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Novel Quantitative Metabolomic Approach for the Study of Stress Responses of Plant Root Metabolism

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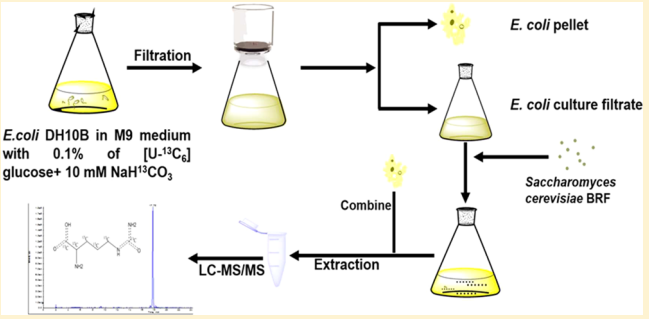
Supporting Information

ABSTRACT: Quantitative metabolomics (qMetabolomics) is a powerful tool for understanding the intricate metabolic processes involved in plant abiotic stress responses. qMetabolomics is hindered by the limited coverage and high cost of isotopically labeled standards. In this study, we first selected 271 metabolites which might play important roles in abiotic stress responses as the targets and established a comprehensive LC−MS/MS based qMetabolomic method. We then developed a novel metabolic labeling method using *E. coli*−*Saccharomyces cerevisiae* two-step cultivation for the production of uniformly ^{13}C-labeled metabolites as internal standards. Finally, we applied the developed qMetabolomic method to investigate the influence of Pb stress on maize root metabolism. The absolute concentration of 226 metabolites in maize roots was accurately quantified in a single run within 30 min. Our study also revealed that glycolysis, purine, pyrimidine, and phospholipids were the main metabolic pathways in maize roots involved in Pb stress response. To our knowledge, this is the most comprehensive qMetabolomic method for plant metabolomics thus far. We developed a simple and inexpensive metabolic labeling method which dramatically expanded the availability of uniformly ^{13}C labeled metabolites. Our findings also provided new insights of maize metabolic responses to Pb stress.



KEYWORDS: *Escherichia coli, maize, Pb stress, quantitative metabolomics, Saccharomyces cerevisiae, uniformly ^{13}C-labeled metabolites*

INTRODUCTION

Metabolites are the direct indicators of biochemical activities and provide the readout of cellular state.[1] Knowing the absolute concentration of the metabolites is always a need for plant biologists, and it empowers the researchers to understand the molecular underpinnings of plant physiology. Unlike human metabolome database (www.hmdb.ca), plant absolute concentration has not been well studied and documented. Quantitative (targeted) metabolomics (qMetabolomics) is considered as a promising tool for quantification of metabolites in a large scale. Compared to untargeted metabolomics, qMetabolomics is the measurement of a subset of chemically characterized and biochemically annotated metabolites. This approach offers the opportunity for the comprehensive understanding of a vast array of metabolic enzymes, their kinetics, end products, and the known biochemical pathways to which they contribute.[2] Attempts have been made to use qMetabolomics for the study of plant hormone signaling under stresses.[3,4] However, only a small number of metabolites were targeted (<100 metabolites).

Precise quantitation of metabolites by qMetabolomics requires the availability of ^{13}C-labeled, or other mass-tagged standards. Stable isotope standards have exactly same chromatographic characteristics with the corresponding unlabeled compounds and thus could normalize the ion suppression in the electrospray ionization of tandem mass spectrometry (ESI-MS/MS).[5] However, the development of qMetabolomics is hindered by the lack of availability and extremely high cost of isotopically labeled standards.[6] It is estimated that the financial expense of purchasing and maintaining an inventory of 500 stable isotope-tagged standards would exceed $0.25 million. Moreover, many metabolites are not commercially available.

Compared to the traditional expensive chemical labeling, metabolic labeling is an alternative cheaper method for the production of stable isotopes. It has been widely used for quantitative proteomics.[7,8] In contrast, the use of metabolic

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labeling to prepare the stable isotopes for targeted metabolomics is limited. By growing *E. coli* in minimal salt medium with uniformly ^{13}C labeled glucose, Bennett et al., obtained 103 U−^{13}C labeled metabolites.[9] A total of 19 amino acids were quantified using U−^{13}C labeled stable isotopes produced by the cultivation of *Pichia pastoris* in a 7-L laboratory fermenter.[10]

In this study, we first selected 271 metabolites that might play important roles in various plant abiotic stresses. A comprehensive LC−MS/MS based targeted metabolomic method was then established. A total of 542 MRMs were monitored, including 271 endogenous metabolites and their corresponding U−^{13}C stable isotopes in a single run. We developed a novel metabolic labeling method using *E. coli*−*Saccharomyces cerevisiae* two-step cultivation. The method permits the in-house preparation of 271 U−^{13}C labeled metabolites by growth of *E. coli* and *S. cerevisae* in minimal medium supplemented with two simple precursors including U−^{13}C-glucose and U−^{13}C-$NaHCO_3$. We applied the developed methods to investigate the influence of Pb stress on maize root metabolism. We provided the absolute concentration of 226 detectable metabolites in maize roots. We also performed multivariate and pathway analysis to further explore the metabolic changes of maize roots metabolism.

MATERIALS AND METHODS

Chemical Reagents and Standards

LC−MS grade acetonitrile, water, ammonium hydroxide, formic acid, uniformly ^{13}C labeled glucose ([U−$^{13}C_6$] glucose, 99% isotopic purity) and sodium bicarbonate−^{13}C (99% isotopic purity) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Unlabeled purified standards were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), Santa Cruz Biotechnology (Dallas, TX, U.S.A.) or Avanti Polar Lipids (Alabaster, AL, U.S.A.) at the highest purity available. Other salts used for bacterial M9 medium and Hoagland's solution were analytical grade and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Mili-Q water was used to prepare the culture medium in this study.

Strains and Media

The bacterial and baker's yeast strains used in this study were *Escherichia coli* DH10B and *Saccharomyces cerevisiae* BRF strain. Both strains were purchased from ATCC, U.S.A. Maize seeds (*Zea mays* spp. *mays*) were provided by National Plant Germplasm System, U.S.A.

M9 minimal medium for *E. coli* contains (per liter): 6 g NaH_2PO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 g $NaH^{13}CO_3$, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$. [U−$^{13}C_6$] glucose (0.1%) was used as the only carbon source. The initial pH was 7.8.

Medium for *S. cerevisiae* growth contains 0.5% uniformly ^{13}C labeled glucose, M9 salts and *E. coli* supernatant.

The full-strength Hoagland's solution for maize growth contains 1 mM KH_2PO_4, 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 92 mM H_3BO_3, 1.5 mM $ZnSO_4$, 0.6 mM $CuSO_4$, 0.2 mM MoO_3, 18 mM $MnSO_4$, and 3.6 mM $FeSO_4$. The pH of Hoagland's solution was controlled at 6.0.

Growth of *E. coli* DH10B in M9 Medium

A metabolic active *E. coli* DH10B colony was inoculated into 3 mL of M9 medium with 0.1% of [U−$^{13}C_6$] glucose as the sole carbon source and grown on a rotary shaker at 37 °C and 180 rpm overnight. The culture was then inoculated into 100 mL of the same medium and grown at the same condition until OD_{600} reached 0.6 (approximately 32 h). The batch culture was centrifuged at 6000 rpm for 10 min at 4 °C. *E. coli* cell pellets were flash-frozen in liquid nitrogen and stored at −80 °C for future use. The supernatant was supplemented with additional [U−$^{13}C_6$] glucose (final conc. 0.5%) and M9 medium (final conc. 1×) and filter-sterilized for the cultivation of *S. cerevisiae*.

Different concentrations of ^{13}C labeled sodium bicarbonate ($NaH^{13}CO_3$) (0%, 0.01%, 0.1% and 0.5%) were added into M9 medium. The effect of $NaH^{13}CO_3$ addition on *E. coli* growth and stable isotopes enrichment was investigated.

Batch Culture of *S. cerevisiae* BRF in *E. coli* Supernatant

A metabolic active colony of *S. cerevisiae* BRF strain was inoculated into 3 mL of *E. coli* supernatant obtained from the previous step and grown on a rotary shaker at 30 °C and 225 rpm agitation overnight. The overnight *S. cerevisiae* culture was then inoculated to 100 mL of sterile *E. coli* supernatant containing 0.5% uniformly ^{13}C labeled glucose and additional M9 salts. Batch cultures were grown at 30 °C and 225 rpm agitation ensuring fully aerated conditions. Cell growth was followed by monitoring the OD_{600} until early stationary phase was reached (about 32 h). The yeast culture was then snap-frozen in liquid nitrogen and stored at −80 °C for future use.

Extraction of ^{13}C Labeled Metabolites

Both yeast culture and *E. coli* cell pellet were lyophilized using a FreeZone −105 °C Benchtop Freeze-Dry System (LABCONCO, U.S.A.) and all the dry powder was mixed together. Extraction of ^{13}C labeled metabolites from yeast and *E. coli* was performed according to a previous report with slight modifications.[11] Briefly, 450 μL of hot extraction buffer (80 °C) containing 80% ethanol in 5 mM ammonium formate, 1 mM EDTA (pH 7.2) was added to 50 mg of dry yeast−*E. coli* powder. The mixture was vortexed for 30 s and incubated 1−2 min on a heating block at 80 °C. The solution was centrifuged for 15 min at 16 000*g* (4 °C). The resulting supernatant was transferred to a new tube and kept at −80 °C for future use.

LC−MS/MS Conditions

LC−MS/MS analysis was conducted using a UHPLC system (LC-20A, Shimadzu) coupled to a Qtrap 6500 hybrid triple quadrupole mass spectrometer (AB SCIEX) operated in both negative (ESI^-) and positive (ESI^+). Multiple reaction monitoring (MRM) with fast polarity switching (20 ms) was used for detection and quantification. This fast switching allows us to monitor both negative and positive modes for up to 1000 MRMs in a single run. The source conditions were as follows: electrospray voltage of −4500 V on negative mode and 5500 V on positive mode, source temperature of 500 °C, curtain gas of 50, CAD gas of 11, gas 1 and gas 2 of 50 and 50 psi, respectively. The values of declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were optimized using the unlabeled standards and set on a compound-dependent basis. A total of 542 MRMs (271 unlabeled metabolites and 271 corresponding U−^{13}C labeled metabolites) were monitored using the Analyst 1.6.2 acquisition software and Advanced Scheduled MRM Algorithm (AB SCIEX).

Chromatographic separation was carried out on an Acclaim Mixed-Mode HILIC-1 column (4.6 × 250 mm^2, 5 μm, 120 Å) (Thermo, CA). Mobile phase A was 95% H_2O with 20 mM ammonium formate and 5% acetonitrile (pH 4). Mobile phase B was 100% acetonitrile. The gradient was as follows: 0 min-

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95% B, 3 min-95% B, 3.1 min-85% B, 6.0 min-85% B, 6.1 min-75% B, 10 min-75% B, 15 min-0% B, 25 min-0%, 26 min-95% B, and 31 min-end. The flow rate was 300 μL/min. The autosampler temperature was 4 °C, and the injection volume was 10 μL.

Retention time check and peak area integration were analyzed using MultiQuant 3.0 (AB SCIEX). The MRM transitions, compound-dependent parameters and retention time on the column were listed in Supporting Information(SI) Table S1.

Isotopic Enrichment Analysis

The AUCs (Peak area under the curve) of uniformly ^{13}C labeled and unlabeled metabolites in the extract were measured by LC−MS/MS using the optimal method described as above in the section of LC−MS/MS conditions. ^{13}C enrichment in the metabolites was calculated using the following equation:

$$^{13}\text{C enrich.}(\%) = \frac{\text{AUC of lbl. metab.}}{\text{AUC of unlbl. metab.}} \times 100\%$$

where "lbl metab." is labeled metabolites and "unlbl. metab." is unlabeled metabolites.

Quantification of Uniformly ^{13}C Labeled Metabolites

The concentration of labeled metabolites in *E. coli*−yeast culture was measured using reverse isotope dilution technique. Eight concentration ranges of unlabeled standard mixes (50 μL) were added into 50 mg of dry *E. coli*−yeast powder before extraction. Metabolites were then extracted as described in the section of "Extraction of ^{13}C labeled metabolites" and analyzed by LC−MS/MS according to the optimal LC−MS/MS conditions. Calibration curves were plotted in MultiQuant 3.0 (AB SCIEX) using the peak area ratio of the unlabeled compounds to the respective labeled compounds (AUCs of unlabeled/AUCs of ^{13}C labeled) against the known concentrations of unlabeled standards. The coefficient (r^2) was also determined from the calibration curves. The concentration of ^{13}C labeled metabolites was calculated through the Y-intercept of the calibration curve and given as ng/mg dry weight.

Evaluation of the Stability of U−^{13}C Labeled Metabolites

The *E. coli*−yeast extract was stored at −80 °C in the sealed glass vials. The stability of uniformly ^{13}C labeled metabolites was evaluated at 0 d, 7 d, 1 month, 6 month, and 1 year of storage. The AUC of each metabolite was measured using the method described in "LC−MS/MS conditions" and log transformed. The relative standard deviation (RSD) of Log transformed AUCs was calculated.

Maize Growth and Pb Treatment

Maize seeds were germinated and the seedlings were grown for 2 weeks on vermiculite germinating mix. Plants were then transplanted to 1.5 L polypropylene chambers. Three plants were placed in each chamber containing 1.25 L full-length Hoagland's solution. After 1 week of acclimation, Plants were treated with Pb by the addition of Pb $(NO_3)_2$ to the medium to achieve the final Pb^{2+} concentration of 400 mg/L. Medium without Pb^{2+} was used for controls. A total of 6 replicates were set up for each group. The plants were maintained in a greenhouse with a photoperiod of 16 h day/8 h dark and light intensity of 18 500 lx. The temperature was controlled at 25 °C and relative humidity was 30% to 50%.

The plants were harvested 7 days after Pb treatment and rinsed in deionized water to remove the attached Pb on the surface of the roots. Maize roots were collected and rapidly frozen in liquid nitrogen and stored at −80 °C for further analysis.

Quantitative Metabolomic Analysis of Intracellular Metabolites from Maize Roots

Metabolites from maize roots were extracted as described before with modifications.[12] Briefly, maize roots were ground into powder under liquid nitrogen in a precooled mortar. About 50 mg of the powdered tissues were weighed. Six replicates were prepared. Ten μL of different concentrations of uniformly ^{13}C labeled metabolites mix was also added before extraction. The amount of standards mix needed for quantification was optimized by the dilution of the *E. coli*−yeast extract into four concentrations including 1x (no dilution), 0.5x, 0.1x, and 0.05x. Four-40 μL of methanol-acetonitrile (50:50) was added and the solution was mixed thoroughly. The metabolites were extracted by sonication on ice for 10 min. The mixture was then centrifuged at 16 000*g* for 15 min at 4 °C, and the supernatant was transferred to a new tube for LC−MS/MS analysis.

The absolute concentration of each metabolite was calculated according to the peak area ratio of unlabeled analyte/^{13}C labeled analyte and the known concentration of ^{13}C labeled internal standard. The calculation was performed using MultiQuant 3.0 (AB SCIEX, U.S.A.) and the concentration was expressed as ng/mg dry weight.

Data Analysis

Peak area, retention time, *S*/*N*, calibration curves, and absolute concentration was automatically calculated using MultiQuant 3.0 software provided by AB SCIEX. Multivariate partial least-squares discriminant analysis and pathway analysis were performed using metaboanalyst (http://www.metaboanalyst.ca) and KEGG pathway (http://www.genome.jp/kegg/pathway.html).

RESULTS

Optimization of LC−MS/MS Conditions

The assembled set of target analytes in this study comprised a broad range of primary metabolites (271 compounds) in maize roots including phospholipids (88), cofactors (10), amino acids and derivatives (38), nucleotides and derivatives (63), organic acids (32), polyamines (4), polyols (6), organic phosphate (10), sugar and sugar phosphates (12), and vitamins (7). The complete list of target metabolites is given in SI Table S1. One of the objectives in this study was to develop a method to quantify all these metabolites in a single chromatographic run.

First, the compound-dependent MRM transitions and instrument-specific parameters (polarity, DP, EP, CE, and CXP) were optimized using each individual unlabeled standard. For each analyte, at least two MRM transitions were developed: one for quantification and the other one for confirmation. The MRM transitions for uniformly ^{13}C-labeled [U−^{13}C] compounds were calculated from the corresponding unlabeled compounds based on the ESI fragmentation. All the optimal settings were also listed in SI Table S1.

We then analyzed three different types of columns for detection and separation of the targeted analytes: (1) Acclaim Mixed-Mode HILIC-1 column (Thermo Scientific, U.S.A.), (2) HILIC Luna NH2 (Phenomenex, CA, U.S.A.) and (3) Synergi Fusion-RP (Phenomenex, CA, U.S.A.). Overall, Acclaim Mixed-Mode HILIC-1, a silica-based column with both reversed phase and hydrophilic interaction properties showed the best performance in terms of the retention and detection of both

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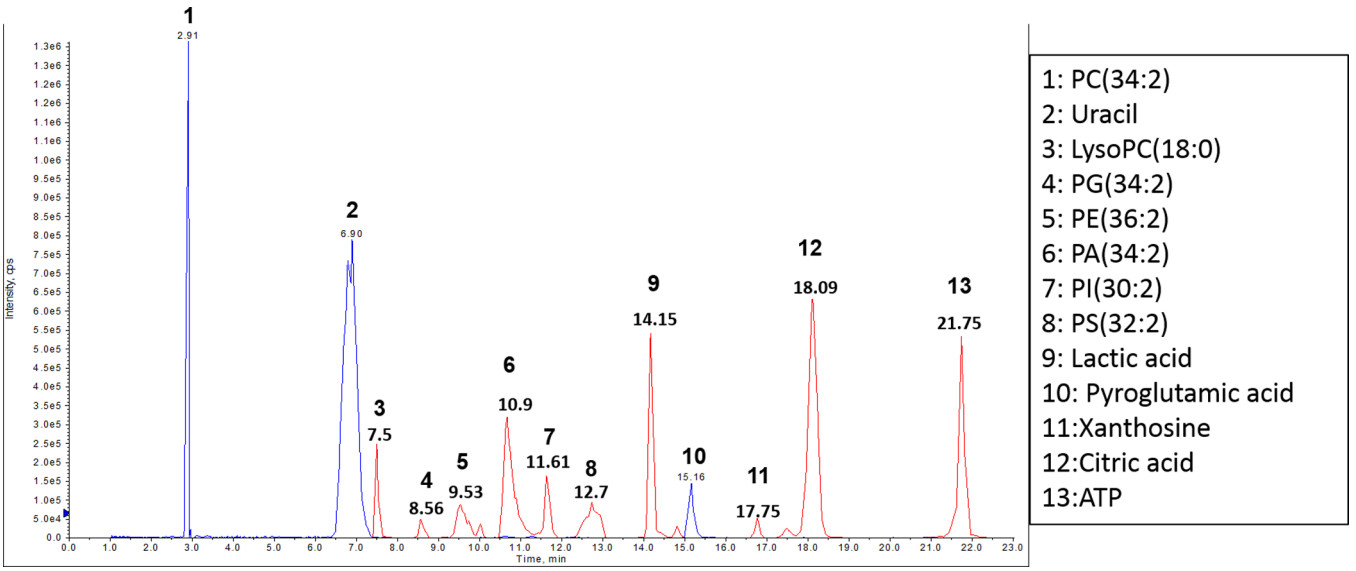

Figure 1. Representative ion extracted chromatograms of 13 targeted analytes on Acclaim Mixed-Mode HILIC-1 column. Separation of class-distinct targeted analytes was achieved using the optimal chromatographic conditions described in M&M section. The full list of compounds and retention time were listed in SI Table S3. PC: phosphatidylcholine; PG: phosphatidylglycerol; PE: phosphatidylethanolamines; PA: phosphatidic acid; PI: phosphatidylinositols; PS: phosphatidylserine.

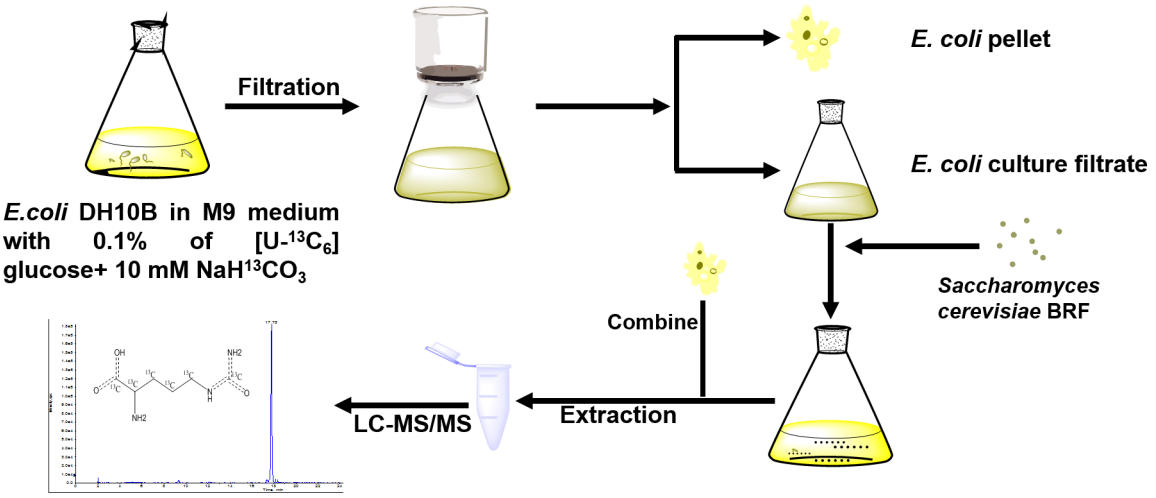

Figure 2. Schematic flow of the procedures used to produce uniformly ^{13}C labeled standards..

polar and nonpolar compounds (SI Figure S1a). Polyamines (putrescine and spermine) had bad peak shape (peak width >1.5 min) on HILIC Luna NH2 column. In addition, coenzyme A and the derivatives including acetyl-CoA, succinyl-CoA were not eluted on HILIC Luna NH2 column due to the strong hydrophilic interactions with stationary phase (SI Figure S1b). Synergi Fusion-RP column did not have good retention on highly polar compounds such as citric acid, ATP and hexose phosphates. All these compounds were eluted in the void time without retention (SI Figure S1c).

Reliable quantification of hundreds of chemically diverse analytes requires optimization of chromatographic separation to reduce ion-suppression effects. Several different sizes of Acclaim Mixed-Mode HILIC-1 columns (4.6 × 250 mm^2; 4.6 × 150 mm^2 and 3.0 × 50 mm^2) were tested and only 4.6 × 250 mm^2 has the resolution power for the separation of nearly 300 compounds. Optimization with respect to mobile phase composition, gradient, pH and run time resulted in the class-distinct separation of 271 targeted compounds using the following conditions: mobile phase A was 95% H_2O with 20 mM ammonium formate and 5% acetonitrile (pH 4). Mobile phase B was 100% acetonitrile. The gradient was as follows: 0 min-95% B, 3 min-95% B, 3.1 min-85% B, 6.0 min-85% B, 6.1 min-75% B, 10 min-75% B, 15 min-0% B, 25 min-0%, 26 min-95% B, 31 min-end. The representative chromatogram of unlabeled standards mix was showed in Figure. 1. For example, phospholipids were baseline separated based on their head polar groups using the optimal conditions. The elution order for phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidic acid (PAs), phosphatidylserines (PSs), phosphatidylglycerol (PGs), and phosphatidylinositols (PIs) was as follows: PCs (2.8−3.1 min) > PGs (8.5 min) > PEs (9.6 min) > PAs (9.7 min) > PIs (11.6 min) > PSs (11.2 min). The full list of compounds and retention time were also listed in SI Table S1.

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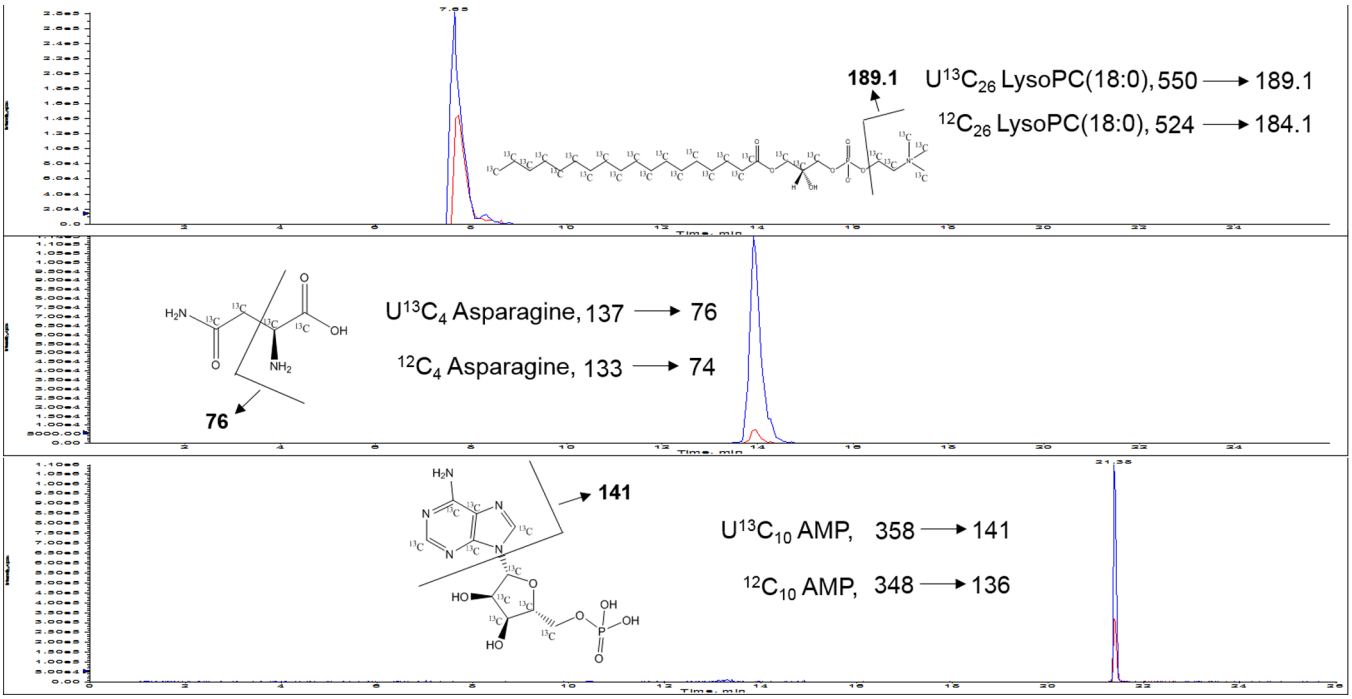

Figure 3. Extracted chromatograms of uniformly ^{13}C labeled metabolites. *E. coli*−yeast extract was spiked with unlabeled standards and analyzed using the optimal chromatographic condition. Blue color: Unlabeled compounds, Red color: ^{13}C labeled metabolites. ESI fragmentation and MRM transitions were also showed on the chromatograms.

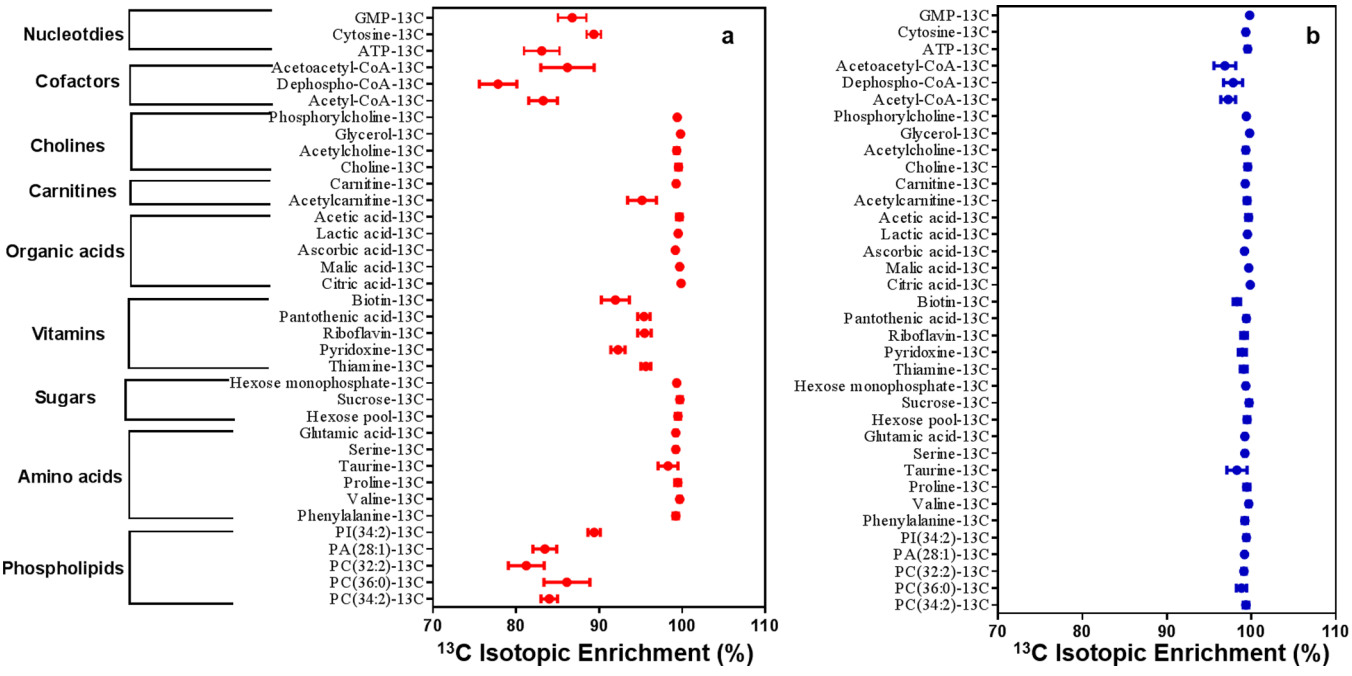

Figure 4. Effect of $NaH^{13}CO_3$ on the enrichment of ^{13}C in the metabolites of *E. coli* and yeast culture. (a) 0 mM $NaH^{13}CO_3$; (b) 10 mM $NaH^{13}CO_3$. Enrichment was calculated by the AUC ratio of labeled and unlabeled analytes in the extract of *E. coli*−yeast culture ($AUC_{[U\text{-}13C]analyte}$/$AUC_{unlabeled\ analyte}$ + $AUC_{[U\text{-}13C]\ analyte}$). AUC of 500 was given if an analyte was not detected ($S/N < 3$). Data points were mean ± SD of three batches of culture.

Metabolic Production of U−^{13}C Labeled Compounds through Two-Step Cultivation

Quantitative metabolomics relies on the availability of isotope standards for accurate absolute quantification. In this study, we developed a two-step cultivation method to produce a total of 271 uniformly ^{13}C labeled metabolites in the proper concentration from two simple stable isotope precursors (U−^{13}C glucose and $NaH^{13}CO_3$) using a novel combination of *E. coli* (*E. coli* DH10B) and yeast (*S. cerevisiae* BRF). The schematic flow of the procedures is shown in Figure 2.

The first step of the strategy was to grow *E. coli* in M9 minimal medium with 0.1% of isotopically labeled glucose (U−^{13}C glucose, 99% isotopic purity) as the sole carbon

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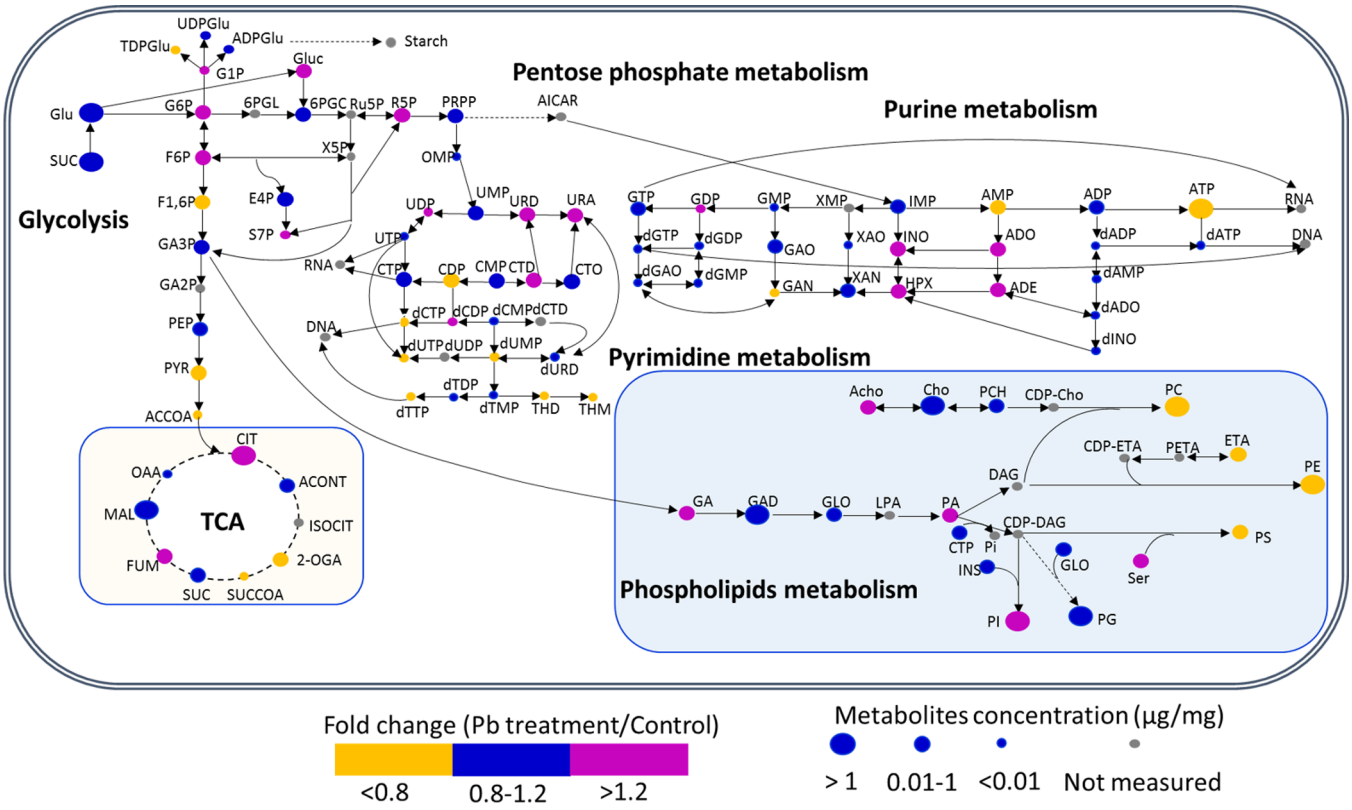

Figure 5. Pathways altered by Pb treatment in maize roots.

source. In order to increase the concentration and coverage of ^{13}C labeled metabolites, we performed a second step of cultivation using a eukaryotic organism *S. cerevisiae*. It is known that the growth of *S. cerevisiae* is limited in M9 minimal medium due to the lack of essential vitamins.[13] Therefore, the supernatant of *E. coli* culture containing ^{13}C labeled metabolites obtained from the first step was used for yeast growth without adding extra isotopic labeled vitamins such as ^{13}C-biotins and ^{13}C-nicotinic acid. Finally, both *E. coli* and yeast culture were pooled together and lyophilized for further analysis. As an example, Figure 3 showed three [U−^{13}C] labeled metabolites (LysoPC (18:0), Asparagine and AMP) and their corresponding unlabeled analogues. All the carbons were substituted by ^{13}C in the [U−^{13}C] labeled metabolites. Both labeled and the corresponding unlabeled analytes were coeluted at the same time on the chromatogram due to the identical chemical properties.

$NaH^{13}CO_3$ Is Required to Ensure the Elimination of Lag Phase Growth and 98−99% of ^{13}C Isotopic Enrichment

U−^{13}C labeled metabolites were extracted for evaluation of isotopic enrichment. The enrichment was calculated by peak area ratios of labeled and unlabeled analytes in the extract of *E. coli*−yeast culture ($AUC_{[U\text{-}13C]\ analyte}/AUC_{unlabeled\ analyte} + AUC_{[U\text{-}13C]\ analyte}$). AUC of 500 was given if an analyte was not detected ($S/N < 3$). Our results showed that amino acids, sugar and sugar phosphates, organic acids and cholines have high ^{13}C enrichment (>98%) (Figure 4a). In contrast, only 75%−85% of ^{13}C enrichment was achieved in a cluster of nucleotides, cofactors, and phospholipids (Figure 4a). Since other sources of ^{12}C contamination has been excluded, it is likely that the ^{12}C in the culture is from CO_2 fixation. We next explored the effect of $NaH^{13}CO_3$ addition on isotopic enrichment. $NaH^{13}CO_3$ (10 mM) was added into M9 medium in addition to U−^{13}C glucose and used for *E. coli* and yeast growth. Other procedures were same as described in Figure 2. LC−MS/MS analysis revealed that all the targeted analytes were uniformly ^{13}C labeled with >98% enrichment (Figure 4b). Interestingly, we also noticed a dramatic effect of $NaH^{13}CO_3$ addition on *E. coli* and *S. cerevisiae*. As shown in SI Figure S2a,b, *E. coli* had a lag period of 4 h after inoculation and *S. cerevisiae* was 2 h. The addition of 10 mM $NaH^{13}CO_3$ completely eliminated the lag period of both *E. coli* and *S. cerevisiae*. This allows the completion of two-step culture procedures within 2 days.

Quantification of U−^{13}C Labeled Metabolites in the Extract of *E. coli*−Yeast Culture

U−^{13}C labeled metabolites were then quantified using the optimal LC−MS/MS conditions described above. The amount of lyophilized *E. coli*−yeast culture used for extraction was optimized. Fifty mg (50 mg) of dry materials was needed to ensure that the *S/N* ratio of all the targeted compounds was greater than 20. The MS/MS parameters were detuned for some compounds with extreme high concentration which are out of linear range. The absolute concentration of U−^{13}C labeled metabolites were quantified using the reverse isotope dilution technique by the addition of different concentrations of unlabeled standards. The standard curves, coefficient *r* and concentrations are presented in SI Table S2. On a weight-to-weight basis, the major classes of U−^{13}C metabolites in *E. coli*−yeast culture were organic acids, amino acids, nucleotides, and phospholipids. Citric acid (58 000.67 ± 1421.56 ng/mg dry weight), acetic acid (30 414.51 ± 3215.93 ng/mg dry weight) and succinic acid (13 123.96 ± 1415.79 ng/mg dry weight) were the main organic acids. The top three U−^{13}C amino acids

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in the extract was valine (19 014.84 ± 432.15 ng/mg dry weight), alanine (14 711.53 ± 781.68 ng/mg dry weight), and glutamic acid (11 905.76 ± 3241.55 ng/mg dry weight). The concentration order of nucleotides was ATP (37 704.52 ± 3108.69 ng/mg dry weight) > GTP (37 260.61 ± 488.31 ng/mg dry weight) > AMP (19 716.16 ± 1582.91 ng/mg dry weight). The dominant phospholipid species in the extract was PE (32:2).

Stability of U−^{13}C Labeled Metabolites

We next evaluated the short-term and long-term stability of U−^{13}C labeled metabolites in the extract stored at −80 °C. The peak area of each metabolite was measured at 0 d, 7 d, 1 month, 6 month, and 1 year of storage. Our results showed that there were no significant changes in AUCs of U−^{13}C labeled metabolites within a year, and the relative standard deviation was less than 1% (SI Table S3).

Quantitative Metabolomic Analysis of Maize Roots in Response to Pb Treatment

Finally, we applied the developed quantitative metabolomic method to investigate the influence of Pb treatment on maize root metabolism. By the addition of U−^{13}C labeled metabolites as internal standards, we were able to accurately quantify 226 metabolites in maize roots out of 271 compounds we targeted in a 30 min run (SI Table S4). Forty-five (45) compounds were not quantified because their concentration in maize roots was below the lower limit of quantification (LLOQ). To our knowledge, the results presented here provided the first comprehensive profile of absolute concentrations of metabolites in maize roots. To maximize the usefulness of the data for the scientific community, we have provided all the details of method validation including MRM transitions, compound-specific MS settings, retention time, standard curves, LLOD, LLOQ, coefficient *r*, concentrations of the metabolites in SI Table S1 and S4.

We then analyzed the metabolomic data by partial least-squares discriminant analysis (PLS-DA) and visualized the results by projection in three dimensions. This revealed dramatic differences in maize roots metabolism between Pb treatment and the control groups (SI Figure S3a). The top 31 significant metabolites were ranked according to their impact by variable importance in projection (VIP) score (SI Figure S3b). We observed that several organic acids and amino acids including citric acid, ascorbic acid, oxaloacetic acid, proline, cysteine, and threonine were significantly increased in response to Pb stress. However, the total hexose pool was decreased after Pb treatment. Further biochemical pathway analysis identified glycolysis, purine metabolism, pyrimidine metabolism, and TCA cycle as the main metabolic pathways changed by Pb stress in maize roots (Figure 5). Moreover, our results revealed that phospholipid metabolism was dramatically altered in response to Pb. Pb stress led to the accumulation of phosphatidylinositol (PI) and phosphatidic acid (PA) in maize roots (Figure 5 and SI Figure S3b). In contrast, among the six main phospholipid species targeted, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) were significantly decreased compared with the control groups ($p < 0.05$).

DISCUSSION

Selection of Target Metabolites

Given the efforts and cost that it needs to develop a targeted metabolomic method, the question of which compounds to measure is of vital importance as the choice of compounds completely shapes the downstream application of the method. In this study, we screened the metabolites that might play important roles in various plant abiotic stresses based on in silico modeling simulation,[14,15] bioinformatic analysis,[16] and wet-lab experimental results.[17,18] Only common metabolites that are present in both plants and *E. coli* or yeast were identified. Due to the dramatic differences of phospholipid composition between plants and *E. coli* or yeast, all the major phospholipids in maize, *E. coli*, and yeast were targeted. Finally, 271 metabolites were selected and a total of 542 MRM transitions were targeted in a single run, including both unlabeled and ^{13}C labeled compounds. To our knowledge, this is the most comprehensive quantitative metabolomic method for plant stress biology so far.

Separation of Diverse Set of Cellular Metabolites

A good chromatographic separation of metabolites prior to their ionization is desirable for increased measurement specificity and quantitative reliability.[1] Phospholipids have been separated on several different column chemistries and in both hydrophilic interaction mode and reversed phase mode.[19−21] Bajad and the coauthors had tested an LC−MS/MS method for the brief separation and reliable quantification of 140 water-soluble metabolites on an aminopropyl column in HILIC mode.[22] In this study, by the optimization of stationary phase, mobile phase and elution gradient, the chromatographic separation of diverse set of cellular metabolites was achieved, including both hydrophilic and hydrophobic compounds. All the 271 metabolites were almost evenly distributed with a 4 min segment in a 26 min run (SI Figure S4).

Significance of *E. coli*−*S. cerevisiae* Two-Step Culture Method and Practical Applications

The reliable quantification of intracellular metabolites is hindered by the availability of stable isotopically labeled compounds used as internal standards. Only about 100 U−^{13}C labeled metabolites that meet the requirement of metabolomics can be produced through either *E. coli*,[23] *Pichia pastoris*,[24] or *S. cerevisiae*.[25] Here, we dramatically expanded the number of U−^{13}C labeled metabolites to more than 250 compounds using *E. coli*−*S. cerevisiae* two-step culture method. The whole procedures are easy to handle and no special bioreactor is needed which is different from the previous studies.[24,25] In addition, this method is relatively inexpensive. Only two ^{13}C uniformly labeled precursors are required which leads to the production of 271 U−^{13}C labeled metabolites. On the basis of our calculation, one batch of culture (200 mL) is enough for about 1000 maize samples or 10000 LC injections (10 μL per injection).

The developed methods have potentially broad applications: The method can be applied to develop the database of the absolute concentrations of metabolites in plants. The method can also be used for the diagnosis of plant stress condition and the study of plant-microbe interaction on roots.

Application of qMetabolomics to Understand Pb Stress Response in Maize Roots

In this study, we identified the comprehensive metabolic responses to cope with Pb stress in maize roots using the

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developed qMetabolomics method. We provided the absolute concentrations of the targeted 271 metabolites. We observed the increase of organic acids, amino acids (citric acid, malic acid, oxalic acid, proline, and polyamine) which might serve as the ligands for metal chelation.[26,27] We also identified the accumulation of ascorbic acid, urate, and glutathione in maize roots (SI Figure S3b and Table S4). The increase of these low-molecular-weight antioxidants protects maize roots against oxidative stress induced by various abiotic stresses.[28] Besides these common plant responses to heavy metal stress, our metabolomic analysis suggested the dramatic changes of phospholipid metabolism in maize roots under Pb stress which has not been reported before (Figure 5). PA plays an important role in membrane biosynthesis and signal transduction.[29] Our results showed that Pb stress triggered the rapid accumulation of PA and PI lipids in maize roots. PA formation acts as a protein-docking site in cellular membrane and provides the cell with spatial and transient information about the external environment.[30]The accumulation of PI increases the concentration of hydroxyl groups and thus helps the protein and membrane stabilization.[31] PC and PE are the main membrane lipids in maize roots. We also observed that PC and PE lipids decreased in response to Pb treatment. It is likely that PE and PC lipids were converted to PA through phospholipase D.[32]

CONCLUSIONS

In summary, in this study, we established the most comprehensive qMetabolomic method thus far for plant root metabolism. We developed a simple and cost-effective metabolic labeling method for the production of uniformly ^{13}C labeled metabolites as internal standards for qMetaboolomic study using *E. coli*−yeast two-step cultivation. We successfully quantified the absolute concentration of 226 metabolites in maize roots in a single run within 30 min using the developed qMetabolomic method. Further pathway analysis revealed that glycolysis, purine, pyrimidine, and phospholipids were significantly altered in maize roots in response to Pb stress.

ASSOCIATED CONTENT

Supporting Information

Figure S1, Extracted ion chromatograms of 10 representative compounds separated by three types of columns. Figure S2, Effect of $NaH^{13}CO_3$ on *E. coli* (a) and yeast growth (b). Figure S3, Changes of maize root metabolism in response to Pb treatment identified by targeted metabolomic analysis. Figure S4, Histogram of metabolites elution order on Acclaim Mixed-Mode HILIC-1 column. Table S1, List of targeted metabolites. Table S2, The absolute concentration of uniformly ^{13}C labeled metabolites in *E. coli*−yeast culture. Table S3, The stability of uniformly ^{13}C labeled metabolites. Table S4, The absolute concentration of targeted metabolites in maize roots. Table S5, The abbreviation of the metabolites listed on pathway map. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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