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Research article

Impact of cluster thinning on transcriptional regulation of anthocyanin biosynthesis-related genes in 'Summer Black' grapes

Xiaojun Xi, Qian Zha, Aili Jiang*, Yihua Tian

Research Institute of Forestry and Pomology, Shanghai Academy of Agricultural Science, Shanghai, 201403, China

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ABSTRACT

Cluster thinning is an agronomic practice that strongly affects anthocyanin biosynthesis in the skin of grape berries. However, the impact of cluster thinning on anthocyanin biosynthesis has not been fully elucidated at the molecular level. Here, we investigated its effects on the berry quality, the biosynthesis of anthocyanins, and the expression levels of related genes from the onset of véraison to harvest in 'Summer Black' grapes. It was observed that the total soluble solid and anthocyanin content in berry skin significantly increased under cluster thinning, whereas the berry weight and titratable acidity showed no differences from the beginning of véraison to harvest. The expression level of most anthocyanin biosynthesis-related genes was significantly up-regulated by cluster thinning from the beginning of véraison and was higher at its end compared to the control. Up-regulation of *flavonoid 3′,5′-hydroxylase* (*F3′5′H*) and *O-methyltransferase* (*OMT*) expression, and down-regulation of *flavonoid 3′-hydroxylase* (*F3′H*) expression were observed, which might be the cause of shift in the anthocyanin profile. These findings provide insights into the molecular basis of the relationship between cluster thinning and anthocyanin biosynthesis in the grape berry skin.

1. Introduction

Consumption of anthocyanins provides health benefits associated with scavenging of free radicals and has protective effects against cardiovascular diseases and cancer (Lin and Weng, 2006). Red grapes and wines are particularly abundant in the bioavailable anthocyanins that are rapidly absorbed as intact molecules and quickly delivered to the brain after ingestion (Passamonti et al., 2005). Moreover, skin color of grapes, which is mainly determined by the content and composition of anthocyanins (Baranac et al., 1997), is one of the most important quality characters that determine its market value. Therefore, there is a great interest in promoting the anthocyanin biosynthesis during grape berry development, for both economic and health reasons.

However, in Shanghai, southeastern China, table grapes such as 'Summer Black', 'Kyoho' fail to achieve the desired level of red color, probably as a consequence of high temperatures and rainy climate in the summertime, which inhibit the accumulation of anthocyanins (Mori et al., 2007; Chorti et al., 2010). In view of this, many agronomic practices are employed to enhance the anthocyanin biosynthesis for high-quality grape production. Among them, fruit load reduction by cluster thinning during berry development, which directly increases the source/sink balance in the grapevines, is one of the most ecological and economical cultivation approaches to improve the grape quality. In previous studies, increasing of soluble sugars in berries was achieved following cluster thinning in different varieties grown at different sites (Guidoni et al., 2002, 2008; Keller et al., 2005; Pastore et al., 2011). Significant increases in the anthocyanin content in berry skins and effects on the anthocyanin profiles have also been reported (Guidoni et al., 2008; Pastore et al., 2011).

Anthocyanins are synthesized via the flavonoid biosynthetic pathway in grapes. In grapevine, the core structural genes encoding anthocyanin biosynthetic enzymes have been cloned and characterized (Sparvoli et al., 1994). Among them, the expression of the gene for UDP-glucose: flavonoid 3-O-glucosyltransferase (*UFGT*) is

Abbreviation: DAA, Days after anthesis; HPLC, high performance liquid chromatography; TSS, Total soluble solid; TA, Titratable acidity; PAD, photodiode array detector; CHS, Chalcone synthase; CHI, Chalcone isomerase; F3H, Flavanone 3-hydroxylase; F3′H, Flavonoid 3′-hydroxylase; F3′5′H, Flavonoid 3′,5′-hydroxylase; DFR, Di-hydroflavonol 4-reductase; LDOX, Leucoanthocyanidin dioxygenase; OMT, O-Methyltransferase; UFGT, UDP-Glucose:flavonoid 3-O-glucosyltransferase; GST, Glutathione-S-transferase; antho-MATE, anthocyanin multidrug and toxic extrusion.

* Corresponding author.
E-mail address: putaojal@163.com (A. Jiang).

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critical for anthocyanin biosynthesis (Boss et al., 1996). Recently, two anthocyanin O-methyltransferases (*AOMT* and *FAOMT*) with ability to methylate anthocyanins *in vitro* and *in vivo*, were identified (Hugueney et al., 2009; Lücker et al., 2010). The degree of 3′- and 5′-methylation of anthocyanins has been explained by the expression level of O-methyltransferases (*OMTs*; Fournier-Level et al., 2011). In addition, anthocyanin biosynthesis is controlled by *MYB*-related transcription factors that activate the expression of structural genes involved in the late steps of the pathway (Kobayashi et al., 2002; Ramsay and Glover, 2005). In particular, the *VvMYBA* genes induce the transcription of *UFGT* and *AOMT* in colored tissues (Cutanda-Perez et al., 2009) and control the anthocyanin accumulation in grapes (Fournier-Level et al., 2011). Two *MYB*-related transcription factor genes that regulate anthocyanin biosynthesis, *VvMYBA1* and *VvMYBA2*, have been isolated from *Vitis vinifera* grapes (Kobayashi et al., 2004; Walker et al., 2007), whereas three functional *MYB*-related genes, (*VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2*) are present in the *Vitis* × *labruscana* genome (Azuma et al., 2011; Kobayashi et al., 2002). Furthermore, *MYB5a*, *MYB5b*, *MYBPA1*, and *MYBPA2* were shown to regulate several genes in the common steps of the flavonoid pathway (Bogs et al., 2007; Deluc et al., 2008; Terrier et al., 2009).

At present, little is known about the molecular mechanisms of anthocyanin biosynthesis when the source/sink ratio is deliberately altered in the field by cluster thinning. In order to increase the berry anthocyanin content in the vineyard, it is fundamental to understand the biosynthesis of these molecules and decipher how the anthocyanin content is affected by this viticultural practice. The main objective of this study was to elucidate the mechanisms leading to the changes in anthocyanin content, in response to cluster thinning.

2. Materials and methods

2.1. Plant material

Experiments were conducted in 2013 on adult 'Summer Black' (*Vitis* × *labruscana* L.) vines, in the greenhouse located in the Shanghai Academy of Agricultural Sciences, Shanghai, China (30°51′N, 121°13′E). All grapevines were placed at a spacing of 2 × 2.8 m in north–south oriented rows and the shoots were trained vertically with 16 shoots per vine. Nine vines per treatment, with the same cluster number at flowering (16 per vine and 60 berries per cluster), were selected in a single uniform row and each vine was randomly assigned to the following two groups: control (no treatment) or cluster thinning [removal of 50% of the total clusters per vine at 56 days after anthesis (DAA)]. The cluster thinning treatment was conducted 7 days before the onset of véraison which occurred at 63 DAA in 2013. As 'Summer Black' grape is a seedless cultivar, 25 and 50 mg/L gibberellic acid was sprayed, first at the time of anthesis and again 10 days later. Pest, nutrition, and water management was the same for the two treatments, which were carried out according to the Shanghai local practices.

2.2. Berry sampling

For each treatment, 30 berries at different positions (top, middle, and bottom) were randomly collected in replicates. Three replicate samples from each treatment were individually collected at seven-day intervals, from the beginning of véraison to harvest. The samples were divided in three parts; 30 berries were processed immediately to monitor berry ripening, 30 were frozen at −20 °C for analysis of anthocyanins by high performance liquid chromatography (HPLC), and the rest were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction.

2.3. Measurement of vine growth, berry growth, and quality

Ten berries from each replicate were weighed on an analytical balance. Total soluble solid (TSS) and titratable acidity (TA) were measured in the juice obtained by crushing the berries of each replicate. TSS was determined by a refractometer (Master-M, Atago, Tokyo, Japan) and expressed as °Brix. TA was measured by titration with 0.1 N NaOH and expressed as % (g tartaric acid/100 ml juice).

The extraction of anthocyanin was performed according to Liang et al. (2011). Frozen berry skin samples were ground in liquid N2 using a mortar and pestle. Then 0.5 g powdery samples were weight and placed in 1.5 ml of extract solution (2:28:70, formic acid:water:methanol). The extracts were shaken for 10 min in a thermo mixer (Eppendorf, USA). Then the extracts were centrifuged at 13,000 g at 4 °C for 10 min. About 1 ml of extract was filtered through a 0.45 μm Millipore filter for analysis. The high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) analysis of anthocyanins was performed according to Liang et al. (2011) using a Waters Acquity UHPLC system (Waters, Milford, MA, USA). MS/MS detection was carried out on a Waters XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) source operated in both positive and negative ionisation modes. The source parameters were set as follows: source temperature of 150 °C; desolvation temperature of 500 °C. The cone and desolvation gas flows were 30 and 1000 L/h, respectively. Multiple reaction monitoring (MRM) mode was developed for quantification of the targeted analyses, and the conditions were optimized for each analyte during infusion. Statistical analysis was performed using MassLynx v4.1 and Targetlynx (Waters). A Waters Atlantis® T3 (Milford, MA, USA) reverse-phase C18 column (250 mm × 4.6 mm, 5 μM) was used for the analysis. The mobile phase consisted of water-formic acid (90:10) as solvent A, and acetonitrile-formic acid (90:10) as solvent B. The gradient profile began at 95% A, to 85% A at 25 min, 73% A at 53 min, then A went back to 95% at 57 min, and was kept there for 5 min. The flow rate was 1.0 ml min^{-1} and the column temperature was set at 30 °C. The injection volume was 20 μl.

The same HPLC protocol was used by the Waters E2695 instrument (Milford, MA, USA) equipped with a 2998 photodiode array detector (PAD). Anthocyanins were identified according to their retention time, molecular and ion fragments weight of their standards as well as the published data (Liang et al., 2011, 2012). Total anthocyanin content was quantified using malvidin 3,5-O-diglucoside chloride (Sigma–Aldrich, St. Louis, MO, USA) as a standard and the quantification equation was as follows: concentration (mg g^{-1}) = 0.003 × [(Area+40,284)/5694.6], with an $r^2 = 0.999$.

2.4. qRT-PCR

Total RNA was extracted using the E.Z.N.A.® Plant RNA Kit (Omega, Bio-Tek, Doraville, GA, USA) according to the manufacturer's instructions. The concentration of each RNA sample was determined using a Nanodrop 2000 instrument (Thermo Scientific, Wilmington, DE, USA). First strand cDNA was synthesized using 1 μg of total RNA as the template and Takara PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Primer sequences were obtained from the literature and amplification efficiencies were tested for the 'Summer Black' samples (Additional File 1). Quantitative real-time PCR (qRT-PCR) was carried out on a LightCycler 480 System (Roche, Mannheim, Germany) using Takara SYBR Premix Ex Taq™ II (Tli RNaseH Plus; Takara, Dalian, China).