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Comparative Metabolomics in *Glycine max* and *Glycine soja* under Salt Stress To Reveal the Phenotypes of Their Offspring

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Supporting Information

ABSTRACT: Metabolomics is developing as an important functional genomics tool for understanding plant systems' response to genetic and environmental changes. Here, we characterized the metabolic changes of cultivated soybean C08 (*Glycine max* L. Merr) and wild soybean W05 (*Glycine soja* Sieb.et Zucc.) under salt stress using MS-based metabolomics, in order to reveal the phenotypes of their eight hybrid offspring (9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590). Total small molecule extracts of soybean seedling leaves were profiled by gas chromatography−mass spectrometry (GC−MS) and liquid chromatography−Fourier transform mass spectrometry (LC−FT/MS). We found that wild soybean contained higher amounts of disaccharides, sugar alcohols, and acetylated amino acids than cultivated soybean, but with lower amounts of monosaccharides, carboxylic acids, and unsaturated fatty acids. Further investigations demonstrated that the ability of soybean to tolerate salt was mainly based on synthesis of compatible solutes, induction of reactive oxygen species (ROS) scavengers, cell membrane modifications, and induction of plant hormones. On the basis of metabolic phenotype, the salt-tolerance abilities of 9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590 were discriminated. Our results demonstrated that MS-based metabolomics provides a fast and powerful approach to discriminate the salt-tolerance characteristics of soybeans.

KEYWORDS: *soybean, salt stress, metabolomics, GC−MS, LC−FT/MS*

1. INTRODUCTION

Salinization of soils caused by anthropic factors (e.g., irrigation) has become a major environmental threat to agricultural production and distribution worldwide.[1] It has been estimated that more than 20% of all cultivated lands around the world is considered to be high salt soils.[2] What's worse, most crop plants such as wheat, rice, maize, and tomato are glycophytic species, and they are vulnerable to salt stress.[3] In order to maintain crop production in high salt areas, development of salt-tolerant crops is needed.

Cultivated soybean (*Glycine max* L. Merr) is an important crop for human and domestic animal nutrition due to its high contents of proteins and fatty acids.[4] It is estimated that about 69% of the world's dietary proteins and 30% of the world's edible oils are from soybean.[5] However, it is important to note that the cultivated soybean, like many other crop species, is a salt-sensitive glycophyte.[6] Salt stress could reduce plant height and leaf size, inhibit nitrogen fixation, and decrease protein content and seed quality, thus causing significant reduction in growth and yield of soybean.[7] In contrast, wild soybean (*Glycine soja* Sieb.et Zucc.), another one of the major varieties of soybean, shows a great potential in the tolerance of soil salinity.[8] In this sense, understanding the mechanisms and identifying genes involved in salt-tolerance of wild soybean would enable breeders to develop new strategies to enhance salt-tolerance of cultivated soybean. Presently, the compete draft sequence of soybean genome has been finished by Jackson et al. in 2010 and more than 46,000 genes are estimated.[9] In 2010, genomes of 31 wild and cultivated soybean varieties were resequenced by Lam et al. using the Illumina Genome Analyzer II platform.[5] However, investigation of salt-inducible genes in soybean is still very limited.[10−12]

In recent years, metabolic phenotype analysis is developing as a new and promising methodology for functional genomics studies.[13] Metabolites are the end products of gene expression, and the corresponding metabolite levels can be regarded as the ultimate responses of biological systems to genetic and environmental changes.[14] Thus, metabolic phenotype reflects genetic morphology and its interactions with environment at the metabolite level, which should be much closer to the genotype of an organism than classic morphological trait that is mainly based upon what we can observe about the characteristics of an organism.[15] Consequently, establishing a direct correlation between metabolic phenotype and genotype would help us understand functional genomics. As a well-known technique, metabolomics offers a powerful approach to describe the metabolic phenotype in a given organism.[16−19] Presently, it has been successfully applied to discriminate different plant phenotypes[20−24] and predict potential phenotypes associated with silent mutations.[25] It is a pity that metabolomics still faces many challenges mainly because of the complexities of metabolite composition in biological samples and the limitations of a given analytical method. Indeed, successful analysis of the metabolome in a biological sample requires analytical methods able to record information on as many

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metabolites as possible. However, no single analytical technique (i.e., GC−MS and LC−MS) alone can provide detection of the total metabolites in the given biological sample, due to their intrinsic limitations in either detection or quantification.[26] For instance, GC−MS is targeted toward the volatile compounds of the primary metabolism, since the derivatization protocols often lose information about secondary (specialized) metabolites. LC−MS is able to measure a wider range of different metabolites, especially for detection of low-concentration compounds that may not be detectable with GC−MS.[27] It is thus conceivable that the combination of GC−MS and LC−MS may offer a complementary advantage for detection of metabolites.[28]

In the present study, we characterized the metabolic changes of one wild (W05) and one cultivated (C08) soybean under salt stress using combined gas chromatography−mass spectrometry (GC−MS) and liquid chromatography−Fourier transform mass spectrometry (LC−FT/MS), in order to reveal the phenotypes of their eight hybrid offspring (9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590). First of all, we investigated the metabolic profiles of all ten soybeans and identified the metabolic variations between W05 and C08. Based on the metabolic variations between W05 and C08, we aimed to establish a fast and powerful model that could be used to discriminate the salt-tolerance abilities of 9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590. Besides, metabolic changes in seedling leaves of W05 and C08 under salt stress were investigated, and potential salt-tolerance mechanisms in soybean were discussed at the metabolite level. To our knowledge, this is the first time that metabolic comparison has been made between *Glycine soja* and *Glycine max* via combined GC−MS and LC−MS analysis.

2. MATERIALS AND METHODS

2.1. Plant Materials. Soybean seeds were kindly provided by Professor Lam Hon-Ming (The Chinese University of Hong Kong, Hong Kong). Two parental soybean varieties (wild soybean W05 and cultivated soybean C08) and their eight hybrid offspring lines (9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590) were selected for this study (Figure S1 in the Supporting Information). Herein, W05, 9H0086, 9H0124, 9H0391, and 9H0736 were salt-tolerant soybeans, and C08, 9H0380, 9H0400, 9H0434, and 9H0590 were salt-sensitive soybeans, which were confirmed based on the morphological traits in growth experiments under salt stress.

2.2. Chemicals. HPLC grade acetonitrile and methanol were purchased from JT Baker (NJ, USA). Spectroscopic grade formic acid, ribitol, methoxyamine hydrochloride, pyridine, and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide were obtained from Sigma/Aldrich (MO, USA). Distilled water was purified using the Milli-Q20 system Millipore (MA, USA).

2.3. Plant Growth and Sample Preparations. Soybeans (n = 3 in each soybean variety/line) were cultured in 1× Hoagland nutrient solution in a green house with natural light. In the salt-treated group, soybeans were treated with 0.6% NaCl for 48 h when they grew into 3−4 trifoliate leaves. In the control group, soybeans were cultivated under normal conditions.

All of the fresh soybean leaves were harvested in the green house on the third day and immediately frozen in liquid N_2. Homogenization was performed with mortar and pestle in liquid N_2, after which 150 ± 5 mg of pooled homogenized plant material was weighed in an Eppendorf tube. Plant materials were extracted with 300 μL of cold MeOH/H2O (80/20, v/v) in a Thermomixer (Eppendorf AG, Hamburg, Germany) during 15 min (1250 rpm, 4 °C). The extraction solvents were spiked in advance with ribitol (internal standard) obtaining a concentration of 180 μg/mL.

All extracts were sonicated for 5 min (Bransonic Ultrasonic Cleaner 1210, Danbury, CT, USA) and centrifuged (Sigma 3-18K, Sartorius AG, Göttingen, Germany) for 15 min (15,000 rpm, 4 °C). The supernatant (300 μL) was isolated and used for LC−MS analysis.

For GC−MS analysis, a 50 μL aliquot of the supernatant was further derivatized by methoxyamination with a 20 mg/mL solution of methoxyamine hydrochloride in pyridine, and subsequent trimethylsilylation with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide.

For the pooled quality control (QC) sample preparation, an aliquot (15 μL) of all prepared sample extracts was mixed in an Eppendorf tube under cold conditions. The QC sample was analyzed at the beginning, at the end, and randomly through the whole analysis batch in both LC−MS and GC−MS systems to evaluate the stability of analytical performance.

2.4. Liquid Chromatography−Mass Spectrometry. LC−MS analysis was performed on a Dionex Ultimate 3000 2D nanoflow LC system (Bruker Daltonics Inc., Billerica, MA, USA) coupled to the Apex Ultra 7.0 Hybrid Qh-FTMS (Bruker Daltonics Inc., Billerica, MA, USA) equipped with an electrospray ionization source. The samples were analyzed in both positive and negative ion modes so as to monitor as many metabolites as possible. The separation was performed on a ZORBAX Eclipse Plus C18 column (3.5 μm, 2.1 × 100 mm, Aglient) at the column temperature of 35 °C. The gradient duration program in positive ion mode was 0−1 min, 5% B; 1−3 min, 5−40% B; 3−8 min, 40−55% B; 8−15 min, 55−70% B; 15−24 min, 70−80% B; 24−32 min, 80−100% B; 32−35 min, 100% B; 35−38 min, 100−5% B (mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively); and in negative ion mode was 0−1 min, 5% B; 1−3 min, 5−40% B; 3−10 min, 40−55% B; 10−17 min, 55−70% B; 17−20 min, 70% B; 20−23 min, 70−5% B (mobile phases A and B were 100% water and acetonitrile, respectively). The flow rate was 0.2 mL/min. Five μL aliquots of sample were loaded for every individual analysis. The capillary voltage and spray shield were set to 4200 and 3500 V, respectively. The dry gas was set to 6 L/min at a temperature of 200 °C. The neb gas was set to 3 L/min. Spectra were acquired over the m/z 50−1000 range. The collision energy in the MS/MS mode was set to 15 V.

2.5. Gas Chromatography−Mass Spectrometry. GC−MS analysis was performed on an Agilent 7683B series injector (Agilent, Santa Clara, CA) coupled to an Agilent 6890N series gas chromatograph system and a 5975 mass selective detector (MSD) (Agilent, Santa Clara, CA). Agilent HP-5 ms capillary GC column (5% phenyl, 95% dimethylpolysiloxane, 30 m × 0.25 mm ID, df = 0.25 um) was used. A constant column flow of 1 mL/min helium was applied. The injector was kept at 230 °C. Samples were splitless injected (1 μL) during 1.5 min using a total flow of 54 mL/min, which was reduced to 35 mL/min after 2 min. The temperature programmed separation started at 60 °C for 5 min, and then ramped by 10 °C/min to 160 °C, 3 °C/min to 220 °C, 10 °C/min to 250 °C, 3 °C/min to 280 °C. After 5 min at 280 °C, the oven was cooled to the initial temperature of 60 °C within 2 min. A temperature equilibration phase of 5 min was allowed before the next injection. The transfer line was kept at 280 °C. The temperature for electron ionization (EI) operation was 260 °C. EI spectra were acquired between 45 and 600 Da.

2.6. Data Analysis. Extraction of LC−MS and GC−MS raw data was performed on XCMS online. Briefly, mass spectra obtained in LC−MS and GC−MS were respectively exported by Data analysis 4.0 software (Bruker Daltonics Inc., Billerica, MA, USA) and MSD ChemStation Data analysis software (Agilent, Santa Clara, CA), and saved as the netCDF files. The files were uploaded to XCMS through a secure SSL connection. For the feature detection, the XCMS *centWave* algorithm was used with the following parameters: signal/noise threshold = 10, ppm = 30, peakwidth = (8, 20), prefilter = (3, 200). The feature alignment was performed with the default parameters in XCMS with bw = 5 and mzwid = 0.025. The retention time correction was performed with the standard *obiwarp* algorithm in XCMS with prfostep = 1. To correct the MS response shift during the run, the raw data were normalized against total integration values. After this operation, the sum of the ion peak area within each sample was set to

10,000. Principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and hierarchical cluster analysis (HCA) were performed by SIMCA-P 12.0 version (Umetrics AB, Umeå, Sweden). The significance was expressed by using Student's *t* test of the SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). *P* value less than 0.05 was considered significant.

2.7. Metabolite Identification. AMDIS with NIST 05 standard mass spectral database was used to identify metabolites out of the GC−MS chromatograms. A potential metabolite was identified based on retention index and mass-spectral similarity (more than 80%) match. For LC−MS analysis, the putative identities of each ion were first given within XCMS by matching features in the METLIN database with the following parameters: ppm = 30, adducts = $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$ in the positive ion mode; ppm = 30, adducts = $[M - H]^-$ and $[M + Cl]^-$ in the negative ion mode. Furthermore, the potential molecule formulas of each ion were calculated by Data analysis 4.0 software (Bruker Daltonics Inc., Billerica, MA, USA) based on accurate mass. Additionally, the MS/MS information (fragment pattern) of each ion was obtained from MS/MS mode analysis. Finally, the metabolites were identified based on accurate mass, fragmentation pattern, and retention time.

3. RESULTS

3.1. Method Validation. In this study, ribitol was added into samples as an internal standard to monitor the performance of GC−MS and LC−MS systems. In parallel, the QC sample was analyzed regularly throughout both the GC−MS and LC−MS analysis sequences to evaluate the GC−MS and LC−MS system stabilities for large-scale sample analysis.[29−32] As described in Materials and Methods, the QC sample was prepared from aliquots of all samples, which provided a representative "mean" of the investigated samples. For GC−MS, the retention time shift ranged from 0.01 to 0.06 min, and the relative standard deviations (RSDs) of peak areas ranged from 3.18 to 15.03%. For LC−MS, the retention time shift ranged from 0.01 to 0.08 min, the mass accuracy deviation ranged from 0.1 mDa to 3.0 mDa, and the RSDs of peak areas ranged from 2.58 to 13.47%. We thus concluded that both LC−MS and GC−MS analytical methods provided measurement stability for the duration of the analysis sequence.

3.2. Metabolic Profiles of W05 and C08. In this study, metabolic profiles of two parental soybean varieties W05 and C08 were first investigated. The extracts of soybean seedling leaves were analyzed via the combined GC−MS and LC−FT/MS analysis. The representative GC−MS total ion current (TIC) chromatographs of leaf extracts obtained from W05 and C08 are displayed in Figure 1. Over 100 peaks were manually assigned and quantified in each sample. At last, 27 of these peaks were tentatively identified as metabolites in soybean leaves, including carboxylic acids, sugars, alcohols, amino acids, and fatty acids (Table 1). The full information of 27 identified metabolites is shown in Table S1 in the Supporting Information. Figure 2 demonstrates the representative LC−MS TIC chromatographs of leaf extracts obtained from W05 and C08. With XCMS online, more than 5,000 ions were finally extracted from each sample: over 3,000 ions were detected in the positive ion mode; and over 2,000 ions were monitored in the negative ion mode. Finally, 74 metabolites (57 metabolites in the positive ion mode and 17 in the negative ion mode) were temporarily identified, including carboxylic acids, amino acids, sugars and phosphates, alcohols, fatty acids, isoflavonoids, flavonoids, amines, and pigments (Table 1). The full information of identified metabolites is shown in Table S2 in the Supporting Information.

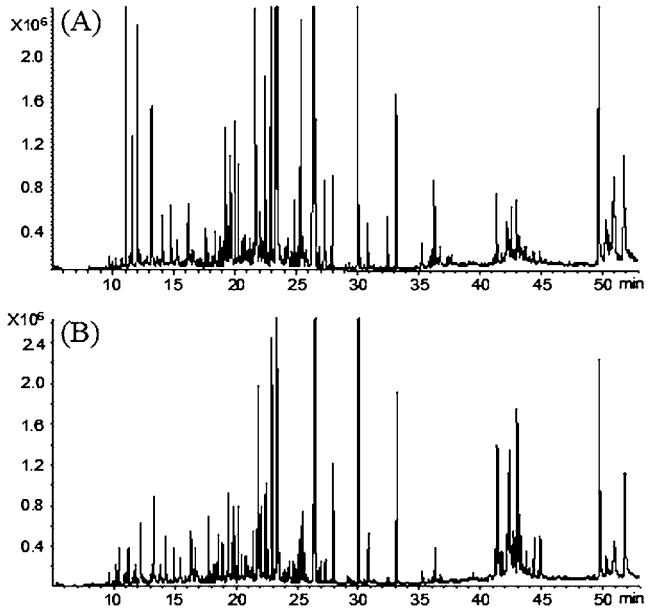

Figure 1. TIC chromatograms of soybean seedling leaf extracts obtained from GC−MS: (A) C08, (B) W05.

In summary, we concluded that the metabolome of soybean seedling leaves was mainly dominated by 98 metabolites including carboxylic acids, amino acids, sugars and phosphates, alcohols, fatty acids, isoflavonoids, flavonoids, amines, and pigments. More importantly, most of the metabolites detected in our study were mapped onto general biochemical pathways by searching Plant Metabolic Network (http://www.plantcyc.org/) and Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), such as glycolysis, TCA cycle, and amino acid metabolism (Figure 3).

3.3. Metabolic Differences between Salt-Tolerant and Sensitive Soybeans. In order to investigate the metabolic variations between salt-tolerant and sensitive soybean varieties, we compared the metabolic profile of W05 with that of C08. In this work, we focused on the 98 identified metabolites.

First, the mean levels of 98 metabolites in W05 and C08 were respectively calculated and compared. The significance of these metabolites was expressed by using Student's *t* test, and *P* value less than 0.05 was considered significant. At last, the levels of 24 metabolites were significantly different between the two parental soybean varieties (Table S3 in the Supporting Information). For instance, we found that the contents of carboxylic acids in W05 were much lower than those in C08, such as biotin, jasmonic acid, malonic acid, and pantothenic acid. However, the level of abscisic acid (ABA) was much higher in W05. Significantly higher levels of glutathione and acetylated amino acids (e.g., *N*-acetylglutamine and *N*-acetyltryptophan) were observed in seedling leaves of W05. The major sugars in soybean leaves were glucose, fructose, maltose, and sucrose. The contents of glucose and fructose were much higher in C08. Conversely, a significantly higher level of sucrose was observed in W05. Sugar alcohol such as mannitol was richer in W05, while sphinganine was richer in C08. As would be expected, the level of unsaturated fatty acid such as linolenic acid in W05 was lower than that in C08. A summary of some metabolomic variations observed within the two parental soybeans are schematized in the pathway diagram as shown in Figure 3.

Table 1. 98 Metabolites Detected in Soybean Seedling Leaves by GC−MS and LC−MS Systems[a]

Carboxylic Acids							
1	abscisic acid	6	chlorogenic acid	10	galactonic acid[b]	14	pantothenic acid
2	acetic acid[b]	7	cinnamic acid[b]	11	jasmonic acid	15	propanoic acid[b]
3	biotin	8	citric acid/isocitric acid	12	malic acid[b]	16	ribonic acid[b]
4	butyric acid[b]	9	ferulic acid[b]	13	malonic acid[b]	17	tartaric acid
5	caffeic acid						
Amino Acids							
18	alanine[b]	22	glutathione disulfide	26	*N*-acetylmethionine	30	threonine[b]
19	asparagine	23	glycine[b]	27	*N*-acetyltryptophan	31	tryptophan
20	ornithine	24	*N*-acetylglutamine	28	*N*-acetyltyrosine	32	tyrosine
21	glutathione	25	*N*-acetyllysine	29	serine[b]	33	valine[b]
Sugars and Phosphates							
34	1-kestose	37	glucose/fructose 6-phosphate	41	ribose[b]	45	sucrose-6-phosphate
35	galactose[b]	38	maltose[b]	42	sedoheptulose	46	xylose[b]
36a	glucose[b]	39	methylglucoside	43	sorbopyranose[b]	47	xylulose[b]
36b	glucose/fructose	40	rhamnose	44	sucrose		
Alcohols							
48	cholesterol	52	kushenol B	56	phytosphingosine-1-phosphate	60	sphingosine
49	glycerol[b]	53	lactitol/maltitol	57	sitosterol	61	sphingosine-1-phosphate
50	inositol[b]	54	mannitol	58	sphinganine	62	xylitol[b]
51	kushenol A	55	phytosphingosine	59	sphinganine-1-phosphate		
Fatty Acids							
63	arachidic acid	66	linoleic acid	68a	octadecanoic acid[b]	70	palmitic acid
64	dodecanoic acid[b]	67a	linolenic acid[b]	68b	octadecanoic acid	71	palmitoleic acid
65	lignoceric acid	67b	linolenic acid	69	oleic acid		
Isoflavonoids							
72	daidzein	75	genistein	78	glycitin	81	malonylglycitin
73	daidzin	76	genistin	79	malonyldaidzin	82	ononin
74	formononetin	77	glycitein	80	malonylgenistin		
Flavonoids							
83	isorhamnetin	86	kaemferol/luteolin	88	naringin	90	quercetin-7-(6″-acetylglucoside)
84	isorhamnetin-3-(6″-acetylglucoside)	87	naringenin	89	quercetin	91	quercimeritrin
85	isorhamnetin-3-glucoside						
Amines							
92	adenosine	93	methylguanosine	94	uric acid	95	uridine
Pigments							
96	carotene (α/β)	97	cyanidin	98	cyanidin-3-(6″-acetylglucoside)		

[a]The number of metabolites refers to Tables S1 and S2 in the Supporting Information. [b]These 27 metabolites were detected by GC−MS. Other metabolites were detected by LC−MS.

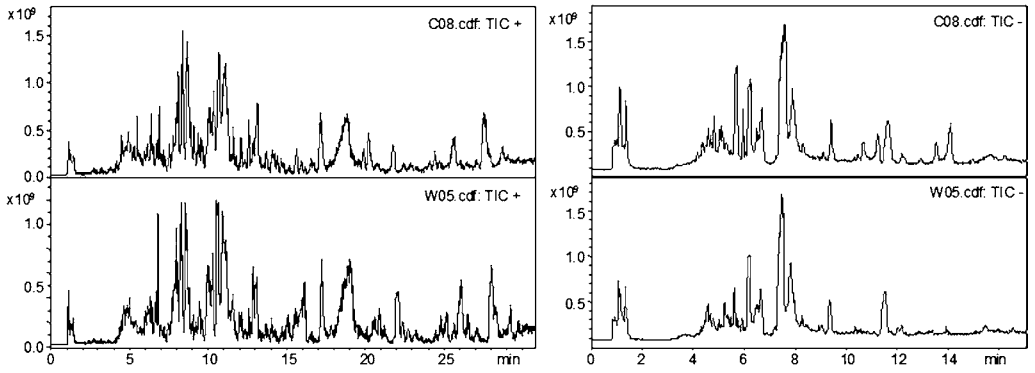

Figure 2. TIC chromatograms of soybean seedling leaf extracts obtained from LC−MS: (left) positive ion mode, (right) negative ion mode.

Moreover, PCA was performed to develop a visual plot for the evaluation of the resemblance and difference in the metabolic profiles between W05 and C08 on the basis of the above 24 significant altered metabolites. PCA is a data visualization method for a rapid means of visualizing similarities or differences within multivariate data, and it can extract chemical information objectively, which has been well established for grouping and discrimination in analyzing the chemical profiles of food and medicines.[33−35] The PCA score plot (Figure 4) showed that W05 and C08 were generally

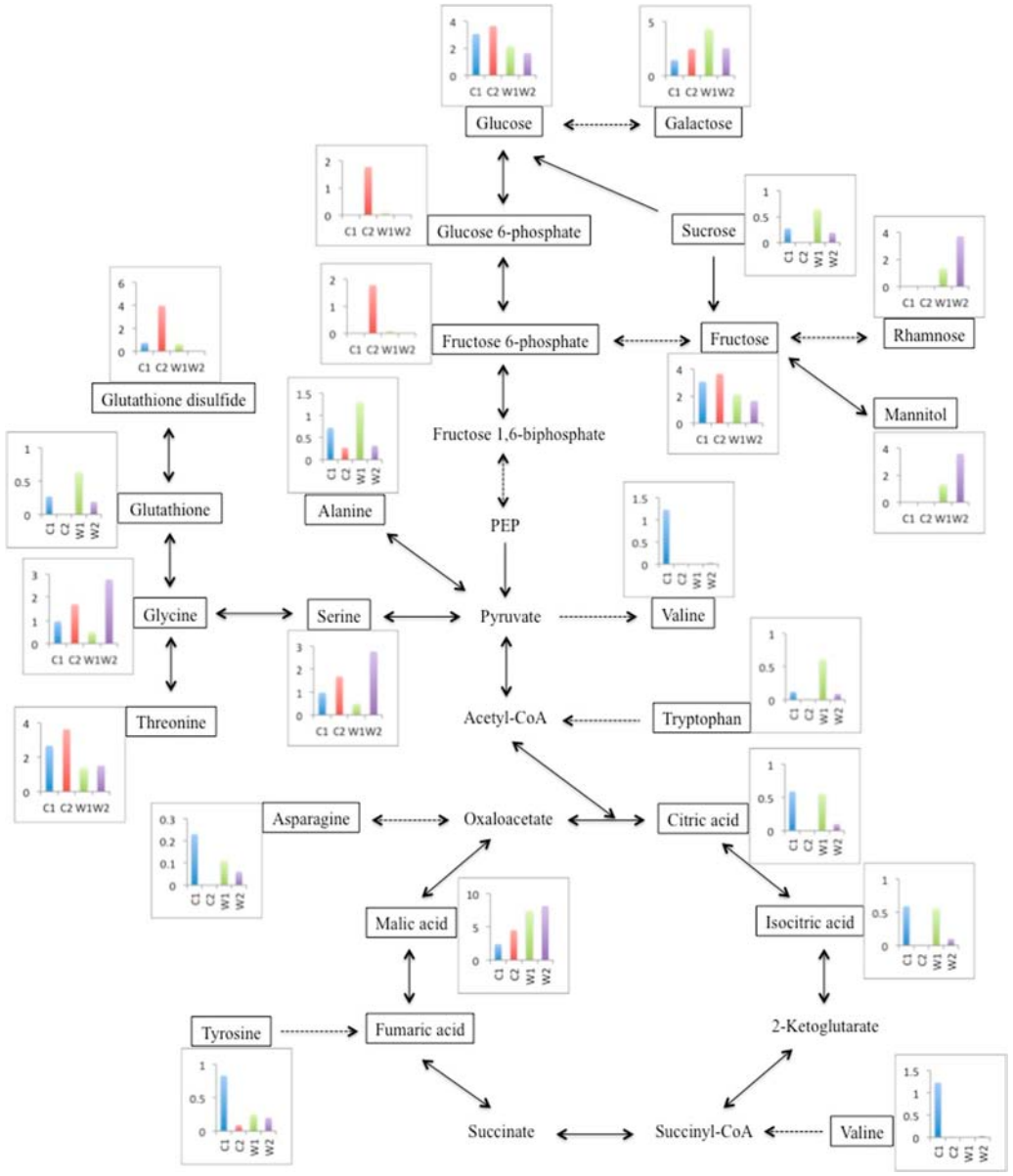

Figure 3. Comparative visualization of 18 identified metabolite (in the squares) levels in the pathways of glycolysis, TCA cycle, and amino acid metabolism. In column charts: C1, C08 under normal conditions; C2, C08 under salt stress conditions; W1, W05 under normal conditions; W2, W05 under salt stress conditions. Full information can be referenced in Table S3 in the Supporting Information.

distributed, which suggested that the metabolic profiles of the two parental soybean varieties were different. The first two principal components (PC1 and PC2) explained more than 94% of variance in the data set. In parallel with PCA, HCA was performed on the same data.[15] Here HCA was calculated by Ward's method with the squared Euclidean distance as a similarity measure. From the dendrogram (Figure 5), we can see that the samples of W05 and C08 were clearly divided into two groups, group A and group B. Our results indicated that W05 and C08 could be successfully distributed on the basis of the 24 significant altered metabolites.

3.4. Metabolic Changes in Soybean Seedling Leaves under Salt Stress. In order to investigate the metabolic responses of salt-tolerant and sensitive soybean varieties under salt stress, W05 and C08 were treated with 0.6% NaCl for 48 h when they grew into 3−4 trifoliate leaves. In this part, we also focused on the 98 identified metabolites.

First, mean levels of 98 metabolites in W05 with and without salt treatment were respectively calculated and compared. The significantly changed metabolites under salt stress were found out by using Student's *t* test. *P* values less than 0.05 were considered significant. At last, we found that 23 out of 98 metabolites in W05 were significantly changed after salt treatment. Similarly, we found that 27 out of 98 metabolites in C08 were significantly changed under salt stress. Full details are summarized in Table S3 in the Supporting Information. Some of the changed metabolites formed part of glycolysis, TCA cycle, and amino acid metabolism, which is shown in Figure 3. Under salt stress, ABA and caffeic acid were accumulated in seedling leaves of W05 and C08. However, jasmonic acid and malonic acid decreased in C08. Some amino acids such as alanine and glutathione decreased in seedling leaves of W05 and C08 after salt treatment, while glycine and serine increased in W05. Surprisingly, the level of sucrose was

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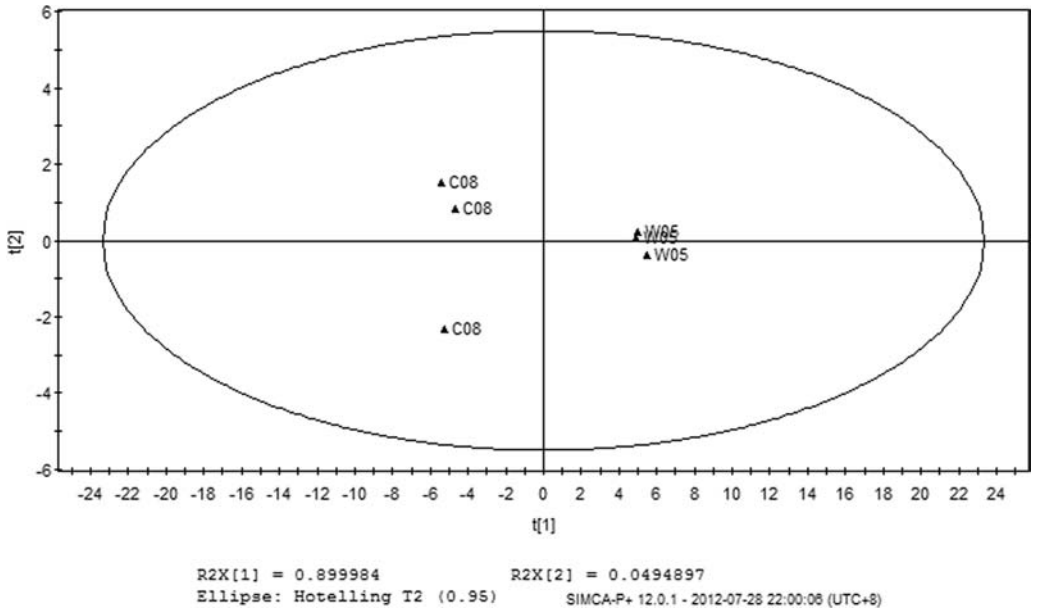

Figure 4. PCA score plot based on 24 significant altered metabolites to illustrate the metabolomic variations between C08 and W05 (R2X = 0.975, Q2 = 0.871).

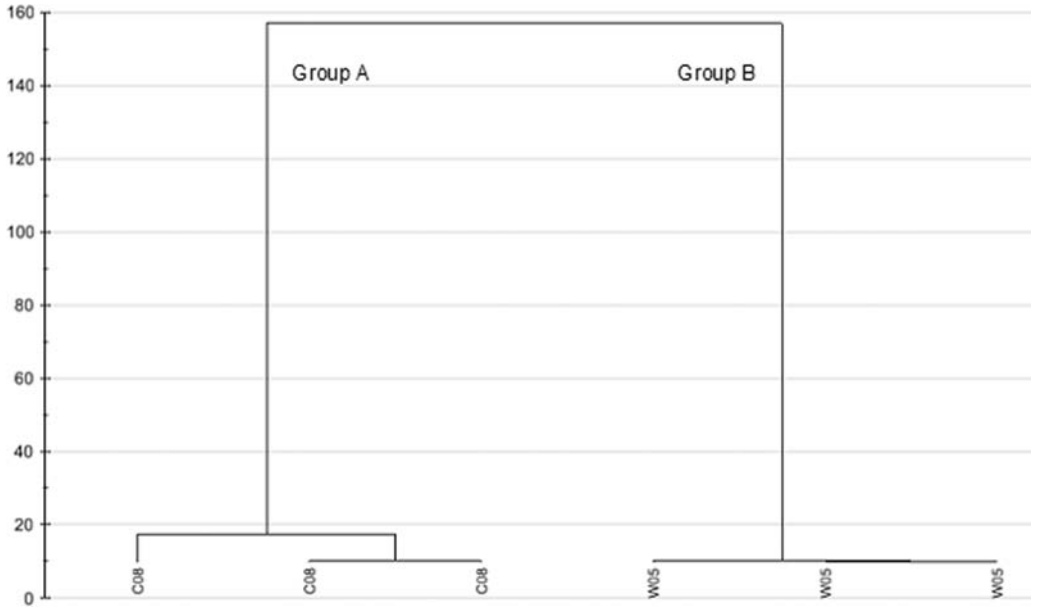

Figure 5. Dendrogram of HCA obtained from two parental soybean varieties (W05 and C08) based on 24 significant altered metabolites. Group A: salt-sensitive soybean. Group B: salt-tolerant soybean.

decreased in seedling leaves of W05 and C08, but the contents of fructose and glucose were not significantly changed. Sugar alcohols such as lactitol/maltitol were accumulated in seedling leaves of W05 and C08 after salt treatment. As would be expected, the levels of unsaturated fatty acid linolenic acid in W05 and C08 were increased after salt treatment. In contrast, saturated fatty acids such as dodecanoic acid and octadecanoic acid decreased in C08.

Furthermore, PCA was performed to develop a visual plot for the evaluation of changes of metabolic profile in the seedling leaves of W05 after salt treatment based on the 23 altered metabolites. As a result, soybean seedling leaf samples obtained from normal and salt stress conditions were divided into two groups in the PCA score plots (Figure 6A). In addition, a similar result was observed in C08 based on the 27 altered metabolites (Figure 6B). All the results suggested that the metabolic profile of soybean seedling leaves was changed after salt treatment, no matter in salt-tolerant or sensitive soybeans.

3.5. Phenotypes of Eight Hybrid Offspring. Our final objective was to reveal the phenotypes (which line is salt-tolerant soybean and which one is salt-sensitive soybean) of 9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590 on the basis of metabolic analysis. As aforementioned, we found that the levels of 24 metabolites were different between salt-tolerant soybean W05 and salt-sensitive soybean C08, which could be considered as the splitters to distinguish the salt-tolerance abilities of soybeans. First, mean levels of the 24 metabolites in two parental soybean

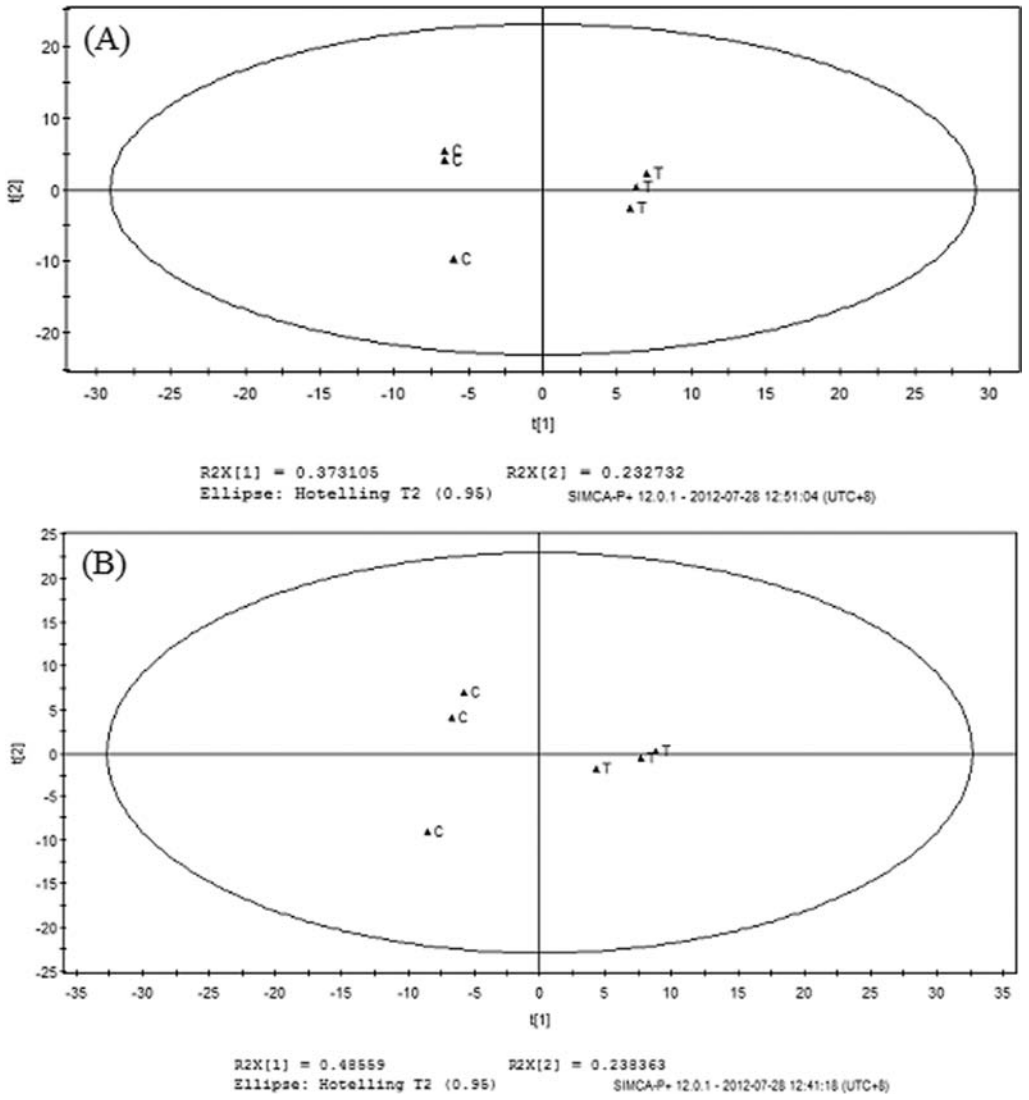

Figure 6. (A) PCA score plot based on 23 altered metabolites in W05 under salt stress, R2X = 0.606, Q2 = 0.352; (B) PCA score plot based on 27 altered metabolites in C08 under salt stress, R2X = 0.724, Q2 = 0.271. C: normal conditions. T: salt stress conditions.

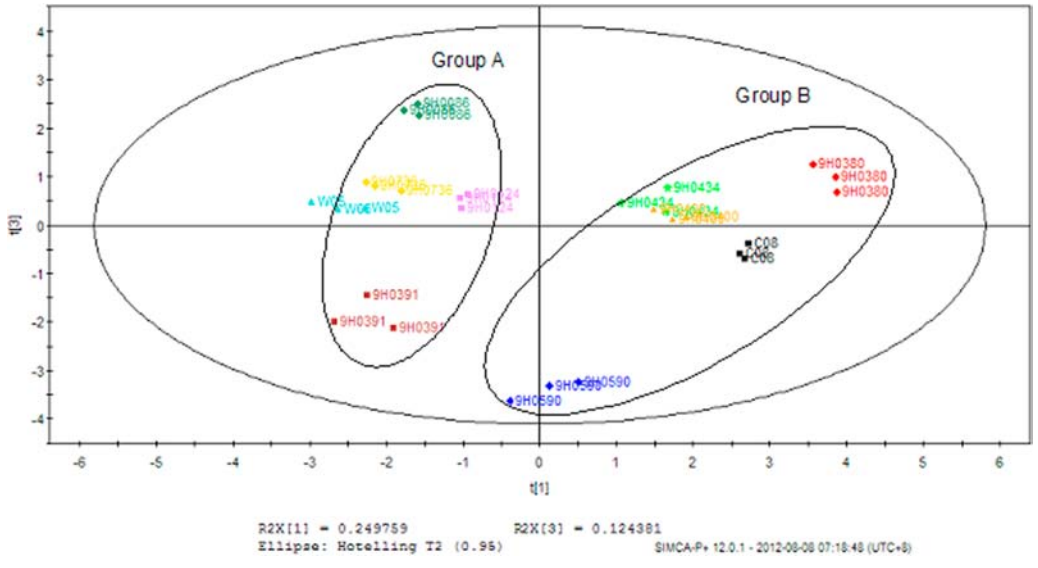

Figure 7. OPLS-DA score plot based on 24 significant altered metabolites between W05 and C08 to discriminate eight hybrid offspring soybean lines (9H0086, 9H0124, 9H0391, 9H0736, 9H0380, 9H0400, 9H0434, and 9H0590) (R2X = 0.942, R2Y = 0.898, Q2 = 0.557).

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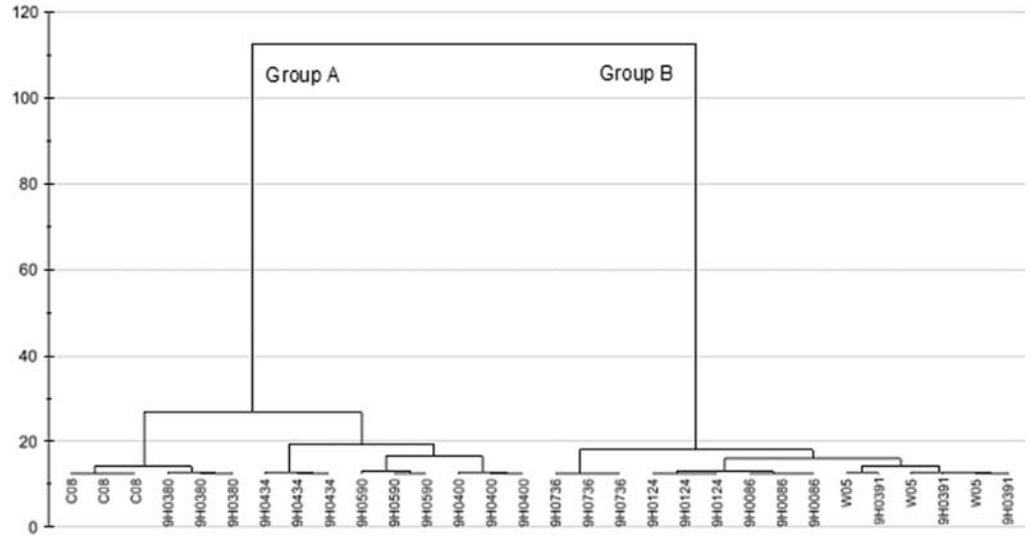

Figure 8. HCA dendrogram of ten soybeans based on 24 significant altered metabolites between W05 and C08. Group A: salt-sensitive soybean. Group B: salt-tolerant soybean.

varieties and eight hybrid offspring soybean lines were calculated and compared (data not shown). However, it was difficult to find out a visual relationship among them from the data set directly. In this case, the OPLS-DA model[36] was established to develop a visual plot based on the mass spectral data of 24 metabolites, to discriminate the salt-tolerance abilities of eight hybrid offspring soybean lines. As a result, the eight hybrid offspring soybeans were separated into two major groups (group A, 9H0086, 9H0124, 9H0391, and 9H0736; group B, 9H0380, 9H0400, 9H0434, and 9H0590) in the OPLS-DA score plot (Figure 7). Soybeans in group A were close to the salt-tolerant soybean W05, and those in group B were around the salt-sensitive soybean C08. Importantly, salt-tolerant (group A) and sensitive (group B) soybeans were clearly separated. The R2X, R2Y, and Q2 of this model are 0.942, 0.898, and 0.557, respectively. R2 and Q2 values close to 1.0 suggest a satisfactory model with a reliable predictive ability. The permutation result (n = 100, Q2 = −0.29) validated the stability and reliability of this OPLS-DA model. In addition, HCA was performed based on the same data. From the dendrogram (Figure 8), we can see that the ten soybeans are divided into two main clusters: group A (salt-tolerant soybeans) included C08, 9H0380, 9H0400, 9H0434, and 9H0590; group B (salt-sensitive soybeans) included W05, 9H0086, 9H0124, 9H0391, and 9H0736. High similarity was observed between the results of OPLS-DA and HCA, suggesting that our results were well consistent with the actual conditions.

4. DISCUSSION

4.1. Metabolite Identification. NIST/EPA/NIH Mass Spectral Library is the most popular mass spectral library for GC−MS analysis, which is copublished by National Institute of Standards and Technology (NIST), Environmental Protection Agency (EPA), and National Institutes of Health (NIH). Here, NIST 05 standard mass spectral database is selected to identify metabolites out of the GC−MS chromatograms. Unlike GC−MS analysis, there is no standard mass spectral library for LC−MS analysis. With more challenges, tens of thousands of peaks (where a peak is defined as an ion with a unique m/z and retention time) are often measured in a single sample. These ions might be metabolites, fragments, adducts, and noise.[29] This further increases the difficulty for metabolite identification. Currently, the exact basis for what constitutes valid metabolite identification is still debated in the community and a consensus is still evolving. However, non-novel metabolites (not being identified for the first time) are often identified based upon the cocharacterization (e.g., accurate mass, retention time, and fragments of ions).[37] In addition, use of literature or external laboratory data also helps us in metabolite identification. In this study, XCMS online with METLIN (Metabolite and Tandem MS) Database was employed for putative metabolite identification first. Then, metabolites were identified on the basis of the elemental composition, fragment pattern, and chromatographic retention behavior of those ions. In addition, literature data[38−41] were referenced as well.

4.2. Salt-Tolerance Mechanisms. Salt stress is a combination of ionic stress due to the chaotropic effects of incoming Na^+ and Cl^- and osmotic stress resulting from a decrease in water potential.[42] In natural environments, plants have developed several protective or compensatory mechanisms to cope with salt stress.[43,44] Known strategies to improve plants' salt stress tolerance include adjusting osmotic potential in cells by accumulating metabolites (compatible solutes), restoring oxidative balance to prevent further damage due to excess accumulation of reactive oxygen species (ROS), and remodeling membrane fluidity by structural modifications of cell wall and membrane composition. Presently, extensive investigations of salt stress responses using metabolomics have been described for tomato,[45] barley,[46] and maize.[33] Our findings were consistent with these studies. A few examples for soybean metabolites involved in salt stress responses include amino acids such as glycine, serine, and glutathione, polyols such as lactitol/maltitol, unsaturated fatty acid such as linolenic acid, and plant hormone such as ABA (Table S3 in the Supporting Information).

To accommodate the osmotic balance between cytoplasm and environment, soybean accumulates low-molecular-weight metabolites termed compatible solutes, which do not interfere with normal cellular functions even when present in high concentrations.[47] Increased concentrations of compatible solutes in the cytoplasm can contribute to reducing the water

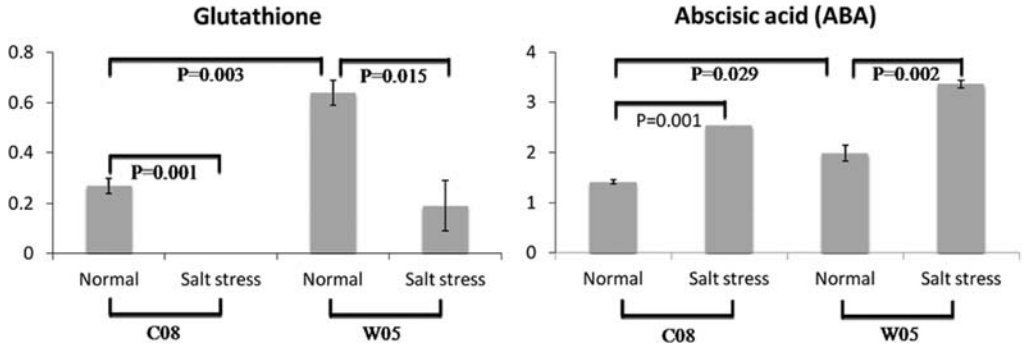

Figure 9. Mean levels of glutathione and abscisic acid in seedling leaves of W05 and C08 under normal and salt stress conditions. *Y*-axis is peak area.

potential in the cytoplasm by balancing the decreased water potential associated with Na^+ accumulation in the vacuoles and the extracellular volume (Widodo 2009).[46] These compatible solutes mainly include amino acids, sugars, and polyols. In this study, glycine and serine in W05 increased in response to salt stress. Generally, sugars such as glucose, fructose, and sucrose accumulate in plants under salt stress.[48] However, the content of sucrose was decreased in both W05 and C08 after salt treatment, and the glucose and fructose contents remained unchanged. Polyols such as lactitol/maltitol increased in response to salt stress.

In soybean, ROS is continuously produced as byproducts of various metabolic pathways, as well as being scavenged by different antioxidative components.[43] Under salt stress, the balance between evolving and scavenging of ROS is perturbed. High levels of ROS can lead to plant cell death. One possible mechanism of salt-tolerance in soybean is to elevate the contents and activities of various antioxidative components in order to restore the oxidative balance. In this study, the content of nonenzymatic ROS scavenger glutathione in W05 and C08 declined in response to salinity stress (Figure 9). This may be because glutathione is used up to scavenge the ROS, while products of glutathione are blocked.

The salt-tolerance of soybean is positively correlated to the stability of plasma membrane under salt stress. The plasma membrane of plant cells is composed of phospholipids, glycolipids, and steroids. Excessive salt could alter the composition of the plasma membrane and cause membrane leakage of electrolytes and organic compounds from the cells. It was reported that plants responded to salt stress by releasing unsaturated fatty acids from membrane lipids.[49] In this study, unsaturated fatty acid linolenic acid in leaf extracts of W05 and C08 increased under salinity stress, which was possibly released from the membrane lipids.

High salt concentration triggers an increase in levels of plant hormones.[50] In this study, salinity stress resulted in increased levels of ABA in seedling leaves of W05 and C08 (Figure 9). ABA has been found to alleviate the inhibitory effect of salt on photosynthesis, growth, and translocation of assimilates.[51]

4.3. Novel Approach To Reveal the Phenotype of Soybean. There is a crucial need to develop more salt-tolerant soybeans to improve production efficiency in the face of a burgeoning world population. Although investigation of salt-inducible genes in soybean is very limited, there is no doubt that wild soybean provides a potential and valuable source of genes for resistance to salt stress. Currently, interspecific hybridization is the major strategy to improve the salt tolerance of soybean.[52] However, the efficiency of traditional breeding is low because of long breeding cycle, since the phenotypic diversity of plants is often evaluated based on morphological traits and seed parameters when they are mature.[53] Our results suggested that metabolic phenotypes of soybean seedling leaves could be used to reveal the salt-tolerance ability of soybean as well. That offers an opportunity to shorten the breeding cycle, since the superior soybean lines with salt-tolerance ability can be picked out at seedling stage.

In summary, the metabolic profiles of soybean seedling leaves were monitored by combined GC−MS and LC−MS analysis. As a result, we found that the metabonome of soybean seedling leaves was mainly dominated by 98 metabolites, including carboxylic acids, amino acids, sugars and phosphates, alcohols, fatty acids, isoflavonoids, flavonoids, amines, and pigments. The levels of glutathione and ABA in W05 were higher than those in C08 even under normal conditions (Figure 9). This is possibly the main reason why W05 is more salt-tolerant than C08. The ability of soybeans to tolerate salt is mainly based on synthesis of compatible solutes, induction of ROS scavengers, induction of plant hormones, and cell membrane modifications. On the basis of metabolic phenotype analysis, eight hybrid offspring soybean lines could be discriminated with OPLS-DA and HCA. Although the present study was investigated within a small sample size, our results demonstrated that MS-based metabolomics possibly provides a fast and powerful approach to discriminate the salt-tolerance characteristics of soybeans.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 depicting 10 soybean seeds used in this study, Table S1 listing information about 27 metabolites detected by the GC−MS system, Table S2 containing MS and MS/MS information of 74 metabolites detected by the LC−MS system, and Table S3 providing the contents of 98 metabolites in soybean seedling leaves. 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.

■ ABBREVIATIONS USED

GC−MS, gas chromatography−mass spectrometry; LC−FT/MS, liquid chromatography−Fourier transform mass spectrometry; ROS, reactive oxygen species; QC, quality control; MSD, mass selective detector; EI, electron ionization; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; HCA, hierarchical cluster analysis; RSDs, relative standard deviations; TIC, total ion current; ABA, abscisic acid; NIST, National Institute of Standards and Technology; EPA, Environmental Protection Agency; NIH, National Institutes of Health

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