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Comparative Metabolic Profiling Reveals Secondary Metabolites Correlated with Soybean Salt Tolerance

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High-performance liquid chromatography−ultraviolet−electrospray ionization mass spectrometry (HPLC-UV-ESI-MS) and HPLC-ESI-MS^n analysis methods were used for metabolic profiling and simultaneous identification of isoflavonoids and saponins in soybean seeds. Comparative targeted metabolic profiling revealed marked differences in the metabolite composition between salt-sensitive and salt-tolerant soybean varieties. Principle component analysis clearly demonstrated that it is possible to use secondary metabolites, for example, isoflavones and saponins, to discriminate between closely related soybean genotypes. Genistin and group B saponins were identified as the key secondary metabolites correlated with salt tolerance. These individual metabolites may provide additional insight into the salt tolerance and adaptation of plants.

KEYWORDS: HPLC-ESI-MS^n; metabolic profiling; soyasaponin; isoflavonoid; soybean

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important legumes, serving as a dietary food and oil in many Asian countries. Soybean seeds are rich in isoflavones (genistein, daidzein, and glycitein) that have been suggested to have antioxidant and estrogenic functions (*1*). Soyasaponins, which constitute an important part of the soybean seed metabolome, have been suggested to exhibit hypocholesterolemic, immunostimulatory, anticarcinogenic, antioxidantative, antidiabetic, and hepatoporotective activities (*2*−*4*). Isoflavones and saponins also play extensive roles in the interaction between plants and the environment, for example, serving as chemical defense compounds against environmental stress to plants (*5*). Research on soybean metabolome analysis has been mostly focused on total isoflavones extraction, quantification, and compound identification (*6*−*10*). The distribution of isoflavononones and saponins in various soybean tissues and exudates has been examined using high-performance liquid chromatography (HPLC) profiling and a liquid chromatography−mass spectrometry (LC-MS) method. The amount of isoflavones present in soybean seed is variable, depending on genetic and environmental factors that are not fully understood (*11*−*15*).

Soil salinity poses an adverse effect on crop yield. In recent years, functional essentiality of ion homeostasis mechanisms in plant salt tolerance has been identified through cell biology and molecular genetics approaches, providing new insight into how plants respond and adapt to salinity stress. Primary metabolites, such as sugars, polyols, amino acids, tertiary and quarternary ammonium, and sulfonium compounds (*16*), are synthesized as osmolytes in response to high salinity stress. These osmolytes mediate osmotic adjustment and reduce cellular oxidative damage caused by free radicals produced in response to high salinity (*17, 18*). These core primary metabolites are also known to provide good metabolite discrimination between genotypes (*19*). However, little is known as to whether secondary metabolites, such as isoflavononones and saponins, can also be used for discriminating between types of closed related plant genotypes with different phenotypes. Furthermore, whether or not these secondary metabolites also contribute to the salt tolerance of plants is still largely undetermined.

Metabolites are the end products of gene expression and subsequent protein translation; thus, the metabolome is most predictive of phenotype (*20, 21*). Consequently, metabolomics, the comprehensive and quantitative study of metabolites, is a desirable tool for studying the metabolic effects of stress tolerance or precise phenotyping (*22, 23*). In this study, HPLC coupled to ion trap MS methods was developed for metabolic profiling and systematic identification of semipolar metabolites in soybean seeds, focusing on isoflavonoids and soyasaponins. The semipolar metabolite profiles of well-selected salt-tolerant vs salt-sensitive soybean varieties were compared. Specifically, the salt-tolerant and salt-sensitive lines are interbred progeny of salt-tolerant wild soybean and salt-sensitive soybean cultivar. We are interested in addressing the question as to whether

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Table 1. Isoflavonoids Detected in Soybean Seed

compounds	R_1	R_2	R_3[a]	R_4	λ_{max} (nm)	MW	t_R (min)	MS^2 main fragments (*m/z*)
biochanin A	OH	H	H	OCH_3	260, 318	284	75.7	283, 268
glycitein	OH	H	OH	OH	258, 315	284	47.3	283, 268
daidzein	H	H	OH	OH	265, 305	254	42.0	253, 225, 207, 161, 135
genistein	OH	H	OH	OH	260, 324	270	56.6	269, 241, 223, 151, 135
glycitin	H	OCH_3	Glc	OH	264, 320	446	21.1	445, 283
daidzin	H	H	Glc	OH	265, 310	416	20.0	415, 253
genistin	OH	H	Glc	OH	260, 325	432	28.0	431, 269
6″-*O*-acetyldaidzin	H	H	AcGlc	OH	265, 321	458	34.5	457, 253
6″-*O*-acetylgenistein	OH	H	AcGlc	OH	263, 320	474	45.9	473, 253
6″-*O*-malonydaidzin	H	H	MalGlc	OH	260, 314	502	31.8	501, 457, 253
6″-*O*-malonygenistin	OH	H	MalGlc	OH	260, 328	518	40.8	517, 473, 269
6″-*O*-malonyglycitin	H	OCH3	MalGlc	OH	265, 323	530	32.0	529, 485, 283

[a] Glc, β-D-glucopyranosyl; AcGlc, 6″-*O*-acetyl-β-D-glucopyranosyl, MalGlc, 6″-*O*-malonyl-β-D-glucopyranosyl.

Table 2. Structure of Some Soyasaponins Detected in Soybean Extract

name	structure[a]	formula	MW[b]	t_R (min)[c]	MS^2 main fragments
Aa	glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara (3←1)xyl(2,3,4-tri-*O*-acetyl)	$C_{64}H_{100}O_{31}$	1364	63.5	1321, 1303, 1279, 1261, 1219, 1201, 1105, 1097, 1039, 863, 473
Ab	glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara (3←1)glc(2,3,4,6-tetra-*O*-acetyl)	$C_{67}H_{104}O_{33}$	1436	65.2	1393, 1375, 1351, 1333, 1291, 1273, 1247, 1211, 1189, 1169, 1151, 1131, 1111, 1105, 1093, 1075, 1033, 973, 935, 879, 473
Ac	rha(1→2)gal(1→2)glcUA(1→3)A(22←1)ara (3←1)glc(2,3,4,6-tetra-*O*-acetyl)	$C_{67}H_{104}O_{32}$	1420	66.7	1377, 1359, 1335, 1273, 1275, 1233, 1111, 1089, 473
Ae	gla(1→2)glcUA(1→3)A(22←1)ara (3←1)xyl(2,3,4-tri-*O*-acetyl)	$C_{58}H_{90}O_{26}$	1202	63.0	1159, 1141, 1117, 1099, 1039, 943, 863, 473
Af	gla(1→2)glcUA(1→3)A(22←1)ara (3←1)glc(2,3,4,6-tetra-*O*-acetyl)	$C_{61}H_{94}O_{28}$	1274	69.4	1231, 1213, 1189, 1171, 1151, 1111, 1093, 1051, 1043, 1205, 1007, 943, 923, 967, 917, 877, 473
Ag	ara(1→2)glcUA(1→3)A(22←1)ara (3←1)xyl(2,3,4-tri-*O*-acetyl)	$C_{58}H_{90}O_{26}$	1172	67.7	1129, 1111, 1087, 1021, 913, 845, 473
Ah	ara(1→2)glcUA(1→3)A(22←1)ara (3←1)glc(2,3,4,6-tetra-*O*-acetyl)	$C_{60}H_{92}O_{27}$	1244	69.7	1201, 1183, 1093, 1009, 1013, 967, 935, 913, 473
Ba	glc(1→2)gal(1→2)glcUA(1→3)B	$C_{48}H_{78}O_{19}$	958	71.4	939, 895, 795, 733, 633, 615, 571, 457
Bb	rha(1→2)gal(1→2)glcUA(1→3)B	$C_{48}H_{78}O_{18}$	942	73.8	923, 879, 795, 777, 733, 633, 615, 597, 525, 457
Bc	rha(1→2)ara(1→2)glcUA(1→3)B	$C_{47}H_{76}O_{17}$	912	74.1	893, 849, 703, 633, 615, 457
Bb′	gal(1→2)glcUA(1→3)B	$C_{42}H_{68}O_{14}$	796	76.3	751, 633, 615, 457
Bc′	ara(1→2)glcUA(1→3)B	$C_{41}H_{66}O_{13}$	766	76.0	615, 457
Bd	glc(1→2)gal(1→2)glcUA(1→3)E	$C_{48}H_{76}O_{19}$	956	74.2	937, 893, 793, 731, 631, 613, 523, 455
Be	rha(1→2)gal(1→2)glcUA(1→3)E	$C_{48}H_{76}O_{18}$	940	76.7	921, 877, 793, 775, 731, 613, 571, 455
αg	glc(1→2) gal(1→2)glcUA(1→3)B(22←2′)DDMP	$C_{54}H_{84}O_{22}$	1084	79.5	1065, 1041, 983, 957, 921, 895, 741, 723, 651, 583, 457
βg	rha(1→2) gal(1→2)glcUA(1→3)B(22←2′)DDMP	$C_{54}H_{84}O_{21}$	1068	81.2	1049, 967, 941, 921, 879, 741, 733, 652, 583, 457
βa	rha(1→2) ara(1→2)glcUA(1→3)B(22←2′)DDMP	$C_{53}H_{82}O_{20}$	1038	81.7	1019, 938, 911, 891, 873, 849, 741, 651, 457
γg	gal(1→2)glcUA(1→3)B(22←2′)DDMP	$C_{48}H_{74}O_{17}$	922	85.0	879, 821, 795, 759, 741, 583, 457
γa	ara(1→2)glcUA(1→3)B(22←2′)DDMP	$C_{47}H_{72}O_{16}$	892	84.6	873, 791, 765, 741, 583, 457

[a] glc, β-D-glucopyranosyl; gal, β-D-galactopyranosyl; glcUA, β-D-glucoronopyranosyl; ara, α-L-arabinopyranosyl; rha, α-L-rhamnopyranosyl; xyl, β-D-xylopyranosyl. [b] MW, molecular weight. [c] t_R, retention time.

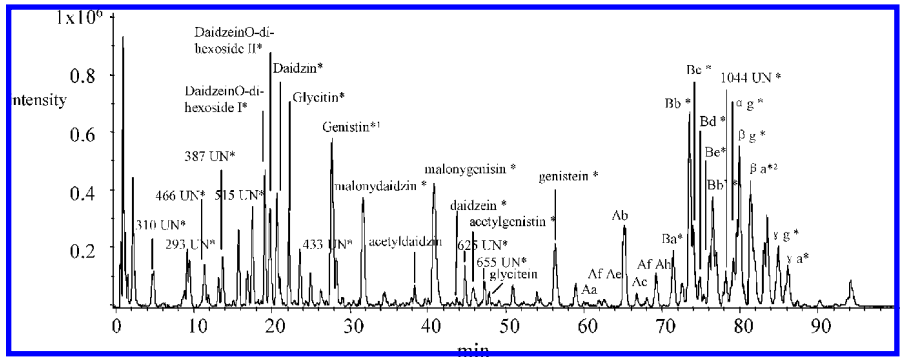

Figure 1. Typical total ion chromatogram of soybean seed semiploar metabolites by HPLC-ESI-MS analysis.

semipolar metabolites can be used to discriminate between closely related soybean genotypes with different phenotypes. Principle component analysis (PCA) was carried out to identify key metabolites with significant impacts on genotype separation.

MATERIALS AND METHODS

Plant Materials and Chemicals. Fully matured seeds of soybean varieties with different salt tolerance properties were selected for this study. These soybean samples include two parental lines (one salt-tolerant wild soybean line and one salt-sensitive soybean cultivar), two salt-tolerant recombinant inbred lines (a7225 and a7593), and two salt-sensitive recombinant inbred lines (c7087 and c7307) produced by the crossbreeding of the two parental lines. Authentic standards daidzein, daidzin, genistin, genistein, biochanin A, glycitein, and glycitin were purchased from Sigma Chemical Co. (St. Louis, MO). The saponins Bb, Bc, and Bb′Bc′ standards were purchased from ChromaDex (Santa

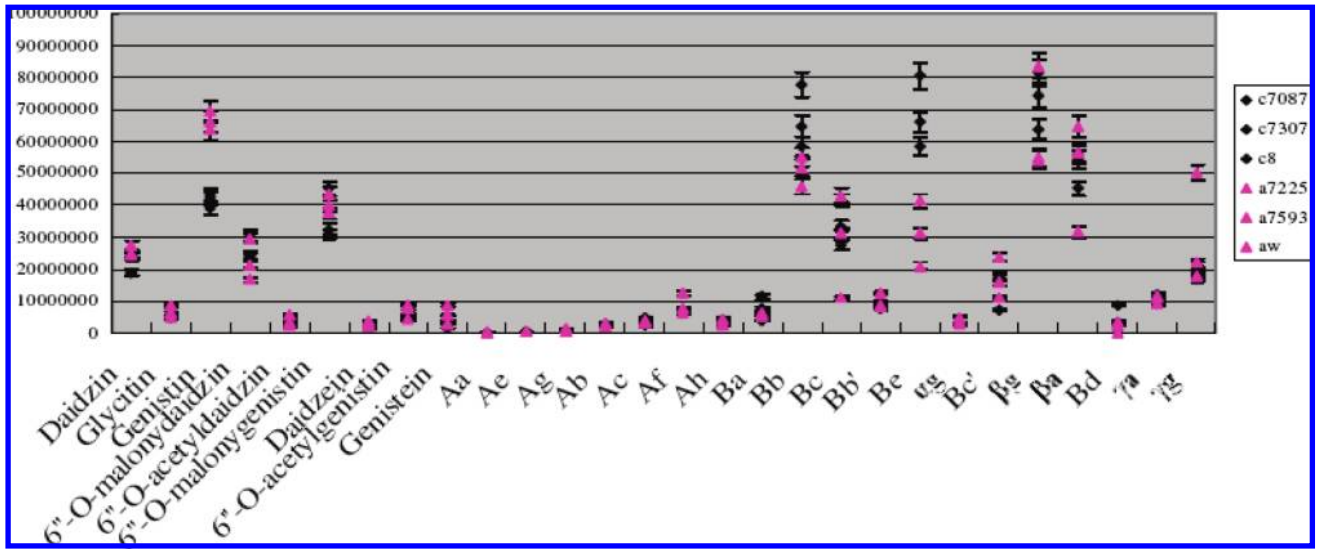

Figure 2. Metabolite variation in different soybean genotypes. aw, parental salt-tolerant wild soybean; c8, parental salt-sensitive line; c7087 and c7307, salt-sensitive recombinant inbred line; and a7225 and a7593, salt-tolerant recombinant inbred line.

Ana, CA). Acetonitrile (HPLC grade) was obtained from Tedia Inc. (Tedia, OH). Water was produced from a Milli-Q system (Millipore, Bedford, MA).

Sample Preparation. The powdered sample of soybean seeds (200 mg) was immersed in 2 mL of 80% methanol for 1 h followed by 30 min of sonication and 3 min of centrifugation at 8000 rpm. Then, 1.0 mL of the liquid extract was collected and filtered through a 0.45 μm PVDF syringe filter before direct chromatographic analysis.

Liquid Chromatography−Mass Spectrometry Analysis. A SymmetryShield RP18 column (150 mm × 2.1 mm i.d., 5 μm) (Waters, United States) was used with an injection volume of 5 μL for the HPLC separation on a HP 1100 system (Agilent Technologies, Palo Alto, CA). The mobile phases consisted of (A) water−0.2% acetic acid and (B) ACN at a flow rate of 0.3 mL/min (0 min 5% B, 15 min 15% B, 65 min 40% B, 105 min 60% B, and 110 min 100% B). Bruker Esquire-4000 ion-trap mass spectrometer (Bruker-Fransen, Bremen, Germany) equipped with electrospray ionization source was used for the sample analysis in negative ion mode. The mass range was m/z 100−1500. Selection of the target mass 500, compound stability 100%, trap drive level 100%, collision energy 1 V, dry temperature 350 °C, dry gas 8 L/min, and nebulizer gas 30 psi were made by examination of the full scan intensity, stability, and the product ions spectra. Data were mean values from three independent experiments.

Data Preprocess and Analysis. The software package XCMS from R platform was used to read the raw MS data. The software enabled finding of peaks in an unbiased way without prior knowledge about their mass spectral characteristics and chemical nature. The retention time shift was taken into account and corrected by calculating the median retention time and the deviation for each sample. Local polynomial regression was used to fit the change from the deviation. A data matrix was formed in which columns represent the samples and rows represent m/z values. PCA and HCA were performed using R statistical package (http://www.r-project.org/).

RESULTS AND DISCUSSIONS

HPLC-ESI-MS Method Development and Peak Assignment. An HPLC-ESI-MS^n method was developed for detection and putative identification of predominant secondary metabolites in soybean seeds. Negative-ion ESI mode total ion chromatograms revealed better sensitivity and more observable peaks than positive ion mode; therefore, it was selected as the main profiling method. The characteristic fragmentations in positive-ion ESI mode provided additional information for compound structural confirmation. The compounds were identified by using the standards' retention time, UV spectra, extracted negative-ion chromatogram, MS/MS data, and references so far identified. The HPLC-MS metabolic profiling revealed many isoflavonoid aglycones and their glucoside, glucoside malonate (acetate) conjugates. Among the detected compounds, isoflavonoids, daidzin, genistin, and their 6″-*O*-malonyl-7-*O*-β-D-glucoside derivatives malonydaidzin and malonygenistin were the major part and glycitin, malonyglycitin, acetyl-daidzein, and acetyl-genistein were the minors. **Tables 1** and **2** summarize the isoflavonoids and saponins detected in this experiment. The typical total ion current chromatogram in negative-ion mode of soybean seed is illustrated in **Figure 1**.

Soybean Genotype Discrimination. Semipolar metabolites from soybean seeds were analyzed using HPLC-ESI- MS as described. Data acquisition and a preprocessing procedure were designed to extract the maximum reliable information from the chromatograms and to identify as many metabolites as possible. Software tools Metalign (*24*) and XCMS (*25*) were used for automated baseline correction and alignment of all extracted mass peaks across all samples. After automatic peak extraction and alignment of samples using XCMS, 1600 mass signals (signal-to-noise ratio >3) were observed. Except for the adducts and fragment ions, about 200 compounds were predicted by retention time and m/z value. As illustrated in **Figure 2**, many metabolite variations were observed between salt-sensitive groups and salt-tolerant groups, with genistins and soyasaponin showing the highest variation. However, virtually all of the observed variation was quantitative rather than qualitative.

To investigate the difference in the metabolomes between genotypes, we carried out a cluster analysis of the mass spectral data. The most commonly used Euclidean distance was chosen to illustrate the results. Calculation of these distance metrics allows the plotting of a hierarchical tree (dendrogram), which illustrates how far apart (or dissimilar) objects are from each other. **Figure 3a** shows a dendrogram produced by calculation of the Euclidean distance. It is shown in this dendrogram that the recombinant inbred lines group more tightly together; that is, all members of the group are in close proximity according to the Euclidean distance. However, no significant grouping in the recombinant inbred lines was detected. The salt-tolerant wild soybean parental genotype, on the other hand, was more distant from other genotypes.

To investigate the roles of different metabolites in discrimination, we carried out a principal component analysis (PCA) of the data. In PCA, data are transformed from a large set of related

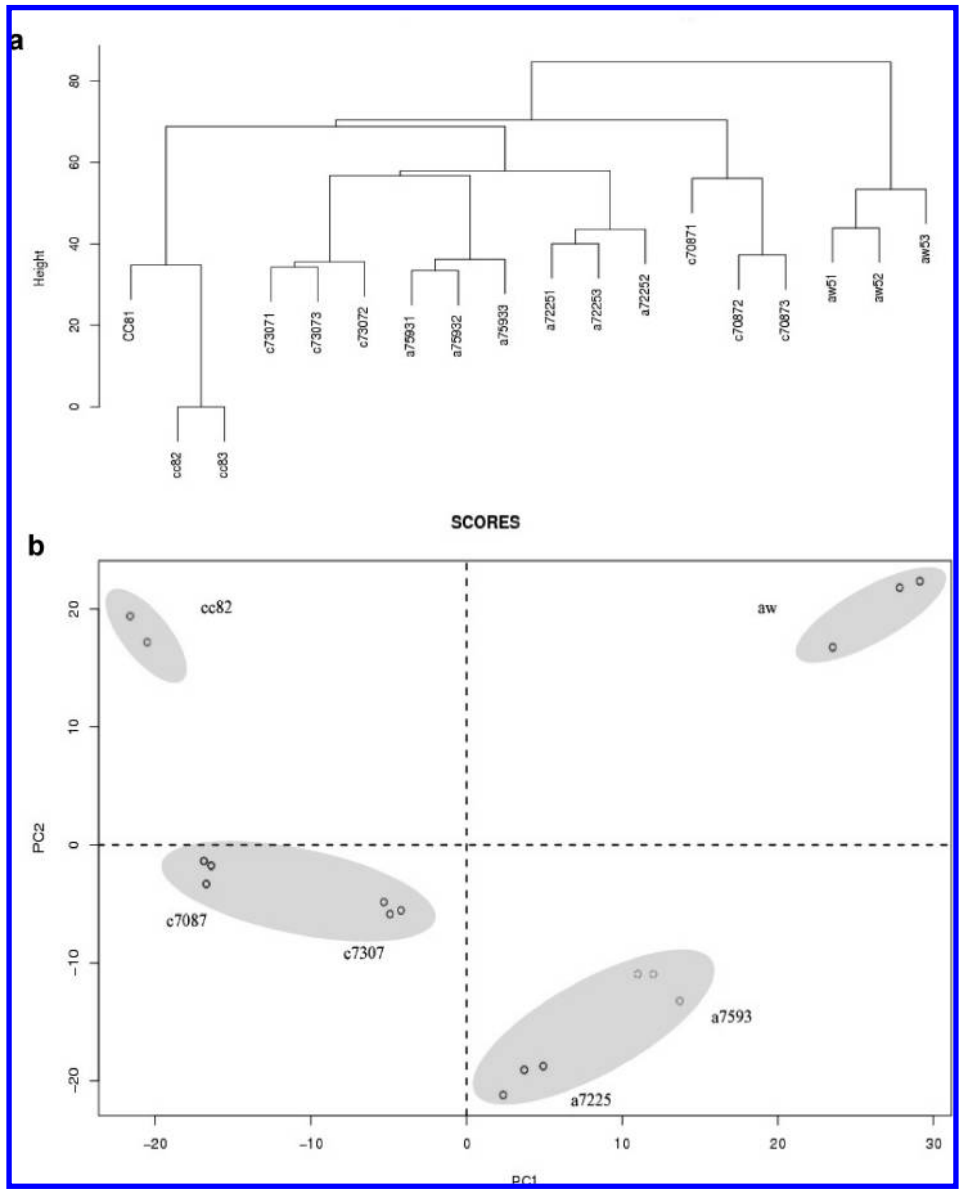

Figure 3. PCA and HCA analysis. (**a**) HCA dendrogram of metabolic profiles according to the Euclidean distance metric. Salt-sensitive parental, CC81-3; wild soybean parental, aw51-3; salt-tolerant progeny, a75931-3 and a72251-3; and salt-sensitive progeny, c73071-3 and c70871-3. (**b**) PCA score plot of MS data representing soybean varieties. The four types of soybean varieties were correctly classified.

variables (e.g., mass spec signals) to a smaller set of uncorrelated variables called principal components (PCs) and are aimed at the expression of maximum variation in the original variables. In a PCA score plot, the same kind of sample could be grouped as independent clusters. As illustrated in the score plot (**Figure 3b**), all of the four types of soybean samples were correctly classified. From these results, it is clear that it is possible to discriminate between the two parental lines and between the parental lines and the recombinant inbred lines. We therefore believe that isoflavonoids and saponins can be used to discriminate closely related soybean genotypes.

Most Discriminatory Metabolites Are Genistins and Group B Saponins. Further to the PCA carried out as previously described, a similar analysis was undertaken using the recombinant inbred line alone, to remove the variation due to the genetic factors. The recombinant inbred lines were derived from the same parental lines, and the major difference is only in salt tolerance property. As illustrated in **Figure 4a**, the 12 soybean samples (six salt-tolerant and six salt-sensitive) were correctly classified, indicating that it is possible to use semipolar metabolites to discriminate between closely related soybean genotypes. It may also be inferred from the PC analysis of data that the first PC partitions the salt-sensitive vs salt-tolerant groups, and the second PC mostly discriminates samples within the same group, using genistin and saponin as the major discriminating metabolites. The first two PCs, which collectively explained 93% of the variation in the original data, were examined in detail.

The contribution of each variable to a PC can also be calculated, giving a loading. Investigation of the relative contribution (loadings) of individual variables in the PC1 dimension revealed metabolite with a significant impact on genotype separation. The top-loaded 30 metabolites in PC1 were labeled (*) in **Figure 1**. Among them, genistin and group B saponins had the highest loading value, contributing more than 37% of the total loadings values for all variables. Group B and DDMP soyasaponins were found as the primary saponins present in soybeans (*26*), while the DDMP-conjugated soyasaponins were the genuine group B saponins present in soybeans (*27*). A relatively low concentration of soyasaponin A exists in the

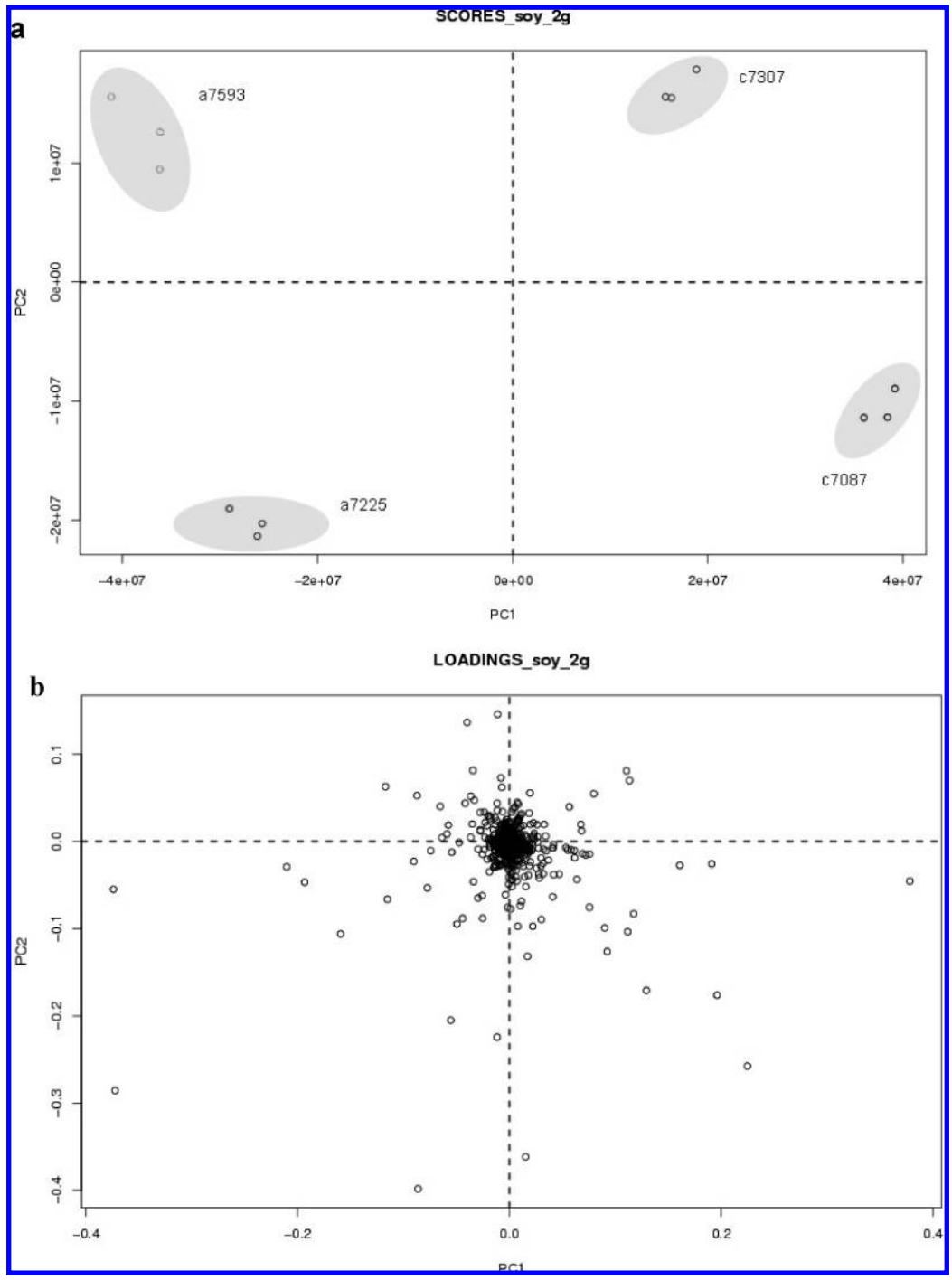

Figure 4. PCA score plot and loading plot of salt-sensitive vs salt-tolerant genotypes. (**a**) PCA score plot of MS data representing salt-sensitive and salt-tolerance varieties. (**b**) Loading plot of PCA vs PC2 of MS data. The relative contribution (loadings) of individual metabolite represents its impact on genotype separation. The top-loaded 30 metabolites (labeled with *) can be found in **Figure 1**.

soybean extraction as compared to soyasaponin B. It is noteworthy that many metabolites appear significant in more than one PC, including some unknown metabolites. Further identification of these compounds may facilitate the development of potential biomarkers related to salt tolerance in soybean crop.

Water deficit and excessive salinity may cause osmotic stress (*28*), which in turn induces the production of reactive oxygen species. Genistin is the glycoside form of genistein and the predominant form found in plants. Plants synthesize compounds that are able to reduce the damaging effects of different stresses. It is known that some of these phenolic compounds are active antioxidants (*29*) and may change membrane permeability or influence respiration and oxidative phosphorylation or protein synthesis (*30, 31*). Glucosyl conjugates of isoflavonoids probably act as a reservoir of aglycons and can be mobilized if needed. Saponins are plant glycosides whose accumulation is usually stimulated in response to challenges by biotic and abiotic stresses (*32*). The complete hydrolysis of saponins yields aglycones and sugars (*33*), which serve in plant cellular oxidative stress protection (*34*). Saponins also act as natural surfactants and thus may enhance the nutrients and water uptakes of the plants (*35*). Because osmotic stress was reported to stimulate saponin

production in *Panax ginseng* cell culture (*32*), the stimulated saponin accumulation was suggested as part of the cell response to osmotic stress.

Conclusions. Genetic and environmental factors are known to have a great impact on plant metabolome composition. Using carefully selected soybean lines, we eliminated the variation due to these two factors and demonstrated the feasibility of using secondary metabolites to discriminate between soybean genotypes. As shown here, the analysis method can distinguish between closely related salt-sensitive and salt-tolerant soybean varieties. Key metabolites correlated with salt tolerance were identified. These individual metabolites may provide additional insight into the salt tolerance and adaptation of plants. Further identification of the metabolites involved and combining proteomic and genomic data to form a holistic, integrated picture of the metabolic pathways implicated in, may provide new insights into soybean salt tolerance and adaptation. Such knowledge will facilitate the identification of new targets for efficient metabolic engineering efforts to improve salt tolerance properties of soybean crop. This approach also has the potential to promote the development of new biomarkers for salt-tolerance traits and therefore has broad applicability in crop breeding.

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