JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Metabolic Differentiation of Diamondback Moth (*Plutella xylostella* (L.)) Resistance in Cabbage (*Brassica oleracea* L. ssp. *capitata*)

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ABSTRACT: The diamondback moth, *Plutella xylostella* (L.), is a major pest responsible for destroying cabbage and other *Brassica* vegetable crops. A diamondback moth-resistant cabbage line was studied by comparing its metabolite profiles with those of a susceptible cabbage. Fourier transform infrared spectroscopy analysis revealed that carbohydrates, aromatic compounds, and amides were the major factors that distinguished the resistant and susceptible genotypes. Gas chromatography−time-of-flight mass spectrometry profiled 46 metabolites, including 19 amino acids, 15 organic acids, 8 sugars, 3 sugar alcohols, and 1 amine in two genotypes and F_1 hybrid cabbages. The levels of glycolic acid, quinic acid, inositol, fumaric acid, glyceric acid, trehalose, shikimic acid, and aspartic acid were found to be very significantly different between the resistant and susceptible genotypes with a P value of <0.0001. These results will provide a foundation for further studies on diamondback moth resistance in cabbage breeding and for the development of other herbivore-resistant crops.

KEYWORDS: *cabbage, diamondback moth, FT-IR, GC-TOF/MS, metabolic profiling*

INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.), is a major pest responsible for the destruction of cabbage and other *Brassica* vegetable crops[1,2] that contribute more than U.S. $26 billion to the world economy.[3] Annual DBM management for *Brassica* crops costs U.S. $1.4 billion worldwide, rising to U.S. $2.7 billion if yield losses are included.[4,5] This insect is characterized by a short life cycle; it reproduces very well in warm temperatures but finds it difficult to adapt to cold temperatures.[2] Therefore, year-round cultivation in areas such as semitropic and temperate-climate areas is severely affected. Climate changes such as global warming are influencing the reproductive potential of pests, resulting in new or increased insect pest incidence.[6]

Classical biological control using larval−pupal parasitoids and insecticides has been used for a long time to control DBM infestation along with crop rotation.[4] Although the use of pesticides in cultivation is the most farmer-friendly, easy, and effective method, it may cause ecological damage as well as chemical contamination of products and the environment. Most importantly, the DBM has developed resistance to most of the pesticides that are currently available[2,7] by evolving rapidly because of its short life cycle (14 days at 25 °C) and high fecundity (up to 300 eggs). Plant−insect control methods have been developed by generating *Bt* gene transgenic crops.[8,9] However, researchers have argued on the safety of genetically modified crops.[8] Although the new crop-breeding methods involving natural selection and traditional crossing do not provide a fast or perfect control method like pesticides and transgenic plants, they have been considered as desirable methods for controlling DBM infestation because they are less labor intensive, involve lower costs, and improve food safety.

The DBM resistance in *Brassica* crops has been reported to be linked to the glossy dark green leaf phenotype by genetic studies on cauliflower[10,11] and cabbage.[12] It was suggested that the DBM resistance of glossy-leaved lines is related to the oviposition preference of female DBMs and to the fact that the compounds in the glossy leaves affect the ability of first-instar larvae to mine the leaf tissue, thereby reducing the survival rate.[12−14] The DBM-resistance traits in crops are considered as

Received: August 2, 2013
Revised: October 21, 2013
Accepted: October 22, 2013
Published: October 22, 2013

dx.doi.org/10.1021/jf403441t | J. Agric. Food Chem. 2013, 61, 11222−11230

partial resistance, instead of complete, because they do not cause the death of DBM larvae.[15] The partial resistance of the DBM in *Brassica* crops might be considered to arise from the preference of its feeding performance or oviposition, implying correlation to the metabolic compositions of the host crops. Glucosinolate is considered as one of the detrimental compounds to insects in plant defense systems.[16] However, it exerts a completely different function in *Brassicaceae* to DBM such as host recognition cues for oviposition.[17,18] It was reported that glucosinolates act as potent stimulants for feeding and oviposition.[19,20] However, the correlation between glucosinolate profiles and feeding performance is not straightforward.[21] Although some secondary metabolomics studies have been conducted, the metabolite compositions of DBM-resistant *Brassica* crops have not been reported yet.

Advances in metabolomics have provided information regarding a set of metabolites involved in biological functions when there are genetic or environmental changes. Various analysis methods are available, including gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to MS (LC-MS), nuclear magnetic resonance spectroscopy (NMR), and Fourier-transform infrared (FT-IR) microspectroscopy.[22−25] FT-IR microspectroscopy has the merits of speed and simple sample preparation for analysis.[26] The GC-time-of-flight (TOF) MS method can help determine the levels of primary metabolites such as amino acids, organic acids, and sugars by employing chemical derivatization of low-molecular-weight hydrophilic metabolites.[27] Therefore, GC-TOF/MS has been used to identify and measure compounds in many biological tissues.

In this study, we compared the metabolites of two different genotypes of cabbage, that is, DBM-resistant and DBM-susceptible cabbages. The compounds associated with resistance were investigated using FT-IR and GC-TOF/MS and will provide a basis for the breeding of DBM-resistant cabbages as biomarkers for the resistant.

MATERIALS AND METHODS

Plant Materials. In this study, we used two distinct cabbage (*Brassica oleracea* L. ssp. *capitata*) inbred lines: a DBM (*P. xylostella* (L.))-resistant line (LN748) (Nunhems Korea Seed Co., Anseong, Korea) and a DBM-susceptible line (FA747) (Nunhems Korea Seed Co.). These lines had been selected from naturally infested cabbages in open fields during several years by the Nunhems Korea Seed Co.

Growing Environments and Sample Preparation for Component Analysis. Two inbred lines (LN748 and FA747) and 1 F_1 hybrid line generated from a cross between LN748 and FA747 were used for component analysis. To analyze the metabolomic differences in the plants by using FT-IR, three seeds of each line were individually sown in seed trays. The plants were grown in a glasshouse for 30 days with a 16 h day and 8 h night period (22 ± 3 °C, 50−60% relative humidity) and then harvested in liquid nitrogen in January 2011. To profile the metabolites of the plants by GC-TOF/MS, five seeds of each line were individually sown in seed trays on August 13, 2011. The plants were grown in the glasshouse for 30 days with a 16 h day and 8 h night period (22 ± 3 °C, 50−60% relative humidity) and then transplanted in the field (Chungnam National University, Daejeon, Korea). All plants were sampled on November 11, 2011. Immediately after the leaf samples were collected, they were frozen in liquid nitrogen. All samples were freeze-dried, pulverized with TissueLyser II (QIAGEN Korea Ltd., Seoul, Korea), and stored in a deep freezer (−70 °C).

FT-IR Analysis of DBM-Resistant and DBM-Susceptible Cabbages. Whole cell extracts were prepared from freeze-dried cabbage powder (20 mg) in 1.5 mL Eppendorf tubes by using 20% (v/v) methanol (200 μL) (Burdick & Jackson, Muskegon, MI, USA). The samples were mixed vigorously by vortexing and inverting and were then incubated in a 50 °C water bath for 20 min and additionally vortexed every 10 min. The mixture was centrifuged at 800*g* for 15 min at room temperature, and the supernatants were transferred into a new tube. A second centrifugation step was performed at 800*g* for 1 min to remove the debris. Extracts (5 μL) were loaded onto a 384-well microplate with 3 repeats and then dried on a hot plate (37 °C). Spectra were detected using the Bruker FT-IR Tensor 27 spectrometer equipped with the HTS-XT module (Bruker Optics GmbH, Ettlingen, Germany). FT-IR spectra were obtained using the OPUS program (Bruker Optics GmbH, ver. 6.5). Each spectrum was recorded from 4000 to 400 cm^{-1} using a spectral resolution of 4 cm^{-1}. To improve the signal-to-noise ratio, 128 interferograms were co-added and averaged with the analytical results. Infrared spectra were obtained by subtraction of the plate spectra (background) used for deposition of the samples. For multivariate analysis, the digitalized original FT-IR spectra were preprocessed, including baseline correction, spectral intensity normalization, and smoothing, using OPUS software (ver. 7.0). Second derivatives (differentiation) of these preprocessed spectra were also performed with OPUS software (ver. 7.0). The processed spectral data, the 1800−800 cm^{-1} region of the FT-IR spectral data, rather than the full spectrum, were then subjected to multivariate analyses (principal component analysis, PCA; partial least-squares discriminant analysis PLS-DA). Each sample was replicated three times.

GC-TOF/MS Analysis of Polar Metabolites in DBM-Resistant and DBM-Susceptible Cabbages. GC-TOF/MS was used to identify and measure the low-molecular-weight hydrophilic compounds in DBM-resistant cabbage samples. The ChromaTOF software was used to support peak findings prior to quantitative analysis and for automated deconvolution of reference mass spectra. The NIST and in-house libraries for standard chemicals were utilized for identification of the compounds. The extraction of polar metabolites was performed as described previously.[28] The metabolites were released from the powdered sample (10 mg) by adding 1 mL of 2.5:1:1 (v/v/v) methanol/water/chloroform and 60 μL of ribitol (0.2 mg/mL H_2O) as the internal standard (IS). They were extracted at 37 °C with a mixing frequency of 1200 rpm for 30 min by using a thermomixer comfort (Eppendorf AG, Hamburg, Germany). The solutions were then centrifuged at 16000*g* for 3 min. The extracted supernatant (0.8 mL) was transferred into a new tube; then, 0.4 mL of water was added, and the solution was centrifuged at 16000*g* for 3 min. The methanol/water phase containing the hydrophilic metabolites was dried in a CVE-2000 centrifugal concentrator (Eyela, Tokyo, Japan) for 2 h, followed by drying in a freeze-dryer for 16 h. For GC-TOF/MS analysis, two-stage chemical derivatization was performed on the extracted metabolites. First, oximation was performed by dissolving the samples in methoxyamine hydrochloride (20 mg/mL, 80 μL) and incubating the solutions at 30 °C for 90 min. Then, *N*-(trimethylsilyl)-trifluoroacetamide etherification was performed by adding 80 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) at 37 °C for 30 min. GC-TOF/MS was performed using an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA, USA) coupled to a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI, USA). A CP-SIL 8 CB low-bleed/MS fused-silica capillary column (5% diphenyl and 95% dimethylpolysiloxane), 30 m × 0.25 mm i.d. × 0.25 μm film thickness phase (Varian Inc., Palo Alto, CA, USA), was installed to the gas chromatograph. The helium gas flow rate through the column was 1.0 mL/min. The split ratio was set at 1:25. The injector temperature was 230 °C. The temperature program was as follows: initial temperature of 80 °C for 2 min, followed by an increase to 320 °C at 15 °C/min and a 10 min hold at 320 °C. The transfer line and ion source temperatures were 250 and 200 °C, respectively. The scanned mass range was m/z 85−600, and the detector voltage was set at 1700 V. Each sample was replicated three times.

Statistical Analysis. The preprocessed FT-IR spectral data of the 1800−800 cm^{-1} region were subjected to PCA and PLS-DA using R statistical analysis program (version 2.7.2) to evaluate similarity among groups of multivariate data. PCA and PLS-DA were conducted according to the nonlinear iterative partial least-squares (NIPALS)

Table 1. Metabolites Identified in GC-TOF/MS Chromatograms for the DBM-Susceptible and -Resistant Cabbages

						quantity	
no.[a]	compound	RT[b]	RRT[c]	mass fragment[d]	quantitation ion[e]	susceptible lines	resistant lines
1	pyruvic acid	4.53	0.425	115, 174, 189	174	1.00 ± 0.06	0.97 ± 0.33
2	lactic acid	4.63	0.433	117, 147, 191	147	1.00 ± 0.20	1.04 ± 0.11
3	valine	5.02	0.470	130, 146, 156	146	1.00 ± 0.16	0.85 ± 0.36
4	alanine	5.13	0.480	116, 147, 190	116	1.00 ± 0.10	0.97 ± 0.20
5	glycolic acid	6.23	0.584	147, 177, 205	147	1.00 ± 0.04	0.11 ± 0.02
6	serine	6.79	0.636	116, 132, 147	116	1.00 ± 0.06	0.75 ± 0.11
7	ethanolamine	6.86	0.643	100, 147, 174	174	1.00 ± 0.17	0.67 ± 0.30
8	glycerol	6.89	0.645	103, 117, 147	147	1.00 ± 0.22	0.89 ± 0.40
9	leucine	6.90	0.647	102, 147, 158	158	1.00 ± 0.64	0.20 ± 0.12
10	isoleucine	7.12	0.667	147, 158, 218	158	1.00 ± 0.69	0.20 ± 0.10
11	proline	7.20	0.675	142, 158, 216	142	1.00 ± 0.58	0.57 ± 0.03
12	glycine	7.26	0.680	147, 174, 248	174	1.00 ± 0.04	1.85 ± 0.45
13	succinic acid	7.33	0.687	129, 147, 247	147	1.00 ± 0.07	1.22 ± 0.11
14	glyceric acid	7.44	0.697	133, 147, 189	147	1.00 ± 0.12	0.37 ± 0.03
15	fumaric acid	7.68	0.719	143, 147, 245	245	1.00 ± 0.15	1.88 ± 0.10
16	threonine	7.96	0.746	101, 117, 219	219	1.00 ± 0.19	0.69 ± 0.31
17	*β*-alanine	8.38	0.785	147, 174, 248	174	1.00 ± 0.08	0.80 ± 0.09
18	malic acid	8.88	0.832	147, 233, 245	147	1.00 ± 0.07	0.81 ± 0.08
19	salicylic acid	9.13	0.855	135, 149, 267	267	1.00 ± 0.04	0.83 ± 0.06
20	aspartic acid	9.14	0.856	100, 147, 232	100	1.00 ± 0.07	0.60 ± 0.11
21	methionine	9.18	0.861	128, 147, 176	176	1.00 ± 0.56	0.74 ± 0.39
22	pyroglutamic acid	9.23	0.865	147, 156, 230	156	1.00 ± 0.15	1.30 ± 0.14
23	4-aminobutyric acid	9.26	0.867	147, 174, 304	174	1.00 ± 0.42	0.11 ± 0.03
24	threonic acid	9.41	0.882	147, 205, 220	147	1.00 ± 0.29	1.58 ± 0.56
25	arginine	9.90	0.928	142, 147, 162	142	1.00 ± 0.16	1.33 ± 0.47
26	glutamic acid	9.94	0.931	128, 156, 246	246	1.00 ± 0.07	0.75 ± 0.08
27	phenylalanine	10.06	0.943	100, 192, 218	218	1.00 ± 0.18	0.80 ± 0.06
28	*p*-hydroxybenzoic acid	10.08	0.945	193, 223, 267	223	1.00 ± 0.23	1.02 ± 0.29
29	xylose	10.15	0.951	103, 147, 217	103	1.00 ± 0.22	1.57 ± 0.90
30	asparagine	10.34	0.969	116, 132, 231	116	1.00 ± 0.14	0.58 ± 0.11
IS	ribitol	10.67	1.000	103, 147, 217	217		
31	glutamine	11.11	1.041	147, 156, 245	156	1.00 ± 0.18	0.74 ± 0.21
32	shikimic acid	11.30	1.058	147, 204, 255	204	1.00 ± 0.04	1.63 ± 0.18
33	citric acid	11.39	1.067	147, 273, 347	273	1.00 ± 0.05	0.74 ± 0.18
34	quinic acid	11.64	1.090	147, 255, 345	345	1.00 ± 0.10	0.17 ± 0.02
35	fructose	11.72	1.098	103, 147, 217	103	1.00 ± 0.04	0.91 ± 0.08
36	galacose	11.86	1.111	147, 205, 319	147	1.00 ± 0.14	1.50 ± 1.04
37	glucose	11.91	1.116	147, 160, 205	147	1.00 ± 0.07	0.78 ± 0.04
38	mannose	12.04	1.129	147, 205, 319	147	1.00 ± 0.14	0.62 ± 0.05
39	mannitol	12.15	1.139	147, 217, 319	319	1.00 ± 0.51	1.87 ± 1.04
40	ferulic acid	12.18	1.142	308, 323, 338	338	1.00 ± 0.16	0.80 ± 0.10
41	inositol	13.19	1.236	147, 217, 305	305	1.00 ± 0.07	0.38 ± 0.05
42	tryptophan	14.03	1.315	202, 219, 348	202	1.00 ± 0.17	1.06 ± 0.40
43	sinapinic acid	14.20	1.330	338, 353, 368	338	1.00 ± 0.25	0.81 ± 0.13
44	sucrose	16.13	1.511	147, 217, 361	217	1.00 ± 0.03	1.28 ± 0.12
45	trehalose	16.66	1.561	147, 191, 361	191	1.00 ± 0.11	0.33 ± 0.10
46	raffinose	19.70	1.846	204, 217, 361	217	1.00 ± 0.37	0.30 ± 0.15

[a]Numbers represent the compound index for chromatogram shown in Figure 2. [b]Retention time (min). [c]Relative retention time (retention time of the analyte/retention time of the internal standard).). [d]First three ions with the highest intensities. [e]Specific mass ion used for quantitation. The quantitative calculations of all analytes were based on the peak area ratios relative to that of the IS.

algorithm[29] and the R program. PCA scores extracted from PCA were used for the calculation of correlation matrices, and PLS-DA was applied for metabolic discrimination. The significant differences among means were determined by Student's *t* test (SAS 9.2; SAS Institute, Cary, NC, USA). Quantitation of the GC-TOF/MS data was performed using selected ions (Table 1). The quantitative calculations of all analytes were based on the peak area ratios relative to that of the IS. The relative quantitation data acquired from GC-TOF/MS were subjected to PCA and PLS-DA by using SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) to evaluate the relationships in terms of similarity or dissimilarity among groups of multivariate data. The PCA and PLS-DA output consisted of score plots for visualizing the contrast between different samples and loading plots to explain the cluster separation. The data file was scaled with unit variance scaling before all of the variables were subjected to PCA and PLS-DA. The significant differences among means were determined by Student's *t* test using SAS 9.2 (SAS Institute).

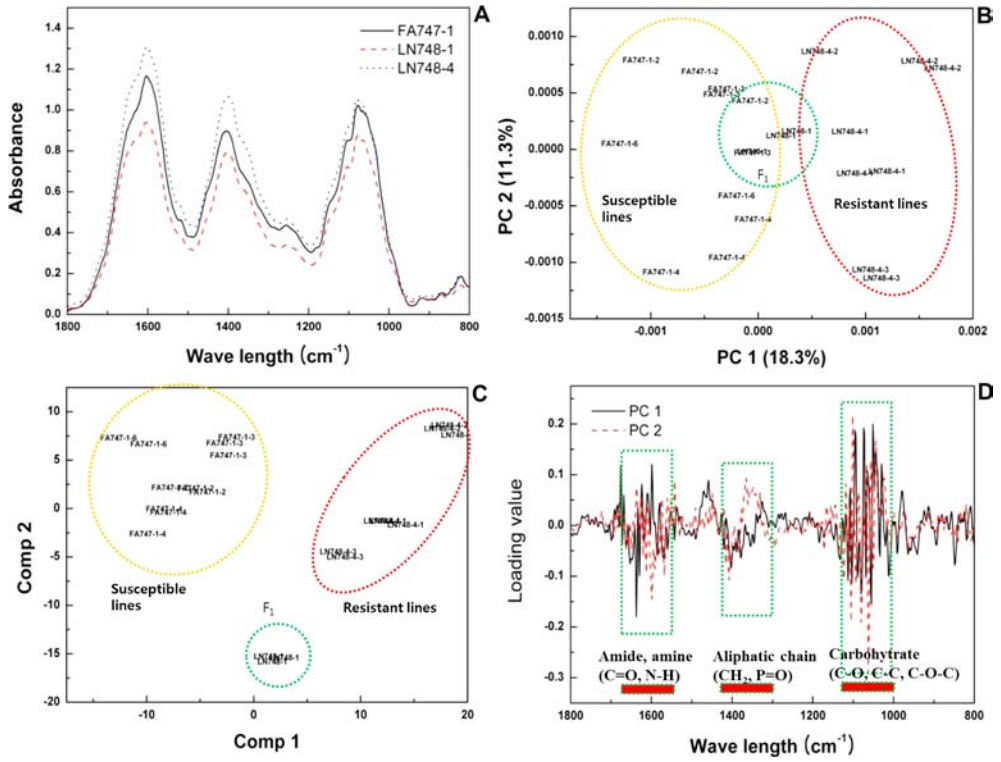

Figure 1. FT-IR metabolite fingerprinting data of the DBM-resistant, DBM-susceptible, and F_1 hybrid lines: (A) average FT-IT spectra of the three different genotypes (tblack solid line represents FA747-1 plants with a susceptible phenotype, the red dotted line represents F_1 LN748-1, and the blue dotted line represents LN748-4 plants with a resistant phenotype); (B) PCA score plot of the FT-IR data; (C) PLS-DA score plot of the FT-IR data; (D) PCA loading plot of the FT-IR data (dotted lines represent the areas grouped in panels B, C, and D).

RESULTS AND DISCUSSION

Comparisons of the Metabolic Components of DBM-Resistant and DBM-Susceptible Cabbages by Using FT-IR Analysis.

FT-IR analysis was performed to clarify the chemical information for DBM-resistant cabbage leaves. In general, the spectroscopic patterns within the region 800−1800 cm^{-1} were similar among the DBM-resistant, DBM-susceptible, and F_1 samples (Figure 1A). The three different genotypes were divided into three groups according to the metabolite differences obtained by PCA. PCA uses an *n*-dimensional vector approach to separate samples on the basis of the cumulative correlation of all component data and then identifies the vector that yields the greatest separation between samples. Principal component (PC) 1 accounted for 18.3% of the variance, whereas PC 2 explained 11.3%, to give a total of 29.6% (Figure 1B). Each sample group belonging to resistant, susceptible, and F_1 groups was successfully separated into discrete boundaries on the PCA score plot (Figure 1B). Furthermore, sample discrimination between each group was more evident in the PLS-DA score plot (Figure 1C). To obtain detailed information regarding the factors that were responsible for the separation of the different genotypes, the correlations between the variables and factors were evaluated. Among the plant genotypes, metabolic differences were detected in the overall wavelength area (800−1800 cm^{-1}), and distinguishable variations were identified in three different regions of the PCA loading plot, that is, 1000−1150, 1300−1430, and 1550−1680 cm^{-1} (Figure 1D). The region 1000−1150 cm^{-1} represented carbohydrates and polysaccharides (C—O ring, C—C, C—O—C).[30] The region 1300−1430 cm^{-1} involved the aliphatic chains (CH_2) and vibrations from nucleic acids (P═O).[31] In the region 1550−1680 cm^{-1}, the 1650 cm^{-1} wavelength corresponded to amides (C═O) and primary amines (N—H). The 1605 cm^{-1} area indicated aromatic rings such as phenolic compounds (C—C), whereas 1525 cm^{-1} represented carote-

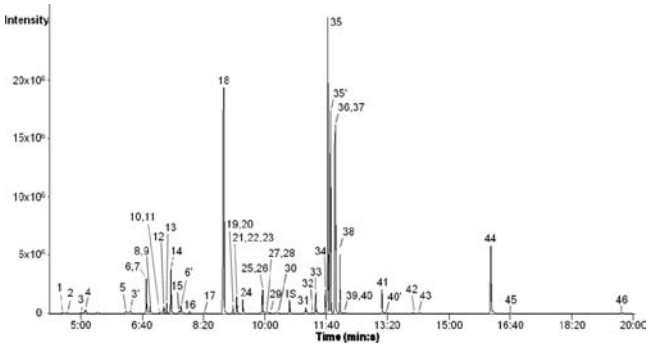

Figure 2. Selected ion chromatogram of metabolites extracted from DBM-susceptible cabbage as MO/TMS derivatives separated on a 30 m × 0.25 mm i.d. fused-silica capillary column coated with a 0.25 μm CP-SIL 8 CB low-bleed stationary phase. Peak identification: 1, pyruvic acid; 2, lactic acid; 3, valine; 4, alanine; 5, glycolic acid; 3′, valine; 6, serine; 7, ethanolamine; 8, glycerol; 9, leucine; 10, isoleucine; 11, proline; 12, glycine; 13, succinic acid; 14, glyceric acid; 15, fumaric acid; 6′, serine; 16, threonine; 17, β-alanine; 18, malic acid; 19, salicylic acid; 20, aspartic acid; 21, methionine; 22, pyroglutamic acid; 23, 4-aminobutyric acid; 24, threonic acid; 25, arginine; 26, glutamic acid; 27, phenylalanine; 28, *p*-hydroxybenzoic acid; 29, xylose; 30, asparagine; 31, glutamine; 32, shikimic acid; 33, citric acid; 34, quinic acid; 35, fructose; 35′, fructose; 36, galactose; 37, glucose; 38, mannose; 39, mannitol; 40, ferulic acid; 41, inositol; 40′, ferulic acid; 42, tryptophan; 43, sinapic acid; 44, sucrose; 45, trehalose; 46, raffinose; IS, internal standard (ribitol).

dx.doi.org/10.1021/jf403441t | J. Agric. Food Chem. 2013, 61, 11222−11230

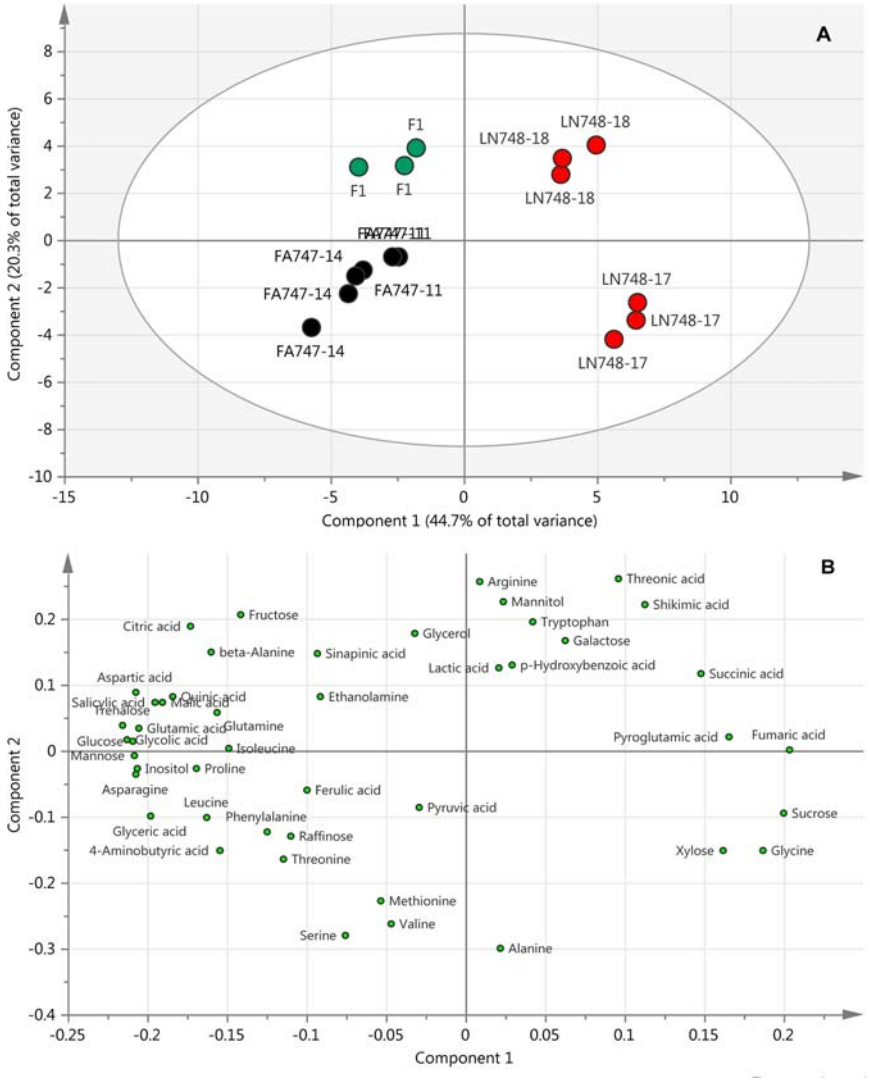

Figure 3. Scores (A) and loading (B) plots of PC 1 and PC 2 of PCA results obtained from polar metabolite data for DBM-resistant, DBM-susceptible, and F_1 hybrid lines. The score plot visualizes the structure of the samples according to the first two principal components with the explained variance in brackets. The eigenvalues of PC 1 and PC 2 were 6.70 and 3.04, respectively, and they accounted for >65% of the total variance. The ellipse represents the Hotelling T2 with 95% confidence in score plot. The loading plot represents the original variables in the space of the principal components. They reveal the magnitude and direction of correlation of the original variables with the first two principal components.

noids (C═C). These results demonstrated that carbohydrates, aromatic compounds, and amides are the major factors that distinguish the resistant and susceptible genotypes. These metabolite differences in DBM-resistant related plants can be used to identify resistant traits for use in breeding programs. To determine what compounds increased the metabolic differences in the plants with the DBM-resistant trait, we analyzed cabbage samples using GC-TOF/MS.

Metabolic Profiles for Cabbages Using GC-TOF/MS Analysis. Primary core metabolites provide good metabolite discrimination between different genotypes. The GC-MS method has been one of the most popular metabolomic techniques because it can determine the levels of primary metabolites such as amino acids, organic acids, and sugars by employing chemical derivatization of these hydrophilic metabolites. In total, 46 metabolites, including 19 amino acids, 15 organic acids, 8 sugars, 3 sugar alcohols, and 1 amine, were detected in the three cabbage genotype samples (Table 1). The corresponding GC-MS and retention times are illustrated in Table 1 and Figure 2.

Evaluation of the GC-TOF/MS Data by PCA. The quantitation data of 46 metabolites normalized to the IS signal intensity were subjected to PCA to outline the differences among the metabolite profiles of the genotype lines. As shown in Figure 3, PC 1 and PC 2 of the PCA score plot represented 44.7 and 20.3%, respectively, of the total variance of the samples. PCA in the present study allowed easy visualization of complex data, and the metabolomes among DBM-resistant, DBM-susceptible, and F_1 lines were separated by PC 1 and PC 2. The PCA results clearly showed the absence of marked variances among samples of the same genotype. It is possible to identify the compounds exhibiting the greatest variance within a population and to determine closely related compounds by using PCA.[28] To further investigate, the contributors to the components, the metabolic loadings in PC 1 and PC 2, were compared. The metabolites contributing to PC 1 were mainly fumaric acid, trehalose, glucose, glycolic acid, mannose, asparagine, aspartic acid, inositol, and glutamic acid (Figure 3B).

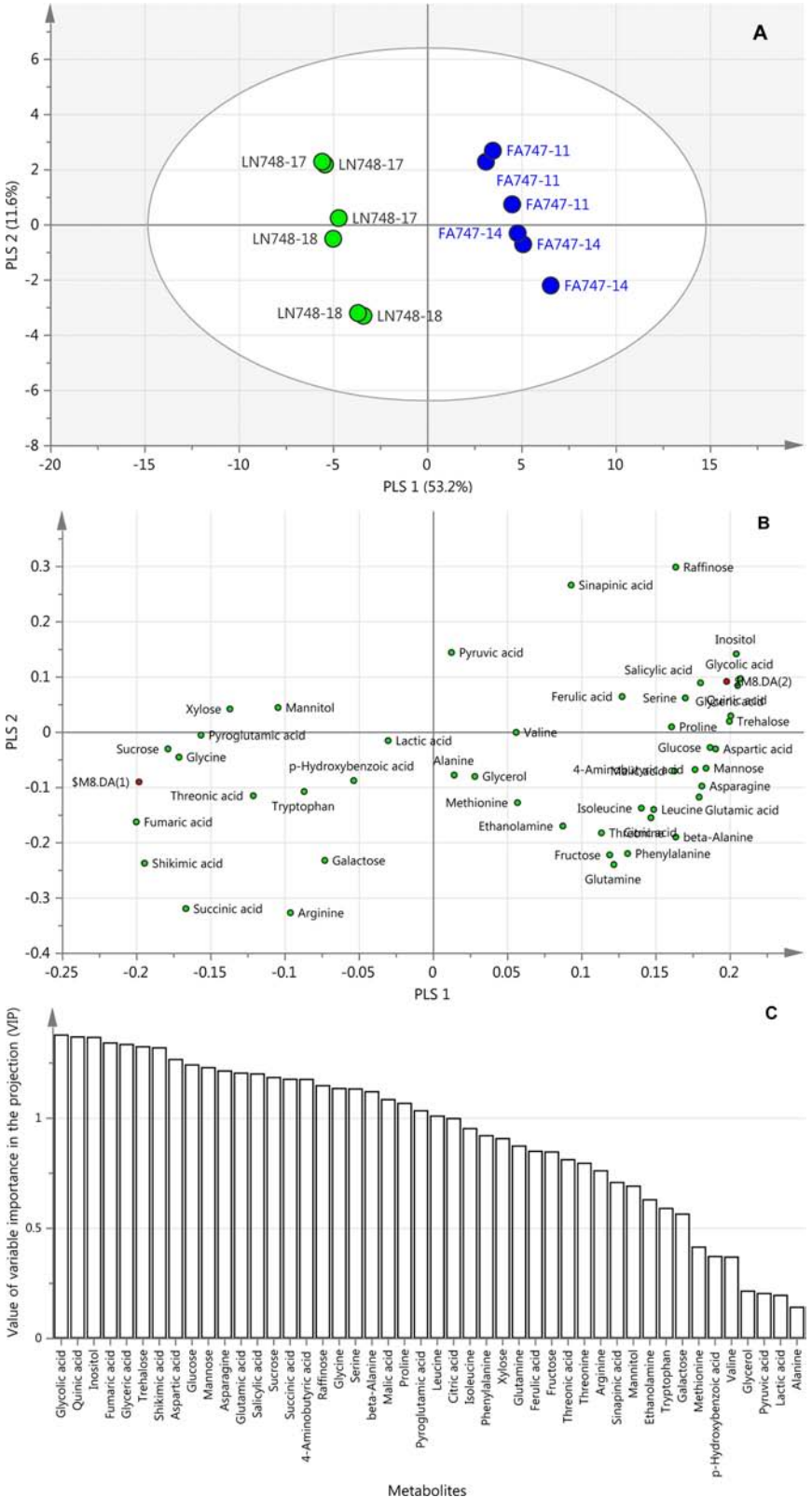

Figure 4. Scores (A) and loading (B) plots of PC 1 and PC 2 of PLS-DA results obtained from polar metabolite data for DBM-resistant and DBM-susceptible lines influence of the variables (C) used to discriminate between DBM-resistant and DBM-susceptible lines from GC-TOF/MS data. The score plot visualizes the structure of the samples according to the first two PLS with the explained variance in brackets. The ellipse represents the Hotelling T2 with 95% confidence in score plot. The loading plot defines the orientation of the PLS planes with the original variables (metabolites and genotype variables). They reveal the magnitude and direction of correlation of the original variables with the first two PLS components. DBM-resistant cabbage is represented by $M8.DA(1) in the loading plot, whereas $M8.DA(2) represents DBM-susceptible cabbage. VIP indicates the relative influence of each metabolite to the grouping; metabolites with higher VIP values are more influential.

dx.doi.org/10.1021/jf403441t | J. Agric. Food Chem. 2013, 61, 11222−11230

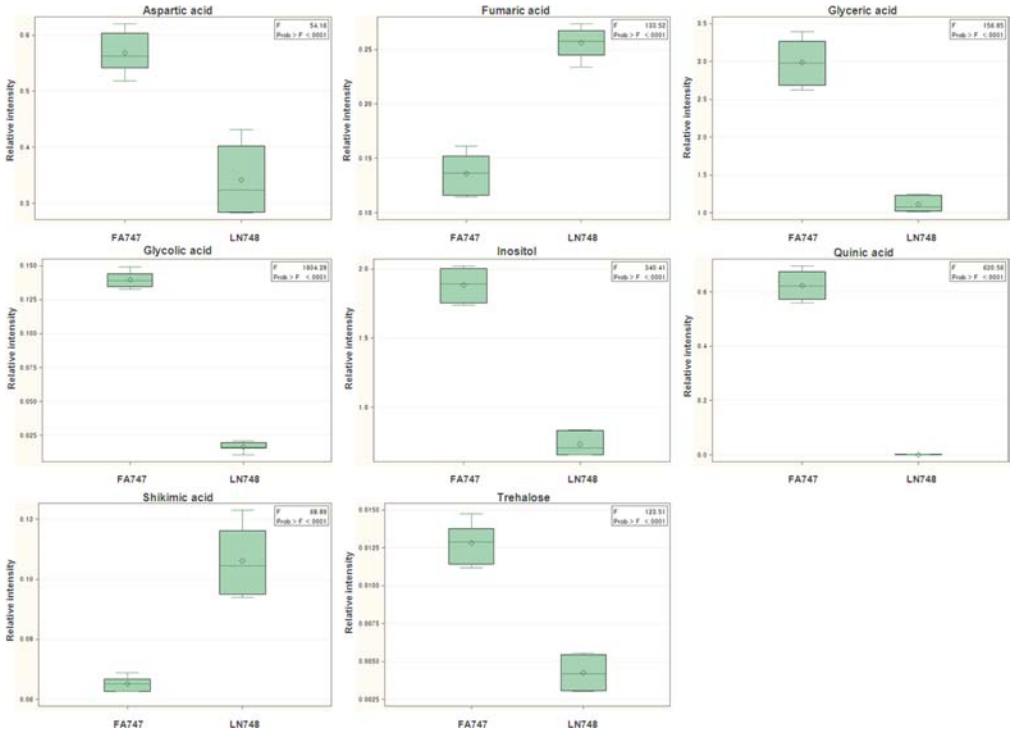

Figure 5. Box plots of metabolites that were significantly different ($P < 0.0001$) between DBM-resistant and DBM-susceptible lines. On the basis of variable importance in the projection (VIP) value of >1.0 in the PLS-DA model and the p values ($P < 0.0001$) in the t tests for all metabolites, eight metabolites were selected.

Classification of Two Cabbage Genotypes by PLS-DA. We employed PLS-DA as the supervised pattern recognition method for the differentiation of the metabolome of DBM-resistant and DBM-susceptible cabbages. As seen in this study, PLS-DA could distinguish between DBM-resistant and DBM-susceptible cabbages (Figure 4). The quality of the model was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the model and indicates the goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates the predictability. The model had an R_y^2 of 0.995 and a Q^2 of 0.986 (if $Q^2 > 0.9$, the model is considered to have excellent predictive ability). In the first component of the PLS-DA, the corresponding loading was mainly positive for glycolic acid, quinic acid, and inositol. The loading plot also indicated that higher levels of fumaric acid, shikimic acid, and sucrose were present in the DBM-resistant cabbage than in the susceptible one. The contribution of variables in the projection could be explained using variable importance in the projection (VIP) values (Figure 4C). Variables with VIP >1 have the greatest influence on the model. Among the metabolites identified, glycolic acid, quinic acid, inositol, fumaric acid, glyceric acid, trehalose, shikimic acid, and aspartic acid levels were found to differentiate significantly ($P < 0.0001$) between the two genotypes (Figure 5). The quantities of all analytes are summarized in Table 1. In the result of GC-TOF/MS analysis, 25 metabolite components had a significant VIP value (>1), as seen in Figure 4C. These findings suggest that the primary metabolism was significantly different between DBM-resistant and DBM-susceptible cabbages. It is supported by the report that differences in herbivore references are due to differences in the primary metabolism of the plants rather than their contents of typical defense compounds.[32]

Glycolic acid, an intermediate in photorespiration, was found to be the most significant component in creating the PLS-DA model for cabbage genotypes (Figure 4); however, the functional correlation of it to DBM resistance is not reported yet. A previous study showed that for oviposition, female *P. xylostella* adults preferred mutant plants that lacked PsbS (a protein that plays a key role in qE-type nonphotochemical quenching) and showed reduced sucrose and fumaric acid levels.[32] In the present study, although the accumulation of these metabolites cannot be explained by their ecological functions, DBM-resistant cabbage contained high levels of sucrose and fumaric acid. The building blocks for the secondary metabolites are derived from primary metabolism. Shikimic acid is an important building block employed in the biosynthesis of phenylpropanoids. Among the phenylpropanoids, the subgroup of hydroxycinnamic acids is very common in plants, usually occurring as esters of sugars, organic acids, or amino acids. Previously, it was reported that thrips-resistant chrysanthemums contain high amounts of the phenylpropanoids chlorogenic acid and feruloylquinic acid.[33] The alteration of the components caused by DBM herbivores in *Brassica rapa*, using NMR spectroscopy, emphasized that DBM stimulated the plants to synthesize more feruloyl malate and sinapoyl malate.[34] These phenylpropanoids are known to play an important role in cell wall extension. For example, feruloylmalate coupled to coniferyl alcohol was proposed as an intermediate that is transesterified to polysaccharides in the cell wall,[35] forming a physical barrier around the site of the pathogen or herbivore attack, hampering further spreading of the infection. Among our 25 significant metabolites, the level of salicylic acid was higher in the susceptible cabbage. Generally, when the insect gnaws the leaf, the plant initiates the jasmonate response defense mechanisms, with which salicylic acid interacts negatively. Thus, the accumulation of salicylic acid

interrupts the function of the jasmonate-inducible defense pathway.[36] In this study, the DBM-resistant genotype was obviously distinguished by the profiled primary metabolites. Further studies on these metabolites and DBM resistance will provide key information on the implementation of not only DBM-resistant cabbage breeding but also herbivore-resistant breeding in other important crops.

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Funding

This research was supported by Cabbage Genomics Assisted Breeding Supporting Center (CGsC) research programs (610008-05-4-WT111) and Golden Seed Project (2013003-04-1-SB330) funded by the Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

FT-IR, Fourier transform infrared spectroscopy; GC-TOF/MS, gas chromatography−time-of-flight mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; PCA, principal component analysis; PLS-DA, partial least-squares discriminant analysis; VIP, value of variable importance in the projection; DBM, diamondback moth; NMR, nuclear magnetic resonance spectroscopy

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