Research article

Anthocyanin accumulation and biosynthesis are modulated by regulated deficit irrigation in Cabernet Sauvignon (*Vitis Vinifera* L.) grapes and wines

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ABSTRACT

Anthocyanins contents and compositions play an important role in grape berries and wines. Grapevines are widely cultivated in arid and semi-arid areas, and water shortage restricts the development of wine industry. The aim of this work was to gain insight on the effect of regulated deficit irrigation (RDI) on the accumulation and biosynthesis of anthocyanins in Cabernet Sauvignon (*Vitis Vinifera* L.) grapes and wines. High-performance liquid chromatography (HPLC) was used for anthocyanins profiles analyses and real-time quantitative PCR (qRT-PCR) was used for the genes expressions measurement. The grapevines were treated with 60% (RDI-1), 70% (RDI-2), 80% (RDI-3), 100% (CK, traditional drip irrigation) of their estimated evapotranspiration (ETc) respectively. RDI treatments significantly reduced titration acid and increased pH with higher total soluble solids. RDI-1 treatment increased total anthocyanins contents in berries and wines in both two vintages. RDI-1 and RDI-2 treatments significantly increased the contents of acylated anthocyanins in berries and wines, especially Malvidin-3-acetly-glucoside. RDI treatments significantly increased non-acylated anthocyanins contents in wines, such as Delphinidin-3-gliucoside and Malvidin-3-glucoside. RDI treatments upregulated the expressions of *VvPAL*, *VvC4H*, *VvCHS*, *VvF3'H*, *VvF3'5'H*, *VvLDOX*, and *VvOMT* in both two vintages. Correlation analysis showed the accumulation of anthocyanins was closely related to the key genes expressions, including *VvPAL*, *VvF3'H*, *VvF3'5'H* etc. The present results provided direct evidence and detailed data to explain that RDI treatments regulated the accumulation of anthocyanins by regulating genes expressions in the anthocyanin synthesis pathway.

1. Introduction

Anthocyanins are an important secondary metabolite in grape berries and wines, synthesized by the phenylpropane metabolic pathway and are widely found in different tissues of plants and have important biological and biochemical activities (He et al., 2008). They provide color and antioxidant activity to grape berries and wines; the composition and contents of anthocyanins play an important role in wine color stability (Li, 2006).

The biosynthesis and accumulation of anthocyanins are affected by many factors, such as variety, light, and moisture. Moisture plays a crucial role in the biosynthesis of anthocyanins in grape fruit. Appropriate water deficit is beneficial to the accumulation of anthocyanins in grape fruit. In the absence of water, the content of endogenous hormone of grape fruit changes, which promotes the synthesis of anthocyanins (Ferrandino and Lovisolo, 2014; Fortes et al., 2015; Pilati et al., 2017). In recent years, the shortage of irrigation water resources has seriously restricted the healthy development of the wine industry. Regulated deficit irrigation (RDI) is an efficient water-saving irrigation technology, which is of great significance for scientifically and rationally utilizing water resources in semi-arid areas, such as California, South Australia, Northwest China, and South Mediterranean Europe, improving grape quality and ecological environment (Cao, 2002; Costa et al., 2016).

In recent years, more and more studies have shown that a certain degree of water stress during viticulture contributes to the

Table 1
General parameters of the ripe grapes and its wines of Ningxia for the two vintages studied (2015, and 2016).

Treatments	Total soluble solids (˚Brix)	Titratable acidity (g/L)	pH	Berry Weight (g)
Grapes of 2015				
CK	22.0 ± 0.41b	5.42 ± 0.35a	3.66 ± 0.05c	120.0 ± 1.53a
RDI-1	23.9 ± 0.30a	4.31 ± 0.25b	4.12 ± 0.08b	104.9 ± 1.83b
RDI-2	23.5 ± 0.60ab	4.80 ± 0.02ab	4.28 ± 0.04a	109.4 ± 1.74ab
RDI-3	23.5 ± 0.42ab	5.02 ± 0.01ab	3.69 ± 0.04c	117.8 ± 2.50a
Grapes of 2016				
CK	22.85 ± 0.07b	3.72 ± 0.20a	3.84 ± 0.01ab	124.8 ± 1.68a
RDI-1	23.95 ± 0.07a	3.15 ± 0.12a	3.88 ± 0.03ab	110.0 ± 2.63b
RDI-2	23.55 ± 0.28ab	3.11 ± 0.06a	3.73 ± 0.06b	121.8 ± 1.05a
RDI-3	23.20 ± 0.35ab	3.49 ± 0.24a	3.92 ± 0.04a	122.0 ± 0.12a

Treatments	Total sugar (g/L)	Titratable acid (g/L)	pH	Alcohol (% vol.)
Wines of 2015				
CK	1.09 ± 0.05ab	5.91 ± 0.07a	3.74 ± 0.01b	10.44 ± 0.46b
RDI-1	1.13 ± 0.04a	5.44 ± 0.06b	3.79 ± 0.10ab	11.03 ± 0.55a
RDI-2	1.12 ± 0.07a	5.57 ± 0.09ab	3.77 ± 0.06ab	10.81 ± 0.96a
RDI-3	1.1 ± 0.08a	5.83 ± 0.10a	3.82 ± 0.05a	10.57 ± 0.66ab
Wines of 2016				
CK	1.78 ± 0.04ab	6.96 ± 0.03a	3.18 ± 0.06b	10.75 ± 0.65b
RDI-1	1.92 ± 0.06a	6.21 ± 0.04b	3.51 ± 0.09ab	11.44 ± 0.90a
RDI-2	1.82 ± 0.11a	6.36 ± 0.12ab	3.21 ± 0.06b	11.13 ± 0.89a
RDI-3	1.91 ± 0.05a	6.33 ± 0.08ab	3.78 ± 0.03a	10.98 ± 0.57ab

Different letters in the row indicate significant differences ($p \leq 0.05$) among treatments for the same vintage.
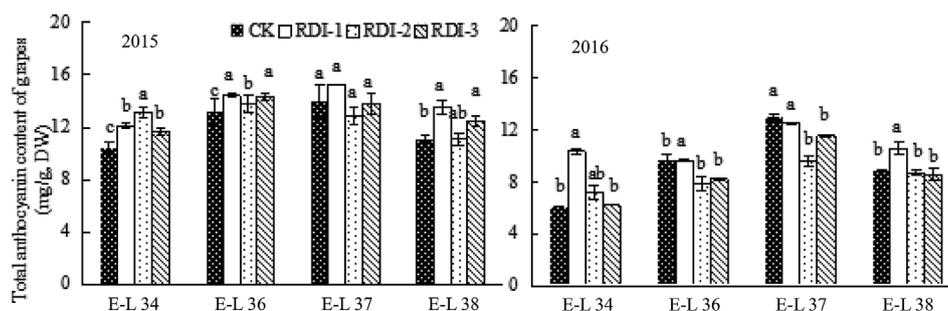

Fig. 1. Contents of total anthocyanin in grape skins (mg/g dry weight). Different letters indicate significant differences among treatments at the same date using Duncan's test ($p \leq 0.05$).

improvement of grape quality (Ji et al., 2015). RDI has a great impact on the water use efficiency (WUE), growth and development, yield, and wine quality (Ji et al., 2015; Chaves et al., 2010; Flexas et al., 2010). Zhang et al. (2014) found that RDI increased the net photosynthetic rate of grapevine. Treated with 25% ETc reduced the starch content in the leaves and increased the soluble sugar content, which led to the conclusion that RDI could significantly affect the distribution of carbon sources in soluble sugars and starches (Dayer et al., 2016). RDI also limited the vegetative growth of grapevine (Xu et al., 2015). Fang et al. (2013) found that RDI significantly reduced the incidence of shoot growth of grapevine without significant effected on Cabernet Sauvignon grape size and yield. Compared to conventional irrigation, RDI increased anthocyanins, total phenolics and total tannin contents in berries (Edwards and Clingeleffer, 2013). Basile et al. (2011) reported that water stress affected the growth and compositions of Cabernet Sauvignon grape berries and the effects were closely relative to phenological stage. Romero et al. (2010) used Monastrell grapevines as materials and conducted two RDI treatments: 30% ETc from fruit set to harvest and 45% ETc post-harvest (RDI-1); 15% ETc from fruit set to harvest (RDI-2), and the results revealed that polyphenol and anthocyanin contents decreased significantly in RDI-2 compared with RDI-1. Santesteban et al. (2011) performed two RDI strategies: RDI-1 and RDI-2 treatments were just after fruit set and, in RDI-2, vines were subjected to an additional stress period shortly after veraison, and found that RDI treated at the green stage significantly increased the contents of

anthocyanin and phenolics in Tempranillo grapes. Ju et al. (2018a) found that RDI (from fruit set to two weeks before harvest) could regulate the fatty acid composition of grape berries, and increase the aroma characteristics of berries, flowery and fruity aroma in wines in arid and semi-arid areas. In addition, studies have reported that RDI optimized the spectral properties of wines and improved the tasting properties of wines (Terry and Kurtural, 2011). Plant water potential could regulate the accumulation of metabolites and simultaneously altered the expression of key genes in the synthesis of secondary metabolites in grape berries (Zarrouk et al., 2012; Genebra et al., 2014). Some previous studies reported that water deficit could regulate total anthocyanin content in some grape varieties, including Shiraz, Cabernet Sauvignon, Chardonnay, Meski, Tempranillo and Graciano (Pinillos et al., 2016; Hochberg et al., 2015; Cáceres-Mella et al., 2017; Niculcea et al., 2015), however, much future works are needed to reveal the effects of RDI on the regulation of the accumulation and biosynthesis of anthocyanin in grape berries.

Previous studies have focused on the effects of RDI on the growth and development total phenolics, aromas of grape berries, but the effects of different irrigation practices on the accumulation and biosynthesis of anthocyanin components are not clear yet. Therefore, this work mainly studies the effect of RDI on accumulation of anthocyanin components in grape berries and wines, and studies the effect of RDI on the expressions of key genes, such as *VvPAL, VvC4H, VvCHS, VvF3′H, VvF3′5′H* etc, involved in anthocyanin biosynthesis. The results can
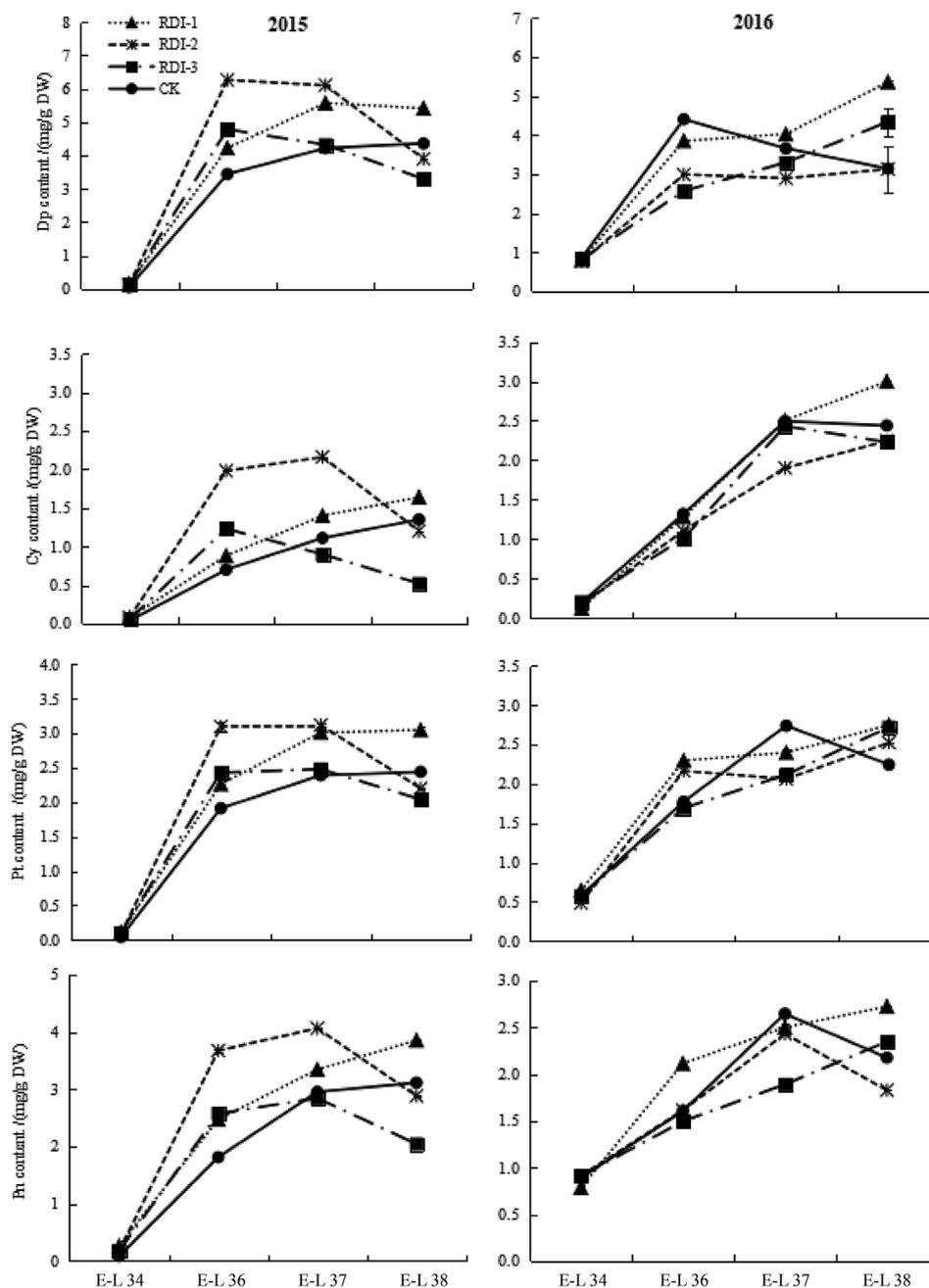
Fig. 2. Anthocyanins profiles in grape skins (mg/g dry weight). Values presented are means ± SE. Dp: Delphinidin-3-gliucoside, Cy: Cyanidin-3-glucoside, Pt: Petunidin-3-glucoside, Pn: Peonidin-3-glucoside, Mv: Malvidin-3-glucoside, Pn-ac: Peonidin-3-acetly-glucoside, Mv-ac: Malvidin-3-acetly-glucoside, Pn-c-g: Peonidin-3-coumayl-glucoside, Mv-c-g: Malvidin-3-coumayl-glucoside.

provide a theoretical basis for the application of RDI in vineyard, located in arid and semiarid regions.

2. Materials and methods

2.1. Field conditions and materials

The experiment was conducted during two consecutive seasons, 2015 and 2016, in a commercial vineyard located in Yinchuan, NingXia, China (38°34′N 106°1′E). Six-year-old own-rooted Cabernet Sauvignon (*V. Vinifera* L.) vines were used in this study. Vines were spaced at 3.0 m × 0.6 m and drip irrigated. A randomized block design was carried out with three blocks and four treatments, and each treatment in the block consisted of five hundreds individual vines. The

RDI strategies used in this study were according to our previous methods (Ju et al. 2018a, 2018b), briefly, the grapevines were watered with 60% (RDI-1), 70% (RDI-2), 80% (RDI-3), 100% (CK, traditional drip irrigation) of their estimated evapotranspiration (ETc) respectively. The amount of irrigation was controlled by controlling the irrigation time, as the vines received water with two 2.4 L/h drippers per plant and the irrigation amount was calculated by ETc according to Pennman-Monteith method (Allen et al., 1998). The treatments were conducted from fruit set until approximately two weeks prior to harvest. Six-hundred berries were sampled for each replicate at E-L 34, E-L 36, E-L 37, and E-L 38 stages according to Coombe (1995) and grapes for vinification were harvested until the berries sugar content reached 190 g/L- 230 g/L. All samples were frozen in liquid nitrogen immediately and stored in −80 °C for further analysis.
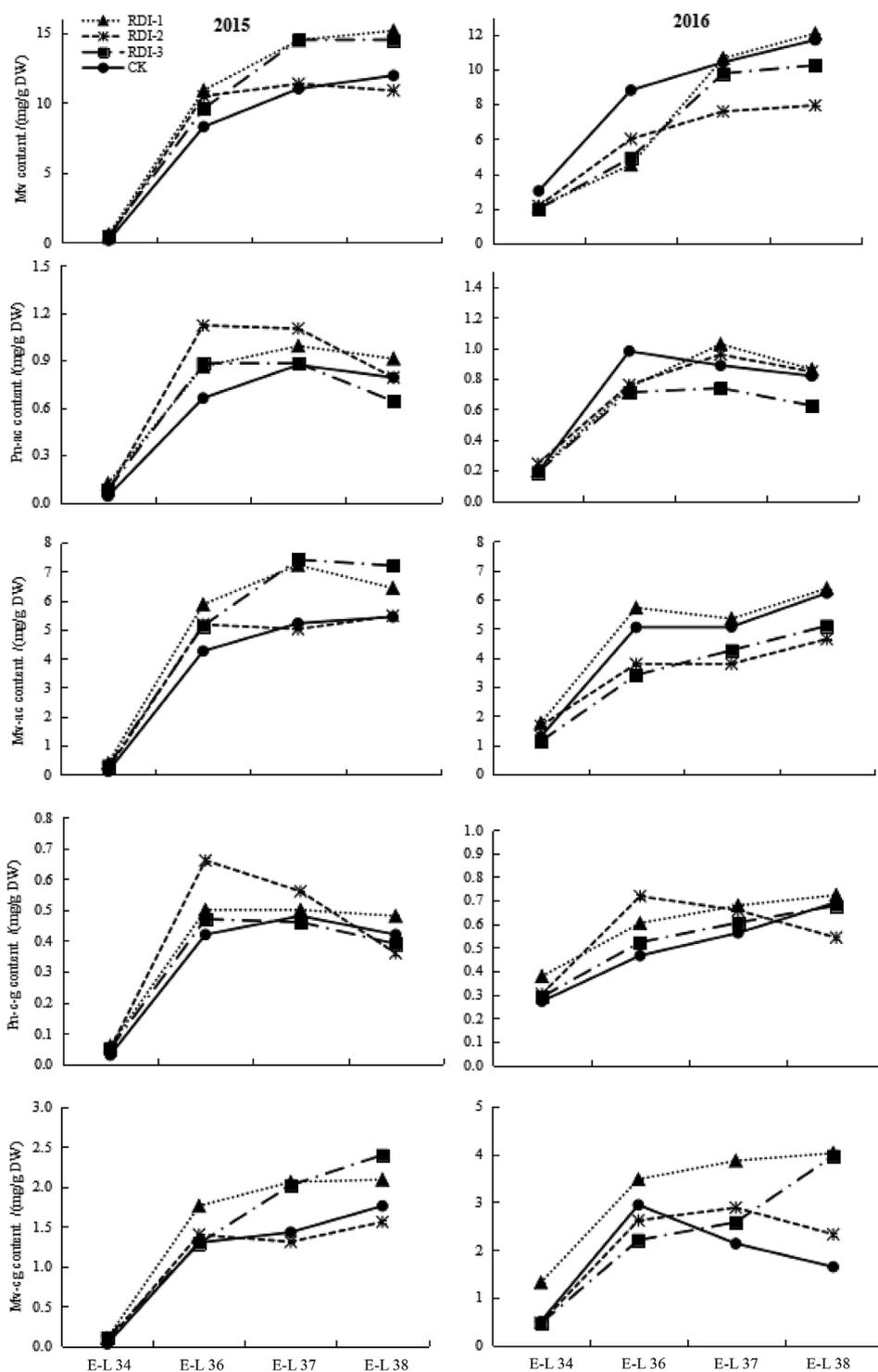
Fig. 2. (*continued*)

2.2. Vinification

One hundred kilograms of Cabernet Sauvignon grapes of each treatment were crushed and destemmed by hand, and the must was collected in a 100 L stainless-steel tanks. The must was treated with sulfur dioxide (SO_2, 50 mg/L) and pectinase (30 mg/L). Musts were inoculated 24 h after crushing and then added a commercial yeast *Saccharomyces cerevisiae* strain BO 213 (200 mg/L, Laffort, Bordeaux, France). During the maceration, the cap was punched down every 8 h and the temperature was maintained at 25 ± 1 °C, the must density were recorded. After completion of alcohol fermentation, the wines

were adjusted to 50 mg/L SO_2 and bottled to mature at 20 °C.

2.3. Determination of physicochemical indices of berries and wine

Brix values were determined with a hand-held digital Atago PAL-1 meter (Atago Co. Ltd., Japan), the titratable acidity, total sugar and alcohol of wine were determined by the method of OIV (2012), the pH was measured with a Mettler Toledo FE20 Desktop pH Meter (Mettler Toledo Instruments Co. Ltd., Shanghai, China), the 100-berry weight was recorded after washing with distilled water and drying with filter paper.
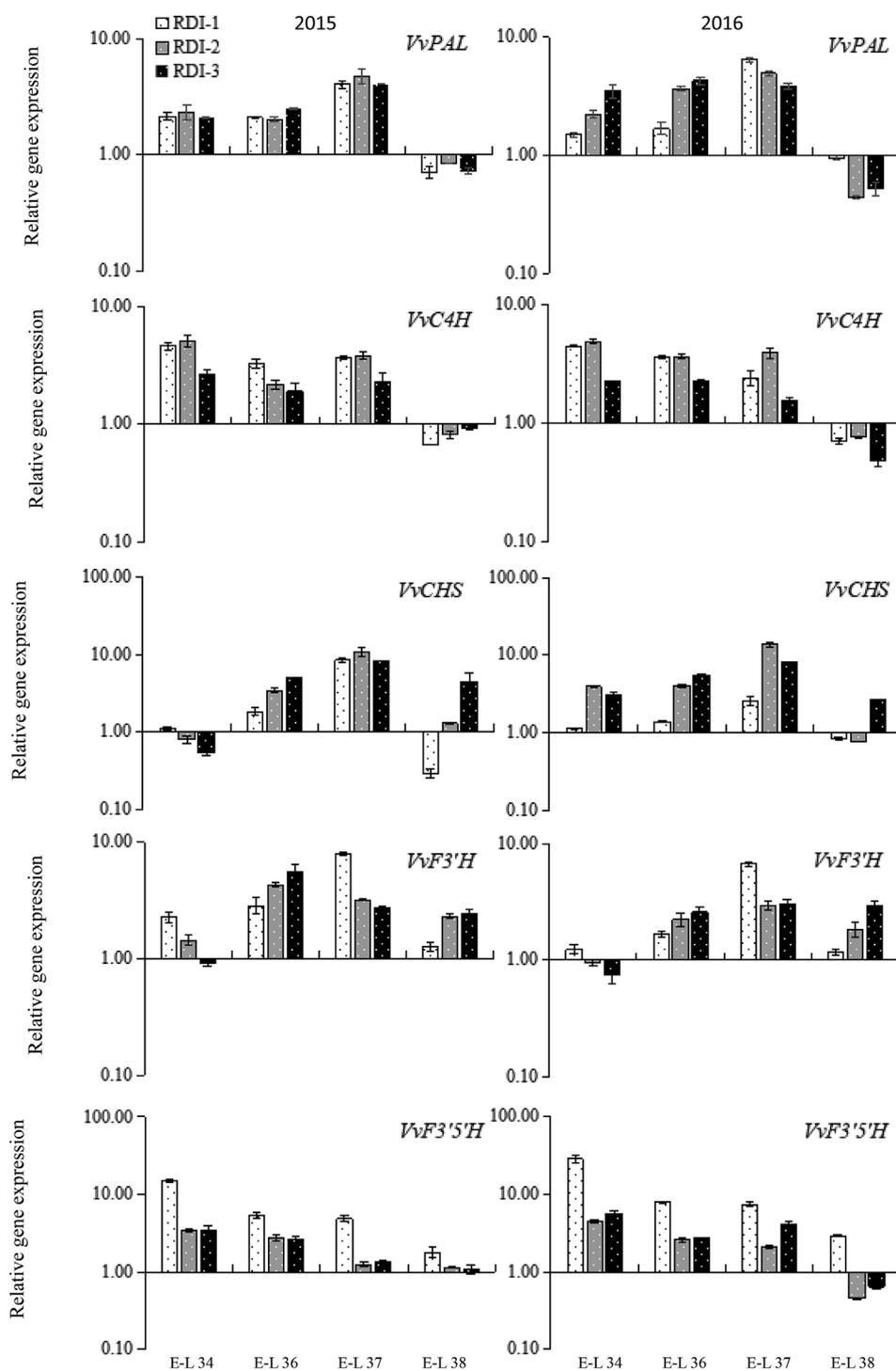
Fig. 3. Expressions of the genes *VvPAL*, *VvC4H*, *VvCHS*, *VvF3'H*, *VvF3'5'H*, *VvDFR*, *VvLDOX*, *VvUFGT*, *VvOMT*, *VvGST* in grape skins of CK and RDI treatments in 2015 and 2016 vintages. Values presented are means ± SE (n ≥ 3). CK was set as a reference group in each period, and the gene expression level was recorded as 1. In the figure, the ordinate value is multiple of the gene expression amount of each treatment group relative to the gene expression amount of the CK group, and the scale is logarithmic scale.

2.4. Determination of antthocyanins

2.4.1. Determination of total anthocyanin content (TAC) in berries and wine

The TAC was estimated according to the pH differential method reported by Meng et al. (2012).

2.4.2. Determination of individual anthocyanins
2.4.2.1. Extraction of individual anthocyanins. About 50 berries were

selected from each treatment, skins were peeled and powered under liquid nitrogen, then freeze-dried at −50 °C. The anthocyanins were extracted according to the methods of Cheng et al. (2014) with some modified, briefly, 0.50 g powder was mixed with 10 mL of HCl/methanol/water solution (1:80:19, v/v/v, 1 mol/L). The mixture was extracted under ultrasonic waves of 100 Hz for 10 min, and then shaken at a rate of 150 rpm for 30 min at 25 °C in the dark. Then, the sample was centrifuged at 10,000 rpm and 4 °C for 10 min using a high speed refrigerated centrifuge and the supernatant was collected. The residue
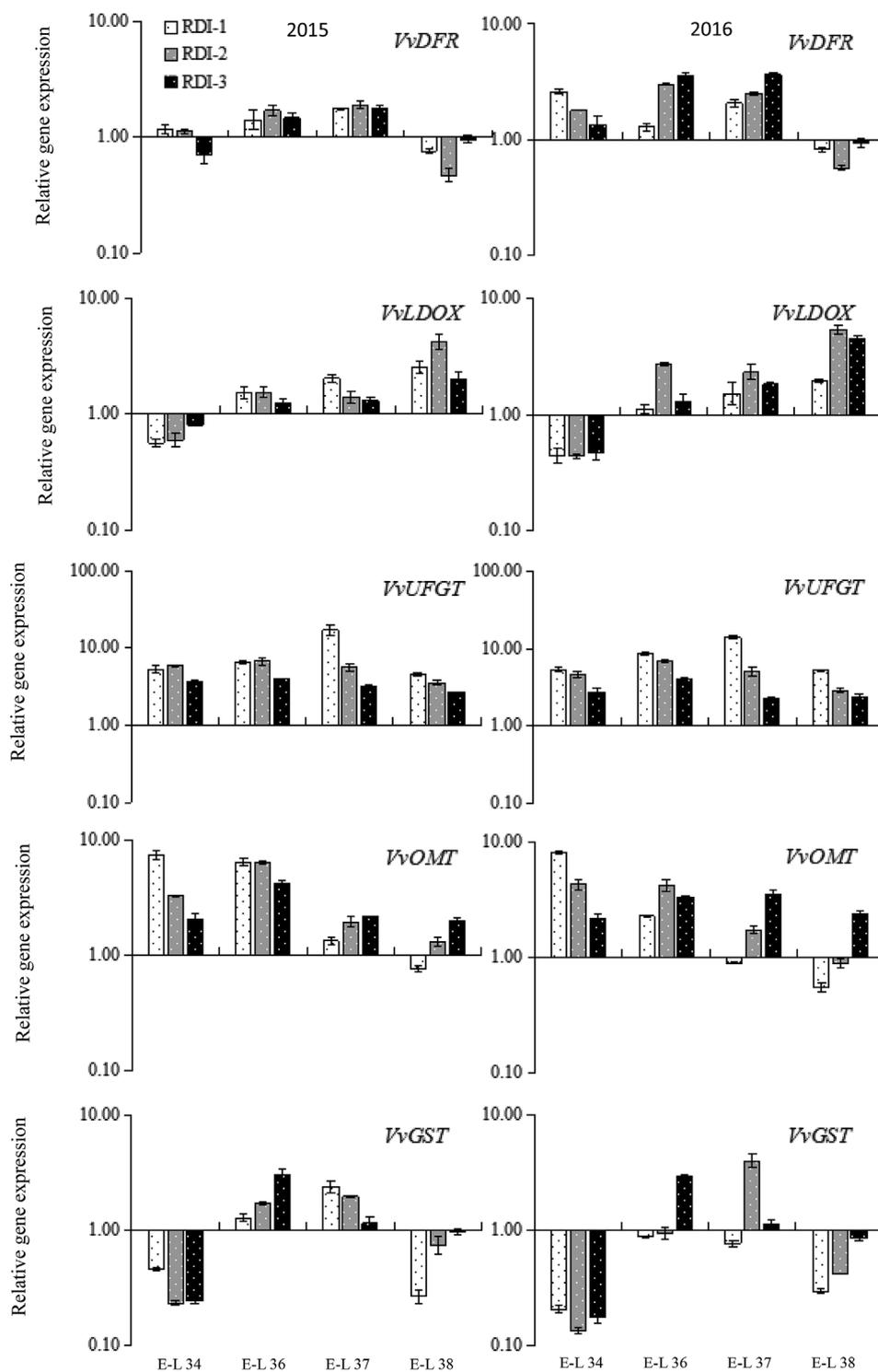
Fig. 3. (*continued*)

was re-extracted four times. Finally, all supernatants were dried using a rotary evaporator, and then redissolved in 10 mL with mobile phase A (as bellow). All extracts were filtered with 0.45 μm filter prior to HPLC analysis. All extractions have three repetitions.

2.4.2.2. HPLC analysis of individual anthocyanins. The analysis of anthocyanins was performed using the method reported by Ju et al. (2016). The high performance liquid chromatography system (HPLC, SHIMADZU, Japan) fitted with a C_{18} column (250 × 4.6 mm; Japan) was used for analysis. The column was operated at 30 °C. Phase A:

water/acetonitrile/formic acid (80:10:7 v/v/v); phase B: water/acetonitrile/formic acid (40:50:7 v/v/v). Samples were eluted according to the methods described by Wang et al. (2008) with some modifications. Briefly:a 1 mL/min flow rate was used, from 0 to 30% B in 15 min; 50% B for 25 min; 35 min, 0% B, with an injection amount of 20 μL. The anthocyanins were identified by comparing their UV-VIS spectra from 200 to 600 nm to known standards and their retention times; they were detected and quantified at 535 nm. Malvidin-3-O-glucoside (Sigma, Shanghai, China) was used as internal standard.
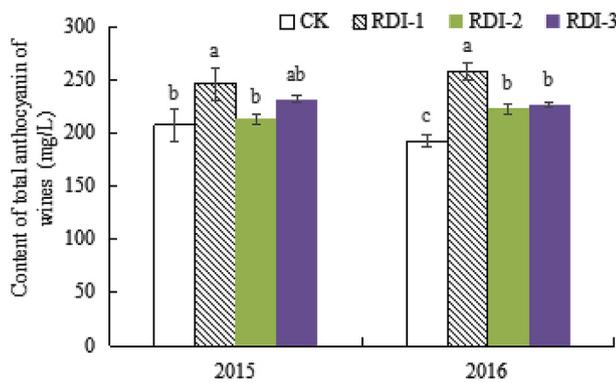
Fig. 4. Contents of total anthocyanin in wines (mg/L). Different letters indicate significant differences among treatments at the same date using Duncan's test ($p \leq 0.05$).

2.5. Expressions of anthocyanins biosynthesis genes

Expression levels of the anthocyanins biosynthesis genes including *VvPAL*, *VvC4H*, *VvCHS*, *VvF3'H*, *VvF3'5'H*, *VvDFR*, *VvLDOX*, *VvUFGT*, *VvOMT*, and *VvGST* in grape skins were measured by real-time quantitative PCR (qRT-PCR). Total RNA Extraction Kit (Bioteke # RP3302, Beijing, China) was used to extract total RNA. HiscriptIIQ RT SuperMix for qPCR (Vazyme #R223-01) was used to synthesize the first-strand cDNA. ChamQTM SYBR qPCR Master Mix (Vazyme #Q311) was used for qRT-PCR. IQ5 System (Bio-Rad, Hercules, CA, USA) was used to perform qRT-PCR. The primers used in this study were shown in Table S1. *VvGAPDH* was used as internal reference gene. qRT-PCR data was analyzed using the $2^{-\triangle\triangle CT}$ method. The reaction mixture contained 2 μL cDNA, 0.8 μL primers (10 μmol/L), 10 μL 2 × ChamQTM SYBR qPCR Master Mix, and 7.2 μL ddH$_2$O. The conditions of reaction was follows: 90 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s. Three technical replicates of each sample were analyzed for qRT-PCR.

2.6. Statistical analysis

Data analysis was performed using SPSS 17.0 software, and all data were determined in triplicate and the results were expressed as mean ± standard deviation (SD). The Duncan's t-tests of these data were used to analysis the differences between the means. Values of $p < 0.05$ were considered as significant. Heat maps were performed with the R pls package.

3. Results and discussion

3.1. Vintage and berry attributes

To examined the effect of RDI on the development of grape berries, the soluble solids, total acidity, pH, and berries weight the grapes were determined. As shown in Table 1, RDI treatments significantly increased the soluble solids of grape berries. The 100-berries weight of the RDI-1 treatment group was significantly lower than that of the control group. RDI-2 and RDI-3 had no significant effect on the 100-berries weight of the berries. In 2015 vintage, RDI-1 and RDI-2 treatments significantly reduced fruit titration acid and increased pH (Table 1). For wine, RDI treatments had no significant effect on wine residual sugar. RDI-1 and RDI-2 significantly increased the alcohol content of wine (Table 1). RDI-1 significantly reduced the titratable acid content of wine, but had no significant effect on pH, and RDI-3 treatment was significantly improved wine pH (Table 1).

Table 2
Anthocyanins profiles of wines from untreated (CK) and treated vineyards (mg/L).

Anthocyanins	2015 CK	RDI-1	RDI-2	RDI-3	2016 CK	RDI-1	RDI-2	RDI-3
Delphinidin-3-glucoside	2.39 ± 0.67b	10.16 ± 1.6a	3.56 ± 0.64b	6.07 ± 0.13b	2.69 ± 0.13c	16.01 ± 2.91a	10.83 ± 1b	10.94 ± 1.88b
Cyanidin-3-glucoside	3.31 ± 0.4ab	4.21 ± 0.6ab	4.54 ± 0.76a	2.15 ± 0.24b	1.43 ± 0.34a	2.01 ± 0.24a	1.73 ± 0.39a	1.72 ± 0.41a
Petunidin-3-glucoside	17.28 ± 1.57ab	20.51 ± 1.05a	17.47 ± 1.58ab	13.65 ± 1.1b	16.63 ± 0.18ab	19.84 ± 2.15a	15.39 ± 1.4b	17.17 ± 1.66ab
Peonidin-3-glucoside	6.85 ± 0.76a	5.31 ± 0.99a	7.6 ± 0.65a	6.23 ± 0.8a	6.2 ± 1.11ab	6.02 ± 0.65ab	4.72 ± 0.22b	6.78 ± 0.37a
Malvidin-3-glucoside	159.96 ± 11.36b	180.86 ± 13.33a	163.82 ± 7.77b	162.85 ± 6.45b	193.48 ± 17.93bc	237.93 ± 18.85a	208.9 ± 21.17b	210.3 ± 21.86b
Total Non-acylated anthocyanins	189.79 ± 11.11b	221.05 ± 2.21a	196.99 ± 1.02b	190.95 ± 1.32b	220.43 ± 1.05b	281.81 ± 3.21a	241.57 ± 2.16b	246.91 ± 1.82b
Peonidin-3-acetly-glucoside	7.72 ± 1.46a	9.94 ± 0.99a	8.26 ± 0.91a	2.93 ± 0.52b	5.83 ± 0.43ab	5.31 ± 0.43ab	6.29 ± 0.31a	4.97 ± 0.08c
Malvidin-3-acetly-glucoside	69.58 ± 10.68b	87.56 ± 6.04a	85.12 ± 4.51a	92.68 ± 6.51a	81.82 ± 7.38b	97.57 ± 11.24a	86.51 ± 11.41ab	84.39 ± 7.15b
Peonidin-3-coumaryl-glucoside	2.99 ± 0.56a	2.87 ± 0.58a	2.49 ± 0.33a	1.99 ± 0.27a	1.57 ± 0.41a	1.73 ± 0.39a	2.12 ± 0.47a	1.36 ± 0.13a
Malvidin-3-coumayl-glucoside	14.81 ± 1.86a	12.41 ± 0.68a	11.12 ± 0.99a	13.47 ± 2.06a	13.63 ± 0.85b	19.08 ± 5.95a	17.93 ± 7.97a	14.11 ± 2.14b
Total Acylated anthocyanins	95.1 ± 1.03b	112.78 ± 1.67a	106.99 ± 1.05a	111.07 ± 2.04a	102.85 ± 1.34b	123.69 ± 2.13a	112.85 ± 1.08a	104.83 ± 2.01b

Different letters in the row indicate significant differences ($p \leq 0.05$) among treatments for the same vintage.
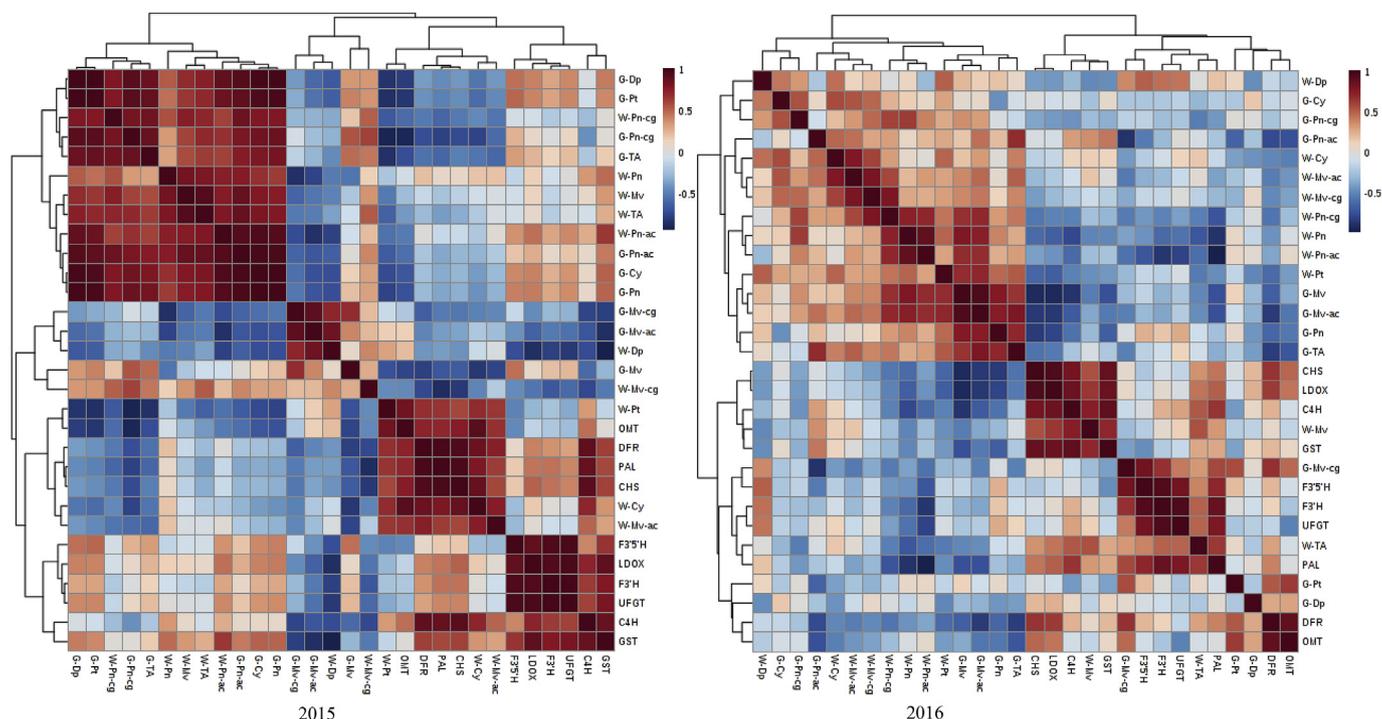
Fig. 5. Correlation analysis of anthocyanins and genes expression in this study from grapes and wines with different treatments. Data was normalized by a pooled sample from control groups.

3.2. Effects of RDI treatments on total anthocyanin content (TAC) in grape skins

As Fig. 1 showed, in 2015 vintage, the total anthocyanin content gradually increased from the E-L 34 stage to the E-L37 stage, and decreased from the E-L 34 to the E-L 38 stage. At the E-L 38 stage, the total anthocyanin contents of RDI-1 and RDI-3 treatments groups were significantly higher than the control group. In 2016 vintage, the RDI-1 treatment group was significantly higher than the other treatments groups at the E-L 34 stage. The total anthocyanin content in the control group and the RDI-1 group reached the maximum at the E-L 37 stage. At the E-L 38 stage, the total anthocyanin content in the RDI-1 treatment group was significantly higher than that of the control group, but the RDI-2 and RDI-3 groups were not significantly different from the control group. Above all, the RDI-1 treatment significantly increased the total anthocyanin content in grape skin in both two vintage.

3.3. Effects of RDI treatments on anthocyanins profiles of grape skins

A total of 9 kinds of anthocyanins including Dp: Delphinidin-3-gliucoside, Cy: Cyanidin-3-glucoside, Pt: Petunidin-3-glucoside, Pn: Peonidin-3-glucoside, Mv: Malvidin-3-glucoside, Pn-ac: Peonidin-3-acetly-glucoside, Mv-ac: Malvidin-3-acetly-glucoside, Pn-cg: Peonidin-3-coumayl-glucoside, Mv-cg: Malvidin-3-coumayl-glucoside were detected. As Fig. 2 shown that all kinds of individual anthocyanins began to accumulate rapidly at the E-L 34 stage. In the mature red (*Lacryma*) or white pulp (*Gamay*) fruit, the anthocyanin content decreased, probably due to covalent attachment to glutathione transport to vacuoles under GST catalysis (Ageorges et al., 2006). From the E-L 34 stage to the E-L 37 stage, RDI treatments groups had higher anthocyanin contents than control group (Fig. 2). In the mature fruit, RDI-1 treatment increased the contents of all kinds of individual anthocyanins in two vintages, which indicated that severe water shortage promotes the accumulation of anthocyanins in grape skin (Fig. 2). In the mature fruit, RDI-2 treatment increased the content of Pt and the Mv-cg in 2016 vintage, however, other anthocyanin levels were not significantly

different from the control group (Fig. 2). In 2015 vintage, RDI-3 treatment significantly increased the contents of Mv, Mv-ac, and Mv-cg in grape skins. In 2016 vintage, RDI-3 treatment significantly increased the contents of Dp, Pn, and Mv-cg in grape skins (Fig. 2). RDI treatments increased the contents of glycosides anthocyanins in fruit, which might be beneficial to increase color stability. The difference in rainfall between 2015 and 2016 vintages might be the cause of the difference in anthocyanin accumulation (Table S2). In 2016 vintage, the rainfall was less before veraison, and the rainfall was more during veraison, which may be the reason for the earlier and longer time for veraison (Table S2).

3.4. Relative expression of anthocyanins biosynthesis genes

In order to better understand the effect of RDI on the anthocyanin biosynthesis in gape skins, the relative expression levels of anthocyanins biosynthesis genes, including *VvPAL*, *VvC4H*, *VvCHS*, *VvF3′H*, *VvF3′5′H*, *VvDFR*, *VvLDOX*, *VvUFGT*, *VvOMT* and *VvGST*, were determined. PAL, C4H and CHS enzymes act on the upstream of anthocyanin synthesis pathway. PAL is one of most important enzymes in the phenylalanine metabolic pathway, and its expression was not significantly different among different tissues of grape fruit, but the difference in its expression between different varieties (including *V. vinifera*, Ruby Okuyama (Ru) and Flame Muscat (Fl)) was significant (Boss et al., 1996a; Kobayashi et al., 2004). While CHS catalyzes the formation of chalcone, which is the first step in the metabolic pathway of flavonoids (Boss et al., 1996b; Castellarin and Di, 2007; Castellarin et al., 2010; Azuma et al., 2012). Therefore, *VvPAL*, *VvC4H*, and *VvCHS* are key genes for anthocyanin synthesis. Compared with the control group, RDI treatments increased the expressions of *VvPAL* and *VvC4H* at E-L 34, E-L 36, and E-L 37 stage in both two years (Fig. 3). The expression of *VvCHS* was significantly higher in RDI treatments groups than that of control groups at E-L 36 and E-L 37 in both two years (Fig. 3). The up-regulation of these genes might be one of the important reasons for the total anthocyanin contents were higher in RDI treatments groups than that of the control group.

F3′H and F3′5′H enzymes act on the precursor flavonoids of anthocyanins, and previous studies have shown that these two enzymes directly affect the type of group of 3′ and 5′ of anthocyanin B ring, and thus affect the type and proportion of individual anthocyanins (Sophie et al., 2006; Holton and Cornish, 1995). The expression of *VvF3′H* was up-regulated at the E-L 36, E-L 37 and E-L 38 stage in RDI treatments groups, while the expression of *VvF3′5′H* was up-regulated at the E-L 34, E-L 36, and E-L 37 stage, especially in RDI-1 treatment group the expression level was up-regulated more significantly in both vintages (Fig. 3). The expression of *VvF3′5′H* was significantly increased during veraison, which is consistent with previous studies (Jeong et al., 2006; Bogs et al., 2006). At the E-L 38 stage, the expression of *VvF3′5′H* was decreased in RDI-2 and RDI-3 treatment groups in 2016 vintage (Fig. 3). These results indicated that RDI might result in changes in the composition and proportion of individual anthocyanins in the skin when the grapes mature. The changes in Cy and Dp contents after RDI-1 treatment may be closely related to the changes of the expression levels of *VvF3′H* and *VvF3′5′H* (Castellarin et al., 2007).

DFR and LDOX are important synthesis enzymes in anthocyanin synthsis pathway (Boss et al., 1996b; Bogs et al., 2006). *DFR* and *LDOX* genes are highly expressed in leaves, seeds and tendrils of plants, but are weakly expressed in the skin and pulp at maturity (Boss et al., 1996b). The expression of *VvDFR* was up-regulated from the E-L 34 to E-L 37 after RDI-1 and RDI-2 treatments in both two vintages (Fig. 3). However, the expression of *VvDFR* was down-regulated at the E-L 38 stage, and the expression of *VvDFR* in RDI-2 group was the most significantly down-regulated (Fig. 3). Compared to control group, the expression of *VvLDOX* was down-regulated at the E-L 34 stage in RDI treatments groups (Fig. 3). After veraison, the expression of *VvLDOX* was up-regulated in RDI treatments groups. At the E-L 38 stage, the expression of *VvLDOX* reached the highest level, and the expression of *VvDOX* in RDI-2 group was the most significantly up-regulated (Fig. 3).

From the E-L 34 to the E-L 38 stage, *VvUFGT* was up-regulated in RDI treatments groups, and the up-regulation was the most significant in the RDI-1 group (Fig. 3). *VvUFGT* plays an important role in the synthesis of anthocyanins (Kobayashi et al., 2001). The up-regulation of *VvUFGT* in grape skin from veraison to maturity might also be one of the important reasons for the increase of total anthocyanin contents after RDI treatments (Fig. 3). Previous studies have shown that water and temperature factors could affect the expression of *VvUFGT* (Castellarin et al., 2010). The expression levels of *VvOMT* in the fruit of RDI groups were increased at the early stage of veraison, and the expression of *VvOMT* in RDI-1 group was the most significantly up-regulated (Fig. 3). The increase in Pt and Mv-c-g content in RDI-1 treatments might be related to the up-regulated expression of *VvOMT*. At the same time, the expression of *OMT* gene leads to O-methylation of the anthocyanin skeleton structure, which changes its structural polarity and thus affects its compartmentalization in cells (Castellarin et al. 2007, 2010; Kim et al., 2006). Compared with the control group, the expression of *VvGST* showed down-regulated at the E-L 34 and E-L 38 stage in RDI treatments groups (Fig. 3). At the E-L 36 and E-L 37 stage, the expression level of this gene was up-regulated in 2015 vintage (Fig. 3).

3.5. Effect of RDI treatments on total anthocyanin contents (TAC) and anthocyanins profiles in wines

Anthocyanins affect the color and stability of wine directly. The content of anthocyanin in wine has an important influence on the sensory evaluation and quality of wine. Total anthocyanin contents were significantly higher in RDI treatments groups than that of control group in 2015 and 2016 wines (Fig. 4). The RDI-1 treatment had the highest total anthocyanin content in wines in both two vintages (Fig. 4).

The anthocyanin compounds in wines are mainly derived from grape berries. Therefore, there is a correlation of the individual anthocyanin contents between grape berries and wines. In this study, the

contents of five non-acylated individual anthocyanins and four acylated individual anthocyanins in wines was also determined (Table 2). Compared to control group, RDI treatments were significantly increased the concentration of Dp in wines of 2015 and 2016, and RDI-1 treatment had the highest Dp content in both vintages (Table 2). The content of Cy in wines increased after RDI-1 and RDI-2 treatments, but no significant differences were observed (Table 2). Mv occupied the most contents of non-acylated anthocyanin, which performed higher in RDI treatments than control group in 2015 and 2016 wines, and RDI-1 treatment had the highest Mv content in both vintages (Table 2). Mv-ac occupied the most content of acylated anthocyanin in wine which is acylated by Mv and anthocyanin acyl transferase (Nakayama et al., 2003). Wines from RDI treatments had higher content of Mv-ac than wines from control treatment in both vintages (Table 2). Wines from RDI-3 had the highest content of Mv-ac in 2015, while wines from RDI-1 had the highest content of Mv-ac in 2016 (Table 2). In both 2015 and 2016 vintages, the total non-acylated anthocyanins contents of RDI wines were higher than that of control wines and RDI-1 had the highest content (Table 2). What is more, RDI-1 and RDI-2 treatments wines showed higher content of total acylated anthocyanins than control wines in both two vintages and RDI-1 treatments wines had the highest content of total acylated anthocyanins (Table 2). RDI-3 treatment was significantly increased the content of total acylated anthocyanins in 2015 wine, but no difference in 2016 wine (Table 2).

3.6. The correlation analysis between gene expression and anthocyanins in grapes and wines

The correlation analysis of individual anthocyanins and structural anthocyanin relative genes expression in grape berries and wines with different treatments was shown in Fig. 5. There was a positive correlation between Pn content in wines and Pn content in grape berries. The same phenomenon was observed for Pn-ac and Pn-cg in both two vintages. It was further suggested that the contents of individual anthocyanins composition in wines were closely related to grape berries.

The accumulation of individual anthocyanins in grape berry was regulated by structural genes in the anthocyanin synthesis pathway. The accumulation of Pn and Pn-ac had a positive correlation with its synthetic upstream key enzymes F3′H and UFGT (Gong et al., 1997). In this study, the accumulation of Pn, Pn-ac and Pn-cg in grape berries showed closely correlation with the expression of its upstream key enzyme synthesis genes, including *VvF3′H* and *VvUFGT* (Fig. 5). The accumulation of Pt in grape berries performed high correlation with the expression of *VvF3′5′H*, which was consistent with the results of Jeong et al. (2006). The higher contents of Pn and Pn-cg in RDI-1 treatment group were consistent with the higher relative expressions of *VvF3′H* and *VvUFGT* in RDI-1 treatment group. As a result, the up-regulation of the expression of key genes leads to an increase in the contents of related individual anthocyanins in grape berries, which gave rise to an increase in the contents of Pn and Pn-cg in RDI-1 wines. The expressions of genes could be affected by many external environmental factors, such as moisture and temperature (Castellarin et al., 2010). The change of experimental water quantity will also lead to the different growth quality of plant root, which will change the soil micro-environment of the root part and finally affect the composition of grape berries (Santos et al., 2007). Regulatory genes could regulate the specific expression of related structural genes during anthocyanin biosynthesis, which in turn affects anthocyanin accumulation and composition (Holton and Cornish, 1995). As present results, RDI treatments regulated the accumulation of anthocyanins by regulating genes expressions in the anthocyanin synthesis pathway.

4. Conclusions

The effects of RDI treatments on the accumulation and biosynthesis of anthocyanins in Cabernet Sauvignon grapes and wines were

evaluated for two consecutive vintages. RDI-1 treatment significantly increased the total soluble solids and decreased berry weight, while RDI-2 and RDI-3 had no significant effects. RDI-1 treated grapes and wines had the highest total anthocyanins contents compared to that of RDI-2 and RDI-3 treatments. RDI-1 treatment enhanced all kinds of individual anthocyanins in grape berries and wines in both two vintages. RDI-2 treatments increased the contents of Pt and the Mv-cg in berries and RDI-3 treatments significantly increased the contents of Mv, Dp, Mv-ac, and Mv-cg in grape berries. RDI-1 and RDI-2 enhanced the total non-acylated anthocyanins and total acylated anthocyanins contents in wines of both vintages. RDI treatments upregulated the expressions of key genes involved in anthocyanins biosynthesis pathway in both two vintages. Correlation analysis showed that the accumulation of anthocyanins was closely related to the key genes expressions, including *VvPAL, VvF3'H, VvF3'5'H* etc. Overall, RDI treatments regulated the accumulation of anthocyanins by regulating genes expressions in the anthocyanin synthesis pathway.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Yan-lun Ju: Writing – original draft, Data curation, Writing – review & editing. **Bo-han Yang:** Writing – original draft, Data curation. **Shuang He:** Investigation, Methodology. **Ting-yao Tu:** Investigation, Methodology. **Zhuo Min:** Writing – review & editing. **Yu-lin Fang:** Resources, Supervision, Writing – review & editing. **Xiang-yu Sun:** Resources, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2018.11.013.

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