Identification of Genes Involved in Root Growth Inhibition Under Lead Stress by Transcriptome Profiling in *Arabidopsis*

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Abstract

Lead (Pb) is a heavy metal with high toxicity to plants. Root is the major organ to respond to Pb stress. However, little is known about how plant roots perceive Pb stress signaling. Here, we describe the transcriptome of *Arabidopsis* root tips under Pb stress using the RNA-seq assay. A total of 703 differentially expressed genes (DEGs) were identified and expressed at every time points. Some early-responsive DEGs (1 h) were predicted to be negatively involved in cell elongation and cell expansion, while some late-responsive DEGs (24 h) positively participated in defense of oxidative stress. Hydrogen peroxide (H$_2$O$_2$) and super-oxide (O$_2^-$) were increased significantly in root tips under Pb stress. Cell wall extension related gene *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 18* (XTH18) was induced in root tips, and *xth18* showed reduced root growth inhibition by Pb stress. Our results revealed the potential mechanism of root growth inhibition by Pb stress and shed light for the further study.

Keywords  Lead (Pb) stress · Root growth · RNA-seq · Cell wall modification · *XTH18*

Introduction

Lead (Pb), a heavy metal with high toxicity to plants, is regarded as the first one smelted by humans (Kalisińska 2019). In daily life, Pb is everywhere because of its corrosion resistance and plasticity, such as fertilizers, pesticides, and pigments (Hadi and Aziz 2015). The contamination of soil by Pb is mainly caused by mining, chimneys of factories using Pb, the storage battery, and smelting. In accordance with ATSDR (Agency for Toxic Substances and Disease Registry) Priority Substance List published in 2019, Pb was labeled as the second most dangerous environment poison due to its toxicity (https://www.atsdr.cdc.gov/SPL/index.html#2019spl). In terms of plants, Pb is not essential for plants’ growth and development, but Pb toxicity sharply occurs even in very low concentration. Pb toxicity brings morpho-physiological and biochemical changes and disturbs normal functioning of the plants from cell to organ level (Ashraf and Tang 2017).

Roots, the first line of defense against Pb stress, secrete exudates into the rhizosphere to chelate Pb and prevent their uptake into root cells. In addition, roots also uptake large amounts of Pb through the NRAMP (natural resistance-associated macrophage protein), ZIP (Zrt/IRT-like protein), and cation transporters, and immensely restrict its translocation to aerial parts. Simultaneously, Pb particles can be fixed on cell wall by extracellular carbohydrates such as callose and mucilage. It is found that callose synthesis could be induced by Pb stress, which formed an efficient barrier for Pb penetration (Samardakiewicz et al. 2012). In cytoplasm, the...
compartmentalization and chelation of Pb proceeds with a faster kinetics to preclude their participation in toxic reactions. Nevertheless, the burst of reactive oxygen species (ROS) attributed to Pb toxicity results in cell damage and reduced root length. To confront oxidative stress, antioxidant system was activated, including non-protein thiols (cysteine and glutathione), ascorbic acid, proline, as well as antioxidant enzymes, such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT).

In this study, root tips of Arabidopsis treated without and with Pb stress for different time points were used for RNA-seq assays. Root growth under Pb stress was apparently reduced, and reactive oxygen species (ROS) was significantly increased by Pb stress in root tips. RNA-seq data showed that a total of 703 differentially expressed genes (DEGs) were expressed at all the time points and mainly implicated in transporter activity, ion binding, catalytic activity, plant growth and development, cell wall modification, and abiotic stimulus. Further results demonstrated that the cell wall extension related gene XTH18 was up-regulated by Pb and xth18 showed reduced sensitivity to Pb stress. These results will enrich our understanding of how plant roots perceive Pb stress signaling and provide a solid foundation for breeding of Pb-tolerant crop varieties.

Materials and Methods

Plant Material and Root Imaging

The Arabidopsis wild genotype (Columbia ecotype, Col-0) was served as experimental materials in the current study. Seeds were surface-sterilized in 10% bleach for 10 min and washed 6 times with double-distilled water to remove residual bleach. The pre-treated seeds were plated on half-strength Murashige and Skoog (MS) medium. Plants were stratified at 4 °C for 2 days in darkness and then transferred to a phytotrone set at 22 °C with a 16-h-light/8-h-dark photoperiod (light intensity 100 μmol m⁻² s⁻¹), which were vertically placed in a plant incubator (22 °C, day/night). The ImageJ was applied to measure root length of 6-day-old seedlings from images captured with a Canon EOS 760D camera. The assay had three biological replicates with ten seedlings per replicate.

Meristem Size Measurements and GUS Staining

The length of root meristem was measured on a ZEISS Axio Imager Z2 microscope using six-day-old seedlings treated with or without 1 mM Pb. Histochemical GUS staining was performed as described previously (Zhang et al. 2018). These experiments were replicated three times for each of ten seedlings.

RNA-Seq Assay

Five-day-old wild-type Seedlings cultured on 1/2 MS medium were transferred to medium supplemented with 1 mM Pb(NO₃)₂ for 1 h, 6 h, 12 h, and 24 h. Root tips (0.5 cm) were collected and quickly frozen with liquid nitrogen, and seedlings on 1/2 MS medium were used as control. Total RNA was isolated using TRIZol reagent (Invitrogen, 15596026). Sequencing libraries were constructed with a total of 3 μg RNA and implemented on Illumina HiSeq 2500 platform (Berry Genomics). All samples were run in three biological replicates. The Kallisto (v 0.44.0) was applied to quantify the transcripts expression levels for each read. The HISAT2 (v 2.0.5) was used to map reads to the Arabidopsis genome. DEGs were analyzed with the DESeq2 package, and genes (P_adj < 0.01 and |Log₂FoldChange| > 0.1) of which were regarded as the significant DEGs and picked for gene ontology (GO) enrichment analysis.

Reverse Transcription Quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) assays were performed to validate the dependability of RNA-seq. All RT-qPCR analyses were conducted with three biological replicates and 3 technical replicates within each biological replicate on a Bio-RAD CFX96™ optics module. The relative expression level was computed by the method of comparative △Ct, and Actin7 (At5g09810) was picked to normalize all results. Specific primers are listed in Supplementary Table 1.

Histochemical Detection of O₂⁻ and H₂O₂ in Root

The 4-day-old seedlings were transferred to the medium with or without 1 mM Pb(NO₃)₂ for 24 h. The seedlings were immersed in 3,3′-diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) staining solution for detection of H₂O₂ or O₂⁻ in light of the methods described in the former study, respectively (Dunand et al. 2007). Three biological replicates with ten seedlings per replicate.

Results

Root Growth Inhibition by Pb Stress

In this study, seedlings of Col-0 were constantly fostered with or without 1 mM Pb(NO₃)₂ for 6 days. The results showed that root elongation was apparently inhibited, and root length was decreased by 64.37% under Pb stress compared with that of mock treatment (1/2 MS), while shoot growth was
relatively less affected (Fig. 1a and b). Postembryonic root growth of higher plants is maintained by the root meristem. The reduction of root meristem size by Pb could result from its negative effect on cell division (Fig. 1c). The CYCB1;1 encodes a cyclin-dependent protein kinase and plays an important role in the G2/M phase of the cell cycle. Therefore, the expression of CYCB1;1pro: GUS in roots can be used to mark mitotic activity in meristems (Colon-Carmona et al. 1999). The CYCB1;1pro:GUS was expressed in the actively dividing cells of the root meristem, and the activity was markedly reduced by Pb stress (Fig. 1d), suggesting that Pb stress represses the cell division activity of the transit amplifying cells in the root meristem.

**RNA Sequencing and Identification of DEGs Under Pb Stress**

To further explore the underlying mechanism of root growth inhibition in response to Pb stress, RNA-seq assays were carried out with Pb treatments for different time points (0 h/1 h/
6 h/12 h/24 h). To test the repeatability and reliability of the sequencing data, the principal component analysis (PCA) was implemented and the results demonstrated that all samples could be divided into five groups (Supplementary Fig. 1), representing that the datasets were available for further analysis. Statistically, a total of 443,708,633 raw reads were obtained from RNA sequencing. The amount of clean reads ranged from 22,626,671 to 33,541,052 among 15 root samples, of which between 78.93% and 94.569% mapped to the Arabidopsis genome (Supplemental Table 2). The reads uniquely mapped to the genome varies between 61.37 and 85.09%. In addition, the minimum Q20 and Q30 of those clean reads were 99.86% and 82.77%, respectively.

The dataset has been uploaded to NCBI database (https://dataview.ncbi.nlm.nih.gov/object/PRJNA593333?reviewer=85.09%). In addition, the minimum Q20 and Q30 of those clean reads were 99.86% and 82.77%, respectively.

Identification and quantification of genes (especially DEGs) expressed under the specific conditions are two vital characters of transcriptome sequencing analysis. In this study, DEGs in response to Pb stress were screened with the threshold of adjusted P value < 0.01. A total of 4284 DEGs were identified at 1 h after Pb stress. With Pb treatments from 0 to 24 h, the number of DEGs reached a peak at 6 h. The up- and down-regulated DGEs reached 2358 and 2739, respectively. However, the amount of DEGs sharply dropped to the minimum value at 12 h. With Pb treatment for 24 h, the amount of up-regulated DEGs was slightly more than that of down-regulated DEGs, and the total number of DEGs was 3498 (Fig. 2a and Supplementary Table 3). Subsequently, a total of 703 expressed DEGs were identified at all the time intervals (Fig. 2b). Hierarchical clustering analysis of 703 DEGs indicated that they could be divided into five groups, and the expression pattern of which was largely different. The result of cluster analyses among four groups with Pb treatments showed that the expression pattern of DEGs at 12 h was similar with that of 24 h, and the difference of which between Pb-1 h and other groups was quite distinct (Fig. 2c). In addition, individual comparisons with a loop design at temporal scale were performed. A total of 3448, 1127, 996, and 3893 DEGs were identified at 1–6 h, 6–12 h, 12–24 h, and 1–24 h, respectively (Supplementary Table 6). For validation of RNA-seq data, a total of 14 DEGs were picked for RT-qPCR assay, and the results of correlation analysis referred that correlation coefficient between RNA-seq and RT-qPCR is 0.751, indicating that the expression trend is consistent (Supplementary Fig. 2).

**GO Enrichment Analysis of Commonly Expressed DEGs**

RNA-seq provides a quick and feasible method for gene function study. Furthermore, the functional relevance of candidate genes could be analyzed with GO tools to separate gene products into three categories, including molecular function (MF), biological process (BP), and cellular component (CC). Here, the commonly expressed DEGs were also divided into three groups (Supplementary Table 4). In terms of molecular function, the processes represented by the GO terms “oxidoreductase activity”, “transmembrane transporter activity,” “metal ion binding,” and “lipid binding” were significantly enriched (Supplementary Fig. 3), indicating that a variety of detoxification metabolisms were activated after Pb stress for few hours. In the biological process ontology (Supplementary Fig. 4), the major terms were “developmental growth involved in morphogenesis,” “root hair cell development,” “cell wall organization or biogenesis,” and “response to abiotic stimulus,” suggesting that plant growth (especially root development) was affected by Pb stress and genes related to abiotic stimulus were activated. Regarding cellular component (Supplementary Fig. 5), the major terms were “plasma membrane,” “vacuolar membrane,” “cell wall,” and “endomembrane system,” demonstrating that cell membrane system performed vital roles in Pb transport, chelation, and sequestration. Although many GO terms related to Pb stress were enriched, it still needs more information to clarify their relationship in further study.

**Identification of Early- and Late-Responsive Genes Under Pb Stress**

To investigate the early- and late-responsive genes related to Pb defense, GO enrichment analysis of DEGs at 1 h and 24 h was performed, and the top 20 GO items (biological process) are shown in Supplementary Figs. 6 and 7, respectively. Interestingly, the terms named “regulation of cell size” and “cell growth” were specifically enriched at 1 h. This result suggests that the DEGs at the early stage (1 h) of Pb stress were negatively involved in cell elongation/expansion and root growth, which demonstrated that cell elongation inhibition in root is a fast response to Pb stress. Furthermore, “root development,” “ion transport,” and “response to abiotic stress” related genes were enriched at 1–6 h (Supplemental Fig. 8). Genes (Expansin, etc.) related to cell elongation were also isolated as DEGs and involved in root development, indicating that cell elongation participated in defense responses of Pb stress at the early stage. At 6–12 h, “cell cycle,” “cell division,” and “response to metal ion” related genes were enriched, suggesting that Pb stress still affected cell division (Supplemental Fig. 9). Moreover, “oxidation-reduction process,” “response to oxidative stress,” and “response to metal ion” related genes were enriched at 12–24 h (Supplemental Fig. 10), demonstrating that oxidation-reduction process was progressively activated by Pb stress. “Response to oxidative stress” and “plant-type cell wall organization” related genes were uniquely enriched at 24 h (Fig. 3a). Similarly, “oxidation-reduction process” and “response to
oxidative stress” related genes were also enriched at 1–24 h (Supplemental Fig. 11), implying that late-responsive DEGs were positively participated in defense against oxidative stress. Furthermore, heavy metal stress could result in the overproduction of ROS inside plant cells (Fahr et al. 2013). Accordingly, 4-day-old seedlings were used to estimate the content of hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$).

As shown in Fig. 3 b and c, the staining was significantly strengthened after Pb treatment for 24 h, indicating that Pb indeed greatly promotes ROS production in root tips.

**XTH18 Plays an Important Role in Pb-Induced Root Growth Inhibition**

Xyloglucan endotransglucosylase/hydrolases (XTHs), one kind of cell wall-modifying enzymes, are correlates with hemicellulose modification and influences cellular expansion and cell wall loosening. In general, there are 33 members of the XTH gene family in *Arabidopsis*, and 10 of them exhibited root-specific expression (XTH-5, XTH-12, XTH-13, XTH-14, XTH-17, XTH-18, XTH-19, XTH-20, XTH-26, and XTH-31). Our RNA-seq data showed that the expression of XTH5, XTH18, XTH20, and XTH31 was quickly and consistently induced by Pb stress (Fig. 4a). According to previous study, XTH18 is also considered as one of major contributors to root growth and for normal root development (Vissenberg et al. 2005). Subsequently, we examined the effect of Pb on XTH18 expression by expressing the GUS reporter gene under the control of the XTH18 promoter (proXTH18::GUS) in 6-day-old Col-0 seedlings. The proXTH18::GUS/GFP was mainly expressed in root meristem and differentiation zone, and its expression was significantly enhanced by Pb treatments for 6 h, 12 h, and 24 h, compared with the mock (0 h) treatment (Fig. 4 b and c). We also compared Pb-induced root growth inhibition between Col-0 (wild-type) and xth18 mutant. Root growth of xth18 showed reduced sensitivity to Pb stress compared with that of Col-0 (Fig. 4 d and e). These results indicated XTH18-mediated cell wall modification was involved in inhibition of root growth under Pb stress.

**A Putative Working Model of Pb Stress**

To better understand the molecular mechanism of plant defense against Pb stress, a supposed model was constructed based on our RNA-seq data (Fig. 5). Perceiving Pb stress signal was the first principle step for plants subjected to Pb stress. In this study, many signaling molecules were identified (Supplementary Table 5). Two calmodulin genes (At2g41110 and At3g56800) were identified and up-regulated after Pb stress for 1 h, while they were constantly down-regulated at
6, 12, and 24 h, indicating that they could be rapidly induced for responding to Pb stress and probably function as the sensor of Pb stress. In addition, three mitogen-activated protein kinase kinase kinase (MPKKK, At2g30040, At2g32510, and At4g36950) were commonly up-regulated by Pb stress at all time intervals and proved to be involve in stress-activated protein kinase signaling cascade in Arabidopsis. Thereafter, Pb was transported from rhizosphere to root and shoot through transporters. Nramp3 (At2g23150), one of the metal transporters, was induced by Pb stress at all time points, and expression of which reached a peak at 6 h. We also identified three ABC transporters (At2g39350, At3g47780, and At4g01830), and two genes of which were continuously induced by Pb. Simultaneously, Pb particles could be absorbed by cell wall. In the cytoplasm, the majority of Pb ion, with or without chelation by phytochelatins, were finally compartmented in vacuole. One cation/H+ antiporter (At5g41610) was isolated and upregulated at all time points except stress for 12 h. Meanwhile, the increased Pb in the cell was responsible for generation of ROS (Ashraf et al. 2017). Under Pb stress condition, a large deal of ROS was generated in chloroplast by photosynthetic system and mitochondrion by respiratory electron transport chain (RETC). Three DEGs encoding NADPH related reductase (At1g10310, At2g17420, and At4g30210) were isolated, while expressed pattern of which was different under Pb stress. Two nitrate reductase (NADH, At1g37130 and At1g77760) were consistently induced by Pb stress, and their expression level reached peaks at 1 h. Furthermore, ROS were also generated through respiratory burst oxidase homolog (RBOH) located on cell wall.

Fig. 3 Expression patterns of early- and late-responsive DEGs and physiological changes of Arabidopsis roots in response to lead (Pb) stress. a Hierarchical clustering heatmap of partial DEGs at 1 h and 24 h. b Detection of hydrogen peroxide in roots stained with DAB. Bar = 50 μm. c Detection of superoxide in roots stained with NBT. Bar = 50 μm.
membrane. To balance the cell metabolism, the activity of oxidoreductases was enhanced to scavenge ROS. Two genes encoding SOD (At2g28190 and At5g23310) were rapidly upregulated at 1 h and their expression level reached a peak after Pb stress for 12 h. A protective enzyme POD (At4g08770) was also induced by Pb stress at all time points. Genes encoding CAT (At4g35090) and peroxidase (At2g48150) were upregulated under Pb stress to transform hydrogen peroxide to water. As shown in Supplemental Figs. 12 and 13, genes involved in Pb stress were differentially expressed and their correlation coefficient between RNA-seq and RT-qPCR is 0.805.

Meanwhile, the ROS signaling pathway was activated to stimulate the defense responses.

**Discussion**

Over the years, heavy metal contaminants attributed to anthropogenic activities are becoming more and more severe. As a nonessential element for plant growth and development, the release of Pb into the environment results in declined crop yield and threatens human health. Accordingly, it is urgent to understand the underlying molecular mechanism of Pb tolerance.
for breeding new varieties. In this study, genes and metabolism pathways related to Pb tolerance were identified via RNA-seq, which lay a soil foundation for further study on Pb stress.

In terms of plant morphology under Pb stress, previous studies found that an inhibitory effect of root growth and shoot growth was pronounced, and the extent of which was enhanced with the increasing concentration of Pb (Seneviratne et al. 2016). In the present study, root length significantly declined under Pb treatment compared with the control, which is consistent with the former studies. However, the shoot growth of Col-0 was not much influenced at the same level of Pb stress, which might be due to the duration and concentration of Pb treatment. Another possible reason is that root is more sensitive than other organs in response to abiotic stresses. Cell division activity and root meristem size were greatly reduced by Pb stress, which implies inhibition of root growth by Pb stress is a complex process.

RNA-seq has been widely applied to identify the DEGs among different conditions in Arabidopsis (Loraine et al. 2016).
study, the expression of XTH18 expansion and cell wall loosening. In addition, relate with hemicellulose modification and influence cellular response. During this process, a great quantity of signaling molecules indicated that cell wall modification is involved in lead stress mutant shows reduced sensitivity under Pb stress; these results were identified in the current study, demonstrating that the strategies for confronting Pb stress were similar among different species (Tian et al. 2015). As shown in Fig. 3a, the expression level of partial DEGs at 1 h and 24 h was displayed and the changes were significant, which could be regarded as the marker genes at early and late stages of Pb stress. The accumulation of ROS resulted from heavy metal stress has been reported in previous study (Ashraf et al. 2017). As shown in Fig. 1 c and d, the color of root was markedly deepened after Pb stress, suggesting that large amounts of ROS were produced, which could be an important indicator of Pb stress.

When plants are exposed to environmental stress conditions, cell wall functions as a barrier against stress factors including metals. Under Pb stress, Pb particles can be fixed on cell wall, which affects the cell wall loosening. XTHs correlate with hemicellulose modification and influence cellular expansion and cell wall loosening. In addition, XTH18 is considered as one of major contributors to root growth and for normal root development (Vissenberg et al. 2005). In this study, the expression of XTH18 was induced and the xth18 mutant shows reduced sensitivity under Pb stress; these results indicated that cell wall modification is involved in lead stress response.

Signal transduction is a key step for plant defense system. During this process, a great quantity of signaling molecules and stress-related proteins were synthesized to activate specific genes for dealing with the stimulus. Calmodulin and mitogen-activated protein kinase kinase kinase (MAPKKK) have been recognized as essential signal molecules in response to stresses (Loraine et al. 2013). In our study, three MAPKKK genes were always up-regulated after Pb treatment at four time points, while two calmodulin genes were only up-regulated at 1 h and down-regulated at 6 h, 12 h, and 24 h, indicating that signal transduction of stress was coordinated. According to previous study, Pb is likely transported by non-specific transporters (Loraine et al. 2013). Therefore, Pb was absorbed into root and finally transported into vacuoles via membrane transporters, such as genes of ABC, ZIP, NRAMP, zinc transporter families, and antiporters. As shown in Supplementary Table 5, a total of 20 significant DEGs related to Pb transport, chelation, and sequestration were isolated for Pb metabolism. Pectin is considered as one of the Pb-chelating agents, and the expression level of its lyase decreased sharply at four time points, suggesting that synthesis of pectin was activated under Pb stress. Krämer et al. (2007) reported that the NRAMP transporters existed in root and shoot, and played an essential role in transport of metal ions through the plasma membrane and the tonoplast (Krämer et al. 2007), which was up-regulated at four time points after Pb treatment. In addition, the content of ROS increased markedly when exposed to abiotic stress (Huang et al. 2019). In this study, two photosystem elements were up-regulated to form much ROS with the extension of the stress time. Two genes encoding respiratory burst oxidase homolog (RBOH) protein were isolated and also up-regulated at 6 h, 12 h, and 24 h. To improve the adverse circumstance attributed to ROS, a large amount of antioxidant enzymes was compounded to release of ROS, such as SOD, CAT, and APX. Furthermore, the ROS signaling was simultaneously motivated for further response to Pb stress.

Conclusion

In summary, our study is mainly focused on the root growth inhibition of model plant Arabidopsis in response to Pb stress at morphological, physiological, and molecular levels and found that the early-responsive genes and the late-responsive genes, and the content of hydrogen peroxide (H2O2) and superoxide (O2−) increased significantly under Pb stress. In addition, XTH18, which encodes a xyloglucan endotransglycosylase, was induced by Pb and xth18 mutant seedling showed increased resistance to Pb stress, which indicated that cell wall modification was involved in of root growth inhibition under Pb stress. Additionally, a putative model was constructed for better understanding the defense mechanism in response to Pb toxicity.
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Compliance with Ethical Standards

Compliance with Ethical Standards  This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest  The authors declare that they have no conflicts of interest.

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