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Metabolomics analysis reveals potential mechanisms of tolerance to excess molybdenum in soybean seedlings

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ABSTRACT

Most plants exhibit strong tolerance to excess molybdenum (Mo). However, the metabolic profile and tolerance mechanisms of plants in response to excess Mo remain unknown. We comprehensively analyzed changes in the metabolic profiles of leaves and roots in soybean (*Glycine max* L.) seedlings cultured under normal-Mo and excess-Mo conditions by using ultra performance liquid chromatography (UPLC) combined with MS/MS (mass spectrometry). There were 42 differential metabolites in the roots and 19 differential metabolites in the leaves in response to excess Mo stress. In roots, the organic acids, levels of gluconic acid, D-glucarate and citric acid increased by 107.63-, 4.42- and 2.87-folds after excess Mo exposure. Several hormones (salicylic acid, jasmonic acid) and lipids (PG, MG, DG etc) also increased significantly under excess Mo condition. Metabolites related to ascorbate–glutathione metabolism and flavonoid and isoflavone biosynthesis notably accumulated in roots. Only lipid metabolism and salicylic acid accumulation were induced in leaves under excess Mo stress. It is speculated that organic compounds such as 2-oxoarginine, L-nicotine, gluconic acid, D-glucurate, and citric acid played important roles to chelate Mo and reduce its toxicity. Signaling molecules (JA, SA, and some lipids) and non-enzyme antioxidants such as flavonoids/isoflavones act synergistically to detoxify ROS and contribute to Mo tolerance in soybean seedlings. More metabolic pathways were induced by Mo excess in roots than in leaves, suggesting that roots play more implant role in Mo tolerance.

1. Introduction

Molybdenum (Mo) is an essential micronutrient for plants growth and development, which played an indispensable role in biosynthesis process of molybdate-dependent enzymes (Kisker et al., 1997). The molybdoenzymes included nitrate reductase, sulfite oxidase aldehyde oxidase, xanthine oxidase and nitrogenase (Kisker et al., 1997). These enzymes have an important role in metabolic processes of nitrogen, carbon and sulfur in plants (Ide et al., 2011; Mendel, 2009). Mo occurs naturally in soil at concentrations between 0.2 and 6 mg kg^{-1}, while metal-rich soils may contain 10–100 mg Mo/kg soil (He et al., 2005). The plant remand little Mo for regular growth, the Mo content in the healthy plant issues was 0.2–300 mg kg^{-1} DW (Gupta, 1997). There was a positive correlation between the concentration of Mo in soil and the concentration of Mo accumulated in plants. The previous studies have found that the Mo concentration in contaminated sites ranged from 304.91 to 1071.52 mg kg^{-1} (Xiao et al., 2010; Cong et al., 2009). However, the Mo content in plant tissues exceeded 100 mg kg^{-1}, most plants exhibited no molybdenosis symptoms (Lu, 2003). For instance, the plants showed no toxic symptoms while the Mo concentration in the leaves of soybean, cotton and carrot were up to 80,1585 and 1800 mg kg^{-1} separately (Adriano, 1986). The chlorophyll content of the *Axonopus compressus (Sw.) Beauv.* exhibited no remarkably change when treated with 100–600 mg kg^{-1} Mo (Tow et al., 2016). The chlorophyll content of cabbage seedlings also exhibited no significant change when exposed to 10 mM sodium molybdate (Kumchai et al., 2013). When the Mo content of the plant issues exceeded the toleration, it would alter the plant physiology processes. For instance, the net photosynthetic rate in winter wheat would decreased notably under 1000 mg kg^{-1} Mo treatment (Lu et al., 2016). Under excess Mo condition, the soybean seeds also exhibited a notable decrease in contents of protein, vitamin C, indispensable amino acids and total amino acids

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(Liu and Yang, 2003). These results suggested that the plants had a high ability of excess Mo accumulation, and the photosynthesis rate processes and soybean seeds quality would decrease under excess Mo condition. The other metabolic processes in response to excess Mo need be explored in the future study.

When the plants grown under excess trace metals condition, the plants physiological and metabolic processes would be altered at the cellular and molecular level. (Rascio and Navari-Izzo, 2011). These different plant physiological processes contained enzymes inactivation, amino acids degradation, lipid peroxidation and DNA-strand cleavage (Villiers et al., 2011). In addition, the plants have also developed active and passive strategies to reduce the heavy metals toxicity, such as chelation of organic ligands and metal ions, antioxidation of enzymes and non-enzymes and regulation of signal molecule (Sytar et al., 2013). Most of these physiological metabolism processes in response to excess heavy metals were mainly concluded from cadmium, nickel and copper. However, there were only some researches focused on physiology metabolism mechanisms of excess Mo accumulation. Hale et al. (2001) found that anthocyanidin might enhance the accumulation of excess Mo in the vacuoles of Brassica napus roots. Moreover, Boojar and Tavakkoli (2011) have revealed that phytochelatins, cysteine and glutathione could enhance the ability of excess Mo tolerance in the Achilla tenuifolia (hyperaccumulator plants). These findings only expressed the characteristics of Mo accumulation in plants. However, the metabolic profile and tolerance mechanisms of plants in response to excess Mo have not been comprehensively investigated now.

Here, we aimed to identify some biomarkers of soybean in response to excess Mo based on ultra performance liquid chromatography (UPLC) combined with MS/MS (mass spectrometry) and multivariate statistical analysis. Then we would comprehensively investigate the potential mechanisms of tolerance to excess molybdenum in soybean seedlings by referring to these differential biomarkers and their related metabolic pathways. To our knowledge, this is the first report on the metabolomic profile of soybean under excess-Mo conditions. These results provide new and overall understanding about the mechanisms of excess Mo tolerance in plants.

2. Materials and methods

2.1. Experimental design

The experimental material was the soybean cultivar “Dongnong53″, which was defined as a high Mo accumulation soybean cultivar (Xu et al., 2016). The growth condition of plants was as followed: relative humidity, 65–75%; 16-h light/8-h dark photoperiod; temperatures, 30 °C days / 25 °C nights. The nutrient solution was aerated for 15 min with an air pump at every two hours. The nutrient solution formulation and method referred to previous research (Xu et al., 2018).

First, the soybean seedlings were cultured in the standard nutrient solution for 21 days. Then, half soybean seedlings were culture in 100 mg/L Mo level, which considered as the excess-Mo treatment (Excess Mo; Mo_{100}), and the other soybean seedlings were cultured in 0.1 mg/L Mo level, which considered as the control (Standard Mo; $Mo_{0.1}$). These seedlings were cultured in above two treatments for 48 h, we harvested the leaves of top third leaf and roots from both treatments for further analysis separately. Each treatment had six replicates. The plants were weighed to determine biomass, and then samples were stored at − 80 °C until further analysis.

2.2. Analysis of Mo concentration

Leaves and roots were digested for 4 h at 250 °C in 10 mL HNO_3/$HClO_4$ (9:1 v/v), and then the mixture was dissolved and diluted by deionized water. The quantification of Mo concentrations in these samples were analyzed by inductively coupled plasma mass spectrometry (EXPEC 7000; FPI, Hangzhou, China). In this study, we selected the Biological reference materials for composition analysis - soybeans (GBW10013(GSB-4)) as certified reference materials, and its Mo content is 0.71 mg kg^{-1}. Here, the Mo content obtained was 0.67 mg kg^{-1} which analyzed by using ICP-MS. Then we calculated the recovery rate and obtain the final data multiplied by recovery rate.

2.3. Non-targeted analysis of metabolites

Frozen root and leaf tissues (approximately 50 mg fresh weight) were homogenized in 0.8 mL methyl alcohol using a JXFSTPRP-II (Fstgrd-24) mixer mill (Shanghai Jingxin Industrial, Shanghai, China) and zirconia beads. The mixture was vortexed for 30 s and then centrifuged at 12,000*g* for 15 min at 4 °C. Then, 200 µl supernatant was transferred into a glass vial for further analyses. A 4-µl aliquot of each sample was separated and its components identified by chromatography-mass spectrometry (LC-MS) (Ultimate 3000LC, Orbitrap Elite; Thermo Scientific, Rockford, IL, USA). Each sample was separated on a Hyper Gold C18 column (100 × 4.6 mm × 3 µm) at 4 °C. The chromatographic separation conditions were as follows: column temperature, 40 °C; flow rate, 0.3 mL/min; mobile phase composition A: water + 0.1% methanoic acid; mobile phase composition B: acetonitrile + 0.1% methanoic acid. The gradient program is shown in Supplementary Table S1.

The mass spectrometer was operated in positive (ESI+) and negative (ESI-) ion modes. The parameters for ESI+ were as follows: heater temperature, 300 °C; sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.0 kV; capillary temperature, 350 °C; S-Lens RF level, 30%. The parameters were the same for ESI- except that the spray voltage was 3.2 kV, the capillary temperature was 350 °C, and the S-Lens RF level was 60%. Six biological replicates were analyzed for each treatment (Smith et al., 2006).

2.4. Data processing and statistical analysis

Data were processed as described previously, with minor modifications (Chen et al., 2015). The raw LC-MS data for all samples were first processed into a common data format by SIEVE software (Thermo Scientific, Waltham, MA, USA). Then, the data from each sample (retention time, mass-to charge ratio (*m/z*), and peak area) were normalized using Excel 2010 software (Microsoft, Redmond, WA, USA), the methods used were peak area normalization (Yu et al., 2014). Then, the normalized data (retention time, *m/z*, normalized peak area percent) were imported into SIMCA-P (ver. 11.0, Umetrics, Umea, Sweden) for principal components analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The quality of the PCA models was evaluated by the relevant R^2X and Q^2, which were regarded as valid only if $R^2X > 0.4$. OPLS-DA models were regarded as valid only if $Q^2 > 0.4$. The values of variable importance in the projection (VIP) are obtained from the model of OPLS-DA. Student's *t*-test was used for two groups comparisons in SPSS 20.0. We used the VIP > 1 and $p < 0.05$(*t*-test) as screening criterion to obtain differential metabolites between two groups. Univariate analysis was performed using SPSS 20.0. A *p*-value less than 0.05 was considered significant. The difference in metabolite concentrations between the two groups is expressed as fold change. For metabolite identification, the exact molecular mass and *m/z* of detected featured peaks were first matched to metabolites from the database of Metlin (http://metlin.scripps.edu) (Abagyan and Siuzdak, 2005). The qualitative of metabolites were based the retention time and accurate mass of parent mass and fragments generated by collision-induced dissociation (CID) MS/MS (Vorkas et al., 2015). Metabolic pathway analysis was performed using tools at the KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html). Biomass data are expressed as means ± SD, and significant differences were detected by independent samples *t*-test in SPSS 20.0.