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ARTICLE

Targeted metabolite profiling to evaluate unintended metabolic changes of genetic modification in resveratrol-enriched rice (*Oryza sativa* L.)

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Abstract Resveratrol-enriched rice (RR) includes the stilbene synthase gene for resveratrol synthesis and the phosphinothricin-*N*-acetyltransferase gene for glufosinate tolerance. To investigate unintended effects resulting from RR's genetically modified chemical composition, 56 polar and nonpolar secondary metabolites were analyzed with gas chromatography–mass spectrometry in RR and conventional non-transgenic rice. Rice was cultivated during two seasons along three representative climatic regions in the Republic of Korea. Principal components analysis was used to visualize chemical composition differences among rice samples. The results showed that chemical composition was more influenced by growing year and location than by whether or not the rice was transgenic. Pearson's correlations and hierarchical clustering analysis also indicated no difference in the biochemical structures of RR versus non-transgenic rice. In addition, the glufosinate-ammonium treatment did not significantly change RR chemical composition.

Keywords Gas chromatography–mass spectrometry · Metabolomics · Multivariate analysis · Resveratrol · Safety assessment

Introduction

Genetically modified (GM) versions of major crops (e.g., beans, corn, cotton, and rapeseed) have been developed since the mid-1990s to overcome problems such as reductions in agricultural land and productivity. Through the introduction of new genes, crops were enhanced for insect, disease, and herbicide resistance or to increase nutritional value. According to a recent report from the International Service for the Acquisition of Agri-biotech Applications, the global cultivation area of GM crops has increased by 100-fold since 1996, from 1.7 million ha to 179.7 million ha [1]. Because GM crops are now closely intertwined with modern standards of living, research on the safety of GM plants for human consumption is becoming increasingly important.

Rice is a major grain consumed by over half of the world's population. Extensive research has been undertaken to improve rice productivity and nutritional value [2, 3]. For example, Baek et al. [4] developed the resveratrol-enriched rice (RR) through introducing a stilbene synthase gene derived from the Palkwang peanut (*Arachis hypogaea*) variety into Dongjin (DJ), a commercial rice variety. Resveratrol (3,4′,5-trihydroxystilbene) is polyphenol produced as a phytoalexin by some plants (e.g., grape and peanut) in response to injury.

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Resveratrol has many health benefits, decreasing oxidative stress, inflammation, and the risk of cardiovascular disease [5–8]. In addition, RR has stronger anti-obesity and skin depigmenting effects than normal rice [9, 10]. Moreover, tests on UVB-irradiated reconstructed skin indicated that RR downregulates matrix metalloproteinase and upregulates procollagen type 1 production, preventing skin photoaging [11].

Safety assessments of GM crops stem from the concept of substantial equivalence, published in 1993 by the Organization for Economic Cooperation and Development. Genetically modified crops are compared with closely related, safe-to-eat conventional crops to obtain their compositional equivalence (based on substantial equivalence). However, most previous assessments of GM crop compositional equivalence, including those on RR, examine only key nutrients and anti-nutrients, including proximates (e.g., ash, carbohydrates, proteins, fat, and starch), amino acids, fatty acids, minerals, vitamins, and phytic acid [12–14]. Although an ideal safety assessment involves analyzing all components of GM crops, this is not feasible. Gas or liquid chromatography–mass spectrometry (GC/MS, LC/MS) is able to analyze more components than just the key nutrients. Thus, recent GM safety evaluations have turned to a metabolomics approach with GC/MS and LC/MS [15–17]. For example, unintended effects stemming from the chemical composition of transgenic crops could be characterized and assessed via profiling low molecular weight compounds in GM plants [18, 19].

In this study, we used GC/MS to analyze polar and nonpolar secondary metabolites for the evaluation of unintended changes to chemical compositions in RR, compared with DJ. For example, RR is herbicide-tolerant because one of its selection markers is a bar gene encoding a phosphinothricin-*N*-acetyltransferase enzyme. European Union guidelines for GM crop safety suggested that compositional equivalence should be demonstrated with comparisons across two growing seasons and multiple, environmentally representative geographical locations. Furthermore, herbicide-tolerant GM crops should be treated with herbicides and compared with untreated crops to assess any unintended effect from herbicides. Thus, in this study, our data were collected from three locations during two seasons (2013, 2014), and RR field plots were treated with glufosinate-ammonium herbicide to assess environmental and herbicide effects on RR. The rice data were discriminated using multivariate statistics with principal component analysis (PCA), as well as Pearson's correlations and hierarchical clustering (HCA).

Study goals were first to determine whether the environment or the insertion of the stilbene synthase gene is responsible for compositional differences in polar and nonpolar secondary metabolites of rice samples. Second, we aimed to evaluate the effects of herbicide treatment on RR using GC/MS and multivariate statistical analysis.

Materials and methods

Rice sample preparation

The RR (cv. DJ) was developed using *Agrobacterium tumefaciens*-mediated transformation, following Baek et al. [4]. Compositional equivalence was assessed with comparisons to non-transgenic commercial DJ. The Rural Development Administration (RDA, Jeonju, Korea) provided certified reference materials [20]. Both transgenic and non-transgenic rice were cultivated at three different sites (Suwon, Iksan, and Miryang, Korea) during April to October of 2013 and 2014 (Supplementary materials and Fig. 1). In 2013, RR field plots were treated with non-glufosinate-ammonium herbicide and glufosinate-ammonium herbicide to evaluate herbicide effects on rice chemical composition. The glufosinate-ammonium herbicide (Basta, Bayer Crop Science, Germany) was applied at 108 g active ingredient/ha as a single application. Except for this herbicide treatment, RR and non-transgenic DJ were cultivated with standard commercial agricultural practices at every site. Specifics of the experimental design were described previously [13].

Compositional analyses

Polar metabolites were extracted according to previously described methods [21]. For analysis, 0.3 g powdered rice sample was extracted with 1 mL of a methanol/water/chloroform solution (2.5:1:1 by volume). Ribitol solution (60 μL, 0.2 mg/mL) was added as an internal standard. Extraction was conducted in a thermomixer comfort (Eppendorf, Hamburg, Germany) set at 37 °C and a mixing frequency of 1200 rpm. Solutions were centrifuged at 16,000×*g* for 3 min before 0.8 mL of the polar phase was transferred into a new 2-mL tube, and deionized water (0.4 mL) was added. Tube contents were centrifuged at 16,000×*g* for 3 min. The methanol/water phase was also transferred into a new 2-mL tube and dried in a centrifugal concentrator (CC-105, TOMY, Tokyo, Japan) for 2 h, and then in a freeze-dryer for 16 h.

For methoxime derivatization, 80 μL of methoxyamine hydrochloride (20 mg/mL) in pyridine was added and shaken at 30 °C for 90 min. Subsequently, 80 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was added and shaken for 30 min at 37 °C to perform trimethylsilylated (TMS) etherification. A GCMS-QP2010 Ultra system with autosampler AOC-20i (Shimadzu, Kyoto, Japan) was

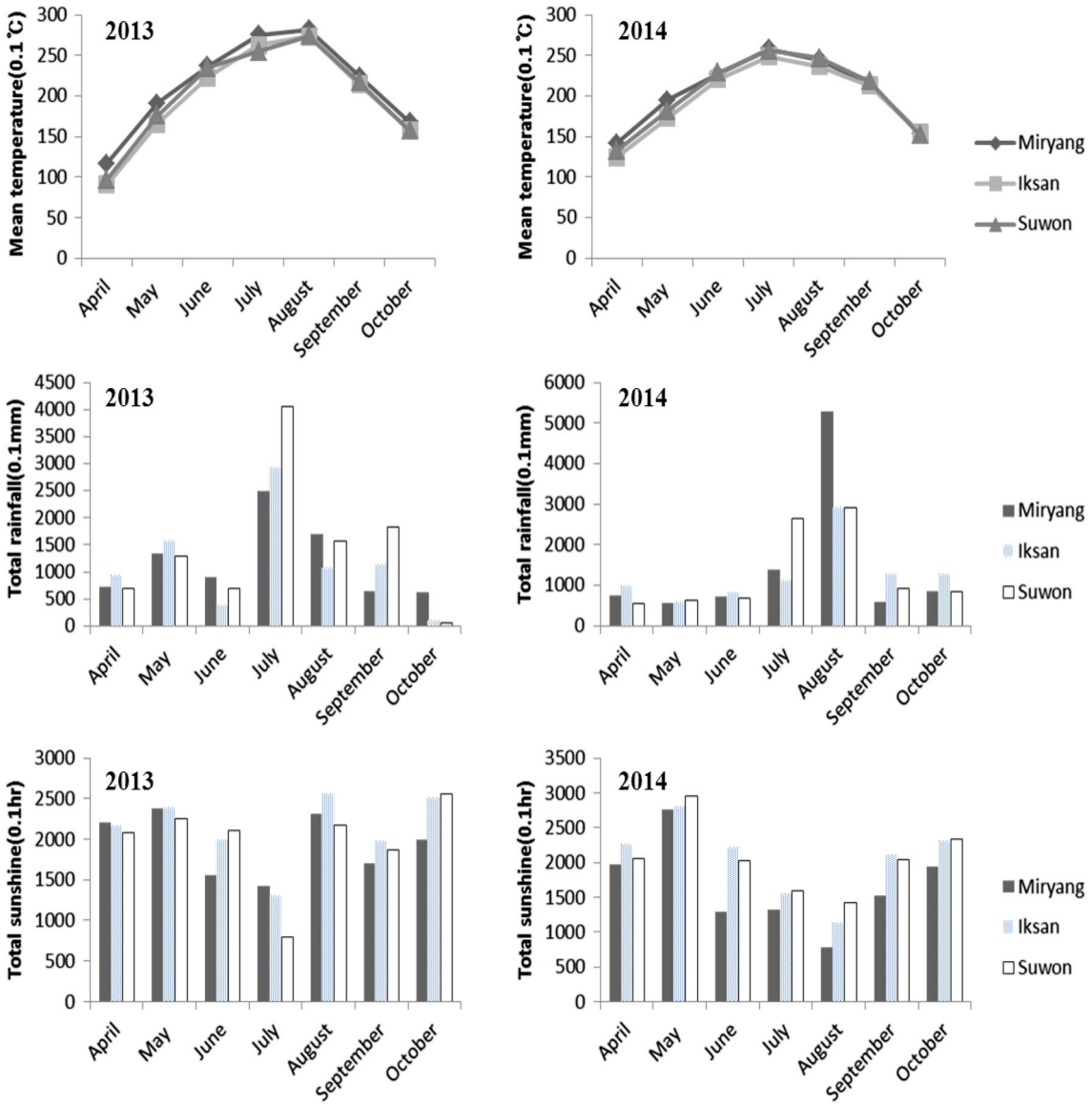

Fig. 1 2013 and 2014 climate data at the study sites: Miryang, Iksan, and Suwon

used for GC/MS. The derivatized sample (1 μL) was separated with a DB-5 column (30 m × 0.25 mm, 1 μm thickness). The injector temperature and split ratio were set at 280 °C and 1:10, respectively. Helium gas flow rate was 1.1 mL/min, and temperatures were programmed to begin at 100 °C for 4 min before increasing by 10 °C/min to 320 °C and maintained for 11 min. Runtime was 4–37 min. Ion source temperature was set at 200 °C. Mass scan range was 45–600 m/z. Peak identification was confirmed in the Wiley9, NIST11, and OA TMS DB5 mass spectral libraries (Shimadzu Corp.).

Nonpolar secondary metabolites were extracted according to previously described methods [21]. Powdered rice samples (0.1 g) were mixed with 3 mL of ethanol containing 0.1% ascorbic acid (w/v) and 0.05 mL of 5α-cholestane as an internal standard (10 μg/mL), vortexed for 20 s, and incubated in an 85 °C water bath for 5 min. After incubation, the extract was saponified with 120 μL of potassium hydroxide (80% w/v), followed by vortexing for 20 s and incubation in the 85 °C water bath for 10 min. Samples were instantly placed on ice after saponification, and deionized water (1.5 mL) was added. Each sample was then extracted twice with hexane (1.5 mL) and dried in a centrifugal concentrator (CC-105, TOMY). For derivatization, 30 μL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide plus 30 μL pyridine were added to the extracted samples, before incubation for 30 min at 60 °C and a mixing frequency of 1200 rpm. Previously described GC–MS conditions were followed [22]. Derivative samples were analyzed in the GCMS-QP2010 Ultra system

Table 1 Chromatographic and spectrometric data of polar and nonpolar compounds in transgenic (resveratrol-enriched) and non-transgenic rice

Compound	RT[a]	RRT[b]	Mass fragment[c]	Quantification ion[d]
Polar compounds				
Caproic acid	8.44	0.462	117, **173**	**173**
Glycolic acid	8.50	0.466	147, **177**, 205	**177**
Alanine (Ala)	9.11	0.499	116, 147, **190**	**190**
Valine (Val)	11.13	0.610	144, 156, **218**	**218**
Urea	11.41	0.625	147, 171, **189**	**189**
Ethanolamine	11.43	0.626	100, 147, **174**	**174**
Benzoic acid	11.62	0.636	105, 135, **179**	**179**
Glycerol	12.05	0.660	147, 205, **218**	**218**
Phosphoric acid	12.07	0.661	299, **314**	**314**
Isoleucine (Ile)	12.40	0.679	147, **158**, 218	**158**
Nicotinic acid	12.44	0.681	106. 136, **180**	**180**
Proline (Pro)	12.48	0.683	142, 158, **216**	**216**
Succinic acid	12.58	0.689	147, 172, **247**	**247**
Glycine	12.62	0.691	147, 174, **248**	**248**
Glyceric acid	12.96	0.710	133, 189, **292**	**292**
Fumaric acid	13.05	0.715	143, 217, **245**	**245**
Uracil	13.07	0.716	241, **255**	**255**
Serine (Ser)	13.38	0.733	204, 278, **306**	**306**
Threonine (Thr)	13.80	0.756	117, 218, **291**	**291**
β-Alanine	14.36	0.786	147, **174**, 248	**174**
Malic acid	15.17	0.831	189, **233**, 245	**233**
Aspartic acid (Asp)	15.59	0.854	232, 306, **334**	**334**
Methionine (Met)	15.61	0.855	**176**, 250, 293	**176**
4-Aminobutyric acid (GABA)	15.75	0.863	147, 174, **304**	**304**
Pyroglutamic acid	15.70	0.860	147, **156**, 230	**156**
Threonic acid	16.17	0.886	205, 220, **292**	**292**
Glutamic acid (Glu)	16.83	0.922	128, 348, **363**	**363**
Phenylalanine (Phe)	17.00	0.931	100, 192, **218**	**218**
Xylose	17.40	0.953	103, 277, **307**	**307**
Asparagine (Asn)	17.47	0.957	231, 333, **348**	**348**
Ribitol[e]	18.26	1.000	217, 307, **319**	**319**
Glutamine (Gln)	18.65	1.022	245, 347, **362**	**362**
Citric acid	19.24	1.054	273, 347, **363**	**363**
Fructose	19.94	1.092	217, 277, **307**	**307**
Fructose	20.02	1.097	217, 277, **307**	**307**
Mannose	20.27	1.110	147, 205, **319**	**319**
Mannitol	20.57	1.127	205, 217, **319**	**319**
Glucose	20.93	1.147	147, **204**, 217	**204**
Inositol	22.19	1.215	217, 305, **318**	**318**
Tryptophan	23.27	1.275	**202**, 219,377	**202**
Sucrose	27.05	1.482	217, 361, **437**	**437**
Trehalose	28.07	1.538	217, 271, **361**	**361**
Nonpolar compounds				
Eicosanol (C20)	10.43	0.782	**355**	**355**
Heneicosanol (C21)	10.98	0.823	**369**	**369**
Docosanol (C22)	11.50	0.862	**383**	**383**
Tricosanol (C23)	12.02	0.901	**397**	**397**
Tetracosanol (C24)	12.49	0.936	**411**	**411**

Table 1 continued

Compound	RT[a]	RRT[b]	Mass fragment[c]	Quantification ion[d]
5α-Cholestane[e]	13.34	1.000	**217**	**217**
Hexacosanol (C26)	13.43	1.006	**439**	**439**
γ-Tocopherol	13.84	1.037	263, **223**	**223**
Heptacosanol (C27)	13.89	1.041	**453**	**453**
Octacosanol (C28)	14.40	1.079	**467**	**467**
γ-Tocotrienol	14.50	1.087	**223,** 261	**223**
α-Tocopherol	14.65	1.098	**237,** 277	**237**
Campesterol	15.56	1.166	**343,** 367, 382	**343**
Triacontanol (C30)	15.59	1.169	**496**	**496**
Stigmasterol	15.76	1.181	255, 355, **394**	**394**
β-Sitosterol	16.20	1.214	255, **357,** 396	**357**

[a] Retention time (min)

[b] Relative retention time (retention time of analyte/retention time of internal standard)

[c] List of ions, with the specific mass ion highlighted in bold

[d] Specific mass ion used for quantification

[e] Internal standard

(Shimadzu), with a Rtx-5MS column (30 m, 0.25 mm inner diameter, and 0.25 μm film thickness).

Statistical analysis

Principal components analysis was performed on data from 56 polar and nonpolar components, reducing them to two variables and visualizing them on a bi-dimensional plane for clear discrimination between rice samples (SIMCA-P version 13, Umetrics, Umeà, Sweden). A PCA allows large datasets to be organized in a way that describes the relationships among samples. Pearson's correlation analysis was conducted to investigate significant relationships between chemical composition levels in SAS 9.3 (SAS Institute, Cary, NC, USA). Finally, HCA and heat map visualization of the correlation coefficients for all 56 analytes were conducted in MultiExperiment Viewer, version 4.4.0 (http://www.tm4.org/mev/).

Results and discussion

Multivariate analysis for the assessment of compositional differences among 56 polar and nonpolar secondary metabolites

Genetic background, cultivation method, and environmental conditions are several factors that cause chemical compositions to vary in crops [15, 23, 17]. We used PCA to determine whether differences in polar versus nonpolar metabolite composition among rice samples is due to the environment or the insertion of stilbene synthase gene. Both PCA and HCA with Pearson's correlation analysis were performed with peak area ratio data from 41 polar components and 15 nonpolar components; quantitative calculations of peak area ratios were relative to IS values. Table 1 specifies retention times and fragment patterns for each compound.

Effect of growing seasons and gene modification on chemical composition

To assess the effects of growing seasons and gene modification, RR and DJ were planted in 2013 and 2014 at Suwon, Iksan, and Miryang. Two principal components explained 47.4% of the total variance within the dataset (PC1, 28.6%; PC2, 18.8%). Regardless of growing season, RR could not be discriminated from DJ. In addition, the PCA score plot (Fig. 2A) indicated that in PC1, rice was separated more by growing year (2013 and 2014) than by planting site. The load plot (Fig. 2B) was analyzed to determine the primary chemical components contributing to between-season rice differences. In PC1, policosanols and phytosterols exhibited positive loading values, whereas tocopherols and tocotrienols had negative loading values. Tocopherol and tocotrienol composition in rice is more dependent on growing environmental conditions during growth than on genotype [24]. The PCA results confirmed that growing season had a stronger effect on chemical composition differences between rice samples than gene modification or growing sites.

Effects of growing sites, gene modification, and herbicide treatment on chemical composition

We performed PCA to evaluate how growing sites, herbicide treatment, and gene modification affected the

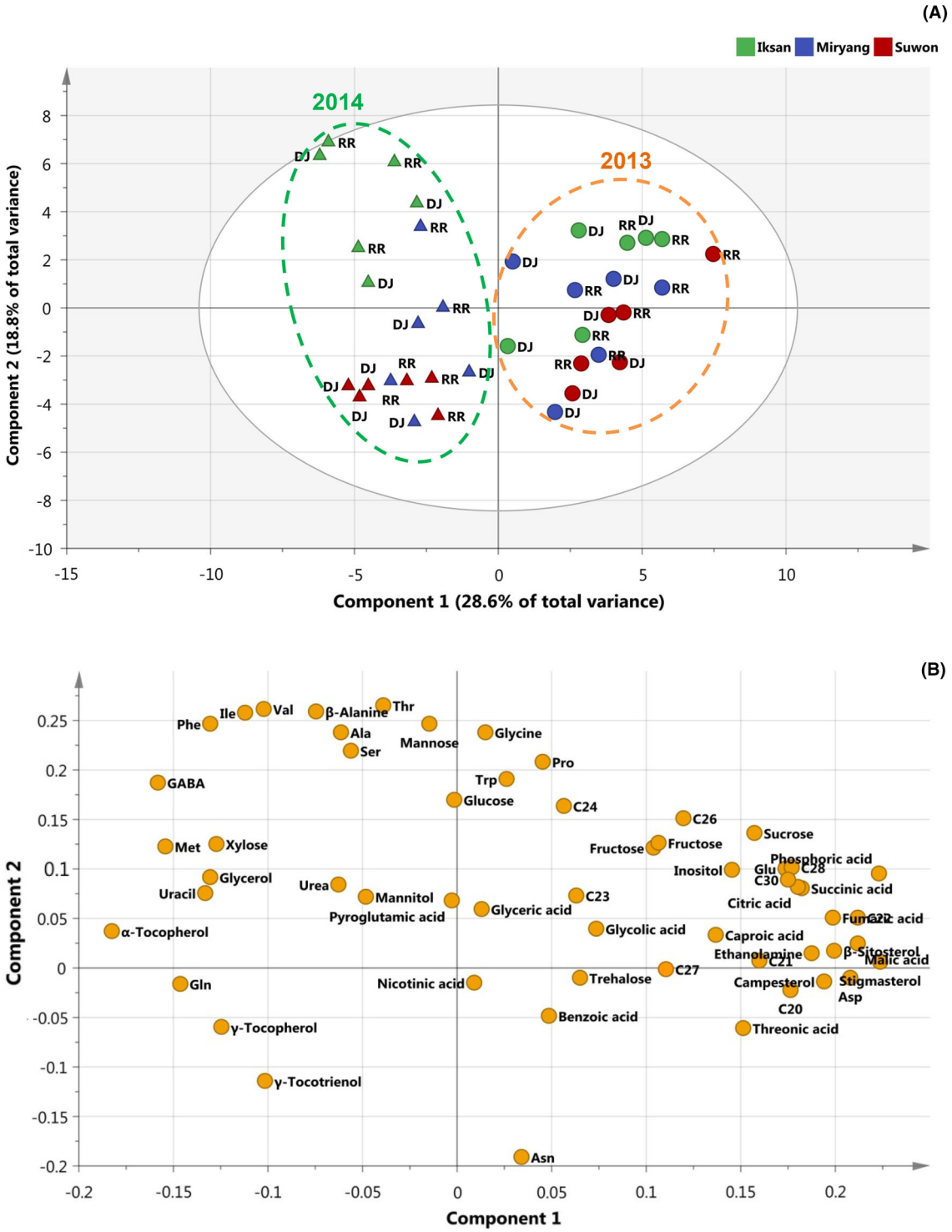

Fig. 2 PCA model of resveratrol-enriched rice (RR) and its parent cultivar (Dongjin, DJ) cultivated at three different sites (Suwon, Iksan, and Miryang) during 2013 and 2014. (**A**) PCA score plot showing the % contribution of each component to total variance; the top two principal components accounted for 47%. *Circle* cultivated in 2013, *triangle* cultivated in 2014. (**B**) Loading plot. The *dotted circle* indicates that rice was separated more by growing year (2013 and 2014) than by planting site

chemical composition of RR and DJ planted at three different sites during 2013. The first two components accounted for 44.2% of the total variation (Fig. 3A) and did not distinguish rice samples based on RR versus DJ. However, PCA score plots, combining PC1 and 2, separated rice samples according to site location (Suwon, Iksan,

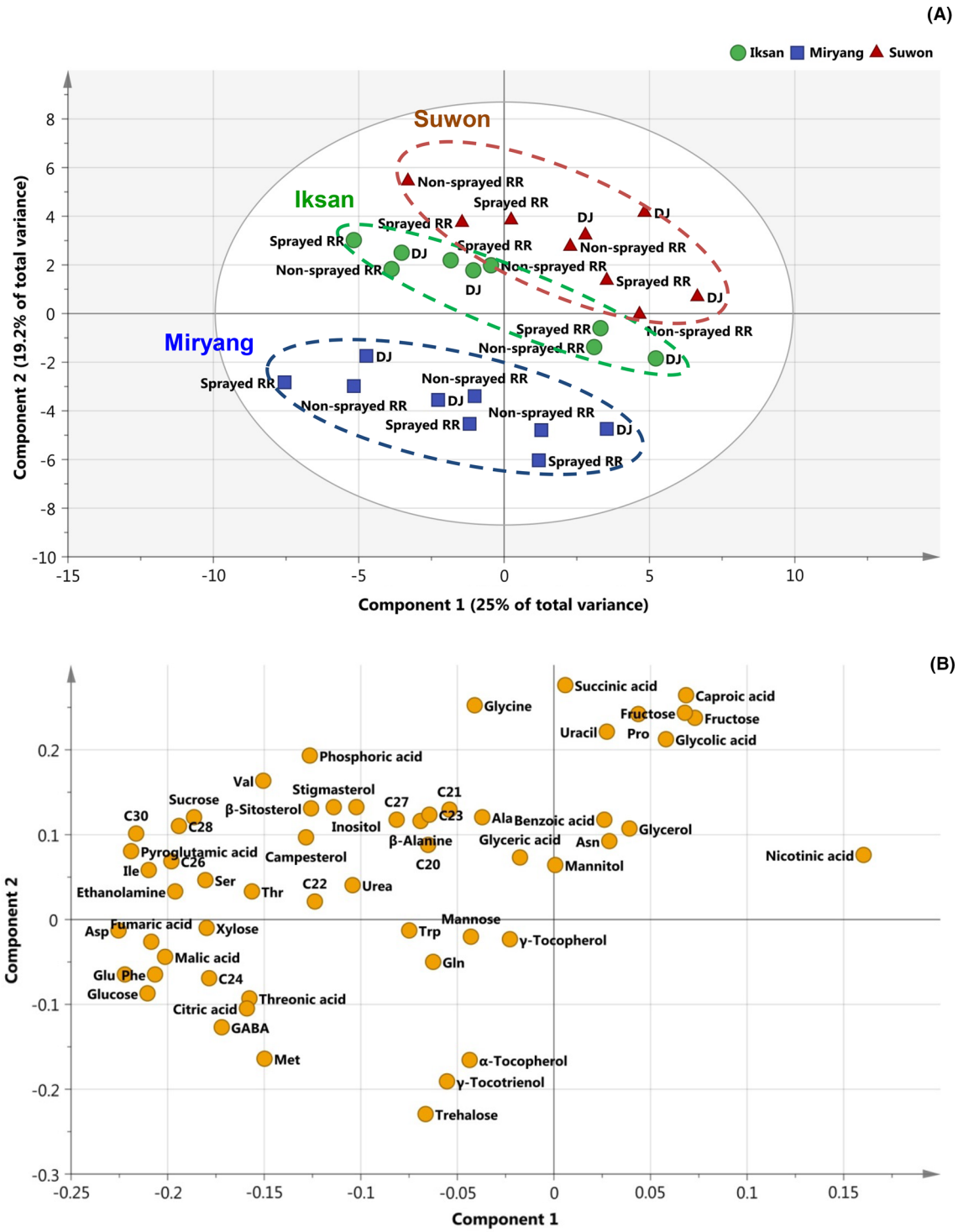

Fig. 3 PCA model of resveratrol-enriched rice (RR), herbicide (glufosinate)-treated RR, and its parent cultivar (DJ), cultivated in three different sites (Suwon, Iksan, and Miryang) during 2013. (**A**) PCA score plot showing the % contribution of each component to the total variance; the top two principal components accounted for 44.2%. *Triangle* cultivated in Suwon, *circle* cultivated in Iksan, and *square* cultivated in Miryang. (**B**) Loading plot. The *dotted circle* indicates that PCA resolved the metabolic profiles of rice based on site location

and Miryang). In addition, RR (herbicide-treated and untreated) was not separated from DJ in the PCA score plot (Fig. 3A). The loading plot indicated that the concentrations of several organic acids (succinic acid, caproic acid, glycolic acid) and proline were higher in rice from Suwon than from Iksan and Miryang. In contrast,

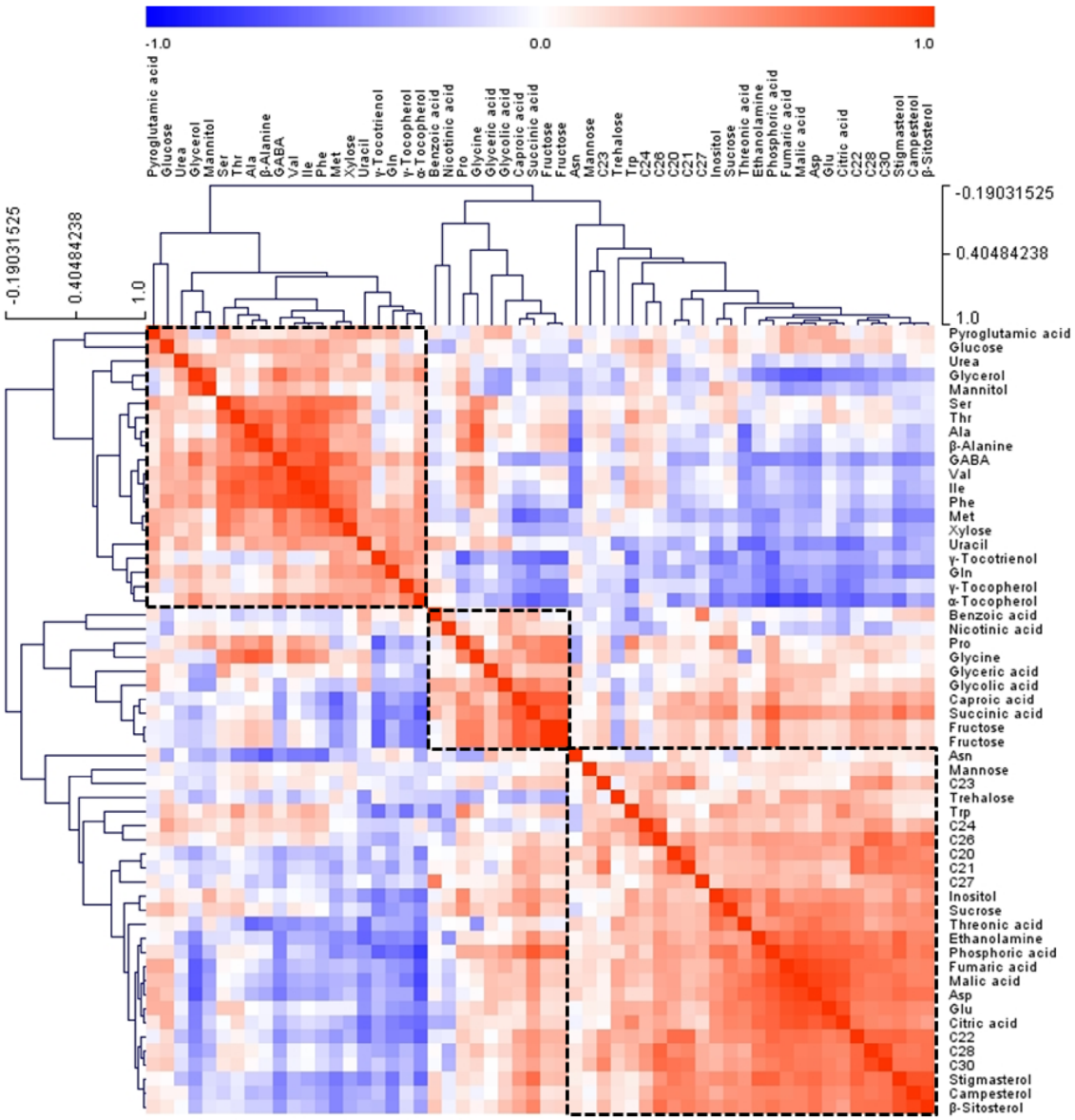

Fig. 4 Correlation matrix of 56 polar and nonpolar secondary metabolites from rice seeds. Each *square* indicates the Pearson's correlation coefficient of a compound pair, and the value of the coefficient is represented by the intensity of *blue* or *red*, as indicated on the *color scale*. Hierarchical clusters are represented in a *cluster tree*

trehalose, GABA, and methionine concentrations were highest in Miryang rice. These results corroborated previous research showing higher proline content in Suwon rice [25]. Under salt stress or dehydration, plants experience increased proline synthesis as a defense mechanism; the amino acid is thought to act as an osmoticum for adjusting water potential [26]. In addition, our previous study indicated that soil condition was the biggest factor dividing rice grown in Suwon from those grown in other locations [13]. Our current results and previous data thus combine to demonstrate that soil conditions are the most influential factor separating Suwon rice from rice of other sites. The concentrations of GABA and several sugars (glucose, trehalose, xylose, mannose) were higher in rice from Miryang than from Iksan and Suwon. This outcome may be due to high temperature from Miryang's location. Overall, our data demonstrated clearly that between-site environmental differences are the primary influence on rice chemical composition, rather than gene modification or herbicide treatment.

HCA and Pearson's correlations between 56 metabolites in rice seeds

The biochemical structures of 56 polar and nonpolar metabolites in rice seeds were evaluated with HCA and

Pearson's correlations (Fig. 4). Three major composition groups were described with HCA (Fig. 4, boxed within dotted lines). Pearson's correlations identified relationships between compounds involved in closely related metabolic pathways. For example, one group—including most organic acids, sterols, and all policosanols—was negatively correlated with the group containing tocopherols and tocotrienols. This outcome corresponds to the PCA loading values (Fig. 2B) indicating negative correlations between the two groups of compounds. Thus, the HCA results support previously reported strong correlations between compounds participating in linked metabolic pathways. Camacho et al. [27] had also reported strong correlations between them. For example, alanine was positively correlated with β-alanine ($r = 0.8410$, $p < 0.0001$), valine ($r = 0.7510$, $p < 0.0001$), and isoleucine ($r = 0.7202$, $p < 0.0001$). Additionally, sterol and tocol were strongly correlated with each other, and both are in the terpenoid pathway. Finally, each cluster from HCA appeared to exhibit a common chemical function.

In conclusion, we applied a metabolomics approach, analyzing polar and nonpolar metabolites with GC/MS, to detect significant chemical composition changes in transgenic RR compared with non-transgenic rice. We also investigated the effects of environment, genotype, and herbicide treatment on rice chemical composition. The results of PCA demonstrated that growing year and site were the major factors influencing variation in rice chemical composition; no effects of genotype or herbicide treatment were detected. Moreover, HCA showed that RR and DJ biochemical structure do not differ from each other. Thus, genetic modification did not trigger detectable unintended effects in RR, although more research is still necessary to fully evaluate the safety of this transgenic rice. Our targeted approach focusing on 56 polar and nonpolar secondary metabolites will provide a solid cornerstone for future RR safety assessments.

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