Grafting with rootstocks promotes phenolic compound accumulation in grape berry skin during development by integrative multi-omics analysis

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Running title:

Multi-omics study of effects in grapes by grafting

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Abstract
In viticulture, grafting has been practiced widely and influences grape development as well as berry and wine quality. However, limited knowledge is present about the effects of rootstocks on grape phenolic compounds, which locate primarily in the berry skin and contribute to certain sensory attributes of wine. In this study, scion-rootstock interactions were investigated at green-berry stage and veraison stage when grapevines were hetero-grafted with three commonly used rootstock genotypes (5BB, 101-14MG, and SO4). The physiological investigations showed that hetero-grafts especially for CS/5BB presented higher concentrations of total proanthocyanidins (PAs) and various PA components in the berry skins when compared to the auto-grafted grapevines. Further metabolomics analysis identified 105 differentially accumulated flavonoid compounds, and the majority of them including anthocyanins, PAs and flavonols were significantly increased in the berry skins of hetero-grafted grapevines compared to the auto-grafted control. In addition, transcriptomic analysis using the same samples totally identified several thousand differentially expressed genes between hetero-grafted and auto-grafted vines. The three rootstocks not only increased the transcription levels of stilbene, anthocyanin, PA, and flavonol synthetic genes, but also affected the transcript abundance of a large number of transcription factors. Taken together, our results jointly supported that hetero-grafting could promote phenolic compound accumulation in the grape berry skin during development, which provide new insights for improving the application value of grafting by enhancing the accumulation of the nutritious phenolic components in the grape.
Introduction

The grapevine (*Vitis vinifera* L.) as an economically important fruit crop is well adapted to grow in a wide range of climatic conditions, thus is cultivated worldwide\textsuperscript{1,2}. Grapes have a variety of economic values, mainly reflected in that they are not only fresh, but also can used for juice and wine making. Additionally, grapes contain rich nutrients with health benefits to humans\textsuperscript{3}. In China, the scale of grape cultivation has increased rapidly in the past few decades. Due to the unique geographical location, light and rich variety resources, Xinjiang has become the largest grape producing area in China. The grapevine cultivation area has reached 26,000 hectares, and the Xinjiang government plans to develop grape industry on a larger scale in the next decade. However, it is frustrating that the quality of grapes in Xinjiang generally has the problem of low content of phenolic compounds such as proanthocyanidins.

Grape berry skin is enriched in phenolic compounds, which contain non-flavonoid compounds such as stilbenes, as well as flavonoid compounds such as flavonols, proanthocyanidins (PAs), and anthocyanins\textsuperscript{4,5}. These phenolic compounds have multiple biological functions including protection against biotic and abiotic stresses, contributing to the taste and astringency of wine, and also acting as potential dietary antioxidants with health benefits to humans\textsuperscript{6}. In grapevine, anthocyanins play a decisive role in the red color and mainly accumulate during ripening in berry skins\textsuperscript{7}. Flavonols are responsible for protection against UV radiation, and are synthesized in berry skins during early stages of berry development and during ripening\textsuperscript{8}. PAs are polymers of flavan-3-ol units such as catechin, epicatechin and epigallocatechin, and contributed to the taste and astringency of wine\textsuperscript{9}. PAs are synthesized in the early stages of berry development and are completed around veraison. These phenolic compounds are synthesized via the flavonoid pathway, which has been widely reported. Accumulation of phenolic compounds in grape skins is a complex physiological and biochemical process, and many factors including grape variety, yield, and exogenous stimuli, influence the accumulation\textsuperscript{10,11}. It has been reported that a series of transcription factors such as R2R3-MYB, WRKY, and bHLH proteins are involved in the transcriptional regulation of flavonoid biosynthetic pathway genes\textsuperscript{12-14}. 
In recent years, some studies have revealed that rootstock genotype has a possible influence on the accumulation of some phenolic compounds\textsuperscript{15-17}. Our lab previously found that the rootstock genotypes such as 5BB and 101-14MG affected the tannin accumulation of Cabernet Sauvignon variety. Therefore, the study on the influence mechanisms of rootstocks on PAs would have a good foundation and application significance to improve the problem of low PA content of Xinjiang grapes.

In the late 19th century, grape phylloxera (\textit{Daktulosphaira vitifoliae} Fitch) spread in Europe and caused serious damages to worldwide vineyards\textsuperscript{18}. Later on, phylloxera-resistant rootstocks derived from American \textit{Vitis} species have been successfully used and represent the most prolonged use of a biological control strategy\textsuperscript{19}. Although the grape cultivation area in China continues to expand, most are own-rooted cultivars that are not resistant to pathogens and pests, which seriously restrict the development of grape industry. Grape grafting cultivation in China started late and began in the 1960s. Grape grafting is a widely used technique with many advantages. Rootstocks are not only used to treat various diseases and pests, but also are tolerant to diverse abiotic stress conditions such as salinity, drought and alkalinity\textsuperscript{20,21}. Additionally, rootstocks can improve the internal and external quality of scion varieties, regulate grape maturity and increase yield\textsuperscript{22}. Rootstock, as a nutrient transport channel of grapevine, also plays a very important role in mineral element metabolism, which can make the vine show differential growth or significant changes in fruit quality and yield. Some rootstocks can advance or delay the grape harvest time. However, studies investigating the molecular processes governing scion-rootstock interactions are still scare. Therefore, the study of the effects of rootstocks on grape growth and PA accumulation would provide important basis for improving PAs and other phenolic compounds in Xinjiang grapes.

In recent years, a series of -omics techniques such as transcriptomics, proteomics, and metabolomics, have been used to study rootstock-mediated effects involved in the modifications of gene expression and secondary metabolites in the grape. An earlier microarray study conducted on the berry skin of Norton revealed that transcripts levels of \textit{PR-1} and stilbene synthase genes clearly increased, which might contribute
to the developmentally regulated resistance. In another study, the analysis of metabolome data showed that compared with Cabernet Sauvignon cultivar, Shiraz cultivar had increased organic acid, sugars, and precursors of phenolic compounds. Accordingly, transcript profiling showed that Shiraz contained a large number of up-regulated genes related to the entire polyphenol pathway and stress processes. Furthermore, a study combining transcriptome and metabolome on leaves of Gaglioppo variety showed that metabolites and genes involved in defense responses exhibited differential variation by diverse rootstocks. However, limited data are available regarding the effects of different rootstocks on dynamic changes in the metabolome and transcriptome in grape berry skins during different developmental stages.

This study aimed at investigating how different scion-rootstock combinations influence the physiological parameters, gene expression, metabolites in the grape berry skin, to find out the actual effects on phenolic compound accumulation during berry development. The climate in Xinjiang is arid, and the soil salinity is high. The rootstocks of 5BB and SO4 have been reported to confer tolerance to drought and salinity, while 101-14MG can promote grape fruit coloring, and they are presently world-wide used for grapevine grafting. The selection of these three rootstocks meets the needs of grape producing area in Xinjiang. Therefore, this study was set up using Cabernet Sauvignon variety grafted on three commonly used rootstock genotypes (5BB, 101-14MG, and SO4), as well as auto-grafted with itself, respectively. At green-berry stage and veraison stage, sugar, organic acid, and PA content were measured. Then flavonoid accumulation variation was evaluated by the metabolomics approach. Alongside the metabolic analysis, gene expression changes affected by rootstocks in the berry skin were analyzed using the transcriptomic approach.

Results

Rootstocks promote the accumulation of sugar and organic acid content of grape berry during development
Vitis vinifera cv. Cabernet Sauvignon (CS), a widely planted variety of red grape, was used in this study. CS was hetero-grafted with three commonly used rootstock genotypes (5BB, 101-14MG, and SO4) and auto-grafted with itself (Fig. 1a), respectively. The grape berries of each scion-rootstock combination were harvested at green-berry stage (45 days after flowering) and veraison stage (75 days after flowering) (Fig. S1). At green-berry stage, the berry size of the hetero-graft CS/5BB was significantly larger than those of CS/101-14MG, CS/SO4 and the auto-grafted control (Fig. 1b). The color of berry skin was shifting from green to purple at veraison stage, and the berry size of CS/5BB was still the largest, whereas CS/SO4 had a little bit smaller berry size compared to the auto-grafted control (Fig. 1b). The results showed that the rootstock 5BB promoted the development of grape berries, while SO4 was opposite.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Effects of rootstocks on the physiological index of grape berries.

a. Grafting process used in this study. b. Effects of rootstocks on the grape berry size at two developmental stages. c. Effects of rootstocks on the concentrations of organic acids in grape berries. d. Effects of rootstocks on the concentrations of fructose and glucose in grape berries. * indicates the statistically significant difference at p < 0.05 between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control, and ** indicates the statistically significant difference at p < 0.01 between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control.
Sugars and acid content determine the organoleptic quality and flavor of the grape\textsuperscript{26}. Thus, we detected sugar and organic acid content of grape berries to investigate the influence of rootstocks on grape quality. Initially, we detected the contents of malic acid, tartaric acid and citric acid. As shown in Fig. 1c, the concentrations of two important organic acids, tartaric acid and malic acid, were largely higher at green-berry stage than that at veraison stage, which was in line with conventional phenomenon that organic acids were synthesized and reached maximal concentrations during initial berry growth (Phase I)\textsuperscript{27}. At green-berry stage, the amounts of citric acid and malic acid were much higher by hetero-grafting with three rootstocks than the auto-grafted control, while tartaric acid was largely decreased in the hetero-grafts (Fig. 1c). Intriguingly, the rootstock genotype SO4 exhibited higher concentrations of malic acid and tartaric acid than the auto-grafted control, whereas 5BB and 101-14MG imparted slight influences at veraison stage. All the three rootstocks significantly increased the content of citric acid compared to the auto-grafted control at veraison stage (Fig. 1c). Sugar accumulation begins during phase II which is characterized as a lag phase\textsuperscript{27}. Consistent with this phenomenon, the concentrations of fructose and glucose were relatively low at green-berry stage, and increased crazily at veraison stage (Fig. 1d). In contrast to the auto-grafted control, 5BB and 101-14MG largely increased the contents of fructose and glucose at veraison stage, while SO4 was opposite. Additionally, the rootstock genotype 5BB had the most abundant contents of fructose and glucose among the three tested rootstocks (Fig. 1d).
These results suggested that the three rootstocks promoted the accumulation of organic acids and sugars of grape berries during berry development.

Rootstocks increase proanthocyanidin content of berry skin by physiological analysis

Proanthocyanidins (PAs) are flavan-3-ol oligomers which play an important role in the wine astringency and bitterness, and are located primarily in the berry skins and seeds\(^\text{28}\). To globally evaluate impacts of scion-rootstock interactions on PA content in the berry skins, total PA concentration was measured. As shown in Fig. 2a, the total PA concentration was largely higher by grafting with three rootstocks compared to the auto-grafted control at both developmental stages. CS/5BB produced the highest amount of PA, followed by CS/SO4 and CS/101-14MG. Notably, the total PA content of CS/5BB was more than twice that of the auto-grafted control (Fig. 2a). Meanwhile, the PA concentration of each scion-rootstock combination decreased at veraison stage compared to that at green-berry stage.
Fig. 2 Effects of rootstocks on total PAs and PA components of the berry skin.

a Effects of rootstocks on total PA content at two developmental stages. b Effects of rootstocks on the concentrations of different PA components at veraison stage. ‘*’ indicates the statistically significant difference at $p < 0.05$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control, and ‘**’ indicates the statistically significant difference at $p < 0.01$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control.

Among PAs, the most important ones are flavan-3-ol (catechin) and its condensed forms including gallicatechin, epicatechin, epigallocatechin, epigallocatechin gallate, gallicatechin gallate, ellagic acid, and epicatechin-3-O-gallate. We detected these PA components in the berry skin at veraison stage. Notably, seven components including epigallocatechin, gallicatechin, epigallocatechin gallate, epicatechin, gallicatechin gallate, ellagic acid, and epicatechin-3-O-gallate, exhibited significantly higher concentrations in all the three hetero-grafted grapevines compared to the auto-grafted control (Fig. 2b). The accumulation of catechin was largely higher in CS/5BB and CS/SO4, whereas showed no influence in CS/101-14MG, when compared to the auto-grafted control. Among the three hetero-grafted combinations, we observed that CS/5BB presented the highest concentrations of seven PA components except catechin, which was most abundant in CS/SO4. Further comparisons between CS/101-14MG and CS/SO4 showed that the concentrations of gallicatechin gallate, ellagic acid, and epicatechin-3-O-gallate were higher in CS/101-14MG, whereas the remaining five PA components were higher in CS/SO4 (Fig. 2b). Taken together, the three rootstocks promoted accumulations of PAs compared to the auto-grafted control, and CS/5BB had the most abundant PA components.

Metabolomics reveals differential flavonoid accumulation by rootstocks

PA is one key compound of flavonoids, which also include several other important compounds such as anthocyanin, flavonol, flavanol, and dihydroflavone. We applied a widely-targeted liquid chromatography-mass spectrometry (LC-MS) method to detect the comprehensive profiling of flavonoids in the grape berry skins after grafting with three different rootstocks. In total, 184 flavonoid compounds were identified within the present berry skin samples, including 11 PAs, 51 flavonols, 16 flavanols, 8 chalcones, 12 dihydroflavones, 43 flavonoid, 30 anthocyanins, 2 flavonoid
carbonoside, and 11 tannins (Table S1). All the identified flavonoid compounds were subjected to PCA to visualize the general trend of flavonoid changes in respect to the scion-rootstock combination and developmental stage. Accordingly, PCA not only showed the clear separation between the two developmental stages, but also exhibited that the general flavonoid composition of berry skins was very different between hetero-grafts and the auto-grafted control (Fig. 3a). Comparison of hetero-grafts and the auto-grafted control showed that 105 flavonoid compounds were differentially accumulated at green-berry stage, while 136 flavonoid compounds were differentially accumulated at veraison stage. Among them, the contents of majority of flavonoids were significantly increased by grafting with three rootstocks (Fig. 3b). Intriguingly, all the three rootstocks commonly promoted the accumulation of 82 flavonoid compounds at veraison stage (Fig. 3b, Table S1), and they were mainly composed of PAs and flavonols.

Fig.3 Differential flavonoid metabolites of berry skins affected by rootstocks. a PCA scatter plot of berry skin samples based on metabolomics data. b Venn diagram of counts of differential flavonoid metabolites among three hetero-grafts. The differential flavonoid metabolites were identified by comparing the abundances of metabolites between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control. The number on the left of the red arrow indicates increased metabolite number, and the number on the left of the green arrow indicates decreased metabolite number. c Cluster heatmap of different anthocyanin compounds affected by three rootstocks. d Cluster heatmap of different PA compounds affected by three
rootstocks. Different colors represent the increase (red) or decrease (blue) of the metabolites fold change as indicated in the color index. Fold change and the abundance of each metabolite can be found in Table S1.

Anthocyanins are mainly responsible for color palette, flavonols for UV protection, and wine astringency. In this study, only five anthocyanin compounds were detected at green-berry stage, while 30 anthocyanin compounds were identified at veraison stage (Fig. 3c, Table S1), which was consistent with the phenomenon that grape berry skins usually began to accumulate anthocyanins at veraison. This displayed progressive accumulation of anthocyanins across berry development by grafting with three rootstocks. At green-berry stage, the amounts of all the five detected anthocyanin compounds raised significantly in the hetero-grafts compared to the auto-grafted control. At veraison stage, 63% of anthocyanin compounds exhibited higher amounts in at least two of the three scion-rootstock combinations compared to the auto-grafted control (Fig. 3c, Table S1). Notably, five anthocyanin derivative compounds involving Delphinidin-3-O-glucoside, Delphinidin-3-O-galactoside, Delphinidin-3-O-(6''-O-acetyl) glucoside, Petunidin-3-O-(6''-O-Acetyl) glucoside, and Cyanidin-3-O-glucoside, were the most abundant anthocyanins in all the analyzed hetero-grafts (Fig. 3c, Table S1). These results indicated that the three rootstocks stimulated anthocyanin production especially at veraison stage.

PAs are predominantly found in the grape berry skin, and are the key factors contribute to the quality of wine. We observed that PAs exhibited decreased accumulation at veraison stage compared to that at green-berry stage. A total of 11 PA derivative compounds were identified in this study, and all of them showed high diversity in the accumulation at both developmental stages (Fig. 3d, Table S1). Four PA derivatives including procyanidin B2, procyanidin B4, procyanidin A2, and 2α,3α-epoxy-5,7,3',4'-tetrahydroxyflavan-(4β-8-epicatechin), showed higher levels by grafting with three rootstocks, and the remaining seven PA derivatives were also abundant in at least one hetero-graft at green-berry stage. Although PA contents decreased at veraison stage, there was still significant difference between hetero-grafted grapevines and the auto-grafted control. In contrast to the auto-grafted control, eight PA derivatives showed higher amounts in CS/101-14MG, while four PA
derivatives were more abundant in CS/5BB and CS/SO4 at veraison stage (Table S1), suggesting that the three rootstocks largely promoted PA accumulation at both developmental stages.

Flavonols are one type of flavonoids with a 3-hydroxyflavone backbone, and play a crucial role in the color and bitter taste red wine by stabilizing anthocyanin pigments. Based on our results, 51 flavonol compounds were found (Table S1), which represented the most abundant flavonoids. At the two developmental stages, the majority of flavonols were differentially accumulated between hetero-grafted vines and the auto-grafted control. At green-berry stage, CS/5BB and CS/101-14MG had ten decreased and only one increased flavonol compounds (Table S1). Meanwhile, CS/SO4 had 21 increased flavonol compounds, indicating that the three hetero-grafts exhibited differential accumulation of flavonols at this stage. Notably, 75% of the detected flavonol compounds were differentially accumulated in at least two of the three hetero-grafted combinations, and the majority of them were highly increased by grafting with three rootstocks at veraison stage (Table S1). Taken together, the rootstock genotypes 5BB and 101-14MG reduced flavonol level at green-berry stage and promoted flavonol accumulation at veraison stage, while SO4 increased flavonol content at both developmental stages.

**Transcriptome overview**

To investigate the rootstock effects on grape berry skin transcriptome, and to relate these changes to the observed metabolic changes, RNA-seq was carried out in the same samples used for metabolic analysis. Quality filtered reads were mapped to the *V. vinifera* genome. The majority of clean reads from each library (82.5-93.6%) were successfully aligned (Table S2), and only the uniquely aligned reads were used for the following calculation of gene expression value. On the whole, over 18,000 expressed genes were detected in each sample.

A PCA analysis was performed to evaluate sample correlation. Based on the transcriptional profiles, there was a clear separation of hetero-grafted samples and auto-grafted samples in the plot (Fig. 4a). PCA showed that data of three hetero-grafts were more similar to each other than data of auto-grafted control at each
developmental stage. Moreover, the distance between hetero-grafts and auto-grafted control was much larger at green-berry stage compared to that at veraison stage (Fig. 4a). This plot also clearly showed a high similarity among the three biological replicates, suggesting a good reproducibility. These results indicated that the produced RNA-Seq data had a high quality, and thus all data were used for subsequent analysis.

**Fig. 4 Overview of transcriptomic changes of berry skins affected by rootstocks.**

a PCA scatter plot of berry skin samples based on transcriptomic data. b Venn diagram of DEG numbers in different comparison groups. The DEGs were identified by comparing the expression levels of genes between hetero-grafts (CS/5BB, CS/101-14MG, or CS/5O4) and the auto-grafted control. The number on the left of the red arrow indicates up-regulated gene number, and the number on the left of the green arrow indicates down-regulated gene number. c GO enrichment for Biological Process (BP) domain in the comparison of the transcriptomes affected by rootstocks.

**Identification and functional enrichment analysis of DEGs**

Pairwise comparison between the hetero-grafted samples (CS/5BB, CS/101-14MG, or CS/5O4) and the auto-grafted (CS/CS) sample, at the same developmental stage, were performed to identify differentially expressed genes (DEGs). A great many of genes were found to be differentially expressed in the berry skin. A total of 3,707, 4,235, and 3,640 DEGs were identified between hetero-grafted and auto-grafted samples at green-berry stage, and 1,262, 2,638, and 2,294 DEGs were found at veraison stage (Fig. 4b, Table S3). For each comparison, 57.9-68.9% of DEGs were up-regulated in the hetero-grafts. In general, we found three major trends. First, a large number of DEGs were identified in the three hetero-grafted samples compared to the
auto-grafted sample, suggesting that hetero-grafting had a significant impact on the transcriptome profile. Second, more DEGs were found at green-berry stage compared to that at veraison stage, indicating stronger differences in the transcriptome towards the berry developmental process. Third, the majority of DEGs were present in at least two of the three hetero-grafts (Fig. 4b), suggesting that the three rootstocks might bring out some similar effects to the berry skin.

To highlight the potential biological functions of these DEGs, they were subjected to gene ontology (GO) enrichment analysis. Biological process enrichment analysis showed that 263 GO terms were largely over-represented in the comparisons between rootstocks and the auto-grafted control (Table S4). Of these, GO terms were mainly related to response to stimulus (GO:0050896), small molecule metabolic process (GO:0044281), signal transduction (GO:0007165), and secondary metabolic process (GO:0019748) (Fig. 4c). Considering the 2,774 DEGs in common to all the three hetero-grafts compared to the auto-grafted control at two developmental stages, they were preferably up-regulated (62.3%) and mainly involved in flavonoid metabolic process (GO:0009812) and anthocyanin-containing compound metabolic process (GO:0046283) (Table S5).

MapMan analysis was performed to evaluate metabolic pathways of these DEGs. The result showed that DEGs were mainly involved in phytohormone action, cell wall organization, solute transport, transcriptional regulation, and secondary metabolism (Table S3). Transcription factors were the main genes participated in transcriptional regulation, and the most represented families were MYB, WRKY, AP2/ERF, and NAC. Among secondary metabolism pathways, these DEGs mainly concentrated in flavonoid, terpene, and phenylpropanoid pathways.

To validate expression profiles obtained from the RNA-Seq data, nine genes were randomly selected to be analyzed by qRT-PCR analysis. All the genes chosen were mainly involved in key points of the flavonoid pathway. As shown in Fig. S2, the expression profiles of qRT-PCR exhibited a similar tendency with RNA-Seq results, suggesting that the expression profiles obtained from RNA-Seq data were reliable for subsequent analysis.
Fig S2. Validation of DEGs by qRT-PCR analysis.

‘*’ indicates the statistically significant difference at $p < 0.05$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control, and ‘**’ indicates the statistically significant difference at $p < 0.01$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control.

**Rootstocks especially 5BB activate transcriptional levels of stilbene synthetic genes**

Stilbenes constitute a non-flavonoid class of phenolic compounds that not only act as a powerful defense system in response to biotic stresses, but also have nutraceutical and pharmacologic properties\(^{32,33}\). Stilbene synthesis usually occurs in the grape berry skin\(^{34}\). In the current study, we observed enriched stilbene-related DEGs in the comparisons between hetero-grafted grapevines and the auto-grafted control (Fig. 5a,
Table S6). In particular, these DEGs were almost exclusively up-regulated. Stilbene synthase (STS) is the key enzyme contributing to the biosynthesis of stilbene, and 32 of the total 37 STS genes were up-regulated by grafting with rootstocks (Fig. 5a). At green-berry stage, both of the rootstocks 5BB and 101-14MG induced high activation of these genes, with 27 STS genes showing up to 76-fold up-regulation. In contrast, only 3 STS genes were up-regulated in CS/SO4 at this stage. At veraison stage, there were still 23 up-regulated STS genes in CS/5BB, whereas only 7 and 2 up-regulated STS genes were identified in CS/101-14MG and CS/SO4 (Fig. 5a). These results suggested that STS genes were differentially expressed depending on the rootstock genotype and berry developmental stage. In addition, several known transcriptional regulators of the stilbene biosynthetic pathway were also investigated. The R2R3-MYB gene VvMYB14 (VIT_07s0005g03340) exhibited increased expression by grafting with three rootstocks at both stages (Fig. 5a). The VvMYB15 gene (VIT_05s0049g01020) was highly down-regulated in the three hetero-grafts at green-berry stage, whereas it had an increased expression at veraison stage, indicating that it likely had an initial activation at veraison stage. Besides MYB genes, several WRKY transcription factors such as WRKY3 and WRKY 24 have been reported as potential STS regulators35. We found that the expression levels of VvWRKY3 (VIT_01s0010g03930) and VvWRKY24 (VIT_08s0058g00690) were significantly up-regulated only in CS/101-14MG at green-berry stage (Fig. 5a). At veraison stage, all the three rootstocks induced the high expression of VvWRKY3. Taken together, the high transcriptional levels of STS and regulators reported above were observed in the three hetero-grafts particularly for CS/5BB, suggested a putative increase in stilbene metabolite synthesis and accumulation in the berry skins by scion-rootstock interactions. A previous study investigated the concentration of stilbene of 78 V. vinifera varieties for 3 years, and identified significant differences among genotypes34. The current study found that the rootstock genotype 5BB had the greatest effect on the transcription of stilbene synthetic genes, suggesting induced stilbene content that was beneficial to accumulation of phenolic compounds in the berry skin.
Fig. 5 Transcriptional changes of genes related to stelbene and flavonoid synthetic pathways.

a Expression heatmap of stelbene synthetic pathway genes at two developmental stages. Different colors represent the up-regulation (red) or down-regulation (blue) of the gene expression fold change as indicated in the color index.

b Expression behavior of phenylpropanoid and flavonoid related genes affected by the rootstock genotype 5B at two developmental stages. Each box with colors represents one gene, and the color of the box represents the transcriptional fold change (blue indicates down-regulation and red indicates up-regulation) of this gene between CS/5BB (or other two) and CS/CS. The boxes on the left of the arrow represent transcriptional changes of related genes at green-berry stage, and the boxes on the right of the arrow represent transcriptional changes of related genes at veraison stage. PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3’H, flavonoid 3’-hydroxylase; F3’5’H, flavonoid 3’5’-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; UFGT, UDP-glucose:flavonoid-3-O-glucosyltransferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

Rootstocks promote transcription of genes related to flavonoid biosynthesis pathway

The flavonoid pathway leads to the synthesis of various phenolic compounds including flavonols, anthocyanins, and PAs. Therefore, we detected the expression of early phenylpropanoid, anthocyanin, and PA-related genes between hetero-grafts and the auto-grafted control to corroborate the metabolic data (Fig. 5b, Table S6). Cinnamic acid 4-hydroxylase (C4H), phenylalanine ammonia lyase (PAL), and
4-coumarate: CoA ligase (4CL), are key enzymes to catalyze the first three steps of the phenylpropanoid pathway. In the current study, the majority of above genes were differentially expressed in the comparisons between hetero-grafted grapevines and the auto-grafted control (Fig. 5b). At green-berry stage, two PAL genes, one C4H gene and four 4CL genes, were highly up-regulated in the hetero-grafts particular for CS/101-14MG. Meanwhile, three PAL genes, two C4H genes and one 4CL gene, were significantly induced by grafting with three rootstocks at veraison stage. It was noteworthy that the expression levels of VvPAL2.2 (VIT_13s0019g04460), VvC4H1 (VIT_06s0004g08150) and Vv4CL2.2 (VIT_11s0052g01090), were activated in all the three hetero-grafts at veraison stage. This high expression levels of above genes suggested higher accumulation of the substrate 4-coumaryl-CoA, which ultimately affecting downstream pathways.

Phenolic compounds such as proanthocyanidin and anthocyanin have a common synthetic pathway, and their pathways branch after coumaroyl-CoA. Chalcone synthase (CHS) and chalcone isomerase (CHI) mediate the reaction from coumaroyl-CoA to naringenin. At green-berry stage, three CHS genes (CHS1) and three CHI genes were down-regulated, whereas only one CHI gene VvCHI3 (VIT_19s0014g00100) was highly activated in the hetero-grafts (Fig. 5b, Table S6). On the contrary, the majority of CHS and CHI genes were significantly induced by grafting with rootstocks at veraison stage. Flavonoid 3’5’-hydroxylase (F3’5’H) and flavonoid 3’-hydroxylase (F3’H) participate in catalyzing the hydroxylation of the B-ring of naringenin and dihydrokaempferol. In the current study, transcriptional profiling of F3’5’H genes were totally different between the two developmental stages. In contrast to the auto-grafted control, a lower expression of seven F3’5’H genes was observed in the three hetero-grafts at green-berry stage, however, their expression levels were significantly increased at veraison stage. Usually F3’5’H activity prevails over F3’H, and we indeed observed that only several F3’H genes were differentially expressed between hetero-grafted grapevines and the auto-grafted control. Three up-regulated and three down-regulated F3’H genes were identified in the hetero-grafts at green-berry stage, and only two up-regulated F3’H genes were found at veraison
stage.

Flavonol synthase (FLS) is a key enzyme on the flavonol branch. We observed that VvFLS6 (VIT_02s0012g00400) was highly expressed in the hetero-grafts at both developmental stages, while VvFLS10 (VIT_13s0047g00210) and VvFLS (VIT_18s0001g03470) were only up-regulated at veraison stage (Fig. 5b, Table S6). Dihydroflavonol-4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose:flavonoid-3-O-glucosyltransferase (UFGT) are involved in biosynthesis of anthocyanins. Transcripts of three DFR genes (VIT_18s0001g12800, VIT_13s0064g00290 and VIT_03s0038g04220) increased to higher levels in the hetero-grafts compared to the auto-grafted control. Four down-regulated and one up-regulated LDOX genes were identified in the hetero-grafts at green-berry stage, however, three up-regulated LDOX genes were found at veraison stage. Additionally, transcripts of VvUFGT1 (VIT_16s0039g02230) were very low in the three hetero-grafts at green-berry stage, but they largely increased at veraison stage. In particular, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) provide two separate pathways to synthesize the starting units for PA polymers. VvLAR1 (VIT_01s0011g02960) showed lower transcript value in the three hetero-grafts at green-berry stage, but was significantly up-regulated at veraison stage. Meanwhile, transcripts of VvLAR2 (VIT_17s0000g04150) were largely increased in all the three hetero-grafts compared to the auto-grafted control at both stages. In addition, we observed that VvANR (VIT_02s0025g01260) showed higher transcript levels in the hetero-grafts at green-berry stage. Taken together, the above results indicated that the rootstocks promoted flavonoid synthesis at both developmental stages.

Rootstocks active transcription factors

The functional enrichment analysis of DEGs between hetero-grafted grapevines and the auto-grafted control exhibited a large number of transcription factors (TFs), which were mainly involved in gene regulation, modification, and signaling36. In the current study, a total of 396 TFs, accounting for 42% of total TFs, were identified to be differentially expressed during berry development, suggesting that rootstocks might
utilize abundant TFs to regulate downstream genes. In general, the number of up-regulated TFs was greater at the two stages. Comparison between the two developmental stages showed that more DEGs encoding TFs were observed at green-berry stage, indicating that TFs were more active at this stage. Additionally, among the three hetero-grafted combinations, CS/101-14MG had more differentially expressed TFs at green-berry stage, and CS/101-14MG and CS/SO4 contained more differentially expressed TFs at veraison stage, suggesting that the rootstock genotype 101-14MG promoted the activation of TFs during berry development. Further analysis showed that these DEGs could be divided into 29 TF families, and the most represented families were MYB, AP2/ERF, C2C2, and WRKY TFs (Fig. S3). MYB TFs such as MYB14 and MYB15 have been reported to be involved in the feedback regulation of stilbene biosynthesis. In the current study, they were differentially expressed by rootstocks during berry development. Although the biological functions of the remaining large number of differentially expressed MYB TFs were unclear, we supposed that they might participate in some important pathways such as biosynthesis and regulation of phenolic compound pathways.
Fig S3. Prevalence of differentially expressed transcription factor families.

Discussion

Grafting is a widely used strategy in viticulture, and affects grape development as well as fruit and wine quality. Phenolic compounds primarily locate in the grape berry skin, and play important roles not only in protection against biotic and abiotic stresses, but also in the contribution to taste and astringency of wine. Some studies have revealed that grafting has an influence on berry skin chemical composition and transcriptome at maturity. However, knowledges about effects of rootstocks on berry skin are still limited during berry development. In the current study, we characterized the physiological, metabolic and transcriptomic differences caused by three different scion-rootstock combinations, by comparing with the auto-grafted control. Our results showed that hetero-grafting brought great changes in the berry skin (Fig. 6).
**Fig. 6** Response of grape berry skins at different developmental stages affected by different rootstocks based on combination of physiological, metabolomics, and transcriptomic data. The red or green arrow represents increase or decrease, and the thickness of the arrow indicates the degree of influence.

At green-berry stage, cell division is rapid and berry size undergoes a sigmoidal increase. The grape organic acids are synthesized and reach maximal concentrations at this stage. Consistent with this, we observed that the concentrations of organic acids in the berry were much higher at this stage than that at veraison stage. Furthermore, all the three hetero-grafts contained more malic acid and citric acid compared to the auto-grafted control, indicating that rootstocks could promote the accumulation of organic acids. Oppositely, sugar content was relatively low at this stage, probably because sugar accumulation usually began prior to veraison. Total PA content was much higher by grafting with three rootstocks especially for 5BB. We focused on the effects of rootstocks on phenolic compounds, and performed metabolomics analysis. The results showed that 105 flavonoid compounds were differentially accumulated at green-berry stage, and they were mainly composed of anthocyanins, PAs and flavonols, which confirmed some major trends described before. All the three rootstocks significantly increased the abundance of many PA compounds. However, only five anthocyanin compounds showed higher amounts in the hetero-grafts. We also observed that only SO4 increased the accumulation of...
flavonol compounds. Further transcriptomic analysis identified several thousands of DEGs between hetero-grafts and the auto-grafted control, and many DEGs were involved in the phenolic compound pathways. The rootstock genotypes 5BB and 101-14MG increased the transcription levels of stilbene synthetic genes, while SO4 did not. Notably, the rootstocks slightly decreased the expression of genes involved in biosynthesis of anthocyanins and PAs. Similarly, another rootstock genotype 1103 Paulsen was also found to have a strong influence on ripening processes related to secondary metabolite accumulation in grape berries. As the important regulators, many TFs such as MYB and WRKY genes exhibited higher transcriptional levels by scion-rootstock interactions in this study. Some of these TFs were the key regulators of the phenylpropanoid pathway. More DEGs encoding TFs were observed at green-berry stage, and 101-14MG had more differentially expressed TFs, reinforcing the hypothesis of a great modulation effect coming from this rootstock.

At veraison stage, there is no increase in berry size, but sugar content begins to accumulate. According to the physiological analysis, both of fructose and glucose contents increased a lot compared to that at green-berry stage. Of the three hetero-grafts, CS/5BB and CS/101-14MG exhibited much more abundant sugar content compared to CS/SO4 and the auto-grafted control, indicating that the two rootstocks promoted the accumulation of fructose and glucose. Although organic acid content decreased at this stage, we still found that the rootstocks especially SO4 increased the accumulation of organic acid content in the berry skin. Notably, the three rootstocks not only promoted the accumulation of total PA content, but also increased the concentrations of multiple PA components. Among the three hetero-grafts, we observed that CS/5BB presented the highest concentrations of seven PA components, suggesting that 5BB could significantly promote the accumulation of PAs in the berry skin. Comparative metabolomics analysis showed that all the three rootstocks significantly promoted the accumulation of flavonols and PAs. Consistently, the rootstock genotype SO4 has been reported to largely promote an increase of PAs and anthocyanins in the berry skin. Further transcriptomic analysis showed that the rootstocks induced the transcript levels of genes involved in the synthesis of stelbenes,
anthocyanins, flavonols, and PAs. In addition, a large number of genes encoding TFs were up-regulated by grafting with rootstocks. Taken together, these data suggested that the three rootstocks had an influence on phenolic compound pathways at veraison stage.

In conclusion, our results based on combination of physiological, metabolomic, and transcriptomic approaches jointly supported that grafting with rootstocks had beneficial effects on the grape berry skin, which could promote the accumulation of phenolic compounds including stelbenes, anthocyanins, PAs and flavonols. We speculated that there were two possible reasons. Firstly, rootstocks affected the transcription levels of phenolic compound pathway genes. The expression levels of many genes related to synthesis of stilbene and flavonoid (anthocyanin, PA, and flavonol), were increased in the hetero-grafts compared to the auto-grafted control. Secondly, rootstocks affected the transcription levels of many transcription factors (such as MYB and WRKY) which participated in transcriptional or post-transcriptional regulation of the phenolic compound biosynthetic pathway genes. These results could provide new insights for improving the application value of grafting by enhancing the accumulation of the nutritious phenolic components in the grapes.

Materials and methods

Plant material and sample collection

The experiments were conducted in the Anningqu vineyard of Urumqi, Xinjiang. This area is 480 m above sea level, with temperate arid and semi-arid continental climate and long sunshine time. The annual average temperature is 7.13 °C. The grape scion used in the current study was *Vitis vinifera* cv. Cabernet Sauvignon (CS), and the used rootstock genotypes included 5BB (*V. berlandieri* × *V. riparia*), 101-14MG (*V. riparia* × *V. rupestris*), and SO4 (*V. berlandieri* × *V. riparia*). In the spring of 2020, when the new shoots of CS and rootstocks were in the semi lignified stage, CS was hetero-grafted with three rootstocks, respectively. CS was also auto-grafted with itself.
to be treated as the control. The soil, frame, shaping, and water and fertilizer management of each treatment were consistent.

The grape berry samples of each scion-rootstock combination were collected at green-berry stage (45 days after flowering) and veraison stage (75 days after flowering). At each sampling date, three independent biological replicates were randomly performed, and each included 90 berries belonging to a different group of 6 vines. 30 berries were frozen immediately and stored for subsequent detection of sugar and organic acid content, and the other 30 peeled berries were frozen and stored for detection of total PAs and PA components. For the remaining 30 berries, skin tissues were separated from the pulp and frozen immediately in liquid nitrogen and stored at -80 °C for metabolomic and transcriptomic analyses.

**Measurement of sugar and organic acid, and metabolomics analysis**

The sugar content of grape berries was measured according to “Determination of fructose, glucose, sucrose, maltose, lactose in foods-High-performance liquid chromatography (GB/T 22221-2008)”. The organic acid content of grape berries was measured according to “Determination of organic acids in plant-Liquid chromatography-tandem mass spectrometry (GB/T 40179-2021)”. The total PA concentration of the berry skin was measured according to “Determination of tannin content in fruit, vegetable and derived product-Spectrophotometry method (NY/T 1600-2008)”. The grape berry skin samples were freeze-dried and ground into powder, followed by qualitative and quantitative analysis of flavonoid compounds using the UPLC-ESI-MS/MS system as described by Sun et al. The analysis was performed with SPSS 22.0 statistical software. Differences between two groups were analyzed by the one-way ANOVA followed by Tukey’s multiple comparison test.

**Metabolomic profile detection and analysis**

Briefly, the samples were immersed in liquid nitrogen, ground to a fine powder, and then suspended in 70% methanol, vortexed, centrifuged at 20000 rpm for 20 min at 4 °C. And then the supernatant was filtered through a 0.22 μm (nylon) syringe filter, and was analyzed by UPLC-QTRAP-MS system (SHIMADZU Nexera X2/Applied Biosystems 4500 QTRAP). Each sample was analyzed three times. The analysis was
performed on an Agilent SB-C18 column (2.1 × 100 mm, 1.8 μm). The mobile phase A was water with 0.1% formic and mobile phase B was 0.1% formic in acid acetonitrile solution. The gradient elution procedure was set as follows: 0-9 min, 0-95% B; 9-10 min, 95% B; 10-11 min, 95-5% B; 11-14 min, 5% B. The flow rate was 0.35 mL/min, and the column temperatures were held constant at 40 °C. The injection volume was 4μL. The product ion scan was acquired using multiple reaction monitoring (MRM) mode. The raw data detected by LC-MSMS were loaded on the metabolites database (MWDB METWARE database) for identification of metabolites. The processed data were then imported into SIMCA-P14.1 software (Umetrics, Umea, Sweden) for principal component analysis (PCA) and orthogonal PLS-DA analysis (OPLS-DA). The quantitative of metabolites were analyzed using software Analyst 1.6.3. The metabolites were also analyzed in KEGG (http://www.kegg.jp) to resolve the metabolic pathways.

**RNA isolation and whole-transcriptome sequencing**

Total RNAs of the berry skin tissue samples derived from each scion-rootstock combination were extracted using the RNA simple Total RNA kit (Tiangen, China) based on the manufacturer’s protocol, followed by treating with DNase I to remove DNA contaminations. The obtained RNAs were detected using gel electrophoresis and quantified with NanoDrop. RNA integrity was verified using the Agilent 2100 Bioanalyzer. For each sample, the cDNA library was constructed by TruSeq RNA Sample Preparation V2 (Illumina, USA) following manufacturer’s protocol. After that, the prepared libraries were sequenced using the Illumina sequencing platform.

**Transcriptomic analyses**

The *V. vinifera* reference genome was obtained from NCBI. The RNA-Seq low quality reads were discarded, and adaptors were clipped. High quality reads were then mapped to the *V. vinifera* genome using Hisat2 software with default parameters. Uniquely localized reads were used for subsequent analysis. The expression value of each grape gene was calculated by normalizing to RPKM (reads per kilobase and per million) value. The expression values of each sample were converted to z-scores and subjected to PCA analysis. Differentially expressed genes (DEGs) between two
samples were analyzed using DESeq2 software\textsuperscript{45}. For each gene, the adjusted $p$-value <0.05 and fold-change >2 or <0.5 were considered as the significant threshold. The gene ontology (GO) enrichment analysis of DEGs were performed by Blast2GO software\textsuperscript{46}. The metabolic pathways of DEGs were analyzed using MapMan ontology tool\textsuperscript{47}.

**Quantitative real-time PCR analysis**

To confirm the accuracy of the transcriptome profiling, the expressions of nine randomly selected genes were evaluated by qRT-PCR analysis. Total RNA was extracted from berry skins of each hetero-graft at two developmental stages. Actin was used as the endogenous control. Real-time monitoring of PCR was carried out with ABI 7500 Real Time System (Applied Biosystems) using the following parameters: 95°C for 5 min, 95°C for 10 s, and 60°C for 34 s (40 cycles) to calculate cycle threshold values, followed by a dissociation program of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain melt curves. Data analysis was carried out based on the $2^{-\Delta\Delta Ct}$ method.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (31960575) and the China Agriculture Research System of MOF and MARA.

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F.Z., X.W., and Y.X. designed the research. M.P., J.X., M.W., Y.W., and M.L. performed the experiments. G.L., T.X., and H.Z. analyzed the data. F.Z., H.Z. and X.Z.
prepared the manuscript. Y.X. and X.W. revised the manuscript. All authors read and approved the final manuscript.

Data availability
The raw RNA-Seq reads have been deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the sequence read archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA777448.

Conflict of interest
The authors declare no competing interests.

Supplementary information
Fig S1. Effects of rootstocks on the appearance of grape berries at two developmental stages.

Fig S2. Validation of DEGs by qRT-PCR analysis. ‘*’ indicates the statistically significant difference at $p < 0.05$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control, and ‘**’ indicates the statistically significant difference at $p < 0.01$ between hetero-grafts (CS/5BB, CS/101-14MG, or CS/SO4) and the auto-grafted control.

Fig S3. Prevalence of differentially expressed transcription factor families.

Table S1. Total and differential accumulations of flavonoid compounds between hetero-grafts and the auto-grafted control by metabolomics analysis.

Table S2. Summary of RNA-Seq data and mapping results.

Table S3. DEGs between hetero-grafts and auto-grafted control.

Table S4. Biological process enrichment analysis of all the identified DEGs.

Table S5. Biological process enrichment analysis of up-regulated genes present in all the three hetero-grafts.

Table S6. Transcriptional changes of genes involved in phenolic compound pathways.
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