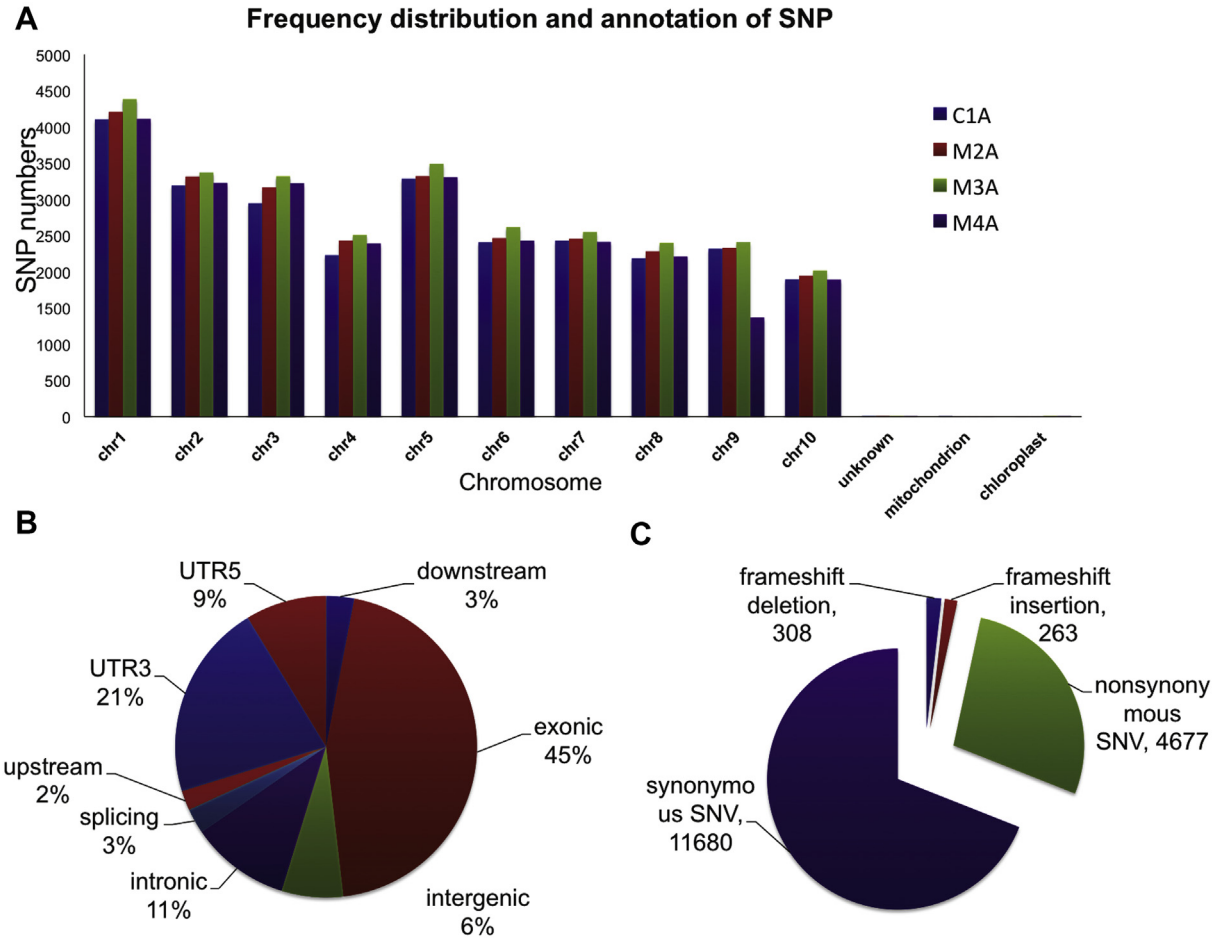

Fig. 1. SNP identified by RNASeq responsive to Pb1000 treatment. Bowtie version 0.12.7 and TopHat version 1.2.0 were used to map the reads against the 5b pseudomolecules (http://ftp.maizesequence.org/) with a unique hit to identify SNP. Based on the 5b annotation (http://ftp.maizesequence.org/), Gene assignment was determined and all identified SNPs were assigned to gene model. (A). Distribution of the number of single nucleotide polymorphisms (SNPs) in maize chromosome. (B). SNPs identified were assigned to gene model. (C). SNPs identified were assigned to synonymous SNV, non-synonymous SNV, frameshift deletion and frameshift insertion.

3.1. Defining the maize root transcriptome

The genic distribution of reads from mRNA-seq was showed as follows: most of them (81%) belong to protein coding genes, followed by known transposable elements (12%), intergenic regions, the splice junctions (3%) and introns (1%) (Fig. 2A). To estimate expressed genes throughout the maize roots responsive to Pb treatment, we detected the successive of gene expression from C1A to M4A: M4A (16,586) < M3A (16,998) < M2A (17,440), as well as mock treat (C1A) (17,707). Of which, 5248 genes are expressed in all four sampled stages of maize roots (Fig. 2B). To check the distribution of reads throughout the body of the transcript, the distribution of reads is plotted relative to cDNA ends in this study (Fig. 2C). Of the splice junctions, contained almost 84% of known and 8% complete novel junctions (Fig. 2D).

To obtain statistical confirmation of the differences in gene expression among the four stages, FPKM is used to normalize the expression level of genes. A hierarchical clustering map of the differentially expressed genes were made based on the three sample's log10FPKM using k-means method to identify the gene expression pattern overall (Fig S2a). The results showed 9 expression patterns (clusters) of 8480 expressed genes were identified in our present study. Of those, the most abundant group was subcluster 2, with 3956 genes whose expression showed a positive slope when treated with Pb1000. These genes were expressed at their highest level at M2A, and then declined; it is similar trend to Subcluster 5. The second most abundant group was subcluster 1, which contained 2820 genes that began to down-regulate at M2A. Subcluster 8 showed a continuous negative slope when treated with Pb1000. Subcluster 6 was composed of 270 genes that showed a continuous positive slope. Subcluster 7 reached to top in M2A, and decreased in M3A, and then increased in M4A, it is contrary to Subcluster 4 (Fig S2b).

Comparison of changes in gene expression between control group and Pb1000 treatment, 157, 202, 195 differential expression genes were detected differentially expressed in three samples compare with mock-treat (C1A) respectively (Fig. 3A, Table S3). Based on deep sequencing of the four DGE libraries in our research, the Venn diagram shows the distribution of expressed genes among the three samples, compare with mock-treat (C1A). Among these genes, 36 were expressed at all three samples, the number of Pb1000 responsive specifically expressed genes were 60 (M2A/C1A), 81 (M3A/C1A), and 121 (M4A/C1A), respectively (Fig. 3B,Table S4). There were 36 differential expression genes (DGEs) expressed in all three samples, which is mostly quantitatively regulated and related to their housekeeping functions (Fig. 3C).

To validate the RNA-seq data, qRT-PCR of 16 randomly selected genes with differential alteration was performed, of which, 4 selected genes was failed to validate, so we only kept 12 candidate genes in this research, As shown in Fig. 4A and Fig. 4B, there was almost similar between RNA-seq data and qRT-PCR data. The qRT-

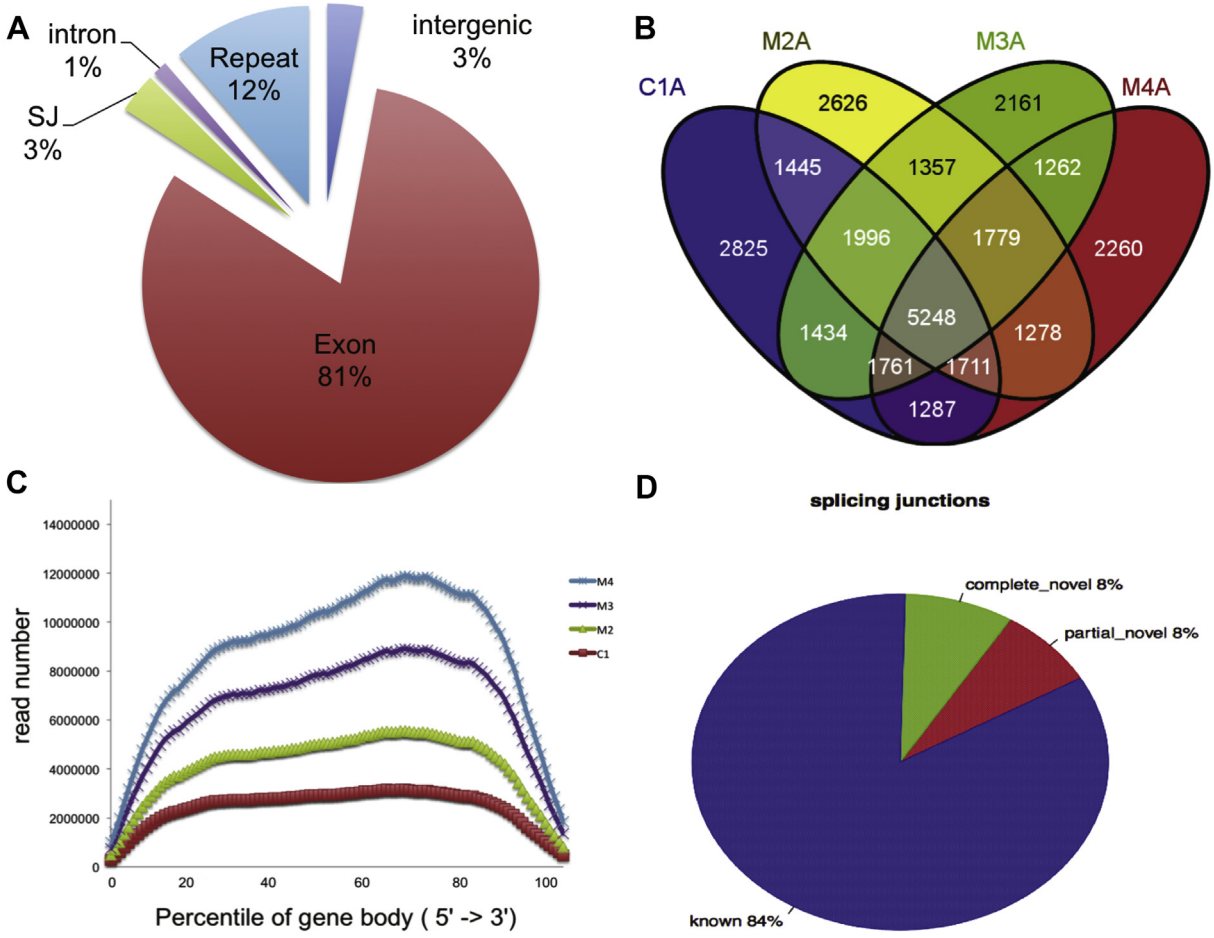

Fig. 2. RNAseq analysis of 9782 roots transcriptome responsive to Pb1000. (A) Distribution of the reads among the gene model and relative to transcript abundance (B) Shared and total reads among successive treatment time points, including mock-treat. (C) Distribution of reads among maize annotated genomics features (D) Splicing junction identified in responding to Pb1000 treatment.

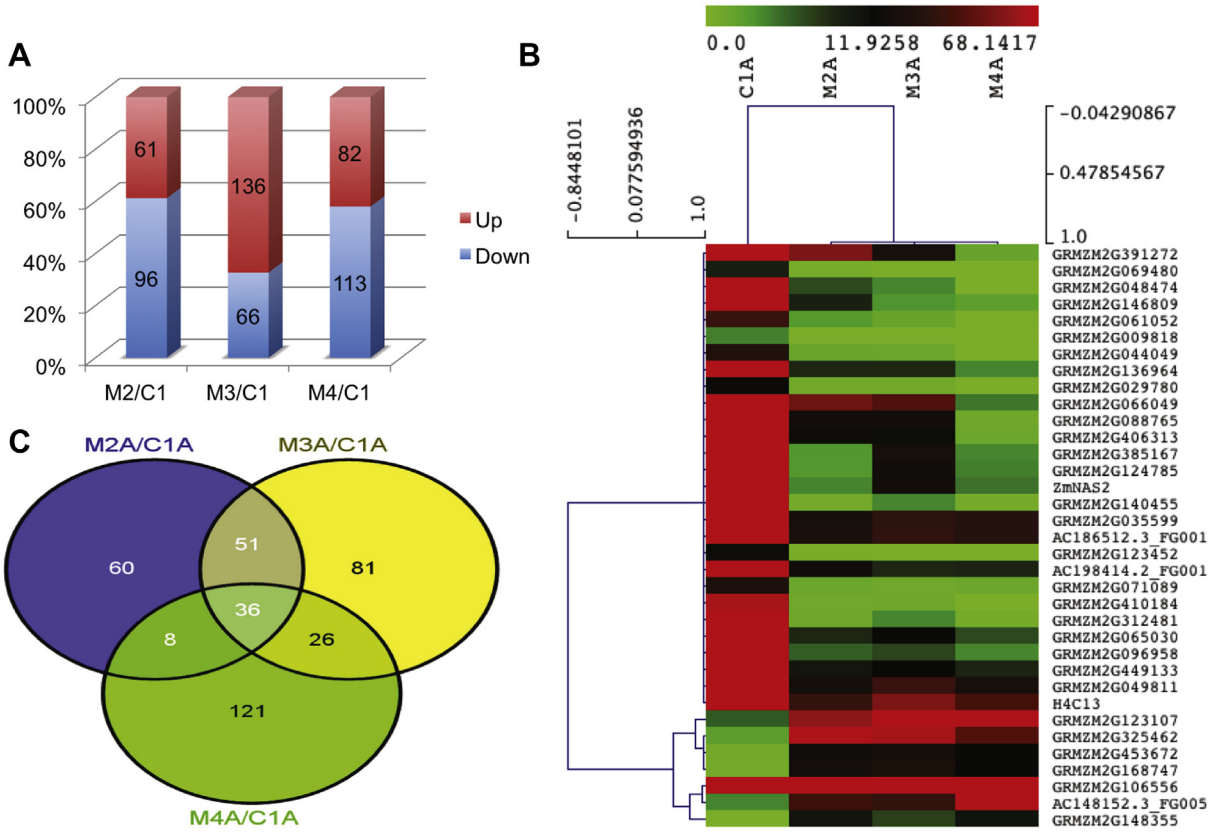

Fig. 3. Mapping information of all expression genes in four successive stages. (A) Numbers of up-regulated genes and down-regulated genes in three Pb1000 treatment stages compared with mock-treat. (B) Venn diagram of all expressed genes from four RNAseq libraries (C1A, M2A, M3A and M4A). The common differential expression genes and stage-special differential expression genes in four successive stages were showed. (C) Visualization of common differential expression genes in four successive stages, including mock-treat and Pb1000 treatment stages.

PCR expression quantities were basically consistent with their transcript abundance changes identified by RNA-seq, it is shown that the RNA-seq data were credible in the present study.

3.2. *Functional annotation of the most abundant transcripts*

AgriGO with Parametric Analysis of Gene Set Enrichment (PAGE) analysis tool were used to facilitate the global analysis of gene expression, with the significantly differentially expressed genes assigned to different functional categories. The functions of the differentially expressed genes between CK and Pb1000 treatment samples (q-value<0.05) were classified by GO assignments and categorized into 37 functional groups that were comprised of three main categories, such as biological process, cellular component and molecular function (Fig. S3a). Of those, the main category of biological process contained metabolic process (GO:0008152) with 164 DEGs and a relative high percentage of genes from biological process response to stress with 42 DEGs and response to stimulus with 45 DEGs were also checked, respectively. Binding, as the main categories of molecular function, consisted of 224 DEGs was found (Fig. S3b, Table S5). In addition, mapman annotation of the differential genes (DEGs) showed that most of them were involved in signal induction, cell wall synthesis, metal transport, as well as protein posttranslaxtional modification (Table S6).

4. Discussion

Lead (Pb) is a common environmental contaminant found in soils and may accumulate to high levels in plants mainly through the roots, followed by leaves, stem, inflorescence and seeds in most plant species [14], which has posed critical concern to human health and environmental safety [3]. Therefore, It has become an urgent issue to control Pb pollution and reduce Pb risks in plant.

In our previous study, we found maize inbred lines 9782, as a non-hyperaccumulator, can accumulate Pb than the other materials under Pb stress, which might reduce the threat for Pb entry into the food chain [27]. In addition, in hyperaccumulator inbred line 178, we found that DGEs were significantly changed association with lipid transport and metabolism pathway under Pb treatment [20]. In this study, we used the RNAseq approaches to investigate the gene expression changes associated with the successive time process induced in maize roots by Pb (Pb1000). A total of 384 transcripts were identified as differentially expressed genes (DEGs) in this research. Similar to previous studies we showed that exposure to Pb1000 induces cellular and oxidative stress, alters expression of genes known to play roles in respond to Pb1000 [9]. Most interestingly, three ZmNAS family genes (ZmNAS2, ZmNAS4, ZmNAS9) were found involved in responding to Pb1000 treatment. Expression analysis by reverse transcription polymerase chain reaction revealed ZmNAS genes are responsive to heavy metal ions (Ni, Fe, Cu, Mn, Zn, and Cd) [28]. Through the statistically analyze the enriched GO terms and pathways association with the DEGs, we found that up-regulated transcripts during Pb1000 stress, with the most significant and high frequently enriched terms and pathways, were predominately involved in the defense response in cellular process (GO:0009987), while for down-regulated genes were mainly related to transferase activity (GO:0016740). Moreover,

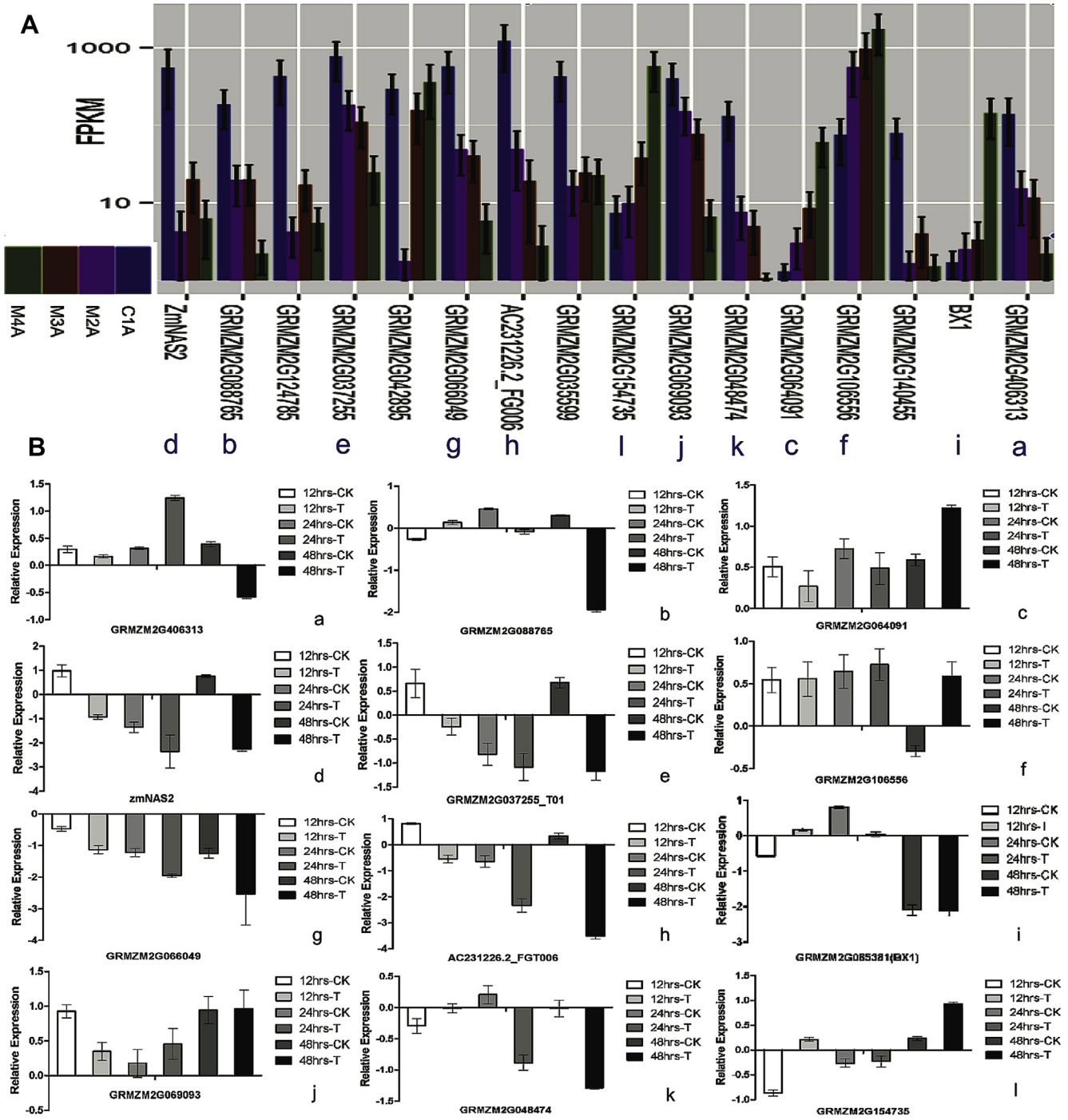

Fig. 4. Bar-plot of 16 differential genes randomly selected from the differential expression genes and 12 of them were validated by qRT-PCR. (A) Bar-plot of 16 differential expression genes randomly selected from the differential expression genes. (B) 12 of differential expression genes were validated by qRT-PCR. The standard error calculated from three biological replicates and the significant ($P < 0.05$) difference identified by uncorrected Fisher's LSD test in multiple comparisons after two-way ANOVA were showed by Error bars and Stars respectively.

Mapman annotation showed that most of differential expression genes (DEGs) were involved in signal induction, cell wall synthesis, metal redox, and metal transport. The qRT-PCR expression quantities were basically consistent with their transcript abundance changes identified by RNA-seq, it is shown that the RNA-seq data were credible in the present study. Moreover, many transcription factor families might act as the important regulators at different developmental stages such as myeloblastosis protein (MYB), basic leucine Zipper (bZIP), ethylene-responsive factor (ERF), and WRKY [7]. In our study, In addition to C2H2 zinc finger family bHLH (bZIP) and AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family (ERF) transcription factors (TF), we also found G2-like transcription factor family (GARP) was involved in response to Pb1000 treatment. These results will expand our understanding of the complex molecular and cellular events in maize root development and provide a foundation for future study on root development in maize under heavy metal pollution and other cereal crops.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.101.

Transparency document

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