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Anthocyanin biosynthesis during berry development in corvina grape

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ABSTRACT

For the production of a highly qualitative wine, such as "Amarone", variations in the grape (Corvina) pigment composition are not negligible. The aim of this work was the investigation of the Corvina anthocyanin profile changes during ripening. The experiment took place in 2015, in two vineyards located in Valpolicella (Italy) with different training systems (Guyot and pergola). Micro-meteorological survey, carpological characterization, chemical analysis and transcriptional studies were carried out to clarify the anthocyanin biosynthesis regulation. Air and grape temperatures and global solar radiation inside the canopy were measured during the season. Grape samples were collected at seven stages of berry development from bunches hang until harvest. Berry growth was followed by volume increase. At the beginning of the lag phase, anthocyanin quantification and characterization by spectroscopy and HPLC were carried out. The expression level of structural and regulatory genes of anthocyanin pathway was studied *via* real-time RT-PCR. At color appearance, the di-substituted anthocyanins prevailed. During ripening, it was observed a progressive increase of tri-substituted, methoxylated, and acylated pigments. A clear correlation among expression of anthocyanin biosynthetic gene, UDPglucose:flavonoid 3-*O*-glucosyltransferase (UFGT), transcription factors, MybA1 and MybA2, and total anthocyanin content during berry development has been identified. Chalcone synthase, flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) genes of the flavonoid pathway showed high correlation as well. The proportion changes between tri- and di-substituted anthocyanins were associated with an increasing ratio of F3′5′H/F3′H gene transcription during ripening. The AOMT genes were expressed with a maximum at the onset of ripening, coherently with the rapid increase of methoxylated anthocyanin proportion in this stage.

1. Introduction

"Amarone", a renowned Italian red wine, is produced by semi-dehydrated grapes. This process is usually carried out after harvesting, under controlled or semi-controlled conditions. Thus, winemakers have a higher flexibility in the harvesting date: the desired equilibrium between sugar, acidity and secondary metabolites is reached by the sum of two different processes (ripening and dehydration) (Failla et al., 2013). For the production of a highly qualitative wine, such as "Amarone", secondary metabolites, including pigments, have a major importance. Grape anthocyanin content and composition could affect the quality and the production strategies of red wines. In fact, differences in the pigment composition modify the color properties in terms of hue (Rustioni et al., 2013a), extractability (Rustioni et al., 2011) and stability (García-Beneytez et al., 2002).

Corvina is the major cultivar used in the production of "Amarone". This grape variety has been already studied (Toffali et al., 2011; Mattivi et al., 2006), however a clear description of the anthocyanin accumulation and the pigment profile changes during ripening is still missing, despite its useful application as a support for the harvest-time decisions.

In anthocyanins, the chromophore is represented by the aglycone anthocyanidin moiety. Nevertheless, due to the limited solubility in water, the rapid degradation by alkali, and the high instability of these compounds (Dao et al., 1998), in nature they are found in their heteroside forms. The detection of free aglycones in plant samples is very uncommon (Macz-Pop et al., 2006), and, thus, the pigment stabilization by glycosylation play a fundamental role in anthocyanins accumulation. As a matter of fact, Boss et al. (1996a) found that the major control point of anthocyanin pathway is UFGT (UDP-glucose:flavonoid 3-*O*-glucosyltransferase) gene, showing an independent regulation in respect to the other genes involved in the pigment biosynthesis. The regulation of this gene is governed by

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different transcription factors: VvMybA1 and VvMybA2 (Ageorges et al., 2006; Walker et al., 2007), two very similar adjacent genes that induce UFGT transcription, and VvMyb5b, showing a marginal effect onto UFGT activation (Deluc et al., 2008). The expression of other genes upstream to the UGFT, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX) was detected up to four weeks post-flowering and their expression increased at the onset of *veraison* (Boss et al., 1996a; da Silva et al., 2005).

Both anthocyanin concentration and profile affect the wine properties. For example, an increased number of substituents in the B ring, as well as their methoxylation, results in a bathochromic shift of the main absorption band (Rustioni et al., 2013a). In grapes, the main anthocyanidins synthesized are: cyanidin and peonidin (di-substituted in the lateral B ring); and delphinidin, petunidin and malvidin (tri-substituted). The di-substituted anthocyanins are synthetized *via* flavonoid 3′-hydroxylase (F3′H), while the tri-substituted anthocyanins *via* flavonoid 3′,5′-hydroxylase (F3′5′H; Boss et al., 1996b) and their expression is consistent with the kinetics of accumulation of di-substituted and tri-substituted anthocyanins during the berry ripening (Castellarin et al., 2006; Jeong et al., 2006). Regarding the anthocyanin methoxylation, three AOMT (O-methyltransferases) that catalyze the conversion of cyanidin-3-monoglucoside into peonidin-3-monoglucoside and delphinidin-3-monoglucoside into petunidin- and malvidin-3-monoglucoside have been identified, but only two, AOMT1 and AOMT2, are shown to be expressed in mature grape berries (Fournier-Level et al., 2011).

In grapes, the sugar moiety could also be esterified by acetic, *p*-coumaric or caffeic acids. This could affect the pigment absorption bands by intra-molecular copigmentation effects (Rustioni et al., 2013a). The enzymes catalyzing the acylation of anthocyanins are anthocyanin acyltransferases (He et al., 2010). Although, in some grapevine cultivars the amount of acylated anthocyanins can account for percentage higher than 60% of the total anthocyanin content, very little is known about the characterization of those genes. Recently, a new QTL (Quantitative Trait Locus) located on linkage group 18 has been identified as a good candidate for explaining the phenotypic variance related to acylation (Costantini et al., 2015).

The present work aims at the characterization of anthocyanins accumulation in Corvina grapes at different levels: total accumulation, pigment profile variations, transcriptional regulation of genes involved in the pathway.

2. Material and methods

2.1. Plant material

The experiment took place in two Corvina vineyards located in the foothills area of Valpolicella (Gargagnago), one of the most important Italian viticulture area, vocated to the production of Amarone. The two vineyards are characterized by different training systems. One (45°31′37.8”N 10°51′36.8”E) is cultivated with a Guyot training system, with 2.5 m distance between rows, 0.9 m gap between stumps. The second vineyard (45°31′39.8”N 10°52′01.55″E) is trained in pergola, and it is characterized by 4 m distance between rows, and 2 m between paired-stumps.

In each vineyard, three biological replications were collected during fruit development, at seven sampling dates. In detail, berries were taken at the following phenological phases: berries pea-sized – bunches hang (BBCH 75; July 14th); berries beginning to touch (BBCH 77; July 24th); majority of berries touching – lag phase (BBCH 79; August 4th); berries change color (BBCH 82; August 13th); advanced ripening (BBCH 84 and BBCH 87; August 25th, September 1st); berries ripe for harvest (BBCH 89; September 7th) (Meier, 2001). For each sample, berry weights, as well as axial and equatorial diameters (20 berries), were measured to calculate the berry volume and characterize the berry growth.

2.2. Micro-meteorological characterization

In order to define the microclimatic features in the two vineyards, air and grape temperatures and global solar radiation inside the canopy were measured. The micrometeorological survey consists of 6 Omega OS-36 Infrared Thermocouple (Omega Engineering, Stamford, Connecticut) for the temperature of grapes measure, 3 HOBO U23 Pro v2 thermistor thermometer (Onset Computer Corporation, Bourne, Massachusetts) for the air temperature in the canopy (HOBO Pro V2) and 2 HOBO S-LIB M-003 silicon pyranometers (Onset Computer Corporation), for the global solar radiation measurements under the canopy.

2.3. Anthocyanin quantification and characterization

Anthocyanin characterization started during the lag phase (BBCH 79). Three biological replication (20 berries) of each vineyard and sampling date were taken. Extractions were performed from the fresh fruit skins to limit anthocyanin degradation in 40 mL of pure methanol (Sigma Aldrich, Steinheim, Germany). After six hours of extraction at room temperature, solutions were stored at −20 °C until analysis (carried out within 4 months). The total anthocyanin content was calculated considering the absorbance at 540 nm of the extract after dilution with ethanol:water:hydrochloric acid (70:29:1) by spectrophotometric analysis (Nagel and Wulf, 1979).

The anthocyanin profile was then characterized by HPLC, following the method proposed by Mattivi et al. (2006) and adapted by Rustioni et al. (2016). 10 mL of extract were dried out in a rotary evaporator at 30 °C. Pigments were then dissolved in an aqueous solution of 0.3% perchloric acid and methanol (73:27 v/v), and kept frozen (−30 °C) until HPLC separation. Anthocyanin profiles were determined at 520 nm by using a Shimadzu HPLC LC-10 AD (Shimadzu Co., Tokyo, Japan) connected to a Shimadzu UV–vis detector SPD-10A. The separation was performed by a column Purospher RP18, 5 mm (250 × 4 mm) (Merck, Darmstadt, Germany). The injection volume varied depending on the sample pigment concentration. The mobile phase comprised methanol (eluent A) and aqueous 0.3% perchloric acid (eluent B). The gradient program was set up as follows: from 27% to 43% A in 32 min, from 43% to 68.5% in 13 min, from 68.5% to 100% in 2 min, then isocratically with 100% A for 3 min; re-equilibration time: 5 min. The flow rate was fixed at 0.45 mL min^{-1}. Molecules were identified according to their retention time and the peak absorption spectrum. The anthocyanin profiles were outlined as the relative levels (%) of delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside. Esterification was considered as acetyl and *p*-coumaroyl derivatives of the main detected anthocyanin.

2.4. Transcript profiling

Total RNA was extracted from berry using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's protocol. Per each sample, 400 mg of tissue were ground under liquid nitrogen and before homogenisation the seeds were removed from berry tissues. The total RNA was further purified by lithium-chloride precipitation. A 10 M lithium-chloride solution was mixed with total RNA to a final concentration of 2.5 M. The final solution was incubated overnight at 4 °C, centrifuged at 13,000*g*, removed