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Transcriptomic Analysis of Root Restriction Effects on Phenolic Metabolites during Grape Berry Development and Ripening

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ABSTRACT: In the present study, the effects of root restriction (RR) on the main phenolic metabolites and the related gene expression at different developmental stages were studied at the transcriptomic and metabolomic levels in "Summer Black" grape berries (*Vitis vinifera* × *Vitis labrusca*). The results were as follows: seven phenolic acid compounds, three stilbene compounds, nine flavonol compounds, 10 anthocyanin compounds, and 24 proanthocyanidin compounds were identified by ultra-performance liquid chromatography−high-resolution mass spectrometry. RR treatment significantly promoted the biosynthesis of phenolic acid, *trans*-resveratrol, flavonol, and anthocyanin and also affected the proanthocyanidin content, which was elevated in the early developmental stages and then reduced in the late developmental stages. The functional genes for phenylalanine ammonia-lyase, *trans*-cinnamate 4-monooxygenase, 4-coumarate-CoA ligase, shikimate *O*-hydroxycinnamoyl transferase, chalcone synthase, chalcone isomerase, stilbene synthase, flavonoid 3′,5′-hydroxylase, anthocyanidin 3-*O*-glucosyltransferase, and the transcription factors MYBA1, MYBA2, MYBA3, and MYBA22 were inferred to play critical roles in the changes regulated by RR treatment.

KEYWORDS: *root restriction, phenol metabolism, RNA-seq, grape berry*

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops cultivated worldwide, as it provides fruit used for wine and table grapes. The size of a grapevine genome is approximately 475−500 Mb.[1] During development and ripening, grape berries undergo a series of complex physiological and biochemical processes that lead to the accumulation of sugars and phenolic compounds. These phenolic compounds are functional biomolecules possessing a specific three-aromatic ring system defined by a C6−C3−C6 structure bearing diverse hydroxyl and nonhydroxyl substituents.[2] The phenolic compounds are synthesized from phenylalanine to coumaroyl-CoA via the core phenylpropanoid pathway (Figure 1). Coumaroyl-CoA serves as a precursor for the biosynthesis of diverse phenylpropanoid compounds, mainly including phenolic acids, stilbenes, flavonols, anthocyanins, and flavanols that confer flavor, color, taste, and nutritional properties, and play important roles in plant growth and resistance to adverse environmental conditions.[3]

Phenolic acids in grapes are derivatives of hydroxycinnamic acids and hydroxybenzoic acids, which are initially synthesized from phenylalanine. These substances are widely distributed in many kinds of plants and occur in the free state and bound forms with esters or glycosides. The hydroxycinnamic acids consist chiefly of *p*-coumaric, ferulic, caffeic, and sinapic acids. They are found in free form or bound to tartaric acid, quinic acid, and shikimic acid. Hydroxybenzoic acids, including gallic acid, protocatechuic acid, and gentisic acid, are mainly found in free form, as well as an acyl substituent of flavan-3-ols.[4] The stilbenes in grape berries biosynthesize from veraison to ripening in the skin via the pathway that branches off from the phenylpropanoid pathway and is induced by biotic and abiotic stress.[5] The stilbene pathway involves four enzymes: phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), and stilbene synthase (STS). The metabolites mainly contain *trans*- and *cis*-resveratrol, which are glucosides known as piceids.[6] Flavonoids are a group of phenolic metabolites that are abundant in grape berry skins. They are synthesized via the flavonoid branch of the phenylpropanoid pathway, starting with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA and carried out by the enzyme chalcone synthase (CHS). The next step is the isomerization of chalcone to flavanone by chalcone isomerase (CHI). From this step onwards, the pathway branches mainly into several flavonoid classes of compounds, including flavonols, anthocyanins, and flavanols.[4,74,7] Flavonols are a class of yellow pigment flavonoids in grape berries in the form of 3-*O*-glycosides of kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin.[5] They play important roles in fresh fruits and fruit products, as they contribute to their taste, quality, and nutrition. Flavonols are synthesized in grape berry skins from flowering to the early stages of development, as well as during ripening. Flavonol synthase (FLS) is the key enzyme in flavonol biosynthesis that determines the flavonol content in grape berry skins.[8] Anthocyanins represent the largest class of

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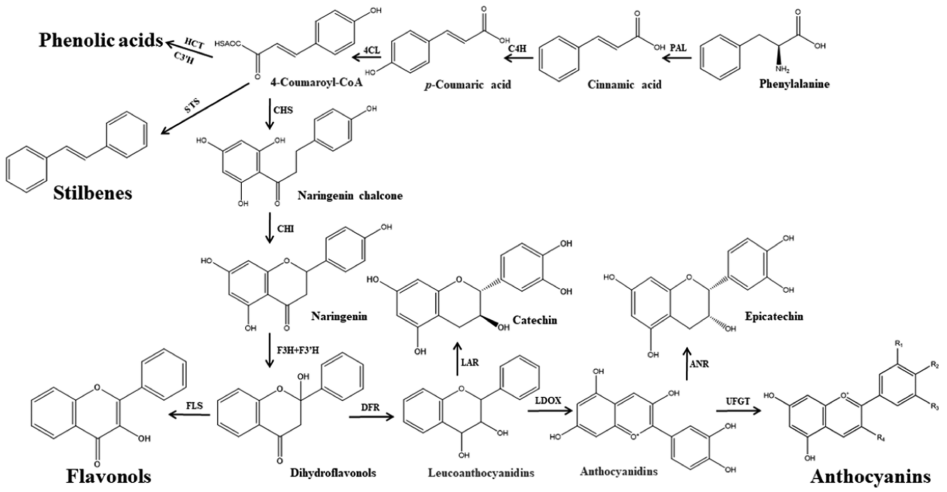

Figure 1. Key steps of the phenylpropanoid pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; HCT and shikimate *O*-hydroxycinnamoyl transferase; C3′H, coumaroylshikimate 3′-monooxygenase; STS, stilbene synthase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3′H, flavonoid-3′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase; LAR, leucocyanidin reductase; ANR, anthocyanidin reductase.

flavonoids responsible for the color of grape berries. They are biosynthesized through the flavonoid pathway and accumulate from veraison until full maturity. The enzyme UDP-glucose:-flavonoid 3-*O*-glucosyltransferase (UFGT) is a determining factor for the anthocyanin composition and content of grape berries. In grape berries, the main anthocyanins of these varieties are cyanidin, delphinidin, peonidin, malvidin, and petunidin, which are present as monoglucoside, acetylmonoglucoside, and *p*-coumaroylmonoglucoside derivatives.[5,9] The final anthocyanin content and composition depend not only on the maturity but also on the response to environmental factors, such as water, temperature, sunlight, and nutrients.[10,11] Flavanols responsible for the bitter and astringent properties mainly include monomeric catechin and epicatechin, and their gallate esters produce oligomers and polymers called proanthocyanidins.[12] These compounds are synthesized before veraison via the flavonoid pathway and share several early steps in the pathway. The first steps in flavanol biosynthesis are catalyzed by leucocyanidin reductase (LAR) and anthocyanidin reductase (ANR) by converting anthocyanidins to catechin and epicatechin, respectively.[13] Generally, flavonols, anthocyanins, and flavanols are synthesized via the flavonoid pathway and regulated by transcription factors, including MYB, basic helix-loop-helix (bHLH), and WD40 proteins.[11,14]

Root restriction (RR) is a novel cultivation technique performed by restricting the root growth in a certain volume to optimize the balance of vegetative growth and reproductive growth. RR treatment improves the efficiency in the use of agricultural resources, controls the shoot size, and assimilates the partition between vegetative and reproductive organs.[15] Recently, RR has been found to improve grape fruit quality. It has been well demonstrated that RR treatment increases the total sugar and anthocyanin contents. After further analysis, RR not only increased the total and individual anthocyanin contents but also enriched the anthocyanin composition compared to the conventional cultivation method. Furthermore, gene expression pattern analysis revealed 16 upregulated genes, coinciding with the increase in anthocyanins in the phenylpropanoid pathway.[16,17] However, the changes of other phenolic metabolites in grape berries influenced by RR treatment during development and ripening are poorly reported. In our study, we integrated transcriptomics and phenolic metabolites to characterize the influence of RR on the development and metabolism of "Summer Black" grape berries under field conditions. We identified metabolic changes and molecular pathways that are triggered by RR treatment during the whole fruit developmental stages to reveal the mechanism by which RR alters phenolic metabolism. The results of the transcriptomic analysis were validated by quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Sample Collection. We performed this study in an orchard greenhouse with 3 year old "Summer Black" (*Vitis vinifera* × *Vitis labrusca*) grapes during 2013−2014 at the Jinhua Academy of Agricultural Sciences (Zhejiang, China). The first group of grapes was planted in 100 cm wide and 40 cm deep ridges separated with a plastic film from the ground around the outside as per the RR treatment procedure. Another group of grapes was planted in a raised 40 cm deep bed with the same soil in open ground to serve as the control. The same fertilizer strategies and watering were applied to the control and RR grapes to avoid different environmental conditions. The fruit was collected from five different developmental stages: S1, fruitlet and 15 days after full bloom (DAFB); S2, immature green and 28 DAFB; S3, before veraison and 42 DAFB; S4, veraison and 53 DAFB; and S5, fully ripe and 74 DAFB. For each treatment, 10 cluster grape berries were picked from at least five plants randomly without evidence of stress symptoms or disease at each sampling time. All samples were picked and selected for the absence of mechanical damage and uniform maturity, then cut into small pieces and frozen immediately in liquid nitrogen, and stored at −80 °C. All experiments were carried out with three biological replicates.

Metabolite Analysis. Secondary metabolites were identified by ultra-performance liquid chromatography−high resolution tandem mass spectrometry (UPLC-HRMS) as follows: 1 g of lyophilized berry powder was extracted with 16 mL of 70% ethanol water (containing 1% formic acid) by sonication for 30 min. The extracts were centrifuged at 10 000 rpm for 10 min at room temperature. The supernatant was collected and the precipitate was re-extracted twice as above. All the supernatants were combined and used for the identification of secondary metabolites. Individual metabolites were analyzed by UPLC-HRMS. Chromatographic separation was used by a UPLC system (Waters Corp.) coupled with an Agilent Eclipse Plus C18

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Table 1. Chromatographic and Mass Spectrometric Parameters of Detected Phenolic Compounds in the Grape Berry

identification	theoretical mass	$[M-H]^-$	fragments	ppm	formula
p-cinnamic acid glucopyranoside	325.0929	325.093	119/163	0.3	$C_{15}H_{18}O_8$
ferulic acid	193.0506	193.0506	134/178	0	$C_{10}H_{10}O_4$
cis-caftaric acid	311.0409	311.0402	179/135	−2.1	$C_{13}H_{12}O_9$
sinapic acid	223.0612	223.0629	193/149/121	3.4	$C_{11}H_{12}O_5$
trans-caftaric acid	311.0409	311.0402	179/135/149	−2.1	$C_{13}H_{12}O_9$
trans-*p*-coutaric acid	295.0459	295.0456	149/119/163	−1.2	$C_{13}H_{12}O_8$
cis-*p*-coutaric acid	295.0459	295.0454	119/163	−1.8	$C_{13}H_{12}O_8$
piceid	389.1242	389.1227	227/185	−3.8	$C_{20}H_{22}O_8$
trans-resveratrol	227.0714	227.0728	227/185/143	2.8	$C_{14}H_{12}O_3$
cis-resveratrol	227.0714	227.0719	227/185/143	1.1	$C_{14}H_{12}O_3$
quercetin	301.0354	301.0371	151/301/121	3.4	$C_{15}H_{10}O_7$
quercetin-3-glucoside	463.0882	463.089	301/271/255	1.4	$C_{21}H_{20}O_{12}$
quercetin-3-glucuronide	477.0675	477.0673	301/477	−0.3	$C_{21}H_{18}O_{13}$
myricetin	317.0303	317.0321	317/151/137	3.6	$C_{15}H_{10}O_8$
myricetin-3-glucuronide	493.0624	493.0612	317/493	−2.4	$C_{21}H_{18}O_{14}$
myricetin-3-glucopyranoside	479.0831	479.0816	479/271/317	−3.2	$C_{21}H_{20}O_{13}$
kaempferol	285.0405	285.0418	151/107	2.6	$C_{15}H_{10}O_6$
kaempferol-3-glucoside	447.0933	447.0916	285/447	−3.8	$C_{21}H_{20}O_{11}$
kaempferol-3-glucuronide	461.0725	461.0718	285/299/257	−1.6	$C_{21}H_{18}O_{12}$
cyanidin-3-glucoside	447.0933	447.093	285/447	−0.6	$C_{21}H_{20}O_{11}$
peonidin-3-glucoside	461.1089	461.1082	299/283	−1.6	$C_{22}H_{22}O_{11}$
peonidin-3′,5′-diglucoside	623.1612	623.1605	301/463	−1.6	$C_{28}H_{32}O_{16}$
peonidin-3-*p*-coumarylglucoside	607.1457	607.1444	299/607	−2.2	$C_{31}H_{28}O_{13}$
delphinidin-3-glucoside	463.0882	463.087	301/463	−2.6	$C_{21}H_{22}O_{13}$
delphinidin-3-acetylglucoside	505.0988	505.0969	505/301/434	−3.7	$C_{23}H_{22}O_{13}$
malvidin-3-glucoside	491.1195	491.1179	329/313	−3.3	$C_{23}H_{24}O_{12}$
malvidin-3-*p*-coumarylglucoside	655.1668	655.1662	329/655/347	−0.8	$C_{32}H_{32}O_{15}$
petunidin-3-glucoside	477.1039	477.1031	315/299	−1.6	$C_{22}H_{22}O_{12}$
petunidin-3-*p*-coumarylglucoside	623.1406	623.1394	315/623/299	−2	$C_{31}H_{28}O_{14}$
catechin	289.0718	289.0713	203/289/109/245	−1.6	$C_{15}H_{14}O_6$
epicatechin	289.0718	289.0714	289/245/203/109	−1.3	$C_{15}H_{14}O_6$
epigallocatechin	305.0667	305.0662	125/305/165/137	−1.6	$C_{15}H_{14}O_7$
gallocatechin	305.0667	305.0662	125/165/305/137	−1.6	$C_{15}H_{14}O_7$
epigallocatechin gallate	457.0776	457.0759	169/125/305/203	−3.8	$C_{22}H_{18}O_{11}$
epicatechin-epicatechin-gallate	1017.2095	1017.2109	1017/287/125	1.4	$C_{52}H_{42}O_{22}$
epicatechin-epigallocatechin-gallate	745.141	745.1404	745/407/495/289	−0.8	$C_{37}H_{30}O_{17}$
epicatechin-gallocatechin	593.1301	593.1285	305/423/593	−2.6	$C_{30}H_{26}O_{13}$
gallocatechin-gallocatechin-gallocatechin	913.1833	913.1797	913/303/423	−3.9	$C_{45}H_{38}O_{21}$
galloylepicatechin-catechin	729.1461	729.1453	729/577/407/289	−1.1	$C_{37}H_{30}O_{16}$
galloylepicatechin-epicatechin	729.1461	729.1459	729/289/407/441	−0.3	$C_{37}H_{30}O_{16}$
proanthocyanidin A1	577.1352	577.1329	289/577/425	−3.9	$C_{30}H_{26}O_{12}$
proanthocyanidin B1	577.1352	577.1332	407/289/425	−3.4	$C_{30}H_{26}O_{12}$
proanthocyanidin B2	577.1352	577.1337	407/289/425	−2.5	$C_{30}H_{26}O_{12}$
catechin-catechin-gallocatechin	881.1935	881.1928	729/881/315	−0.7	$C_{45}H_{38}O_{19}$
catechin-gallocatechin-gallocatechin	897.1884	897.1881	897/289/407	−0.3	$C_{45}H_{38}O_{20}$
proanthocyanidin C1	865.1985	865.1984	865/407/287	−0.2	$C_{45}H_{38}O_{18}$
tetramers B-type procyanidin	1153.2619	1153.2626	1153/575/287	0.6	$C_{60}H_{50}O_{24}$
pentamers B-type procyanidin	1441.3253	1477.305		−0.5	$C_{75}H_{62}O_{30}$
hexamers B-type procyanidin	864.1907	864.1905	864/407/287/289	−0.2	$C_{90}H_{74}O_{36}$
heptamers B-type procyanidin	1008.2224	1008.2224	287/1008/289/407	0	$C_{105}H_{86}O_{42}$
octamers B-type procynidin	1152.2541	1152.2547	1152/575/287	0.5	$C_{120}H_{98}O_{48}$
nonamers B-type procynidin	1296.2858	1296.2853		−0.4	$C_{135}H_{110}O_{54}$
decamers B-type procyanidin	1440.3175	1440.3185		0.4	$C_{150}H_{122}O_{60}$

column (4.6 mm × 100 mm, 1.8 μm). The compounds were eluted with mobile phase A (1% formic acid−water) and mobile phase B (1% formic acid−acetonitrile). The gradient elution was as follows: 0−4 min, 96−92% A; 4−18 min, 92−84% A; 18−22 min, 84−83% A; 22−26 min, 83−79% A; 26−28 min, 79−75% A; 28−34 min, 75−50% A; 34−40 min, 50−5% A; 40−45 min, 5−4% A; a flow rate of 0.75 mL/min. The column temperature was set to 30 °C and the injection volume was 5 μL. The eluents were detected at 200−600 nm. MS analysis was performed using a quadrupole-time-of-flight mass spectrometer (AB SCIEX, Triple TOF 5600$^+$ System) equipped with an electrospray ionization (ESI) source. The optimal MS conditions were as follows: scan range, m/z 100−2000; source voltage, −4500 V; negative ion mode; the source temperature, 550 °C; the pressures of gas 1 (air) and gas 2 (air), 50 psi; pressure of curtain gas (N_2), 30 psi;

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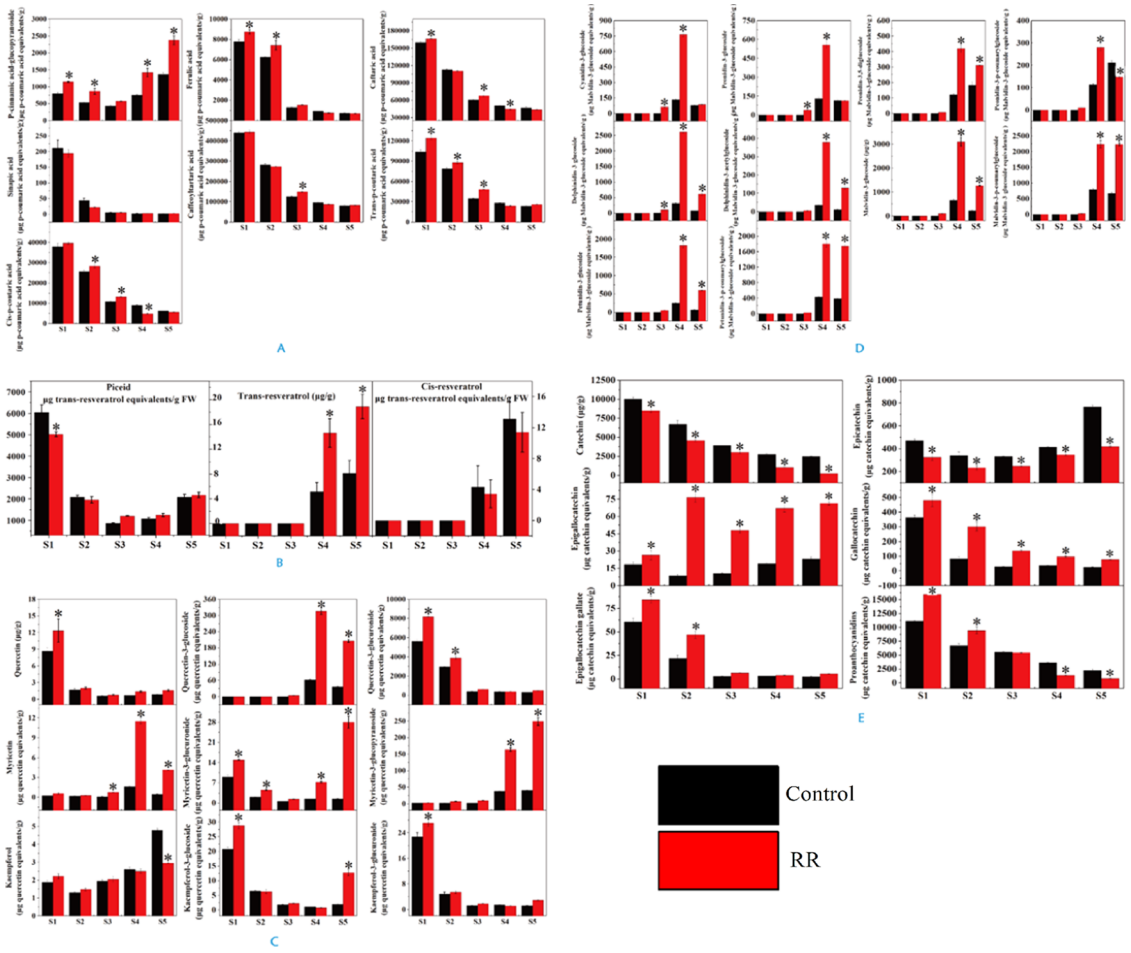

Figure 2. Effects of root restriction (RR) treatment on the contents of phenolic compounds during grape berry development and ripening. (A) Phenolic acids; (B) stilbenes; (C) flavonols; (D) anthocyanins; (E) flavanols.

maximum allowed error, ±5 ppm; declustering potential (DP), 100 V; collision energy (CE), 10 V; and collision energy, 40 V, with collision energy spread, ±20 V. Exact mass calibration was carried out automatically before each analysis using the automated calibration delivery system. The raw data were analyzed using PeakView 1.2.0.3 (AB SCIEX).

RNA Extraction and RNA Sequencing (RNA-Seq). Total RNA was extracted according to our previously published method from frozen whole grape berry powder.[18] After contamination, genomic DNA was removed with a TURBO DNA free kit (Ambion), then the total RNA was quantified using a NanoPhotometer Pearl (Implen), and used for RNA-Seq and qRT-PCR analysis. For RNA-seq, the raw reads with 5 Gb reads per sample were obtained by Shanghai Majorbio Biopharm Biotechnology Co. (Shanghai, China) using an Illumina HiSeq 2000. Raw reads were initially processed by removing the adapter and low-quality sequences to get clean reads using SeqPrep software (https://github.com/jstjohn/SeqPrep). The clean reads were aligned to the *Vitis vinifera* reference genome (http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ ML/data/)[1] using TopHat software (http://tophat.cbcb.umd.edu/)[19] and the RSeQC-2.3.2 program (http://code.google.com/p/rseqc/) was used for saturation analysis, duplicate read analysis, and gene coverage analysis.[20] Cuffdiff program (http://cufflinks.cbcb.umd.edu/) was used for calculating gene expression values by the read per kilobase of exon per million fragments mapped reads (RPKM). EdgeR software was used to analyze the differential expression according to the count values of each transcript in the two libraries. Genes with a false discovery rate (FDR) <0.05 and estimated absolute $\log_2$ fold change (FC) >1 were used as the thresholds for judging a significant difference in transcript expression.[21] KOBAS (http://kobas.cbi.pku.edu.cn/home.do) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed genes.[22]

All of the raw sequence data have been deposited at the NCBI Sequence Read Archive (SRA) with the following accession codes: SRX2234711/SRR4408346, SRX2234711/SRR4408347, SRX2234711/SRR4408413, and SRX2234711/SRR4408414.

qRT-PCR Validation of RNA-Seq Data. Gene-specific oligonucleotide primers were designed (Supporting Information Table S1), and the gene specificity was checked for each pair of primers using the melting curves and by re-sequencing the product twice. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH: NCBI accession number: CBI14856.3) gene was carried out as the internal control to calculate the relative mRNA expression.[23−25] The GAPDH primer sequences are described in Supporting Information Table S1. qRT-PCR was performed using FastStart Universal SYBR Green (Roche), which was started at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 10 min, and was completed with a melting curve analysis program. The PCR mixture (10 μL of total volume) comprised 5 μL of Roche FastStart Universal SYBR Green Master (ROX), 0.5 μL of diluted cDNA, 0.75 μL of each primer (10 μmol/L), and 3 μL of PCR-grade ddH_2O. During each run, each gene was subjected to no-template controls and melting curve analyses.

Statistical Analysis. Statistically significant differences were calculated using single factor variance analysis (ANOVA). The results were analyzed using a data processing system with the SPSS16.0 statistical software package and presented as the mean ± SE. Figures were plotted using Origin 8.0 (Microcal Software Inc.).

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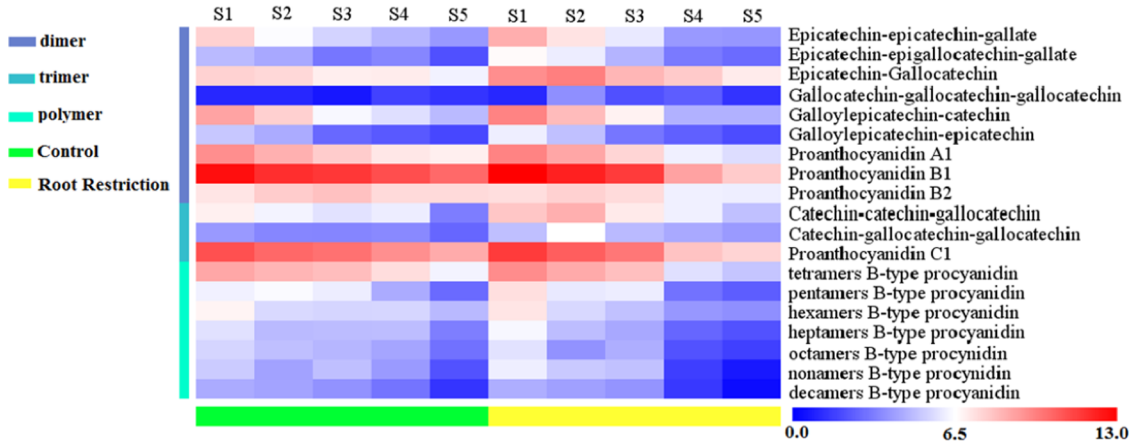

Figure 3. Effects of root restriction (RR) treatment on the contents of procyanidins during grape berry development and ripening.

RESULTS AND DISCUSSION

Differential Gene Expression in the Phenylpropanoid Pathway. The 150 Gb sequence reads were generated from three biological replicates per treatment across all developmental stages using an Illumina HiSeq. 2000 platform. The expression of 29 971 genes was detected after aligning the sequence reads with the grape reference genome.[24,25] Phenolic compounds as the important secondary metabolites in grape berries are derived from phenylalanine via the core phenylpropanoid pathway. In this study, the transcriptomic analysis revealed changes in the expression levels of several genes. A total of 29 genes coding for 10 enzymes were significantly differentially expressed during the growth processes in both groups. One gene coding for shikimate *O*-hydroxycinnamoyl transferase (*VIT*_11s0037g00440) was upregulated at the S1 stage, while other differentially expressed genes were all downregulated at the earlier stages and upregulated at the later stages (Supporting Information Table S2).

Compounds in the Phenylpropanoid Pathway. To match gene expression patterns with phenolic compound profiles, the detection and quantification of metabolites in both grape berry groups from the whole growth process were carried out using UPLC-HRMS. In general, we observed the deprotonated molecule $[M-H]^-$ and its important fragments by MS/MS experiments. A total of 53 phenolic compounds involved in the phenylpropanoid pathway were identified, and their theoretical mass, molecular ions (negative mode), important fragments, ppm (mass error less than 5 ppm), and formula are shown in Table 1. All of the identified compounds were classified into five groups according to their structure and chemical properties: phenolic acids, stilbenes, flavonols, anthocyanins, and flavanols. Then, available standards were achieved for final qualitative and semiquantitative analysis.

Phenolic Acids. Phenolic acids as a kind of small molecular compounds that can be divided into two groups: hydroxycinnamic acids (coumaric acid, caffeic acid, ferulic acid, coumaryl tartrate, caffeyl tartrate, etc.) and hydroxybenzoic acids (*p*-hydroxybenzoic acid, vanillic acid, syringic acid, protocatechuic acid, gallic acid, etc.). In this study, we detected seven phenolic acid compounds and their derivatives in the grape berries (Figure 2A): *p*-cinnamic acid glucopyranoside, ferulic acid, *cis*-caftaric acid, sinapic acid, *trans*-caftaric acid, *trans*-*p*-coutaric acid, and *cis*-*p*-coutaric acid. From the results, we found that *p*-cinnamic acid glucopyranoside content decreased during the initial three stages and then increased until ripening. RR treatment significantly promoted the accumulation of *p*-cinnamic acid glucopyranoside. The other phenolic acids showed a decreasing trend across the whole growth process in both groups. RR treatment slightly increased the content of ferulic acid, *trans*-*p*-coutaric acid, and *cis*-*p*-coutaric acid at the initial stages, but slightly decreased their contents at the later stages.

Stilbenes. Stilbenes occur in the skin of grape berries and can be induced by biotic and abiotic stresses.[3,26,27] In general, it includes *trans*-resveratrol, *cis*-resveratrol, viniferin, and their glucosides, such as piceid. In this study, the effect of RR treatment on stilbene content in grape berries is shown in Figure 2B. We found that piceid is a major stilbene compound in our samples. Piceid concentrations decreased to the bottom at the S3 stage, then increased slowly towards maturity, and were reduced by RR treatment at the S1 stage. On the other hand, *trans*-resveratrol and *cis*-resveratrol accumulated at the later developmental stages. The concentration of *trans*-resveratrol in the RR treatment (19 μg/g dry weight) was much higher than that in the control (8 μg/g dry weight) at the time of maturity. RR treatment greatly promoted the accumulation of *trans*-resveratrol after veraison but had no effect on *cis*-resveratrol content.

Flavonols. Flavonols are a type of flavonoids and play pivotal roles in grape berry taste, quality, and nutrition. Flavonols normally exist in grape berries in the form of glucosides, galactosides, rhamnosides, rutinosides, and glucuronides of quercetin, myricetin, kaempferol, isorhamnetin, laricitrin, and syringetin. In this study, we detected nine flavonol compounds, which were quercetin, kaempferol, myricetin, and their derivatives (Figure 2C). Quercetin-3-glucuronide was a major flavonol compound in our samples. RR treatment significantly decreased kaempferol content at maturity but promoted the accumulation of other compounds at different developmental stages. At maturity, some substances were greatly affected by RR treatment, such as myricetin-3-glucuronide (18.1-fold increase), myricetin (9.2-fold increase), kaempferol-3-glucoside (6.4-fold increase), myricetin-3-glucopyranoside (6.1-fold increase), and quercetin-3-glucoside (5.5-fold increase).

Anthocyanins. Anthocyanins are a class of flavonoids synthesized after veraison in the berry skins and are responsible for the color of grape berries. The principal individual anthocyanins in grape berries are 3-*O*-monoglucosides or 3,5-*O*-diglucosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin, as well as their acetyl-, *p*-coumaroyl-, and caffeoyl-

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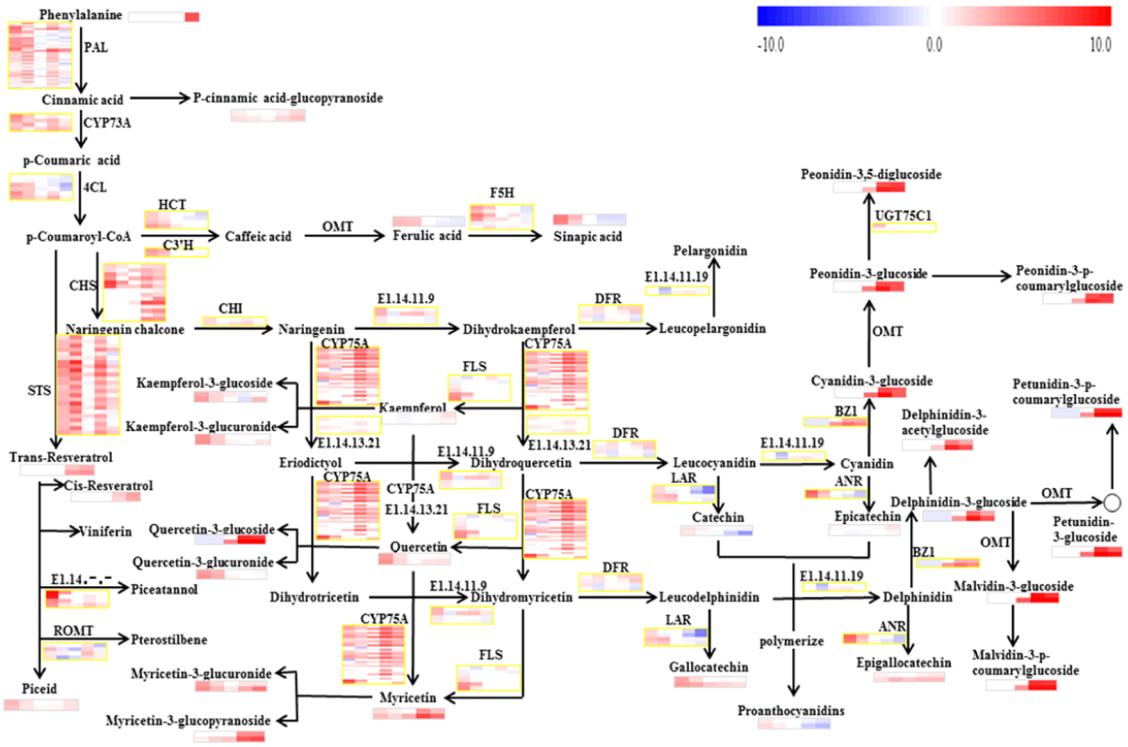

Figure 4. Effects of root restriction (RR) treatment on the phenylpropanoid pathway in grape berry. PAL, phenylalanine ammonia-lyase; DFR, bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase; ANR, anthocyanidin reductase; F5H, ferulate-5-hydroxylase; E1.14.11.9, naringenin 3-dioxygenase; E1.14.11.19, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; CYP73A, *trans*-cinnamate 4-monooxygenase; E1.14.13.21, flavonoid 3′-monooxygenase; C3′H, coumaroylshikimate 3′-monooxygenase; CYP75A, flavonoid 3′,5′-hydroxylase; LAR, leucoanthocyanidin reductase; ROMT, *trans*-resveratrol di-*O*-methyltransferase; CHS, chalcone synthase; STS, stilbene synthase; HCT, shikimate *O*-hydroxycinnamoyl transferase; BZ1, anthocyanidin 3-*O*-glucosyltransferase; UGT75C1, anthocyanidin 3-*O*-glucoside 5-*O*-glucosyltransferase; CHI, chalcone isomerase; 4CL, 4-coumarate-CoA ligase.

esters.[4,28] Anthocyanin accumulation is usually regulated in response to environmental factors, such as UV irradiation, temperature, and water deficit.[29] In this study, a total of 10 anthocyanins were detected and classified into five groups according to their different anthocyanidins (Figure 2D). The concentrations of these anthocyanins were low in control samples but very high in RR treatment samples. Notably, RR treatment greatly affected the content of delphinidin-3-acetylglucoside (12.2-fold increase), delphinidin-3-glucoside (9.2-fold increase), and petunidin-3-glucoside (9.1-fold increase).

Flavanols. Flavanols are present mainly in the form of catechin, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin, gallocatechin, proanthocyanidins, and so on, and are an abundant class of flavonoids in grape berries. Flavanols are synthesized from anthesis to veraison and are responsible for the bitterness, aroma, and astringent properties of foods and beverages.[30] From the results, we observed that catechin is the most abundant flavanol in grapes, with more than 10 000 μg/g dry weight content in young berries that then gradually reduced until maturity. RR treatment not only significantly reduced the contents of catechin and epicatechin at all developmental stages, but also promoted catechin and epicatechin polymerization with gallate. On the other hand, RR treatment significantly increased the total proanthocyanidin content in the young berry stage and reduced their concentrations at the time of maturity (Figure 2E).

Proanthocyanidins, also known as condensed tannins, are a group of polymers comprising flavanol units that arise through the flavonoid biosynthetic pathway from anthesis to veraison and decrease during ripening.[31] In this study, we identified 19 proanthocyanidin compounds in our samples and found that proanthocyanidin B1 is the most abundant proanthocyanidin in the "Summer Black" grape berry (Figure 3).

Integration of Transcriptome and Metabolome Data with the Phenylpropanoid Pathway Map. In this study, UPLC-HRMS and RNA-seq covered the whole grape genome, making it possible to integrate transcriptome and metabolome analyses with the phenylpropanoid pathway map to study metabolic changes in RR-treated grape berries (Figure 4). Phenolic compounds produced by the phenylpropanoid pathway in many fruits during the development and ripening play a critical role in plant growth, as well as in plant interactions with environmental stresses.[3] These compounds share the same synthetic pathway from phenylalanine to coumaroyl-CoA. Phenylalanine ammonia-lyase (PAL), *trans*-cinnamate 4-monooxygenase (CYP73A), and 4-coumarate-CoA ligase (4CL) are common participants in this synthetic pathway. From coumaroyl-CoA, precursors for the biosynthesis of diverse phenolic compounds, including phenolic acids, stilbenes, and flavonoids (flavonols, anthocyanins, flananols) are derived. A close link between various branches of the phenylpropanoid pathway has been reported.[3,32]

Grape berry phenolic acids and their derivatives are synthesized from a branch of phenylpropanoid metabolism.[3] The key phenolic acid biosynthesis-related gene, shikimate *O*-hydroxycinnamoyl transferase (HCT) was proved to be positively regulated under drought stress in the previous

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report.[33] In this study, we found a good correlation between the increase in ferulic acid and *cis*-caftaric acid contents and the upregulated expression of a gene coding for HCT (*VIT*_11*s*0037*g*00440) at the S1 stage. Our results agreed with the previous finding that the HCT enzyme plays a key role in regulating phenolic acid biosynthesis under stress. On the other hand, other enzymes, including PAL, CYP73A, and 4CL also regulated phenolic acid biosynthesis. However, the expression of genes encoding these enzymes was negatively correlated with phenolic acid content. We speculated that these enzymes play a negative regulatory role in the biosynthesis of phenolic acid or that phenolic acid might serve as a precursor for the biosynthesis of volatiles and other phenolic metabolites.

Stilbenes are formed via a pathway that branches off from the phenylpropanoid pathway. This pathway involves four enzymes: PAL, CYP73A, 4CL, and stilbene synthase (STS).[34] The stilbene family contains several compounds that are thought to be synthesized by the modification of *trans*-resveratrol.[32,35] *trans*-Resveratrol generally accumulates from veraison to maturity and can be induced by biotic and abiotic stress in the grape berry, such as UV-C irradiation, water deficit, and hormones.[32,36,37] A high correlation between stilbene content in grape varieties and PAL, CYP73A, 4CL, and STS gene expression has been demonstrated previously.[38] In this report, five genes coding for PAL (*VIT*_06*s*0004*g*02620, *VIT*_13*s*0019*g*04460, *VIT*_16*s*0039*g*01100, *VIT*_16*s*0039*g*01110, and *VIT*_16*s*0039*g*01120) were downregulated under RR treatment. Two genes coding for STS (*VIT*_16*s*0100*g*00950 and *VIT*_16*s*0100*g*01010) putatively involved in piceid biosynthesis were downregulated in the early berry developmental stages. However, we found a high correlation between the upregulation of genes coding for PAL (*VIT*_06*s*0004*g*02620 and *VIT*_13*s*0019*g*04460), CYP73A (*VIT*_06*s*0004*g*08150), and 4CL (*VIT*_16*s*0039*g*02040) under the RR treatment and an increase in *trans*-resveratrol content after veraison. It should be noted that no one gene coding for STS was significantly upregulated under the RR treatment, but the levels of most genes coding for STS were higher than their expression in the control after veraison (Supporting Information Table S3). It was speculated that the genes coding for STS had a combined regulatory effect on the accumulation of *trans*-resveratrol.

Flavonols are synthesized in grape berries from anthesis to before veraison and during ripening, through a branch of the flavonoid biosynthetic pathway and share several early steps with other phenolics in the phenylpropanoid pathway.[30,39] In the later flavonol biosynthesis steps, chalcone synthase (CHS), chalcone isomerase (CHI), naringenin 3-dioxygenase (E1.14.11.9), flavonoid 3′-monooxygenase (E1.14.13.21), flavonoid 3′,5′-hydroxylase (CYP75A), and flavonol synthase (FLS) play important roles in flavonol metabolism.[40] We found one gene coding for CHI (*VIT*_13*s*0067*g*03820), one gene coding for E1.14.11.9 (*VIT*_04*s*0023*g*03370), and four genes coding for CHS (*VIT*_05*s*0136*g*00260, *VIT*_14*s*0068*g*00920, *VIT*_14*s*0068*g*00930, and *VIT*_16*s*0022*g*01020) that were upregulated. Ten genes coding for CYP75A (*VIT*_06*s*0009*g*02810, *VIT*_06*s*0009*g*02830, *VIT*_06*s*0009*g*02840, *VIT*_06*s*0009*g*02860, *VIT*_06*s*0009*g*02880, *VIT*_06*s*0009*g*02910, *VIT*_06*s*0009*g*02920, *VIT*_06*s*0009*g*02970, *VIT*_06*s*0009*g*03010, and *VIT*_06*s*0009*g*03050) had a good correlation with an increase in myricetin, myricetin-3-glucuronide, myricetin-3-glucopyranoside, quercetin-3-glucoside, and kaempferol-3-glucoside contents at the S3 and S4 stages. From the results, we further confirmed that flavonols were synthesized in young berries and after the veraison stages, and were induced to accumulate under stress. Since kaempferol is a precursor to other flavonols, its content in the RR treatment group was lower than the control at maturity. We observed that quercetin, quercetin-3-glucuronide, myricetin-3-glucuronide, kaempferol-3-glucoside, and kaempferol-3-glucuronide were synthesized at the young berry stage, with higher contents in the RR treatment group than in the control. Finally, we speculated that the flavonols tended to exist in the form of glucuronide in young berries.

Anthocyanins are synthesized through the phenylpropanoid pathway and accumulated in grape berry skins after veraison, with an occasional decrease in content towards maturity, especially in hot climates.[10,39] The enzyme UDP-glucose:-flavonoid 3-*O*-glucosyltransferase (UFGT or BZ1) catalyzes the glycosylation of unstable anthocyanidin aglycones into pigmented anthocyanins, and acylation of glucose further increases the stability of anthocyanidins.[40] The BZ1 gene is a critical factor for the final content and composition of anthocyanins in grape berries.[10,40] Beyond that, the other gene levels in the flavonoid pathway show a high correlation with the content and composition of anthocyanidins.[41] The content and composition of anthocyanins were susceptible to environmental factors, such as water deficit, temperature, phytohormones, UV irradiation, sunlight exposure, and so forth.[10,11] We found one gene coding for BZ1 (*VIT*_16*s*0039*g*02230) that had a dramatic upregulation at the S3 and S4 stages, which agreed with the increase of anthocyanins. RR treatment significantly increased the anthocyanidin content through upregulation of BZ1. Meanwhile, we noted a good correlation between anthocyanin content and the expression levels of genes encoding PAL, CYP73A, 4CL, E1.14.11.9, CHS, CHI, and CYP75A. Our results were consistent with previous studies that found that RR treatment significantly induces anthocyanin accumulation,[16,17] and further confirmed that BZ1 plays an important role in RR-induced anthocyanin accumulation in grape berries.

Flavanol monomers are synthesized in the flavonoid pathway from the intermediates leucocyanidin and leucodelphinidin. Leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), and leucoanthocyanidin dioxygenase (E1.14.11.19) are involved in this pathway.[31,42] Proanthocyanidins are synthesized as oligomers or polymers from various combinations of flavanol monomers,[13] but the whole accumulation and polymerization pathway of proanthocyanidins is still unknown. Flavanol concentration decreased progressively towards maturity due to competition with anthocyanin accumulation because they share the same upstream pathway.[43] There was no significant change in the expression of genes, LAR, ANR, and E1.14.11.19 throughout the whole growth process. However, the expression of genes coding for PAL (*VIT*_06*s*0004*g*02620, *VIT*_13*s*0019*g*04460, *VIT*_16*s*0039*g*01100, *VIT*_16*s*0039*g*01110, and *VIT*_16*s*0039*g*01120), CHS (*VIT*_14*s*0068*g*00920 and *VIT*_14*s*0068*g*00930), and CYP75A (*VIT*_08*s*0007*g*05160) were downregulated with the decrease of catechin and epicatechin by RR treatment at the earlier stages. It should be noted that the catechin and epicatechin contents were negatively correlated with proanthocyanidin content at the before-veraison stage. After veraison, when more precursors flow into the anthocyanin biosynthesis branch pathway, the total flavanol levels were significantly lower in the RR treatment grape berries than that in the control.

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Transcription Factors Associated with Accumulation of Secondary Metabolites. Along with MYB, bHLH, and WD40 proteins are the main transcription factors for genes in the flavonoid biosynthetic pathway. In grape berries, recent studies have pointed out that transcription factors, VvMYBA1 and VvMYBA2 regulated the expression of genes encoding BZ1, an enzyme responsible for anthocyanin biosynthesis. VvMYB5A and VvMYB5B regulate the expression of genes encoding enzymes in the flavonoid pathway and lead to the accumulation of anthocyanins and proanthocyanidins. VvMYBPA1 and VvMYBPA2 regulate the proanthocyanidin branch pathway, and VvMYBF1 regulates the flavonol biosynthesis branch.[44−47] The expression levels of these transcription factors are affected by environmental stresses, such as light, temperature, water, and phytohormones, which correlate to the mRNA levels of the structural genes in the flavonoid pathways.[30] In this study, we found 16 genes encoding the transcription factors may be involved in the phenylpropanoid pathway that were significantly changed by RR treatment at the different developmental stages. A good correlation was observed between the upregulated genes, VvMYBA22 (*VIT*_02*s*0033*g*00380), VvMYBA2 (*VIT*_02*s*0033*g*00390), VvMYBA1 (*VIT*_02*s*0033*g*00410) and VvMYBA3 (*VIT*_02*s*0033*g*00450) and the increased anthocyanin levels (Supporting Information Table S4). VvMYBA3 and VvMYBA22 are the homologues of VvMYBA1 and VvMYBA2 genes and might be critical in the regulation of anthocyanin biosynthesis in grape berries.

Validation of Differential Gene Expression Using qRT-PCR. To validate the accuracy and reproducibility of the results obtained by RNA-Seq, five genes involved in the phenylpropanoid pathway were randomly selected for qRT-PCR detection (Figure 5). These genes were randomly selected for being upregulated, downregulated, and unaffected by RR treatment. Linear regression analysis showed an overall correlation coefficient of 0.7639, indicating that the qRT-PCR expression profiles were in agreement with the RNA-Seq values, suggesting the reliability of the RNA-Seq data.

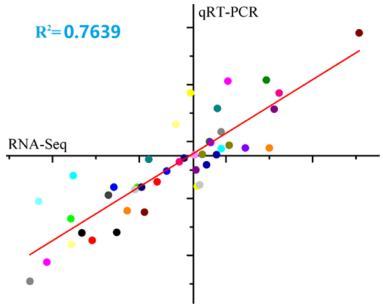

Figure 5. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of differential gene expression between the two treatments of grape berries during development and ripening. Correlation of fold change analyzed by RNA-Seq (x axis) and the data obtained using qRT-PCR (y axis).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02488.

Table S1, primers for real-time polymerase chain reaction; Table S2, differential genes in the primary metabolic pathways under root restriction (RR) treatment during grape berry development; Table S3, differential genes of transcription factors involving the phenylpropanoid pathway under root restriction (RR) treatment during grape development (PDF)

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Author Contributions

F.L. and C.S. designed the experiments; F.L., J.C., Y.W., and C.Z. performed the experiments; F.L., Z.G., Y.Z., and C.S. analyzed the data; S.W., X.L., and C.S. contributed reagents, materials, and analytical tools; F.L. and C.S. composed the paper.

Notes

The authors declare no competing financial interest.

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