Nontarget Metabolomics of Grape Seed Metabolites Produced by Various Scion–Rootstock Combinations

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ABSTRACT. The use of resistant rootstocks is an inevitable trend in the development and production of grapes (*Vitis* sp.). The present study analyzed differences in the metabolites in grape seeds of different rootstock combinations (1103P, 5C, SO4, 3309C, 140R, and control) grafted onto ‘Cabernet Sauvignon’ (CS) wine grape (*Vitis vinifera*) scions (control, CS/CS, self-rooted grafting vines) using liquid chromatography–mass spectrometry (LC-MS) and nontargeted metabolomic techniques. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal-partial least squares discriminant analysis identified 30 significant metabolites and 22 metabolic pathways in the seeds of CS that differed significantly from the control seeds. This study revealed that rootstocks influence metabolite concentrations and metabolic pathways (alanine–aspartate–glutamate pathway, arginine–proline pathway, and the tricarboxylic acid cycle) in the scion onto which they are grafted. The rootstocks increased the concentration of delphinidin-3-(6-acetylglucoside), peonidin 3-(6-p-coumarylglucoside), L-threonine, and D-tartaric in CS seeds. Appropriate rootstock combinations can be used to improve the quality of grape seeds by changing the concentrations of amino acids, organic acids, polyphenols, and vitamin B. This study provides a theoretical basis for selecting grape rootstocks and provides important insights for improving the quality of commercial products derived from grape seeds.

Grapes (*Vitis* sp.) are widely cultivated for fresh or dried fruit, wine making, juice making, and as a components of health foods and medicines. In fact, grapes are one of the most commercially valuable plants worldwide (Harb et al., 2015; Leng et al., 2015). The total land area used for grape cultivation has been increasing over the past 10 years in China, and China is now the leading grape producer worldwide (International Organisation of Vine and Wine, 2018). The Xinjiang region, historically China's largest grape-producing region, covers a vast territory with a diverse array of climates and soils. The region's long history (2400 years) of producing grapes and wines is attributed to its climatic conditions being suitable for grape production. Drought, soil salinity, and cold weather can severely impact the development and production of wine grape (*V. vinifera*), and therefore, a cultivar of rootstocks has been developed in Xinjiang grape to resist such environmental stress. These resistant rootstocks not only improve the stress resistance of scions but also affect the yield and quality of grape berries

(Corso et al., 2016; Suriano et al., 2016). Rootstock and scion combinations can also be used to regulate vine growth, phenological period, fruit yield, and the concentrations of key constituents in the fruit, such as tannins, anthocyanins, and resveratrol, all of which affect grape quality and taste by changing water balance relationships, gas exchange rates, and nutrient absorption capacities. Areas under grape cultivation can be increased in China (and the commercial industry enhanced) if grape cultivars are improved to enable growth under varied soil and climatic conditions, further resist pathogens, and improve the concentrations of key chemical constituents.

Grape seeds comprise flavonoids and other metabolites that improve plant biotic and abiotic resistance. They also have bactericidal and antiviral effects and potential roles in preventing cardiovascular and cerebrovascular diseases (Zerbib et al., 2018). Trad et al. (2017) think polyphenols in grape seeds are key compounds that play a role in selection and domestication, as they offer astringency and bitterness to derived products and promote health safety. Tannins, monomeric flavan-3-ols and other substances in the seed are correlated to wine quality by both consumer and expert tasting panels, which have an effect on the color and taste of wine (Joshua et al., 2020). At the same time, grape seed is the primary by-product of wine production. Because of their high content of phenols, unsaturated acids, and other biologically active compounds, grape seeds have been increasingly used for further processing; for instance, they are processed into high-quality plant oil. It is also believed that

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grape seeds have a health function and an effect of extending the shelf life of food (Gordana et al., 2019; Hela et al., 2018). Approximately 155 cosmetics have “grape seed” in their names in the China Food and Drug Administration database, and 127 types of health foods have been named “grape seed.” Grape seeds have become an important source of chemicals for some foods, health products, and medicines. At present, research on the effects of rootstocks on scion quality is mainly focused on the growth and development of vines and only partially on fruit quality. However, no studies have been conducted on the effects of various rootstocks on the chemical composition of grape seeds or related metabolic processes and pathways. Metabolomic profiling involves the analysis and data integration of a total population of metabolites in a given sample based on high-throughput, multivariate data. Nontarget metabolomics analyzes the broadest possible array of metabolites in a sample from a holistic perspective and has been routinely used in science, including in biology, clinical medicine, pharmacology, and food science (Arapitsas et al., 2016; Narduzzi et al., 2015; Qi et al., 2011). When Bueno et al. (2018) used metabolomic profiling to evaluate organically and conventionally cultivated tomato (*Solanum lycopersicum*), they found that organically cultivated tomato exhibited significantly different chemical sample profiles for six bioactive compounds: L-tyrosyl-L-isoleucyl-L-threonyl-L-threonine, trilobatin, phloridzin, tomatine, phloretin, and echinenone. Harb et al. (2015) used gas chromatography–mass spectrometry and LC-MS to examine the effects of abiotic stress on the metabolism of various grape cultivars and found that stress significantly affected primary metabolite production (especially amino acids) but had no effects on secondary metabolite production. When Son et al. (2008) found significant differences in the chemical compositions of several grape cultivars grown in various grape-producing areas using metabolomics, they suggested that metabolomics could be used to predict wine quality and product origin. Other studies have also demonstrated that metabolomic profiling can be used to effectively analyze variations in grape metabolites and identify differences in metabolic pathways among grape cultivars.

In this study, nontarget LC-MS was used to study the effect of rootstock grafting on the grape seed metabolome of CS, a major wine grape cultivar in China. This study used PCA and partial multivariate statistical analysis methods [e.g., PLS-DA] to screen differences in grape metabolites among various rootstock combinations and analyze their metabolic pathways, and explored patterns in metabolite accumulation to analyze grape seed quality and the nutritional effects of various grape rootstocks on CS seeds. By evaluating the effects of scion–rootstock graft combinations, this study will provide a theoretical basis and provide important insights for improving the quality of commercial grape seed–related products.

Materials and Methods

TEST MATERIALS. This study used CS as scion material. A cultivar of rootstocks was grafted onto CS, which provides excellent resistance: rootstocks ‘SO4’ and ‘5C’ (*Vitis berlandieri* × *Vitis riparia*), rootstocks ‘140R’ and ‘1103P’ (*V. berlandieri* × *V. rupestris*), and rootstock ‘3309C’ (*V. riparia* × *V. rupestris*). The rootstock combinations used in our experiments were CS/‘1103P’, CS/‘SO4’, CS/‘140R’, CS/‘5C’, and CS/‘3309C’, with CS/CS (self-rooted grafting seedlings) used

as the control. All rootstocks were obtained from the National Grape Resources of Zhengzhou Fruit Research Institute.

In Autumn 2015, 10 hard-twig vines with uniform growth were selected for each rootstock scion combination; these were grafted and planted (0.5 m apart) in blocks in the field at the Experimental Station of the Agricultural College of Shihezi University in Xinjiang, China (lat. 86°06’N, long. 44°32’E). Cultivation conditions and management were consistently maintained. The cultivation management conditions were the same, and the control group has similar processing yields. In Sept. 2017, fully ripe grape bunches were randomly selected from near the root and away from the root, with ≈400 g of berries per replicate and five replicates per each rootstock scion combination. Seeds were quickly collected from the fruit in the laboratory by hand, packaged in tin foil, frozen in liquid nitrogen, and stored at –80 °C. This study used ultrapure water [ddH₂O (Arium mini; Sartorius, Göttingen, Germany)] and chromatographically pure methanol, chloroform (Sinopharm Chemical Reagent Co., Shanghai, China), acetonitrile (Merck Chemical Technology Shanghai Co., Shanghai, China), and formic acid (Tokyo Chemical Industry Co., Tokyo, Japan) as reagents.

INSTRUMENTS AND EQUIPMENT. The experimental instruments included a vacuum concentrator (53050; Eppendorf, Hamburg, Germany), a centrifuge (H1650-W; Eppendorf), a vortex mixer (QL-866; Qilinbeier, Haimen, China), a pure water meter (Arium mini), a 0.22-μm polytetrafluoroethylene filter (Jin Teng, Tianjing, China), an ultrasonic cleaner (KW-100TDV; Shumei, Kunshan, China), and a tissue grinder (SCIENTZ-48; Xinzhi, Ningbo, China).

SAMPLE PREPARATION. For each treatment, 0.5 g of grape seed was ground using a high-flux tissue grinder, 100 mg of grape seed powder was placed into a 5-mL centrifuge tube, and then 1000 μL of methanol (–20 °C) was added; this mixture was vortexed for 30 s and the vortexed material was placed in an ultrasonic cleaner for 30 min at room temperature. Then, 750 μL chloroform and 800 μL ddH₂O (at 4 °C) were added to the mixture, which was vortexed for 1 min and centrifuged for 10 min at 12,000 g_n and 4 °C. A total of 1000 μL of supernatant was removed and transferred to a new 1.5-mL centrifuge tube, and it was concentrated using a vacuum centrifugation concentrator. The sample was dissolved in 250 μL methanol/ddH₂O solution [1:1 (v/v) at 4 °C] and filtered through a 0.22-μm membrane filter. Subsequently, 20 μL of each sample was removed and mixed with a quality control sample to correct for any deviations in the mixed samples due to error(s) caused by the analytical instrument. LC-MS was performed on the remaining samples tested. Five replicates were then randomly selected for each treatment (De Vos et al., 2007).

CHROMATOGRAPHY CONDITIONS. This study used an ultra-performance liquid chromatography system [UPLC (Acquity; Waters, Milford, MA)] equipped with a 1.8-μm, 150 × 2.1-mm column (Acquity UPLC HSS T3; Waters) maintained at 40 °C to chromatographically separate metabolites. The temperature of the autosampler was maintained at 4 °C. The gradient elution of analytes was performed using 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow rate of 0.25 mL·min^{–1}. After equilibration, 6 μL of each sample was injected. An increasingly linear gradient of Solvent B (v/v) was used as follows: 2% Solvent B for the first minute, from 2% to 50% Solvent B from min 1 to min 9.5, from 50% to 98% Solvent B from min 9.5 to min 14, 98% Solvent B from

min 14 to min 15, from 98% to 2% Solvent B from min 15 to min 15.5, and 2% Solvent B from min 15.5 to min 17 (Sangster et al., 2006).

MASS SPECTROMETRY CONDITIONS. This study used electrospray ionization mass spectrometry (ESI-MSⁿ) using a mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher Scientific, Waltham, MA) with positive and negative spray voltages at 4.8 and -4.5 kV, respectively. Sheath gas and auxiliary gases were set at 45 and 15 arbitrary units, respectively. The capillary temperature was 325 °C. The positive and negative voltages of the capillary were set at 35 and -15 V, respectively, whereas those of the tube were set at 50 and -50 V, respectively. We used an Orbitrap analyzer (Thermo Fisher Scientific) scanned over a mass range of 50 to 1000 m/z for a full scan. We performed data-dependent acquisition MS/MS experiments with a collision-induced dissociation scan. The normalized collision energy was 30 eV. Dynamic exclusion was implemented with a repeat count of 2 and exclusion duration of 15 s (Want et al., 2010).

DATA ANALYSIS. Data were analyzed using proteomics data analysis software (ProteoWizard version 3.0; ProteoWizard, Palo Alto, CA), and mass spectrometric peaks were filtered, identified, and aligned using statistical analysis software (R version 3.3; R Foundation for Statistical Computing, Vienna, Austria) to obtain data matrices for mass-to-core ratios, retention times, and peak areas. The data were then subjected to batch normalization and adaptive conversion to standardize the peak areas. PCA [SIMCA-P (version 13.0; Umetrics AB, Umea, Sweden) and R language ropes package], PLS-DA, orthogonal PLS-DA (OPLS-DA), and other chemometric principles and multivariate statistical analyses were performed (Thévenot et al., 2015; Want et al., 2010).

Results and Discussion

Differences in the metabolome of CS grape seeds of various rootstock combinations

PCA ANALYSIS. In the present study, the PCA scores reflect the original state of the metabolome data, which was helpful in examining the differences in metabolites produced in grape seeds of various CS scion–rootstock combinations and all the data at once (Fig. 1) (Gromski et al., 2015). There was a wide separation of grape seed samples from the grafts of CS/‘3309C’, CS/‘1103P’, CS/‘SO4’, and CS/CS (control) on the ordination diagram, indicating obvious differences in metabolite production among the various rootstock grafts. The CS/‘140R’ and CS/‘5C’ rootstock combinations did not separate, indicating that there is no significant difference between their metabolites.

PLS-DA ANALYSIS. PCA analysis considers intragroup errors and does not eliminate random errors unrelated to the research purpose, indicating that PCA is not very conducive for detecting differences between groups. Therefore, this study used a PLS-DA to further classify the grape seed metabolites derived from the rootstock combinations (Boulesteix and Strimmer, 2007; Lee et al., 2010).

The PLS-DA score plot and permutation test results of various rootstock combinations of CS grape seed samples were essentially consistent with our PCA results (Fig. 2). The principal component regression coefficient (Q²) for grape seed samples was 0.962, which is much more than 0.5 (maximum value = 1.0), indicating that the model is extremely predictive,

Fig. 1. Principal components analysis (PCA) scores of ‘Cabernet Sauvignon’ (CS) grape seeds of various rootstock combinations.

reflecting the visually obvious geometric separation between samples (i.e., rootstock combinations) in the PCA diagram. The percent contribution (R²Y) of the model of CS grape seed samples was 0.992, indicating that the contribution of the model to the variation in the dependent variables was 99.2%. When combining data from various rootstocks with a CS grape seed model (based on 100 permutation test experiments), the Q² point of the model was moved lower than the original Q² point (from the left to the right) and the R² and Q² values on the rightmost side were both more than 0.9. Furthermore, the intercept of the Q² regression line was -0.594, which indicates that the constructed PLS-DA model is not associated with overfitting, and that the model has good predictive ability, is effectively available, and can combine CS with different rootstocks.

OPLS-DA ANALYSIS. OPLS-DA, which is an extension of PLS-DA, is an analytical method that is commonly used when analyzing metabolomics data (Consonni et al., 2009; Tarachiwin et al., 2008). The plots of OPLS-DA scores (Fig. 3) were generally consistent with the plots of PCA scores. The OPLS-DA model explained 62.5% of the variation in the independent variable X (R²X = 0.625) and 99.2% of the variation in the categorical variable Y (R²Y = 0.992), with a Q² value of 0.932, indicating that the model explained 93.2% of sample variation. These results demonstrate that the OPLS-DA model is stable and has strong predictive power. Although data from the CS/‘140R’ and CS/‘5C’ seeds have partial overlap in PCA or PLS-DA and no overlap in the OPLS-DA score plot, the separation effect is better. All the samples separated from one another in OPLS-DA, suggesting differences in the metabolites produced in seeds owing to the differences in the various rootstocks onto which the CS scions were grafted.

Screening and identification of metabolites

The variable importance in projection values of variables greater than 1.0 (VIP > 1) and one-way analysis of variance were used to identify significant differences ($P \leq 0.05$) in grape seed metabolites among the various rootstocks of CS. Six of the 30 metabolites (Table 1) discovered in grape seeds significantly differed among the various rootstock combinations evaluated (Fig. 4).

Fig. 2. Partial least squares discrimination analysis (PLS-DA) score plot and permutation test results of 'Cabernet Sauvignon' (CS) grape seeds from various rootstock combinations.

Fig. 3. Orthogonal-partial least squares discrimination analysis (OPLS-DA) score plot of 'Cabernet Sauvignon' (CS) grape seeds from various rootstock combinations.

Heat map metabolites

The cluster and heat map analyses of the metabolites for various rootstock combinations of CS grape seeds are shown in Fig. 5. The colors in the figure indicate relative metabolite concentration, with red indicating high concentration and green indicating low concentration. Each rootstock combination shows either significantly high or low areas of expression. The different rootstock combinations can be distinguished based on the relative expression of the above differential metabolite in grape seeds.

The concentration of malic acid was higher in the self-rooted vine (control group) than in other rootstock combinations (Fig. 4). Similarly, the concentration of alvidin-3-O-arabinoside was higher in the CS/'1103P' graft than in other rootstock combinations, and the concentrations of L-lysine, malvidin 3-(6-p-caffeoylglucoside), kaempferol 3-O-beta-D-glucoside, gallate, citric acid, and 5-oxo-D-proline were higher in the CS/'SO4' combination than in other rootstock combinations. The con-

centrations of malvidin 3-(6-p-coumaroylglucoside), N-acetyl-L-glutamate, L-arginine, pyridoxine, and adenosine were higher in the CS/140R rootstock combination than in other combinations, and the concentrations of L-glutamic acid, (-)-epicatechin, L-threonine, myricetin 3-glucoside, and petunidin 3-(6-acetylglucoside) were higher in the CS/'5C' combination than in other combinations. The concentrations of 2-oxoglutaric acid, D-gluconic acid, (R)-pantothenic acid, 1-(3-hydroxy-4-5-dimethoxybenzoyl) pyranose, peonidin 3-(6-p-coumaroylglucoside), 2-deoxy-alpha-D-ribose, and delphinidin 3-(6-acetylglucoside) were higher in the CS/'3309C' combination than in other combinations.

Pathway analysis of metabolic products

This study analyzed the metabolic pathways based on the variations in the metabolites in grape seeds within the various rootstock combinations examined. This study used the Metabolic Pathway Analysis (MetPA) (based on the Kyoto Encyclopedia of Genes and Genomes metabolic pathway database) to identify possible metabolic pathways affected by the various rootstock combinations (Kanehisa and Goto, 2000). This study found 15 substances enriched in metabolic pathways, including lysine, threonine, and glutamic acid, among which three amino acids (lysine, threonine, and glutamic acid) were involved in three or more different metabolic pathways (Table 2). This suggests that these three amino acids are positioned at nodes in metabolic pathways and so are the key substances connecting the various pathways. These three amino acids are precursors for the synthesis of various proteins/enzymes that are associated with tolerance to cold and resistance to closely related diseases.

The metabolic pathways altered by various rootstock combinations in grape seeds mainly involve 22 pathways associated with the metabolism of alanine, aspartic acid, and glutamate; the metabolism of arginine and proline; and the tricarboxylic acid (TCA) cycle (Fig. 6, Table 2). The three metabolic pathways with obvious combination effects (alanine, aspartic acid, and glutamic acid pathways) provide precursors for the synthesis of various amino acids that typically occur in grapes. These amino acids also constitute

Table 1. Various metabolites of ‘Cabernet Sauvignon’ grape seeds of various scion–rootstock combinations.

No.	Metabolite	Mz	Rt (s)	VIP	q-value
1	D-gluconic acid	195.0488	98.2249	1.12367	0
2	2-oxoglutaric acid	145.0128	156.2850	1.04388	0
3	N-acetyl-L-glutamate(2-)	188.0542	211.8410	1.35998	0
4	5-oxo-D-proline	128.0344	189.0410	1.44629	0
5	Peonidin 3-(6-acetylglucoside)	505.1314	426.2520	1.18307	0
6	L-arginine	175.1183	95.4428	1.27279	0
7	(-)-epicatechin	289.0676	372.4270	1.55669	0
8	Gallate	169.0125	271.1590	1.20819	0
9	citric acid	191.0174	172.4240	1.34724	0
10	nicotinic acid	124.0387	158.7510	1.15311	0
11	L-lysine	147.1120	84.1111	1.44138	0
12	L-threonine	120.0649	101.5455	1.27202	0
13	2-deoxy-alpha-D-ribose	133.0496	246.4400	1.23969	0
14	Petunidin 3-(6-acetylglucoside)	521.1257	405.0090	1.25473	0
15	Delphinidin 3-(6-acetylglucoside)	507.1101	382.6990	1.33976	0
16	(R)-pantothenic acid	218.1005	304.4305	1.23284	0
17	kaempferol 3-O-beta-D-glucoside	447.0877	476.3015	1.40111	0
18	pyridoxine	170.0803	189.9590	1.2264	0
19	1-O-(3-Hydroxy-4,5-dimethoxybenzoyl)hexopyranose	359.0936	318.2920	1.02898	0
20	malic acid	133.0131	115.0870	1.17395	0
21	Sequiterpene Lactone 326	265.1421	344.8430	1.10369	0
22	D-tartaric acid	149.0074	105.8605	1.1301	0
23	L-glutamic acid	148.0596	101.8770	1.18473	0
24	Myricetin 3-glucoside	481.0951	418.1780	1.10691	0.00001
25	adenosine	268.1026	248.3980	1.09745	0.00002
26	malvidin-3-O-arabinoside	463.1209	400.6830	1.10058	0.00008
27	taurine	125.9856	84.0558	1.23672	0.00028
28	Malvidin 3-(6-p-caFFEYglucoside)	655.1618	445.4310	1.05048	0.00475
29	Peonidin 3-(6-p-coumarylglucoside)	609.1569	472.9930	1.01011	0.00797
30	Malvidin 3-(6-p-coumarylglucoside)	639.1667	472.9930	1.00033	0.0121

Mz = mass-to-charge ratio, Rt = retention time, VIP = variable importance of the first principal component of orthogonal-partial least squares discriminant analysis, q-value = significant difference *P* value after false-positive correction.

the main trait examined by commercial growers to predict growth characteristics in grapes. The arginine and proline metabolic pathways include amino acids that produce important substances that promote plant growth and development for producing cold-resistant osmotic constituents; and the TCA cycle is a common pathway for the oxidative metabolism of sugars, lipids, and proteins, and its intermediate products are precursors for the biosynthesis of amino acids, proteins, and fatty acids. This study found that plant-related amino acid concentration significantly increased after grafting the CS scion onto rootstock. Altering the metabolic pathways of alanine, aspartic acid, and glutamate, those of arginine and proline metabolism, and the TCA cycle increased the synthesis and enrichment of nutrients in grape seeds.

Analysis of metabolites in grape seed relative to various rootstock combinations

Grapes are widely used in fresh foods, juice and wine making, and pharmaceuticals. Grape metabolites are also important in influencing the quality of grape seed-derived products, while also affecting the growth and development of grapes. To ensure the quality and safety of products that contain grapes, it is necessary to improve our understanding of the nutritional value of metabolites present in grapes.

Amino acids play an important nutritional role in grape seeds, in the taste of grape fruit, and in the timing of phenophases in grapes. For example, lysine is highly correlated with sugar concentration (Jin et al., 2016a), glutamate has a pleasant taste, and the ratio of proline-to-arginine affects the number of days required for grapes to ripen (Jogaiah et al., 2013). Arginine regulates the nitric oxide synthase pathways, which increases the resistance of fruit to diseases (Ji et al., 2018). Arginine and lysine are also purported to improve cardiovascular health, whereas threonine is an essential amino acid that appears to exhibit antibacterial effects in humans (Rieping and Hermann, 2006; Zhao et al., 2015). Some rootstocks have been found to change the amino acid concentration in fruit of ‘Gold Finger’ (Jin et al., 2016b), ‘Summer Black’ (Jin et al., 2016a), and ‘Thompson Seedless’ table grapes (Ji et al., 2018). This study found that the concentrations of arginine, lysine, threonine, and glutamic acid were increased or decreased in seeds in varying degrees depending on the rootstock combination from which they were extracted (Fig. 4); for example, the concentration of those four amino acids in the grape seeds varied in the CS/‘SO4’ rootstock, but other rootstock combinations exhibited high

amino acid concentrations. Therefore, different rootstock combinations can regulate the amino acid concentration in grape seeds.

Organic acids are closely associated with the quality of grapes. For wine grapes, organic acids play an important role in the flavor composition of wine. Low content of organic acids in wine leads to increased acidity and affects the wine taste balance and quality (Batista et al., 2010). Citric acid, tartaric acid, and malic acid are the main organic acids in grape seeds, accounting for more than 90% of their total organic acid concentrations. These acids play a major role in controlling the flavor and acidity of grape fruit, (e.g., determining the sensory perception of wine and its pH) and are widely used in food products. Overall, the acidity of tartaric acid is 90% of citric acid, and the acidity of malic acid is 80% of citric acid. Thus citric acid has a greater effect on acidity (Jin et al., 2016b). In the present study, the concentration of malic acid was lower (in varying degrees) than that in the control (self-rooted grafting) in each grafting combination, whereas the concentration of tartaric acid was higher (in varying degrees) than that in the control (self-rooted grafting). The CS/‘SO4’ rootstock combination had a very significantly higher ($P < 0.01$) concentration of citric acid than the CS/CS combination, and the CS/‘1103P’, CS/‘140R’ and CS/‘5C’ rootstock combinations had a

Fig. 4. Box diagram of differential metabolite concentrations in ‘Cabernet Sauvignon’ (CS) grape seeds from various rootstock combinations.

very significantly lower ($P < 0.01$) concentration of citric acid than the CS/CS combination. All the rootstock combinations, except the CS/‘1103P’ combination had significantly reduced concentrations of 2-oxoglutaric acid. It has been reported that

the organic acid concentrations of the table grapes ‘Gold Finger’ (Jin et al., 2016a) and ‘Summer Black’ (Jin et al., 2016b), and the wine grape ‘Sauvignon Blanc’ (Pulko et al., 2012) were significantly affected by the different rootstocks,

Fig. 5. Hierarchical clustering heatmap of the metabolites of ‘Cabernet Sauvignon’ (CS) grape seeds from various rootstock combinations.

which is similar to the findings of the present study. Therefore, it appears that rootstocks can significantly change the concentrations of organic acids in grape seeds of different cultivars grafted onto them.

Polyphenols affect various biological functions, including protection against predators, pathogens, and abiotic stress (Cheng et al., 2017). Strong antioxidant activity (e.g., because of picatechin and gallic acid esters) maintains normal physiological and biochemical processes in fruit and protects reactive oxygen species produced during abiotic stress (Koundouras et al., 2009). It also has been reported that ingesting foods that comprise polyphenolic antioxidants can provide anti-inflammatory effects, reduce the incidence of cardiovascular diseases, and exert anticancer effects (Morrison et al., 2014; Prince et al., 2016; Yoshioka et al., 2000). Anthocyanins, such as delphinidin glucoside and peony glucoside, affect the color and taste of wine (Vidal et al., 2004). It has been reported that anthocyanins in grape seeds also exert anticancer effects and improve cardiovascular and cerebrovascular functions (Pascual-Teresa and Sanchez-Ballesta, 2008). Chemicals derived from grape seeds also provide active ingredients in medicines.

In the present study, it was demonstrated that all the grafting treatments had higher concentrations (in varying degrees) of delphinidin-3-(6-acetylglucoside) and paeonidin-3-(6-p-coumarin glycoside) in the grape seeds than in the controls and that seeds from the CS/‘SO4’ rootstock combination had significantly higher concentrations of epicatechin

and gallate than the controls. Some studies have shown that the concentration of grape polyphenols is high in other cultivars of rootstocks (Cheng et al., 2017). Furthermore, the expression levels of genes responsible for the synthesis of VviPAL3-like, VviCHS3, VviLAR2, and VviUFGT anthocyanins are reported to be higher in grape seeds of the CS/‘M4’ rootstock combination than in those of the CS/‘1103P’ rootstock combination (Corso et al., 2016), which indicates that the synthesis of polyphenols in grape seeds can be improved by selecting appropriate rootstock combinations. However, the molecular mechanism of this synthesis needs further exploration.

It is well known that grapes are rich in vitamins. Reports on the effects of rootstocks on vitamin metabolism in grapes have mainly focused on vitamins C and E. However, to the best of our knowledge, the effects of rootstocks on the vitamin B family have not been reported to date, despite the vitamin B family playing an important role in plant development. The human body must have, but cannot synthesize, several substances that prevent diseases (Chen and Xiong, 2005; Noctor, 2006; Said and Mohammed, 2006). The present study showed that the CS/‘1103P’ rootstock combination significantly increased the concentrations of nicotinic acid (vitamin B3 or vitamin PP) and pantothenic acid (vitamin B5), whereas the CS/‘SO4’ rootstock combination significantly increased the concentration of pyridoxine (vitamin B6). These results suggest that the health benefits of grape seeds can be improved by using appropriate

Table 2. Various metabolic pathways in ‘Cabernet Sauvignon’ grape seeds of various rootstock combinations.

No.	Metabolic pathway	Total metabolites (no.) ^z	Detected metabolites (no.) ^y	–log(<i>P</i>) ^x	Impact ^w	Pathway ^v	Compounds ^u
1	Aminoacyl–transfer RNA biosynthesis	67	4	4.0604	0	ath00970	C00062,C00047,C00188,C00025
2	Arginine and proline metabolism	38	3	3.9579	0.27008	ath00330	C00062,C00025,C00624
3	Citrate cycle (TCA cycle)	20	2	3.3016	0.16769	ath00020	C00026,C00158
4	Alanine, aspartate and glutamate metabolism	22	2	3.1247	0.42528	ath00250	C00026,C00025
5	Taurine and hypotaurine metabolism	5	1	2.5852	1	ath00430	C00245
6	Flavone and flavonol biosynthesis	9	1	2.0267	0	ath00944	C12249
7	Lysine biosynthesis	10	1	1.9287	0.07407	ath00300	C00047
8	Vitamin B6 metabolism	11	1	1.8406	0	ath00750	C00314
9	beta-Alanine metabolism	12	1	1.7609	0	ath00410	C00864
10	Nicotinate and nicotinamide metabolism	12	1	1.7609	0	ath00760	C00253
11	Pantothenate and CoA biosynthesis	14	1	1.6212	0.15	ath00770	C00864
12	Nitrogen metabolism	15	1	1.5594	0	ath00910	C00025
13	Lysine degradation	17	1	1.4487	0	ath00310	C00047
14	Glyoxylate and dicarboxylate metabolism	17	1	1.4487	0.10544	ath00630	C00158
15	Pentose phosphate pathway	18	1	1.3987	0	ath00030	C00257
16	Butanoate metabolism	18	1	1.3987	0	ath00650	C00025
17	Valine, leucine and isoleucine biosynthesis	26	1	1.0878	0	ath00290	C00188
18	Glutathione metabolism	26	1	1.0878	0.07756	ath00480	C00025
19	Porphyrin and chlorophyll metabolism	29	1	0.99963	0	ath00860	C00025
20	Glycine, serine and threonine metabolism	30	1	0.97272	0.13697	ath00260	C00188
21	Flavonoid biosynthesis	43	1	0.70212	0	ath00941	C09727
22	Purine metabolism	61	1	0.47184	0.00256	ath00230	C00212

^zTotal number of metabolites in the target metabolic pathway.^yNumber of differential metabolites in the target metabolic pathway.^xSignificant difference *P* value.^wMetabolic pathway influence value.^vNumber of metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes [KEGG (Kanehisa and Goto, 2000)].^uNumbering of compounds in KEGG.

Fig. 6. Influence factor map of the metabolites of ‘Cabernet Sauvignon’ grape seeds from various rootstock combinations.

rootstocks. Furthermore, our study shows that various rootstock combinations can increase the concentrations of amino acids, organic acids, polyphenols, and vitamin B in grape seeds. Therefore, grape seed quality can be improved by

selecting specific rootstock combinations for specific purposes, such as developing disparate attributes related to improvements in mouthfeel, nutritional properties, and even the efficacy of grape products.

Conclusions

This study used LC-MS and nontargeted metabolomic profiling to examine 30 metabolites produced in five grafted rootstock combinations and a self-rooted grafting CS grape control to conduct pathway enrichment analysis and screen for differences among treatments of metabolites in grape seeds. It found 22 significant metabolic pathways in the six rootstock combinations of CS grape seeds.

According to the nutrients contained in grape seeds, the SO4 rootstock in Xinjiang is a suitable rootstock for CS grape seeds. Various rootstock combinations change the nutrient composition of grape seeds by influencing several metabolic pathways: alanine-aspartic acid-glutamate metabolism, arginine-proline metabolism, and the TCA cycle.

Various rootstock combinations differ in the degree to which they increase the concentrations of amino acids, organic acids, polyphenols, and vitamin B in grape seeds. When CS is grafted onto the ‘1103P’, ‘140R’, ‘SO4’, ‘5C’, and ‘3309C’ rootstocks, the concentrations of delphinidin 3-(6-acetylglucoside), peonidin 3-(6-p-coumarylglucoside), L-threonine, and tartaric acid increased in the CS grape seeds, and subsequently, the quality,

taste, nutritional concentration, and efficacy of these grape seeds may be improved.

The present study successfully identified characteristic markers for six types of rootstock combinations (all with CS as the scion) and revealed the influence of rootstocks on the metabolites in grape seeds, which provided a basis for determining the influence of various rootstock combinations. The additional breeding of grape rootstocks could provide important insights into improving the quality of derivative commercial products derived from grapes.

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