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Metabolite Profiling of the Response of Burdock Roots to Copper Stress

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Supporting Information

ABSTRACT: *Arctium lappa* L. (Asteraceae), also known as burdock, has a long history of cultivation as a dietary vegetable worldwide. Stress in plants disrupts metabolic homeostasis and requires adjustment of metabolic pathways. Exposure to heavy metals is one of the most prevalent environmental stresses encountered by plants. In this study, metabolite profiling based on ^{1}H NMR and GC−MS was used to obtain a holistic view of the response of burdock roots to copper stress. The principal component analysis model generated from the NMR data showed significant separation between groups. Copper-treated burdock roots were characterized by increased levels of phenols and decreased levels of primary metabolites. These results suggest that copper stress leads to activation of the phenylpropanoid pathway and growth inhibition. GC−MS analyses revealed increased levels of unsaturated fatty acids and decreased levels of sterols in the copper-treated group. Changes in metabolite concentrations were analyzed by UPLC/QTRAP−MS, and the significances were confirmed by two-way analysis of variance and Bonferroni's test. Interestingly, linoleic acid was increased about 2.7-fold, from 316 ± 64.5 to 855 ± 111 ppm, in the group treated with copper for 6 days. This study demonstrates that metabolomic profiling is an effective analytical approach to understanding the metabolic pathway(s) associated with copper stress in burdock roots.

KEYWORDS: *metabolite profiling, burdock, copper stress, ^{1}H NMR, mass spectrometry*

INTRODUCTION

Arctium lappa L. (Asteraceae), also known as burdock, has been used historically as a dietary vegetable and as an ingredient in folk medicines for hypertension, gout, arteriosclerosis, hepatitis, and inflammatory disorders.[1,2] The biological activities and pharmacological functions reported for *Arctium* species include anti-inflammatory, anti-cancer, antidiabetic, antimicrobial, and antiviral effects.[3]

Stress in plants typically disrupts metabolic homeostasis and requires adjustment of metabolic pathways, a process that is usually referred to as acclimation.[4] Exposure to heavy metals is one of the most prevalent environmental stresses encountered by plants. In natural environments, an increase in the concentration of heavy metals occurs mainly as a result of agricultural, manufacturing, mining, or waste-disposal practices. Copper is essential to plant nutrition and acts as a catalyst for many enzyme systems.[5−7] However, excessive copper is highly toxic due to its high affinity for thiol groups. Copper can participate in redox reactions that generate highly reactive hydroxyl radicals, thereby enhancing the production of reactive oxygen species (ROS) through Fenton reactions. Free copper catalyzes the reaction between superoxide anions and H_2O_2, resulting in direct bonds to the free thiols of cysteine residues with consequent catastrophic damage to lipids, proteins, and DNA.[6,8]

Metabolomics has become an important field in plant science and natural products chemistry. Metabolomics is generally defined as both the qualitative and quantitative analysis of all metabolites in a given organism.[9,10] A metabolomic approach is potentially useful for studying the biochemical origins of stress because changes in the metabolic profile are the ultimate result of such external influences.[11]

Unlike other "-omics" technologies, there is no single means of detecting all metabolites present in a single sample. A combination of extraction and detection techniques is required to fully characterize a metabolic profile.[12] Among the technological platforms available, nuclear magnetic resonance (NMR) and mass spectroscopy (MS) have been successfully utilized in metabolite profiling. Complementary use of these two techniques is typically successful given the synergies between their respective advantages and limitations. NMR analyses allow rapid screening of polar metabolites with minimal sample preparation, short acquisition times, and quantitative information. Conversely, gas chromatography (GC)−MS analyses can be used to detect nonpolar metabolites that are not identified by NMR. Therefore, these two analytical techniques provide complementary information for a complete metabolomics approach.

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A metabolomic study can contribute significantly to our understanding of stress in plants and other organisms by identifying various stress-related compounds, such as by-products of stress metabolism, stress signal transducers, and molecules that comprise the acclimation response of plants.[4] This study describes a method that makes use of metabolic profiling and metabolic fingerprinting to obtain a holistic view of stress in plants. This study also includes a detailed pathway analysis. Even with the highest-quality soil and agricultural practices, the impact of stress on a crop can reduce productivity significantly. Thus, diminishing farm land and the potential effects of global climate change on environmental and pathogen or insect-induced stresses provide an increased impetus to understand stress resistance in crop plants.[13] Although much concerning the participation of flavonoids in plant stress is known, the exact function of flavonoid metabolites in a plant's reaction to toxic heavy metals remains a topic of debate.[14] However, it is important to note that not all plants respond to heavy metals in the same manner.[14] This report describes a metabolomic study of burdock roots in response to copper stress.

MATERIALS AND METHODS

Chemicals. Methanol-d_4 (99.8% v/v) and deuterium oxide (99.9% v/v) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Acetic acid, methylnonadecanoic acid, and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt were purchased from Sigma–Aldrich (St. Louis, MO). HPLC-grade methanol, water, and methylene chloride were purchased from Burdick & Jackson (Muskegon, MI).

Plant Materials. Burdock plants were cultivated from March to July 2011 in bare ground after seeding. Stress experiments were performed in pots. Eighty plants were transplanted into four pots and allowed to acclimate for 4 weeks. To elicit stress, two pots were spiked with 100 μM $CuCl_2$, while another two pots received a spike of deionized water (DW; control group). Each of the pots received 500 mL of copper solution every 2 days. Plants were harvested after 1 and 6 days, rinsed immediately with water, and divided into groups of eight plants each. Photographs of burdock roots are provided in Supporting Information, Figure S1. Roots cut from the plants were rapidly frozen in liquid nitrogen, chopped into small pieces, freeze-dried, and ground into a fine powder in liquid nitrogen by use of a pestle and mortar. The resulting tissue powder was stored at −80 °C until required for analysis.

Sample Preparation. To obtain a copper-stress-induced metabolic profile, nonpolar and polar metabolites were sequentially extracted from the same sample. This strategy provides a convenient means of replicating the results and performing multivariate data analysis by accounting for the inherent biological variation of independent samples.

For the extraction of nonpolar metabolites, 4 mL of methylene chloride (MC) and 10 μL of 1 mg/mL methylnonadecanoic acid (internal standard) were added to 100 mg of dry, powdered plant material. The mixture was thoroughly mixed at room temperature (RT) for 1 h. The mixtures were centrifuged at 3000 rpm for 10 min at 4 °C. The resulting supernatants were passed through a 0.2-μm poly(tetrafluoroethylene) syringe filter (Merck Millipore, Billerica, MA). The extracts were dried with N_2 gas and dissolved in 200 μL of chloroform for GC–MS analysis. The remaining tissue pellets were extracted with 4 mL of MeOH containing 15 μL of 2 mg/mL sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in order to profile the polar metabolites. The MeOH extracts were prepared in the same way as the MC phase. Aliquots (500 μL) of the MeOH extracts were transferred to a separate vial for additional analyses. The remaining MeOH extracts were dried with N_2 gas. For NMR analyses, the resulting residue was dissolved in a mixture of 300 μL of methanol-d_4 and 300 μL of 0.2 M sodium phosphate buffer (pH 7) in D_2O. D_2O and DSS provided a field frequency lock and a chemical shift reference (^{1}H, δ 0.00), respectively.

GC–MS Analysis and Data Preprocessing. GC–MS analyses were performed on a 6890 Plus gas chromatograph equipped with a 5973N quadrupole mass spectrometer system (Agilent, Palo Alto, CA). A DB-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenyl–95% dimethylsiloxane phase) was obtained from J&W Scientific (Folsom, CA). The GC oven temperature was maintained at 60 °C for 2 min, then ramped to 320 °C at 10 °C/min and maintained for 10 min. The sample was injected with a splitting ratio of 1:10. The temperatures of the GC injection port and MS interface were 250 and 280 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The mass spectrometer was run in electron impact (EI) mode, with an electron energy of 70 eV. Mass spectra were acquired at 1 scan/s with a full scan range of 50–800 atomic mass units (amu). A freely available mass spectral deconvolution algorithm (automated mass spectral deconvolution and identification system, AMDIS) was used to analyze the data in combination with a filtering algorithm (SpectConnect) for processing of multiple data sets. All GC–MS data were normalized by use of methylnonadecanoic acid as an internal standard.

NMR Analysis and Data Preprocessing. ^{1}H NMR spectra were acquired on a VNMRS 600-MHz NMR spectrometer using a triple-resonance HCN salt-tolerant cold probe (Agilent Technologies Inc., Santa Clara, CA). A NOESYPRESAT pulse sequence was applied to suppress the signals from residual water. For each sample, 64 transients were collected into 67 568 data points with spectral width of 8445.9 Hz, relaxation delay of 2.0 s, acquisition time of 4.00 s, and mixing time of 100 ms. A 0.5-Hz line-broadening function was applied to all spectra prior to Fourier transform (FT). The assignments of NMR signals were based on total correlation spectroscopy (2D ^{1}H–^{1}H TOCSY), heteronuclear single quantum correlations (2D ^{1}H–^{13}C HSQC), and spiking experiments.

All NMR spectra were phased and baseline-corrected by use of Chenomx NMR suite version 7.1 (Chenomx Inc., Edmonton, Alberta, Canada). The regions corresponding to water, methanol, and DSS (4.78–4.85, 3.29–3.37, and 0.00–0.70 ppm, respectively) were excluded, and the remaining spectral regions were divided into 0.005 ppm bins. The spectra were then normalized to the total spectral area and converted to ASCII format. The ASCII files were imported into MATLAB (R2006a; Mathworks, Inc., Natick, MA) and all spectra were aligned by use of the correlation optimized warping method.[15]

NMR Data Processing. The resultant NMR data sets were imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for chemometric analyses. All imported data were Pareto-scaled for multivariate analyses. Pareto scaling, in which each variable is divided by the square root of the standard deviation, gives greater weight to the variables with larger intensity but gives results that are not as extreme as those obtained with unscaled data. Pareto scaling is typically used when a very large dynamic range exists in the data set.[16,17] Principal component analyses (PCA) were performed to examine the intrinsic variation in the data set and to obtain an overview of the variation among the groups. The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.[18]

Metabolites were identified by use of Chenomx Profiler, a module of Chenomx NMR Suite version 7.1, and by analysis of the 2D NMR experimental data. The identities of those metabolites deemed important on the basis of chemometric analyses were confirmed by use of spiking experiments and other references. The quantification of primary metabolites was achieved by use of the 600-MHz library from Chenomx NMR Suite version 7.1, which uses the concentration of a known reference signal (in this case DSS) to determine the concentration of individual compounds. The library is based on a database of 260 individual metabolite spectra acquired with the NOESYPRESAT sequence. Because the secondary metabolites were not contained in this library, their intensities were subjected to two-way analysis of variance (ANOVA).

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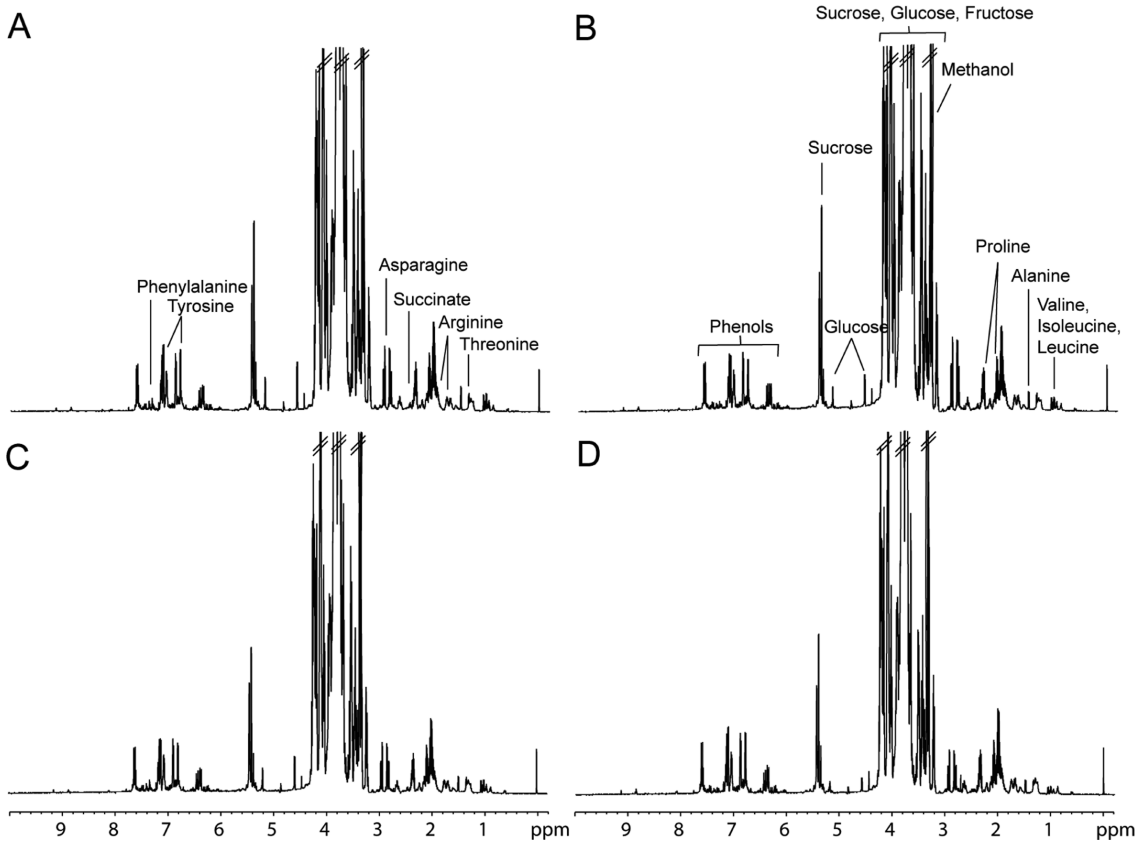

Figure 1. Representative ^{1}H NMR spectra of burdock root extracts. (A) DW treatment for 1 day, (B) DW treatment for 6 days, (C) 100 μM $CuCl_2$ treatment for 1 day, and (D) 100 μM $CuCl_2$ treatment for 6 days.

Targeted Analysis by UPLC/QTRAP-MS. Two plant hormones (jasmonic acid and salicylic acid) and seven fatty acids (myristic acid, palmitic acid, stearic acid, palmitoleic acid, oleic acid, linoleic acid, and linolenic acid) of burdock extracts were analyzed by ultraperformance liquid chromatography (UPLC)/QTRAP-MS. Nine targeted metabolites were prepared by dilution of MC and MeOH fractions of the burdock extracts. Separation of these analytes was performed with an Ekspert ultraLC 100-XL system (Eksigent, Dublin, CA) equipped with a high-strength silica (HSS) T3 C18 column that was 50 × 2.1 mm with a 1.8 μm particle size (Waters, Milford, MA). The column temperature was 40 °C and the autosampler was maintained at 4 °C. Aliquots (5 μL) of sample were injected via the partial loop method. The mobile phase consisted of (A) HPLC-grade water with 0.1% acetic acid and (B) methanol with 0.1% acetic acid. The column was eluted at a flow rate of 0.45 mL/min. For separating plant hormones, the mobile phase was changed from 70% A/30% B at time zero to 1% A/99% B over 3.5 min. To separate the fatty acids, the initial composition of the mobile phase was 15% A/85% B, changing to 1% A/99% B over 3.5 min. In both cases the end gradients remained constant for 0.5 min and then returned to the initial composition over 0.1 min and maintained that mixture for an additional 0.9 min.

This UPLC system was connected to a QTRAP 6500 MS/MS (AB Sciex, Framingham, MA) equipped with an IonDrive Turbo V source operating in negative-ion mode. Two plant hormones and seven fatty acids were detected individually by LC−MS/MS using multiple reaction monitoring (MRM) for the transition of the parent ion to the product ion. The concentration of each compound was then calculated according to calibration curves. The MRM transitions were acquired with the following operational parameters: capillary voltage of −4500 V, nebulizer pressure of 60 psi, drying gas pressure of 60 psi, curtain gas pressure of 30 psi, source temperature of 500 °C, entrance potential (EP) of −10 eV, and dwell time of 20 ms for the monitored transition. To obtain reliable results, specific values of collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) were used for each targeted metabolite. Data acquisition and processing were performed with Analyst version 1.6 software and MultiQuant version 2.1 software (AB SCIEX, Framingham, MA).

Statistical Methods. Two-way ANOVA was performed with GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA) to test the significance of differences in metabolite levels among the control and copper-exposed groups on day 1 versus day 6. Bonferroni's test was performed to determine differences between specific groups. Differences were tested at the 95% probability level ($p < 0.05$).

RESULTS

Polar Metabolite Profiling by Use of NMR. Representative one-dimensional ^{1}H NMR spectra of aqueous burdock extracts are shown in Figure 1. Signals in the sugar region of the NMR spectra were higher than those in the aliphatic and aromatic regions. The majority of the detected metabolites were amino acids and sugars, although some organic acids and phenols were also assigned. The following assignments are based on comparisons with the chemical shifts of standard compounds by use of NMR Suite software (version 7.1; Chenomx) and were confirmed by 2D NMR analyses and spiking experiments: alanine at δ = 1.48 and 3.78; arginine at δ = 1.71, 1.93, 3.22, and 3.76; asparagine at δ = 2.81, 2.94, and 4.02; fructose at δ = 3.48−4.11; glucose at δ = 3.20−3.91, 4.58, and 5.19; isoleucine at δ = 0.95 and 1.02; leucine at δ = 0.97; phenylalanine at δ = 7.33 and 7.38; proline at δ = 2.03, 2.34, 3.32, 3.41, and 4.07; succinate at δ = 2.40; sucrose at δ = 3.41−4.18 and 5.40; threonine at δ = 1.33; tyrosine at δ = 6.79 and 7.12; and valine at δ = 1.00 and 1.05. The characteristic signals in the aromatic region of the spectrum were attributed, by 2D

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NMR, to phenols having structures similar to that of caffeic acid (Figure 2).

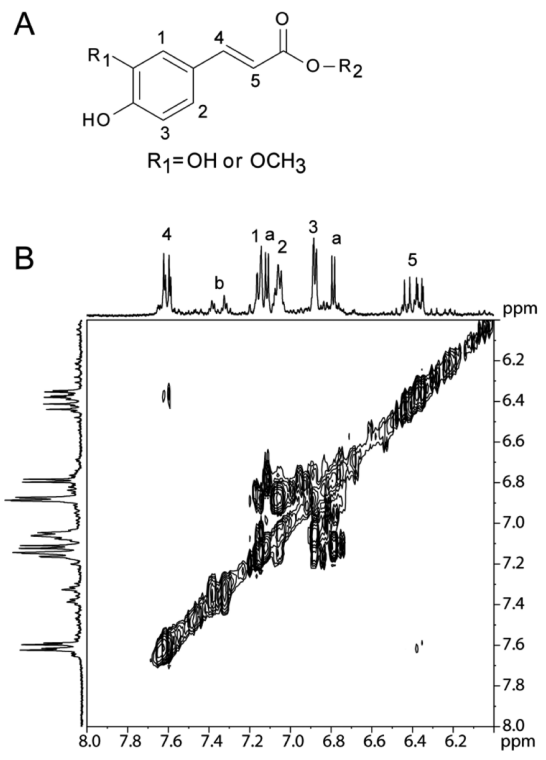

Figure 2. (A) Structure of phenols; (B) expanded TOCSY spectrum. a and b indicate tyrosine and phenylalanine, respectively.

To provide comparative interpretations and a means of visualizing the intrinsic variations and metabolic differences in the burdock root extracts that had been exposed to copper stress, PCA was applied to the ^{1}H NMR data set. The PCA score plot shows significant separation between control and copper-treated groups at different exposure times (Figure 3). The PCA model was established with five components and yielded R^2 and Q^2 values of 0.778 and 0.558, respectively. In this score plot, control groups of burdock root extracts showed no differences between day 1 and day 6. However, groups that had been exposed for 6 days are separated from the other groups by the first component.

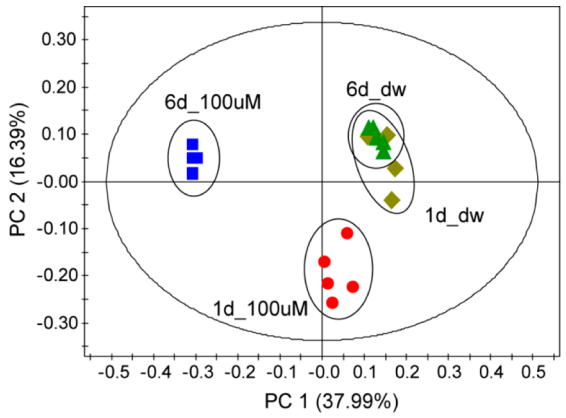

Figure 3. PCA score plot derived from NMR spectra of burdock extracts (R^2 = 0.778, Q^2 = 0.558). The ellipse represents the 95% confidence region for Hotelling's T^2.

To identify the differences in metabolic levels, the aqueous metabolite concentrations were determined by use of the 600-MHz library from Chenomx 7.1. This method compares the integrated intensity of a known reference signal (DSS) with signals derived from a library of compounds containing chemical shifts and peak multiplicities for all of the resonances of that known reference. However, since phenol concentrations could not be determined with Chenomx, peak areas were used instead of compound concentrations.

PCA analyses were performed with quantified metabolites of burdock extracts. The resulting score plot also showed significant separation between control and copper-treated groups (Figure 4). The PCA model was established with three components and yielded R^2 and Q^2 values of 0.926 and 0.803, respectively. In the score plot, control groups of burdock root extracts are separated from copper-treated groups by the first component. Groups with different exposure times (1 or 6 days) are separated by the second component. The loading plot of PCA, in which each data point represents a component (variable), reveals that metabolites that significantly contributed to the intergroup differences were those farthest from the main cluster of metabolites (Figure 4B). Exposure groups were characterized by a lower concentration of all metabolites, except phenols.

Two-way ANOVA was used to assess the significance of observed differences in metabolite levels among the groups. Concentrations of the quantified metabolites and ANOVA results are presented in Table 1. ANOVA showed that, between control and copper-treated groups, all metabolites—with the exception of arginine, proline, and sucrose—were present at significantly different levels.

Nonpolar Metabolite Profiling by Gas Chromatography–Mass Spectrometry. A GC–MS method was used in metabolite profiling of nonpolar extracts. A typical total ion chromatogram of the nonpolar fraction of burdock root extract is shown in Figure 5. Based on spectral similarities and corrections for shifts in retention time, integrated signals of the base peak ions of six major peaks, including the internal standard (IS; methylnonadecanoic acid), were generated by SpectConnect. Identification of the five sample peaks was achieved by searching the NIST05 mass spectral library for compounds matching the experimental fragmented ion to base peak (bp) abundance ratio and retention time. Library matches were validated with standard materials. Thus, the five major peaks were attributed to linoleic acid (9,12-octadecadienoic acid), linolenic acid (9,12,15-octadecatrienoic acid), sitosterol, stigmasterol, and campesterol. The sterol group of the latter three compounds was evidenced by a high-intensity molecular ion. Instead of a molecular ion, compounds containing a fatty acid group yielded an $[M - 15]^+$ ion through the loss of a methyl group.

All mass spectral peaks were normalized by use of methylnonadecanoic acid as an internal standard. Two-way ANOVA was performed to determine the significance of differences in normalized peak intensities. Note that the peak areas used in the ANOVA represented the relative concentration of each metabolite, not the absolute concentration. Nevertheless, relative values can be used to compare among samples. The differences between the copper-treated groups and the controls, determined by Bonferroni post hoc tests, are shown in Figure 6. All five of the metabolites displayed a

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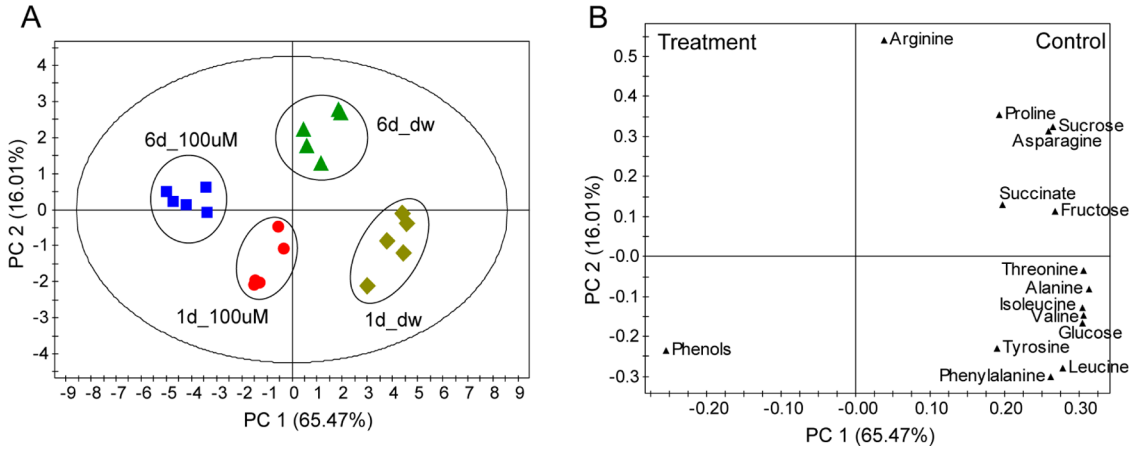

Figure 4. (A) PCA score plot (R^2 = 0.926, Q^2 = 0.803) and (B) loading scatter plot of quantified metabolites in burdock extracts. The ellipse represents the 95% confidence region for Hotelling's T^2.

Table 1. Identification and Quantification of Metabolites from ^{1}H NMR Spectra of Burdock Extracts

	treatment for 1 day			treatment for 6 days					
metabolite[a]	DW[b] (μM)	100 μM $CuCl_2$ (μM)	x-fold change	DW (μM)	100 μM $CuCl_2$ (μM)	x-fold change	interaction (p)[c]	day factor (p)	treatment factor (p)
alanine	591 ± 30.3	382 ± 11.2	0.65***	286 ± 30.2	223 ± 27.4	0.78***	0.0201	<0.0001	<0.0001
arginine	2680 ± 490	2380 ± 432	0.89	3250 ± 316	2890 ± 213	0.89	0.866	0.0056	0.0709
asparagine	7210 ± 353	5360 ± 206	0.74***	8340 ± 345	4290 ± 195	0.51***	<0.0001	0.795	<0.0001
fructose	19000 ± 3230	16600 ± 1310	0.88	20200 ± 1370	7270 ± 456	0.36***	<0.0001	0.0002	<0.0001
glucose	6380 ± 246	4070 ± 391	0.64***	4030 ± 151	1710 ± 156	0.43***	0.981	<0.0001	<0.0001
isoleucine	240 ± 11.9	170 ± 13.5	0.71***	176 ± 14.7	67.9 ± 3.75	0.38***	0.002	<0.0001	<0.0001
leucine	134 ± 18.1	82 ± 11.3	0.61***	64.7 ± 14.4	22.2 ± 7.27	0.34***	0.447	<0.0001	<0.0001
phenylalanine	304 ± 107	259 ± 45	0.85***	207 ± 22.3	147 ± 49.6	0.71**	0.0414	<0.0001	<0.0001
proline	11000 ± 1290	8930 ± 454	0.81	11100 ± 549	9720 ± 643	0.88	0.323	0.251	0.0002
succinate	61.5 ± 1.65	28.5 ± 7.29	0.46***	46.2 ± 4.04	44.5 ± 1.45	0.96***	<0.0001	0.858	<0.0001
sucrose	13300 ± 606	9300 ± 417	0.70	13700 ± 597	9610 ± 718	0.70	0.871	0.210	<0.0001
threonine	345 ± 24.6	283 ± 11.6	0.82*	307 ± 14.2	204 ± 24.6	0.67***	0.0329	<0.0001	<0.0001
tyrosine	3010 ± 110	2300 ± 111	0.77***	2260 ± 69.4	2450 ± 292	1.08	<0.0001	0.0011	0.0034
valine	407 ± 18.1	292 ± 17.1	0.72***	295 ± 10.8	151 ± 14.5	0.51***	0.0510	<0.0001	<0.0001
phenols[d]	4.02 ± 0.107	4.41 ± 0.317	1.10	3.77 ± 0.212	4.92 ± 0.16	1.30**	0.0012	0.165	<0.0001

[a]Identification and quantification of metabolites were performed with the Chenomx software. Concentrations are indicated as mean ± standard deviation. [b]DW, deionized water (control treatment). [c]P values are the result of two-way ANOVA using GraphPad Prism 5.0. [d]Phenols were assigned by use of standard compounds and 2D NMR data with peak intensities instead of actual concentrations.

significance of $p < 0.05$. Levels of fatty acids, including both linoleic acid and linolenic acid, were significantly higher in extracts of burdock roots that had been exposed to copper for 6 days, while levels of sterols such as sitosterol, stigmasterol, and campesterol were significantly lower than those of the control group.

Targeted Analysis by UPLC/QTRAP-MS. Various primary metabolites—including amino acids, organic acids, and sugars—were detected in NMR analyses of methanolic burdock extracts. However, plant hormones that are known to protect plants from oxidative damage associated with a variety of stresses were not evident in the NMR data. Therefore, targeted analyses of jasmonic acid and salicylic acid were conducted by use of UPLC/QTRAP-MS. In addition, among the various fatty acids in the extracts, only linoleic acid and linolenic acid were detected in the GC–MS analyses. To identify not only unsaturated fatty acids but also saturated fatty acids, targeted analyses of myristic acid, palmitic acid, stearic acid, palmitoleic acid, oleic acid, linoleic acid, and linolenic acid were also performed.

MS/MS analyses of standard compounds were first conducted with the QTRAP system with flow injection via syringe pump. The selected fragment ions (Table 2) allowed for selective detection of two plant hormones and seven fatty acids by LC-MS/MS with MRM. MRM transitions were primarily optimized for deprotonated molecular ions $[M - H]^-$. All resulting calibration curves showed high linearity (R^2 > 0.993). The concentrations of metabolites calculated from the calibration curves are presented in Figure 7. A two-way ANOVA test was performed using Prism version 5.0 (GraphPad Software, Inc.) to determine the significance of differences in metabolite levels among the groups. Of the plant hormones, the concentrations of jasmonic acid were lower than the limit of quantitation in all groups and would therefore be less than 5 μg/mL in the original methanolic extract. The concentration of salicylic acid in the copper-treated groups was significantly higher than that of the control groups after the 6-day exposure period. Levels of saturated fatty acids (myristic acid and stearic acid) did not differ significantly between copper-treated and control groups. Only palmitic acid exhibited an increase in

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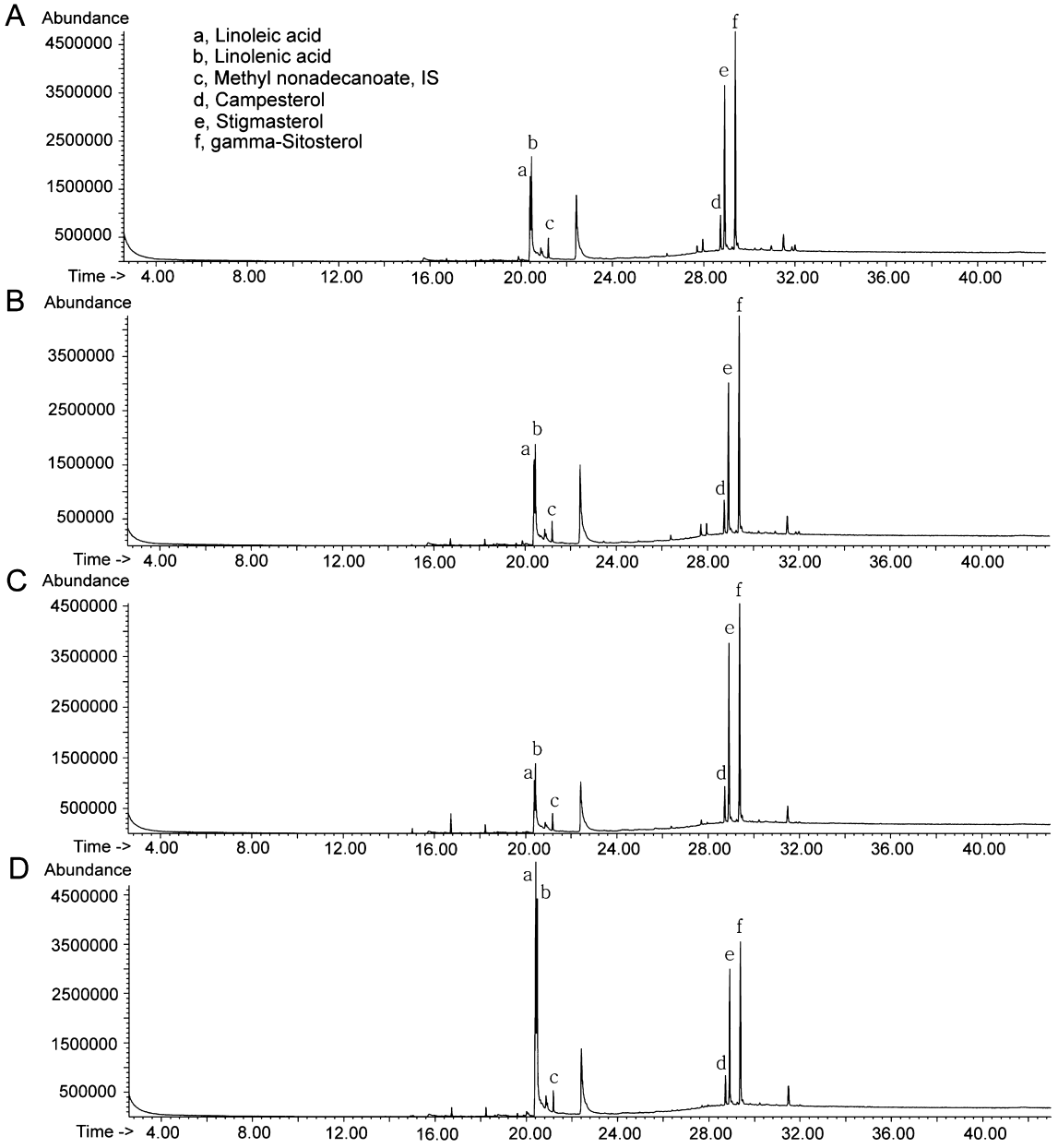

Figure 5. Representative GC−MS chromatograms of burdock extracts. (A) DW treatment for 1 day, (B) 100 μM $CuCl_2$ treatment for 1 day, (C) DW treatment for 6 days, and (D) 100 μM $CuCl_2$ treatment for 6 days.

concentration following copper exposure. Conversely, concentrations of all unsaturated fatty acids (palmitoleic acid, oleic acid, linoleic acid, and linolenic acid) were significantly higher in the copper-treated groups.

DISCUSSION

Generally, the average copper concentration in the dry mass of untreated plant tissue is 10 mg/kg.[19,20] Halasz et al.[21] reported a copper concentration of 21 mg/kg in burdock roots from an uncontaminated site in Hungary. Copper levels above 20−30 mg/kg can be toxic in plants.[20] Roots of *Typha latifolia* contained copper concentrations of 8 and 450−580 mg/kg before and after incubation in a solution of 100 μM copper, respectively.[20] Even though the amount of copper in root extracts was not measured directly, levels of copper concentrations of burdock roots used in this study were expected to be higher than the 20−30 mg/kg toxic level in plants. High copper concentration of 100 μM $CuCl_2$ was selected to induce the metabolic changes in burdock roots under copper stress. It was previously reported that copper concentration of 100 μM or higher was used to identify the effect of copper stress in other plants such as *Vicia faba* L.,[22] *Typha latifolia*,[23] maize,[24] and so on. Growth was significantly inhibited and important cellular processes were impaired in plants containing high copper concentrations. The goals of this study were to examine the metabolic changes in burdock roots in response to copper stress and to understand the metabolic pathway associated with copper stress in burdock roots through polar and nonpolar metabolite profiling.

NMR data acquired on the polar extract showed clear differences between control and exposed groups. In a PCA score plot derived from ^{1}H NMR spectra, the controls were grouped together and the copper-treated groups (6-day exposure) were clearly separated. Conversely, a PCA score

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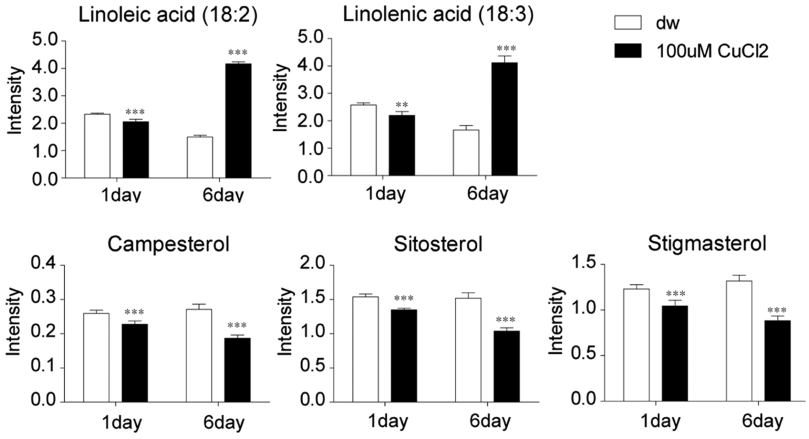

Figure 6. Quantification of identified metabolites by two-way ANOVA of GC−MS data of burdock extracts. Error bars indicate means ± standard deviation. Two-way ANOVA tests (copper treatment × time) yielded significant differences in the levels of all metabolites ($P \leq 0.0002$). ***$p < 0.001$ as determined by Bonferroni post hoc tests using GraphPad Prism 5.0.

Table 2. Specific Fragment Ions and Calibration Parameters for Targeted Metabolites

targeted metabolites	dilution degree	MRM	DP[a] (eV)	CE[a] (eV)	CXP[a] (eV)	retention time (min)	linear range (μg/mL)	R^2
				Plant Hormones				
jasmonic acid	×10	209/58.9	−25	−16	−7	2.58	0.5−50	0.999
salicylic acid	×10	137/92.9	−20	−22	−11	1.78	0.5−50	0.999
				Fatty Acids				
myristic acid	×10	227/209	−95	−28	−21	1.73	20−500	0.997
palmitic acid	×10	255/237	−95	−28	−15	2.4	20−1000	0.999
stearic acid	×10	283/265	−115	−32	−15	3.11	50−2000	0.997
palmitoleic acid	×100	253/235	−105	−26	−13	1.92	10−2000	0.994
oleic acid	×100	281/263	−125	−28	−19	2.58	20−1000	0.999
linoleic acid	×1000	279/261	−105	−24	−29	2.16	100−2000	0.997
linolenic acid	×1000	277/233	−105	−22	−21	1.83	20−1000	0.997

[a]DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

plot derived from specific, targeted profiles showed slight differences in the patterns of control groups. On the basis of NMR spectra, sugars accounted for the majority of polar compounds in the burdock extracts. However, only free sugars, such as sucrose, glucose, and fructose, were identified from the Chenomx library. Thus, it is likely that other bound sugars and polyols were detected in the polar extracts but did not affect the separation between control groups.

The levels of most primary metabolites identified in the NMR data decreased after exposure to copper stress. Generally, heavy metals such as Cu are essential for normal plant growth and development.[25] However, excessive concentrations of heavy metals in the soil can lead to toxicity symptoms, inhibit growth, and induce metabolic disorders.[26] High concentrations of heavy metals in plant tissues can disrupt nutrient metabolism, resulting in abnormal growth.[27] The inhibition of growth may result in the observed decrease in the levels of primary metabolites (free sugars, amino acids, and organic acids) in the copper-treated burdock roots. A plant's response to metal toxicity can be expressed in a variety of ways, such as metal ion chelation. Chelation of metals in the cytosol by high-affinity ligands is a potentially important mechanism of heavy-metal detoxification and tolerance.[25] Histidine is particularly important in the chelation of metal.[26]

Phenolic metabolites were detected in polar metabolite profiling based on NMR. The observed phenolic compounds were found to be similar to the structure of caffeic acid through 2D NMR analysis. Caffeic acid derivatives are typically included in phenylpropanoids, which have important functions in stress defense mechanism, and the levels of caffeic acid derivatives in burdock root increased following copper exposure. Increased production of ROS, followed by primary defense reactions, is associated with production of phytoalexin, activation of the general phenylpropanoid pathway, and induction of lignin biosynthesis.[4,26] Phenylpropanoids are precursors of lignins, which are an integral part of the stress defense mechanism, especially at the root level, where such materials can affect cell wall composition and stiffness.[12] Caffeic acid derivatives of phenylpropanoids would be expected to increase as a part of the burdock's defense response. In addition, salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate, and other small molecules produced as a result of stress can also serve as signaling molecules that activate systemic defense mechanisms and acclimation responses.[4] These plant hormones were not detected in the above NMR analyses because of the low sensitivity of the technique. However, targeted UPLC/QTRAP-MS data showed that levels of jasmonic acid and salicylic acid in the copper-treated groups were twice those of controls.

Nonpolar metabolite profiling of the MC phase of burdock extracts by use of GC−MS showed high levels of two unsaturated fatty acids (linoleic acid and linolenic acid) and three sterols (campesterol, sitosterol, and stigmasterol). Fatty acids and sterols with phospholipids are primary components of the plasma membrane. Because the cell comes into contact with its environment via the plasma membrane, the adaptability and flexibility of the membrane is vital for cell survival.[28] Therefore,

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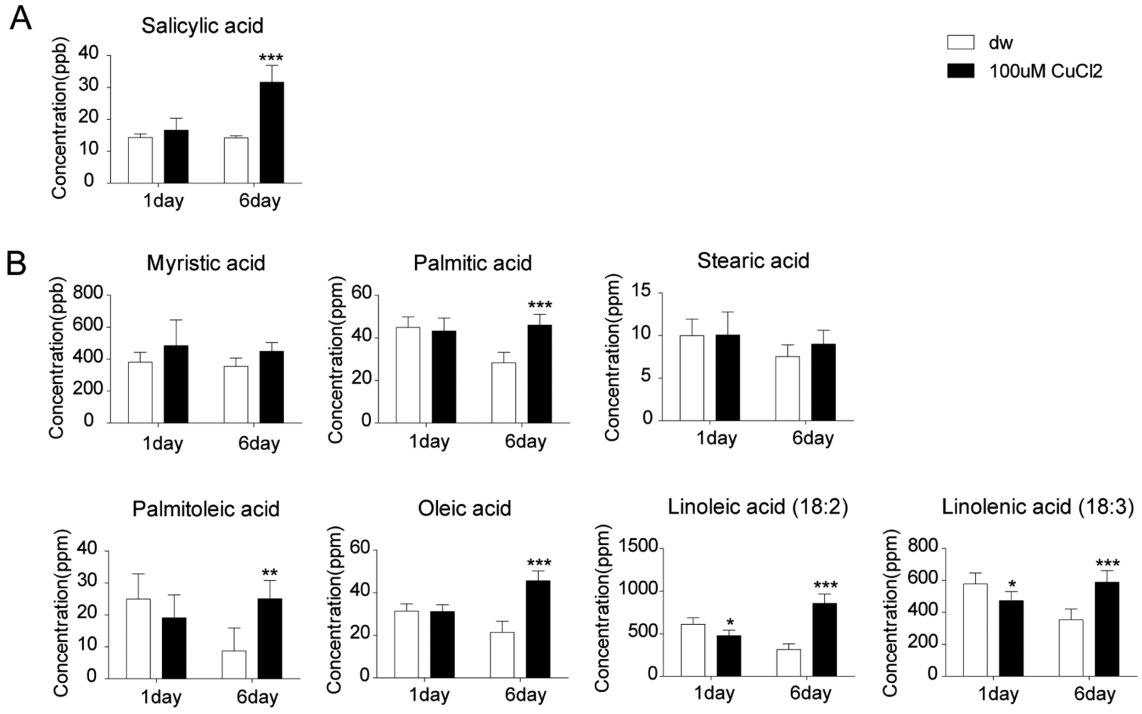

Figure 7. Quantification of identified metabolites by two-way ANOVA on Qtrap-MS data of burdock extracts. Error bars indicate means ± standard deviation. (A) Two-way ANOVA (copper treatment × time) yielded a significant difference in the level of salicylic acid ($P = 0.0001$). (B) Two-way ANOVA revealed significant differences in the levels of five fatty acids ($P \leq 0.0029$). ***$p < 0.001$ as determined by Bonferroni post hoc tests using GraphPad Prism 5.0.

the plant plasma membrane may be regarded as the first "living" structure that is exposed to heavy metal toxicity.[25] The function of the plasma membrane can be rapidly affected by heavy metals, as evidenced by increased cell leakage in the presence of high concentrations of metals, particularly copper.[25] Changes in the composition of the plasma membrane, such as relative levels of sterols and fatty acids, may contribute to changes in membrane fluidity and permeability and can be used as a mechanism of stress adaptation.[28,29]

In the above results, the levels of linoleic acid, linolenic acid, and other unsaturated fatty acids increased following copper exposure, although the presence of other fatty acids was not evident in the GC−MS data. However, targeted UPLC/QTRAP-MS analyses revealed significant changes in the concentrations of various fatty acids as a result of copper exposure. Although the level of palmitic acid increased significantly with copper exposure, the levels of myristic acid and stearic acid were unchanged. However, the levels of all unsaturated fatty acids (palmitoleic acid, oleic acid, linoleic acid, and linolenic acid) increased significantly with copper treatment. This high level of membrane lipid unsaturation is thought to help maintain the fluidity necessary for proper membrane function, which in turn can contribute to cell expansion and plant growth.[29] The ability to adjust membrane lipid fluidity by changing levels of unsaturated fatty acids is a mechanism for stress acclimation in plants. These adjustments are facilitated by the regulated activity of fatty acid desaturases.[13]

Sterol levels (campesterol, sitosterol, and stigmasterol) also decreased following the exposure of burdock roots to copper ion. Sterols are synthesized via the mevalonate pathway of isoprenoid metabolism.[30] The relative levels of stigmasterol, sitosterol, and campesterol have been proposed to influence the properties of cell membranes, particularly in relation to various stresses.[30,31] However, there were no obvious differences in the ratios of these three sterols between the copper-treated and control groups. Although membrane permeability increases according to the relative levels of the three sterols by campesterol < sitosterol < stigmasterol,[30] the observed decrease in concentrations of these three sterols was uniform across all three sterols at approximately 1.5-fold. Therefore, in the copper stress response of burdock roots, sterols may influence the properties of the plasma membrane through changes in their overall levels, not their relative ratios.

In this study, extracts from burdock roots exposed to copper for 1 or 6 days were compared and characterized by metabolite profiling with complementary analyses of ^{1}H NMR, GC−MS, and UPLC/QTRAP-MS data. Changes in primary metabolite and phenol concentrations may result from heavy-metal stress-induced growth inhibition and activation of the phenylpropanoid pathway, respectively. Conversely, changes in levels of salicylic acid and unsaturated fatty acids may result from damage to the cell membrane and/or loss of membrane integrity. This study demonstrates that metabolomic profiling is an effective analytical approach to understand the metabolic pathway(s) associated with copper stress in burdock roots.

■ ASSOCIATED CONTENT

Supporting Information

One figure with pictures of burdock roots following incubation in DW or 100 μM $CuCl_2$ for 1 day or 6 days. 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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