ORIGINAL ARTICLE

Metabolite analysis reveals distinct spatio-temporal accumulation of anthocyanins in two teinturier variants of cv. 'Gamay' grapevines (*Vitis vinifera* L.)

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Abstract

Main conclusion **White-fleshed grape cv. 'Gamay' and its two teinturier variants presented distinct spatial–temporal accumulation of anthocyanins, with uncoupled accumulation of sugars and anthocyanins in 'Gamay Fréaux'.**

Abstract In most red grape cultivars, anthocyanins accumulate exclusively in the berry skin, while 'teinturier' cultivars also accumulate anthocyanins in the pulp. Here, we investigated the teinturier cvs. 'Gamay de Bouze' and 'Gamay Fréaux' (two somatic variants of the white-fleshed cv. 'Gamay') through metabolic and transcript analysis to clarify whether these two somatic variants have the same anthocyanin accumulation pattern in the skin and pulp, and whether primary metabolites are also affected. The skin of the three cultivars and the pulp of 'Gamay de Bouze' begun to accumulate anthocyanins at the onset of berry ripening. However, the pulp of 'Gamay Fréaux' exhibited a distinct anthocyanin accumulation pattern, starting as early as fruit set with very low level of sugars. The highest level of anthocyanins was found in 'Gamay Fréaux' skin, followed by 'Gamay de Bouze' and 'Gamay'. Consistently, the transcript abundance of genes involved in anthocyanin biosynthesis were in line with the anthocyanin levels in the three cultivars. Despite no evident differences in pulp sugar content, the concentration of glucose and fructose in the skin of 'Gamay Fréaux' was only half of those in the skin of 'Gamay' and 'Gamay de Bouze' throughout all berry ripening, suggesting an uncoupled accumulation of sugars and anthocyanins in 'Gamay Fréaux'. The study provides a comprehensive view of metabolic consequences in grape somatic variants and the three almost isogenic genotypes can serve as ideal reagents to further uncover the mechanisms underlying the linkage between sugar and anthocyanin accumulation.

Abbreviations

AOMT	Anthocyanin *O*-methyltransferase
CYTOB5	Cytochrome b5
DAF	Days after flowering
DFR	Dihydroflavonol reductase
F3'5'H	Flavonoid 3′, 5′-hydroxylase
F3'H	Flavonoid 3′-hydroxylase
UFGT	UDP glucose: flavonoid-3-*O*-glucosyltransferase

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Introduction

Grapevine (*Vitis vinifera* L.) is a world-wide cultivated fruit crop with a high economic value. Grape berries can be used as fresh fruit and raisin, but most of them are fermented to produce wine, thus conveying a far greater economic value than that of fresh grapes. The organoleptic properties of berries and wines (i.e. flavor, color and aroma) are determined by the concentration and composition of several primary (e.g. amino acids, organic acids and sugars) and secondary (e.g. isoprenoids and phenolic compounds) metabolites. For example, free amino acids are the major nitrogenous compounds in grape berries and also act as the nitrogen source for yeast during winemaking (Kliewer 1970; Bell and Henschke 2005). In addition, some amino acids such as phenylalanine serves as the precursors of many important secondary metabolites, including anthocyanins and some phenylpropanoid aromas (Hernández-Orte et al. 2002; Saigne-Soulard et al. 2006). The organic acid content of wine plays an important role in regulating color stability and longevity during aging (Robles et al. 2019). Acidity of berry and wine mainly results from the concentrations of tartaric and malic acids, which are the principal organic acids accounting for approximately 90% of organic acids in grape berry (Kliewer 1966). Tartaric acid concentration gradually increases in herbaceous young berry, reaches its peak just before version, and then gradually declines throughout berry ripening (Dai et al. 2013; Sweetman et al. 2014). By contrast, the concentration of malic acid is relatively low in the young berry, reaches its maximum level at the initiation of ripening and consistently declines during the ripening process (Lamikanra et al. 1995). Concomitant with the reduction in organic acid content at the beginning of ripening, the concentration of sugars sharply increases. Grape sugar content plays a key role in shaping berry sensory properties and determining alcohol concentration of wine. In most grape berries, glucose and fructose are the main sugars, with only trace of sucrose (Liu et al. 2006). Glucose and fructose accumulation begins at the veraison stage, and continuously increases reaching a maximum at maturity. The ratio between glucose and fructose also changes throughout fruit development, with glucose being more abundant than fructose at the beginning of maturation but with an opposite pattern at technological maturity (Kliewer 1965, 1967).

The biosynthesis of anthocyanins is the most significant feature in red grape during ripening. Anthocyanins are the most important secondary metabolites in grape and wine, contributing dominantly to their colors. They are synthesized via the flavonoid pathway, one of main branches of the phenylpropanoid pathway. Anthocyanins are the origin of several tones of red, violet, and blue colors in grapes and wines. In red grape cultivars, the skin is the main or only source of anthocyanins, but their biosynthesis is also activated in the pulp of '*teinturier*' (from the French word meaning 'tainted') cultivars (Castellarin et al. 2011; Guan et al. 2012). In general, teinturier cultivars have much higher concentration of anthocyanins per unit of juice volume or fresh mass than normal (white-fleshed) cultivars (Ageorges et al. 2006). Because of their high anthocyanin content, the use of teinturier grapes in wine production exert significant effects on the color parameters of red wines and, therefore, are often used for blending with pale red wine to improve and enhance wine color (Ageorges et al. 2006).

In nature, more than 600 kinds of anthocyanins have been identified, that derive from six widespread anthocyanidins: malvidin, cyanidin, peonidin, petundin, delphinidin and pelargonidin (Castañeda-Ovando et al. 2009; Smeriglio et al. 2016). While pelargonidin is only found with trace concentration in a limited set of grape cultivars (Castillo-Munoz et al. 2009; He et al. 2010), the other five main anthocyanin compounds and derivatives are widely found in most grape cultivars responsible for color ranging from light red to dark blue (Liang et al. 2008). The difference in hydroxylation and methylation patterns results in the five main anthocyanin compounds found in grape (Mattivi et al. 2006; Fournier-Level et al. 2011). Among them, malvidin derivatives are considered as the most abundant anthocyanins in the vast majority of grape cultivars (Mattivi et al. 2006; Liang et al. 2008). The proportion between these five compounds is cultivar specific, so that anthocyanin composition was also characterized as a cultivar fingerprint (Mattivi et al. 2006; Dimitrovska et al. 2011). Previous studies have shown that total anthocyanin concentration in the skin of some teinturier varieties is much higher than that in the pulp, such as in cv. 'Lacryma' (Ageorges et al. 2006), 'Alicante Bouschet' (Castillo-Munoz et al. 2009), and 'Yan-73' (He et al. 2010; Guan et al. 2012) with malvidin and peonidin derivatives being dominant in the skin and pulp, respectively.

The concentration and composition of anthocyanin in grapes is mainly determined by genetic factors (Fournier-Level et al. 2009), but is also affected by internal and external factors, such as sugar (Zheng et al. 2009) and light (Guan et al. 2015). Indeed, the accumulation of anthocyanins is observed simultaneously with the accumulation of sugars, and previous studies demonstrate that sugar accumulation stimulates anthocyanin biosynthesis in different models, such as in vitro intact detached berries or grape cell cultures (Larronde et al. 1998; Zheng et al. 2009; Gambetta et al. 2010; Dai et al. 2014; Olivares et al. 2017). Interestingly, Dai et al. (2014) showed that the sugar mediated increase in anthocyanin production in detached berries occurred whereas the concentration of phenylalanine (the synthesis precursor) decreased, suggesting that the induction of anthocyanin accumulation by sugars does not solely correspond to a metabolic effect but also is most likely coupled

to a signaling process. In addition, this effect of sugar is correlated with transcriptional changes in the expression of structural and regulatory genes of the flavonoid biosynthesis pathway, in the context of a genome-wide transcriptome reprogramming during the vegetative-to-maturation transition of the berry (Fasoli et al. 2012).

Most wine grapevine cultivars grown to date are spontaneous (Laucou et al. 2018; D'Onofrio et al. 2021) and due to the fact that this crop is vegetatively propagated many of them are also the result of somatic variation. Somatic variants present important differences compared to their 'originator' including berry size and texture, anthocyanin, pigmentation or other metabolite compositions (This et al. 2006; Ferreira et al. 2018). A few somatic variants accounting for color variation in berry skin or pulp have been studied in grape (Kobayashi et al. 2004; Walker et al. 2006; Santibáñez et al. 2019). For example, a large fragment deletion in the promoter of the functional *MYBA1* allele in the black-skinned cv. 'Pinot Noir' gave rise to the white-skinned cv. 'Pinot Blanc' and pink-skinned cv. 'Pinot Gris' independently (Yakushiji et al. 2014). Recently, anthocyanin biosynthesis in the pulp of some teinturier cultivars was shown to be associated with a 408 bp repetitive DNA element in the promoter region of the *MYBA1* gene (Röckel et al. 2020).

Somatic variant of grape color provides a valuable system to study the genetic mechanisms of anthocyanin biosynthesis. In the present study, the grapevine cvs. 'Gamay Fréaux' and 'Gamay de Bouze' (two somatic variants of cv. 'Gamay') were investigated at the metabolic and gene expression levels. These somatic variants were originally and mainly identified based on their capacity for accumulating anthocyanins in the berry pulp. However, it is still unknown whether these two somatic variants have the same spatial–temporal pattern of anthocyanin accumulation between the skin and pulp. Further, whether other

metabolites such as sugars, organic acids and free amino acids are also affected in comparison with 'Gamay' is also unknown. Therefore, our objectives were to clarify the consequences of somatic variants in berry metabolite compositions and gene transcriptions by comparing the three aforementioned genotypes that share close genetic backgrounds. Gene expression study was also focused on the structural and regulatory genes involved the pathway of anthocyanin biosynthesis. This study provides comprehensive insights into cultivar- and tissue-specific anthocyanin accumulation in the grape berry.

Materials and methods

Plant materials

All experiments were carried out with grape (*Vitis vinifera*. L) cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux' (Fig. 1). Grape berries were collected from fruiting-cuttings grown in semi-controlled greenhouse (mean seasonal temperature 20–35 °C) (Mullins and Rajasekaran 1981). Grape berries were harvested at 11 successive developmental stages from 29 days after flowering (DAF) to 99 DAF at a 1-week interval. At each sampling date, 15–20 berries were collected from 3 vines to form a replicate with three replicates for each sample of each cultivar. The fresh weight of berries was recorded immediately and then berries were frozen in liquid nitrogen and stored at − 80 °C.

Frozen berries were dissected to separate skin from fresh and remove seeds. To collect the skins, we first gently cut a cross on the berry with a sharp blade, then gently tear the skin with forceps (Fig. 1 and Fig. S1). Special care was taken to make sure no pulp contamination for the skin samples.

Fig. 1 Patterns of pigmentation of berry skin and pulp in cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux' at three stages: before veraison (29 DAF, top), veraison (71 DAF, middle) and maturation (99 DAF, bottom). For each genotype at each stage, three views of the berry were presented, from left to right being berry peeling, cross cut, and longitudinal cut (for detailed annotation see Fig. S1)

Subsequently, skin and pulp were immediately frozen in liquid nitrogen separately. Later, the weight of skin was measured by a Precision Balance (Mettler Toledo PR2003 DeltaRange, Current Directions INC. USA). The skin and pulp were ground into fine powder in liquid nitrogen using a ball grinder MM200 (Retsch, Haan, Germany) and the powder was stored at − 80 °C for later biochemical and molecular analysis.

Quantification of sugars, organic acids and amino acids

Sugars, organic acids and amino acids were extracted from 500 mg (fresh weight) finely ground powder of pulp and from 200 mg of skin. The powder was extracted twice at 80 °C with 2 mL (pulp) and 1 mL (skin) of decreasing concentrations of ethanol successively 80% and 50% (v/v), sequentially. Supernatants were pooled, evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, NY) and the dry residue was resuspended in 2.5 mL deionized water.

Glucose and fructose were measured enzymatically with an automated micro-plate reader (Elx800UV, Biotek Instruments Inc., VT, USA) according to the method of Gomez et al (2007). Tartaric and malic acids were determined with an automated colorimetric method using the TRAACS 800 autoanalyzer (Bran-Luebbe) by reaction with ammonium metavanadate and using an enzymatic kit (R-Biopharm, Darmstadt, Germany) separately as described by Pereira et al. (2006). Amino acid concentration in berries was analyzed according to Pereira et al. (2006).

Quantification of anthocyanins

Anthocyanins were extracted from 50 mg freeze-dried powders, in the presence of methanol containing 0.1% HCl (v/v). Extracts were then filtered through a 0.45 μm polypropylene syringe filter (Pall Gelman Corp., Ann Harbor, USA) for HPLC analysis.

Each individual anthocyanin for all samples was analyzed as described by Dai et al. (2014). Briefly, analysis was performed on the HPLC system consisting of a P680 pump, ASI-100 T™ autosampler and UVD 340U UV–vis detector. Separation was conducted on a reversed-phase Ultrasphere ODS column 250 mm × 4.6 mm, 5 μm particle sizes fitted with an Ultrasphere C18 guard column 45 mm × 4.6 mm purchased from Beckman Coulter Inc. (Fullerton, USA). The solvents were (A) aqueous containing 2.5% formic acid (CNW, Düsseldorf, Germany), and (B) acetonitrile containing 2.5% formic acid. The gradient used was 10–15.8% B for 7 min, 15.8–17.3% B for 12 min, 17.3–20% B for 3 min, 20–22.3% B for 9 min, 22.3–23% B for 9 min, isocratic 23%

B for 20 min, followed by initial conditions for 4 min, at a flow rate of 0.3 mL·min^{-1}. Injection volumes were 5 μL and column temperature was set at 40 °C. The integrated absorbance at 520 nm was used to determine the concentration of individual anthocyanin expressed as malvidin 3-glucoside equivalents (Extrasynthese, Genay, France) calculated from a calibration function prepared from the commercial standard.

Gene expression analysis

Total RNA was isolated from 1 g ground powder as described in Reid et al. (2006). The amount of extracted RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the RNA quality was checked on 1% agarose gels. First-strand cDNA was synthesized from purified RNA using Superscript III enzyme (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA obtained was diluted (target concentration: 0.01 μg/μL) in distilled water for later analysis.

Quantitative real-time PCR expression analysis was carried out using the CFX96 Real-Time PCR Detection system (Bio-Rad). Reaction mixes (10 μL) included 5 μL of iQTM SYBR Green Supermix (Bio-Rad), 0.2 μM of each primer and 2 μL of diluted cDNA. Gene transcripts were quantified upon normalization to *ACTIN* and *GAPDH* as internal standards by comparing the cycle threshold (Ct) of the target gene with those of standard genes. Specific annealing of the oligonucleotides was controlled by dissociation kinetics performed at the end of each PCR run. The efficiency of each primer pair was measured on PCR product serial dilutions. All samples were assayed in technical duplicates. All primers used are listed in supplementary Table 1.

Statistical analysis

Data were analyzed with multivariate analysis methods using the R statistics environment (R Core Team 2007).

Results

Berry weight and water content

The change of berry weight and water content were recorded throughout berry development, as shown in Fig. S2. The developmental profile of berry flesh weight followed the typical double-sigmoid curve (Kennedy 2002). For cv. 'Gamay de Bouze' and 'Gamay Fréaux', berry weight increase had very similar profiles, which peaked at 85 DAF (1.1 g and 0.9 g, respectively) and then declined by 20–0.9 g and by 27–0.7 g, respectively. In contrast, no obvious decrease of

berry weight was observed in 'Gamay' at maturity, resulting in heavier berries for 'Gamay' than for the other two teinturier cultivars (Fig. S2a). Fresh weight of skin fluctuated in an upward trend in the three cultivars (Fig. S2b).

Compared to skin, the water content in pulp stayed at a higher level about 95% before veraison, then rapidly decreased during veraison onwards to 75% at 99 DAF (Fig. S2c). In the skin, the water content was about 83% at 29 DAF in the three cultivars and continually declined following berry development and ripening to ~65% at 99 DAF (Fig. S2d). Despite the proportion of water in pulp was always higher than that of skin throughout berry development, no significant difference in water content was observed in both skin and pulp between the three cultivars (Fig. S2c, d).

Organic acid accumulation

Despite similar accumulation profiles of malic and tartaric acid were observed between the three cultivars (Fig. S3), distinct acid accumulation kinetics were observed between skin and pulp. In the skin, the concentration of malic acid constantly increased from 29 DAF onwards and peaked at 78 DAF in 'Gamay' (17.3 mg/g FW) and 85 DAF in 'Gamay de Bouze' and 'Gamay Fréaux' (22.0 mg/g FW and 22.6 mg/g FW), respectively (Fig. S3a). While the maximum concentration of malic acid was detected 2 or 3 weeks after veraison in the skin, malic acid peaked at 64 DAF (25.7 mg/g FW, 17.2 mg/g FW and 29.7 mg/g FW, respectively) in the pulp, just before veraison, and then steeply declined to its lowest value at about 3 mg/g FW at maturity in the three cultivars (Fig. S3b).

Tartaric acid exhibited more complex profiles during berry development and ripening. In the skin, the concentration of tartaric acid firstly decreased to its lowest value just before veraison then peaked at 78 DAF ('Gamay Fréaux') and 85 DAF ('Gamay' and 'Gamay de Bouze'), and finally declined until maturity (Fig. S3c). Conversely, the concentration of tartaric acid in pulp declined throughout berry development with the exception of a slight increase at 64 DAF. No significant differences were observed among the three cultivars for tartaric acid before veraison, while 'Gamay de Bouze' showed higher concentration than 'Gamay' and 'Gamay Fréaux' after veraison (Fig. S3d).

Soluble sugar accumulation

Glucose and fructose presented similar accumulation profiles in the pulp for the three cultivars. Their concentrations were very low before veraison, at about 5 mg/g FW for glucose and 1 mg/g FW for fructose. From veraison onwards, they sharply increased and peaked at the 99 DAF, at about 100 mg/g FW for both sugars and without clear differences among the three cultivars (Fig. 2b, d, f). Conversely, skin of 'Gamay Fréaux' showed lower glucose and fructose concentrations compared to those of 'Gamay' and 'Gamay de Bouze'. The concentrations of fructose and glucose in 'Gamay Fréaux' were about 50 mg/g FW at maturity, just half of that in 'Gamay' and 'Gamay de Bouze' (Fig. 2a, c, e).

Accumulation of free amino acids

In total, 18 free amino acids were identified in berry skin and pulp. They were sorted in six groups based on their metabolic precursor: α-ketoglutarate family (glutamate, glutamine, arginine, proline and γ-amino butyric acid), oxaloacetate family (aspartate, asparagine, methionine, threonine and isoleucine), 3-phosphoglycerate family (serine, glycine and cysteine), pyruvate family (alanine, valine), aromatic amino acid family (tyrosine and phenylalanine) and ribose 5-phosphate family (histidine) (Fig. 3).

For most amino acids quantified and all cultivars, the concentrations in skin were higher than in pulp. The exceptions included proline, γ-amino butyric acid, phenylalanine and serine, whose concentrations in pulp were higher than in skin. For instant, at 99 DAF, the concentration of proline in pulp of 'Gamay' (8.3 pmol/mg FW) and 'Gamay de Bouze' (7.6 pmol/mg FW) was two and four times higher than those in skin (4.1 pmol/mg FW and 1.9 pmol/mg FW), respectively (Fig. 3).

In the skin, two distinct developmental accumulation profiles were distinguished, with 16 amino acids showing a 'U' shaped accumulation pattern and two amino acids (glycine and phenylalanine) displaying a bell-shaped pattern (Fig. 3). Differences in free amino acids in skin were mainly observed at the beginning (29–43 DAF) and end (85–99 DAF) of berry development among the three genotypes. At 29 DAF, the concentration of most amino acids in the two teinturier cultivars were much higher than in 'Gamay'. The exceptions included asparagine whose concentration was similar in the three genotypes, as well as glycine and phenylalanine whose concentrations were much lower in teinturier cultivars compared to 'Gamay'. At 85–99 DAF, aspartate and methionine were higher in 'Gamay de Bouze', with 'Gamay' and 'Gamay Fréaux' being similar. In contrast, γ- amino butyric acid, proline and alanine were lower in 'Gamay de Bouze', followed by 'Gamay' and 'Gamay Fréaux'. In skin, the remaining amino acids showed similar concentration among the three genotypes.

In the pulp, 14 out of 18 amino acids showed an 'S'-shaped accumulation profile, while serine, glycine and cysteine remained relatively constant and phenylalanine showed a bell-shaped accumulation profile throughout berry development (Fig. 3). The 'S'-shaped trend exposed that these 14 amino acids were kept at a very low level before veraison, increasing at veraison onwards in all

Fig. 2 Seasonal changes in sugar content in the skin (left panel) and pulp (right panel) of cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux'. Values are the mean ± SE of three biological replicates
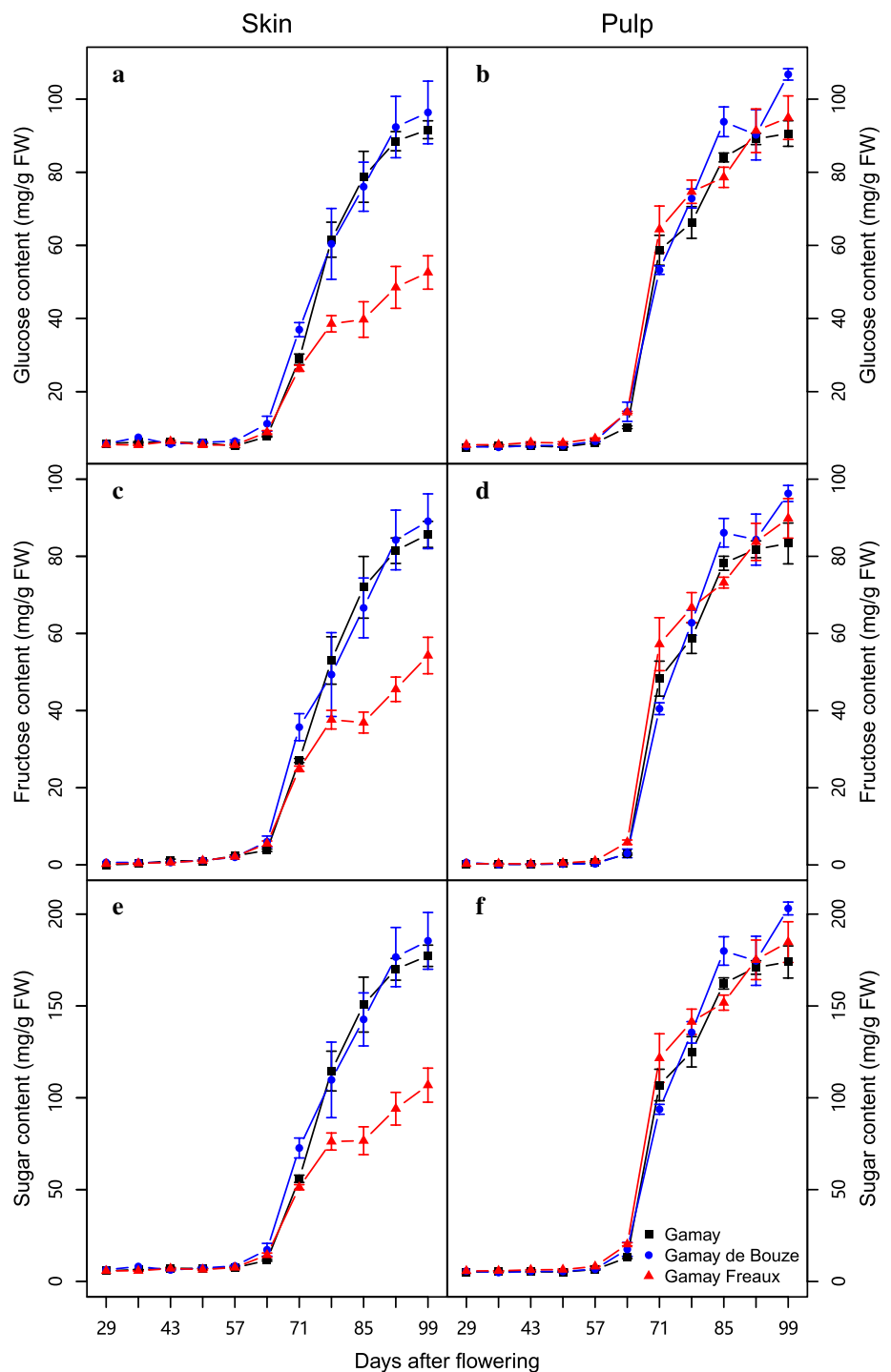
cultivars, especially for proline (Fig. 3). The three amino acids of the 3-phosphoglycerate family were always in a very low concentration with some fluctuation and no remarkable increase was observed during development (Fig. 3). A slight increase in serine content was only found in the 'Gamay' at maturity. Phenylalanine presented its highest concentration at 50 DAF, 2 weeks before veraison,

after which it continuously dropped to around 0.01 pmol/ mg FW at 99 DAF (Fig. 3). Differences in free amino acids in the pulp were mainly observed at post-veraison among the three genotypes. The pulp of 'Gamay Fréaux' showed highest concentrations around maturity for methionine, arginine, alanine, valine, glycine and tyrosine, while the white-fleshed genotype 'Gamay' had lower concentrations.
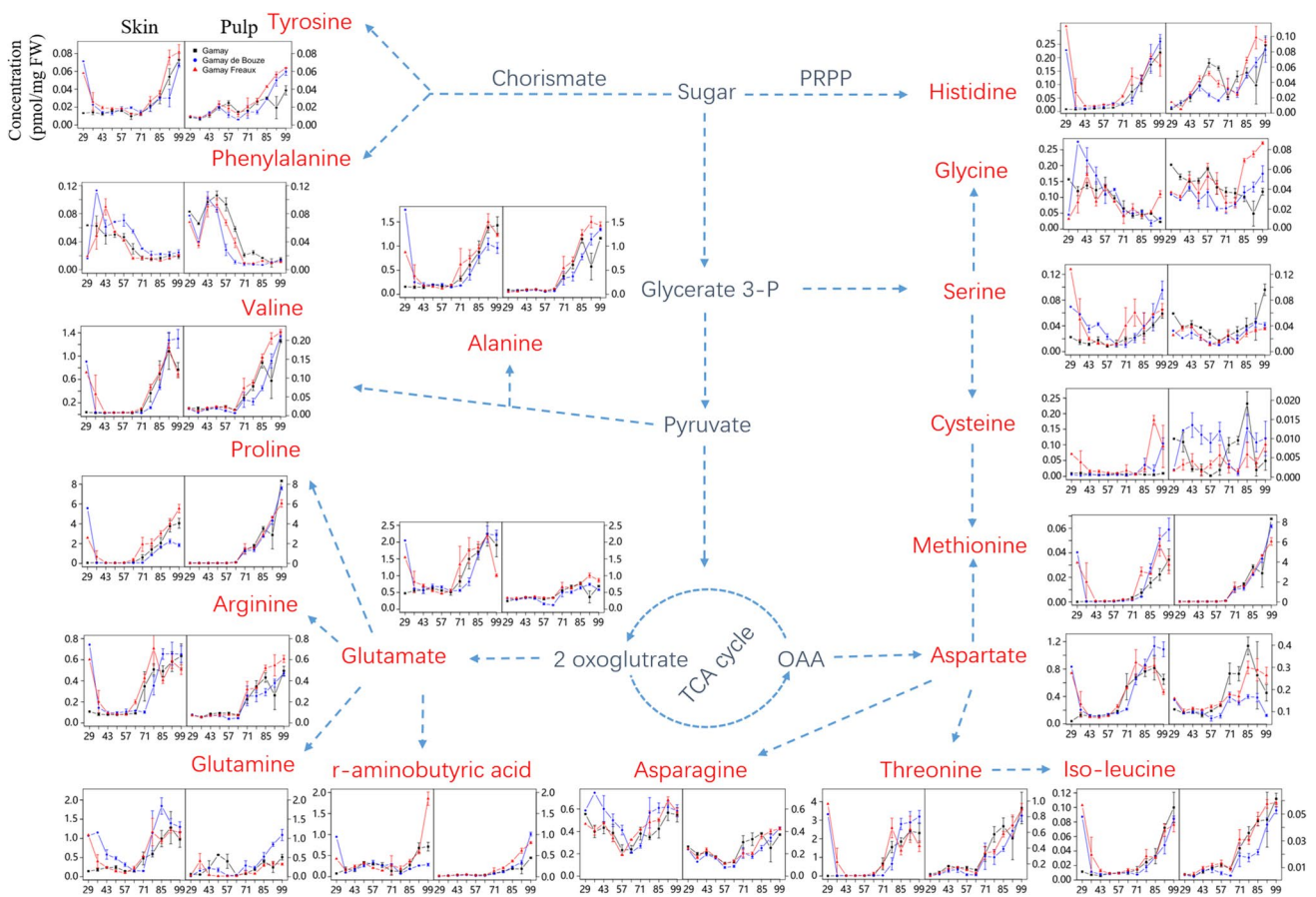
Fig. 3 Seasonal changes in amino acids in the skin (left panel) and pulp (right panel) of cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux'. Values are the mean ± SE of three biological replicates

In contrast, 'Gamay' had the highest concentrations for aspartate and phenylalanine at the 57–78 DAF interval.

Anthocyanin accumulation

The profile of anthocyanin accumulation presented cultivar- and tissue-specific patterns (Figs. 1, 4). In the skin, anthocyanins sharply accumulated after veraison in the three cultivars. At maturity, 'Gamay Fréaux' had the highest concentration of anthocyanins (34.7 mg/g FW), followed by 'Gamay de Bouze' (28.2 mg/g FW) and 'Gamay' (17.2 mg/g FW). Furthermore, the maximum content of anthocyanin in the skin of 'Gamay de Bouze' was detected 1 week later than the other two cultivars.

In the pulp, 'Gamay' did not accumulate anthocyanins, whereas 'Gamay de Bouze' synthesized anthocyanins from veraison onwards and 'Gamay Fréaux' begun to color from the first date measured (29 DAF). In 'Gamay Fréaux', total anthocyanin content was very low (about 0.02–0.04 mg/g FW) until veraison. These low concentrations of anthocyanins in the pulp of Gamay Fréaux before veraison might partially be a result of dilution due to uneven pigmentation

and berry growth. In particular, at 29 DAF, only the inner section of pulp showed red pigmentation, while there was still a large proportion of pulp devoid of anthocyanins (thus looked green in color Fig. 1 and Fig. S1) in Gamay Fréaux. However, we did not separate the green and red sections of the pulp during sampling, therefore, the green pulp section will dilute the anthocyanins in the colored pulp sections. Additionally berry size increased significantly from 29 to 64 DAF in Gamay Fréaux (Fig. S2), and if the anthocyanin accumulation rate is lower than berry growth, then the increase of berry size will further dilute anthocyanin concentrations as well. Anthocyanin sharply increased reaching a peak concentration (1.1 mg/g FW) during the first 3 weeks after veraison in the pulp of 'Gamay Fréaux'. The maximum concentration of anthocyanins in the pulp of 'Gamay Fréaux' was much higher than in 'Gamay de Bouze' (0.3 mg/g FW at 85 DAF, Fig. 4).

Furthermore, the concentration of 3′4′5′-trihydroxylated anthocyanins was always higher than that of 3′4′-dihydroxylated anthocyanins in the skin for all three cultivars. During the first 3 weeks (from veraison to maturity), the ratio of dihydroxylated to trihydroxylated anthocyanins sharply

Fig. 4 Seasonal changes in anthocyanin content in the skin (left panel) and pulp (right panel) of cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux'. Values are the mean ± SE of three biological replicates
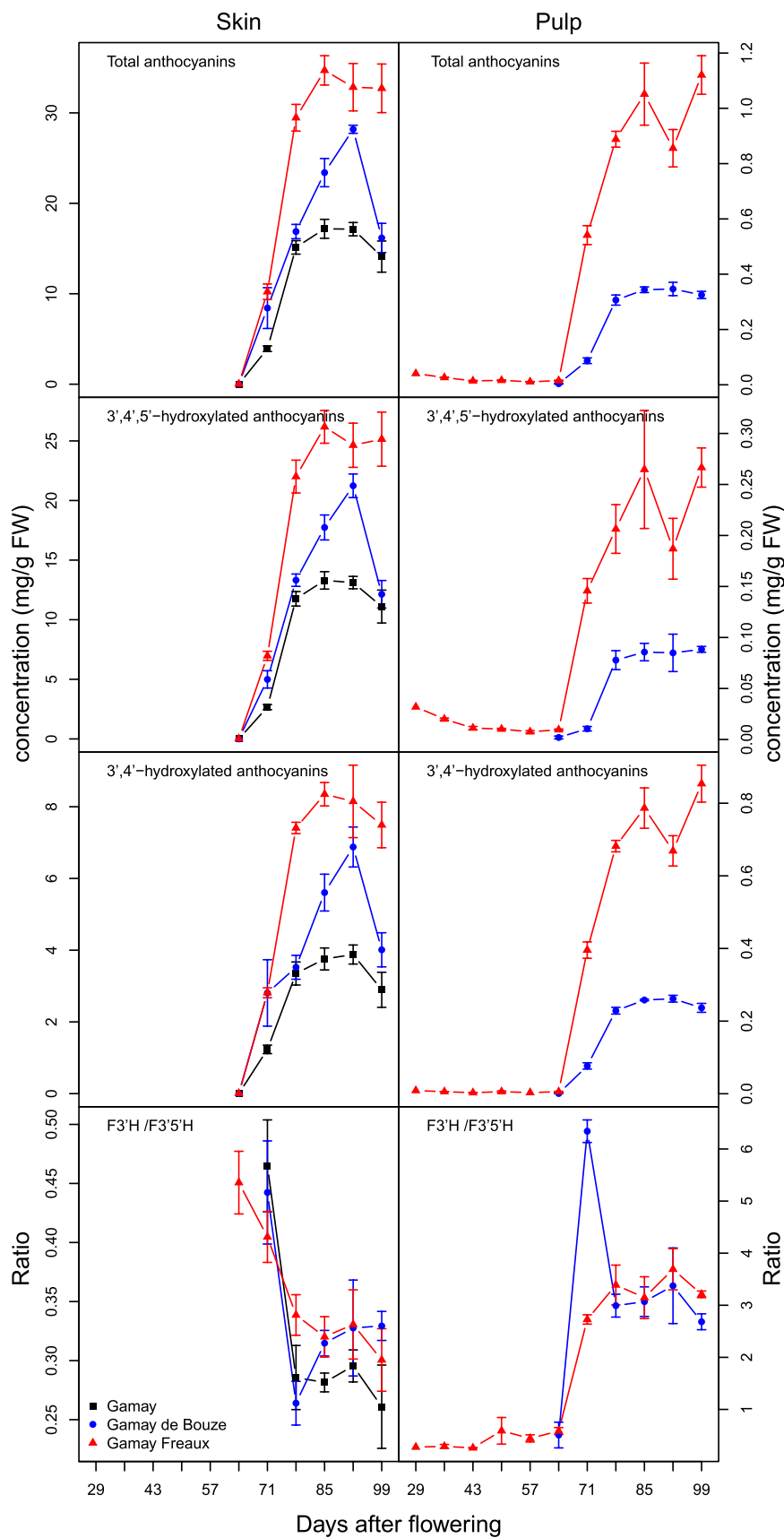
decreased. Conversely, in the pulp of 'Gamay Fréaux', the concentration of 3′4′5′- trihydroxylated anthocyanins was higher than that of 3′4′- dihydroxylated anthocyanins before veraison; but this tendency was inverted after veraison. In 'Gamay Fréaux', their ratios slightly increased from 0.3 to 0.6 before veraison and increased dramatically up to 3.3 in the following 2 weeks. In the pulp of 'Gamay de Bouze', at the very beginning of the transition from unripe to ripening, the concentration of 3′4′5′- trihydroxylated anthocyanins was higher compared to 3′4′- dihydroxylated anthocyanins like in 'Gamay Fréaux'. At veraison, this ratio great increased from 0.5 to 6.3, after which decreased and remained around 3 in the following weeks.

Moreover, the profile of the five main anthocyanins differed in the skin of the three cultivars (Fig. 5). In the skin of 'Gamay' and 'Gamay Fréaux', the concentrations of delphinidin-3-glucoside, cyanidin-3-glucoside and petunidin-3-glucoside increased dramatically from 64 to 78 DAF and sharply decreased thereafter. However, peonidin-3-glucoside and malvidin-3-glucoside increased steadily during ripening and then remained relatively stable or had a very slightly decreased. In the skin of 'Gamay de Bouze', the trend of the five main anthocyanins was similar. Despite their differences in concentration, the five compounds shared similar accumulation pattern in the skin of the three cultivars.

Transcript abundance of structural genes of anthocyanin biosynthesis

Transcript levels of structural genes related to anthocyanin biosynthesis varied among tissue and cultivar throughout berry development (Fig. 6). For the five structural genes detected both in the skin and pulp, their transcription was the highest in 'Gamay Fréaux', followed by 'Gamay de Bouze' and 'Gamay', which was consistent with the total anthocyanin concentrations. In skin, all genes analyzed shared the same profile, with a low transcript abundance in the early development stages before veraison, a dramatic increase at veraison stage (64 and 71 DAF) and a peak at 71 DAF. Similar profiles were detected for *UFGT, F3'HA, DFR* and *AOMT* transcript levels variations in the pulp of 'Gamay de Bouze' and 'Gamay Fréaux'. Interestingly, *F3'5'HI* presented its highest transcript level at 64 DAF in the pulp of 'Gamay Fréaux', just at the beginning of veraison, 1 week earlier than other colored tissues. In addition, as the only white-fleshed cultivar, the transcript level of these five structural genes were detected to very low or null levels in the pulp of 'Gamay'. Furthermore, all the five genes had higher transcript abundances in the skin compared to pulp.

Transcript abundance of regulatory genes of anthocyanin biosynthesis

Expression of regulatory genes also differed among tissues and cultivars (Fig. 7). For most of these genes and many of developmental stages, 'Gamay Fréaux' presented the highest transcript abundance, 'Gamay de Bouze' had lower transcript levels than 'Gamay Fréaux', and 'Gamay' exhibited the lowest.

In the skin, *MYBA1* and *CYTOB5* (an enzymatic regulatory player) presented an increase in transcript abundance at veraison stage in the three cultivars. The transcript profile of *MYC1* was different from them, with a slight decrease at 64 DAF in 'Gamay' and 'Gamay de Bouze' (Fig. 7).

In the pulp of 'Gamay de Bouze' and 'Gamay Fréaux', the transcript abundance of *MYBA1*, *MYC1* and *CYTOB5* increased at the initiation of veraison and peaked at 71 DAF, before decreasing at later stages of berry ripening. In contrast, the three genes were weakly expressed and barely detectable in the pulp of 'Gamay' (Fig. 7).

The transcript abundance of *MYBA6* and *MYBA7* were also studied in the skin and pulp of 'Gamay' and 'Gamay Fréaux'. No difference was detected between these two cultivars. However, in a given cultivar, the transcript abundance of *MYBA6* and *MYBA7* in the skin were much higher than in the pulp (Fig. S4).

Discussion

Distinct profiles of anthocyanin accumulation in the somatic variants of cv 'Gamay'

It has been widely reported that color development begins at the onset of ripening in berry skin (for all red cultivars) and in pulp in the case of teinturier cultivars (Castellarin et al. 2011; Kuhn et al. 2013). Consistently, the skin of 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux' as well as the pulp of 'Gamay de Bouze' commenced to accumulate anthocyanins at the onset of berry ripening (veraison, Figs. 1, 8). Interestingly, the pulp of 'Gamay Fréaux' contained anthocyanins as early as fruit set (the first surveyed stage before veraison, Figs. 1 and 8), a phenomenon that to our best knowledge has not been reported before. Before the onset of ripening, the pulp of 'Gamay Fréaux' preferably accumulated trihydroxylated anthocyanins, in agreement with a higher expression of the *F3'5'HI* compared to *F3'HA*. 'Gamay Fréaux' had the highest concentration in both skin and pulp, followed by 'Gamay de Bouze' and 'Gamay' (only in the skin). This is in line with other teinturier cultivars such as 'Alicante Bouschet' and 'Yan73', which also show a higher accumulation of anthocyanins compared to non-teinturier cultivars (Castillo-Munoz et al. 2009; He et al.

Fig. 5 Seasonal changes of the five main anthocyanins in the skin (left panel) and pulp (right panel) of cvs. 'Gamay', 'Gamay de Bouze' and 'Gamay Fréaux'. Values are the mean ± SE of three biological replicates
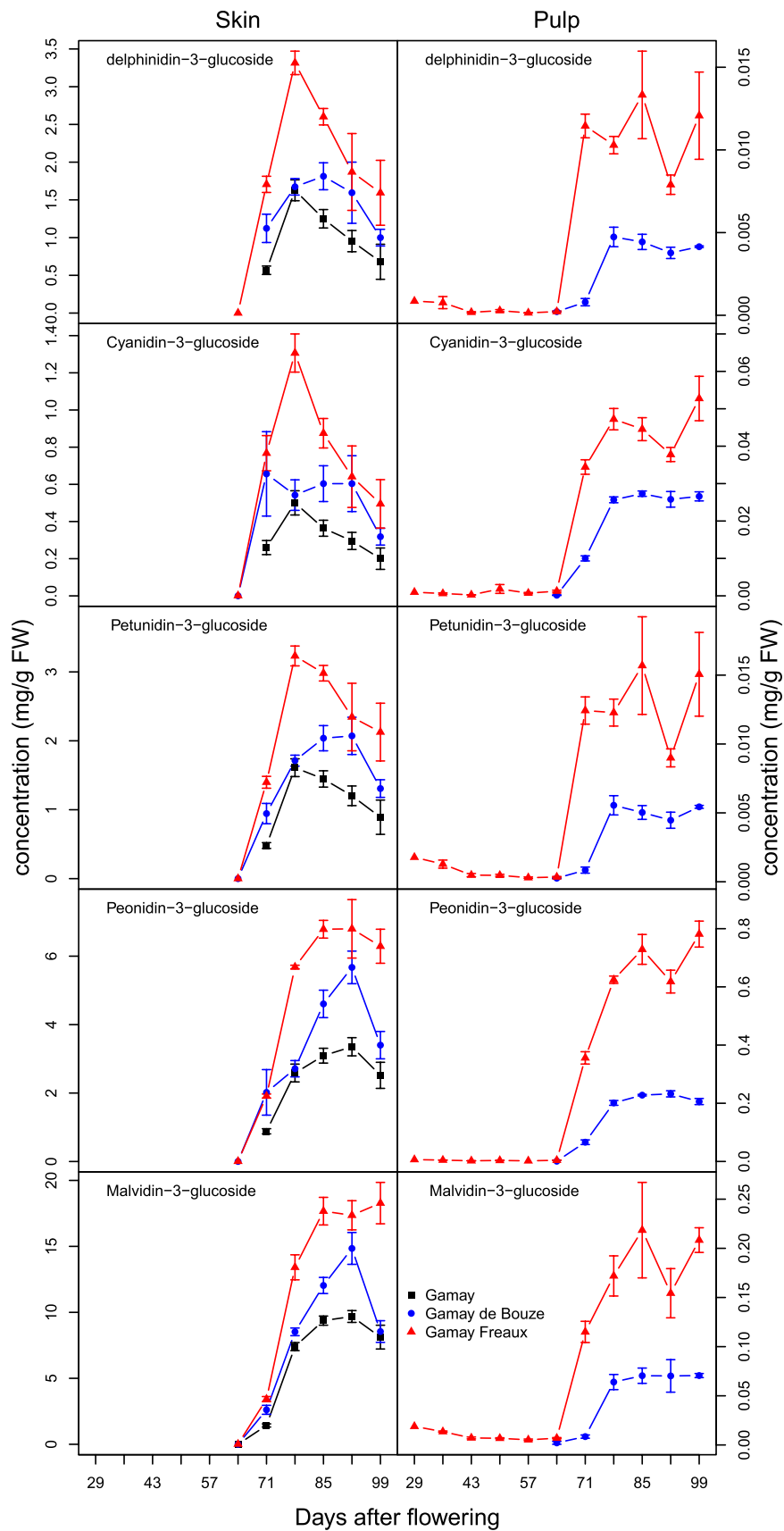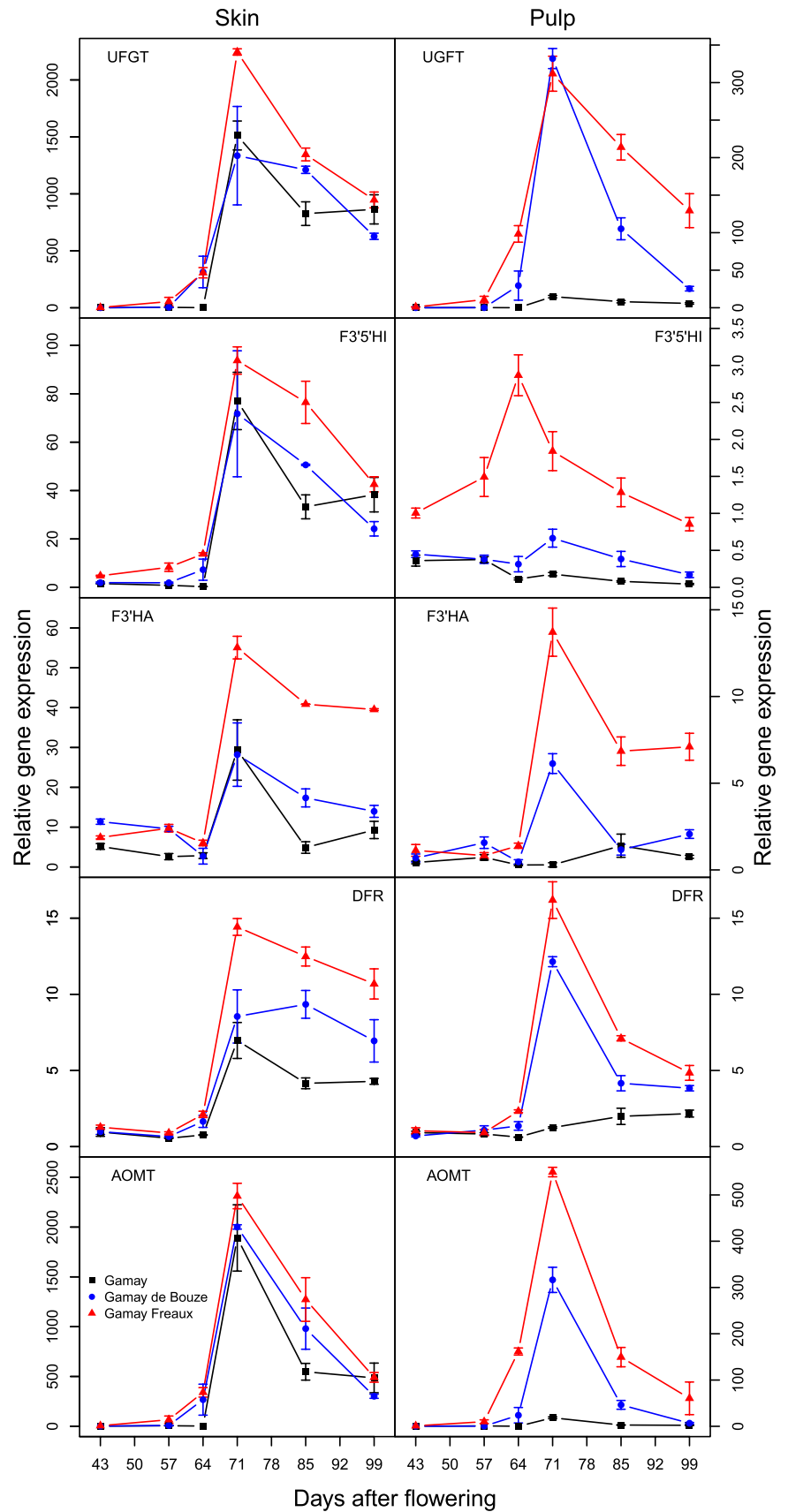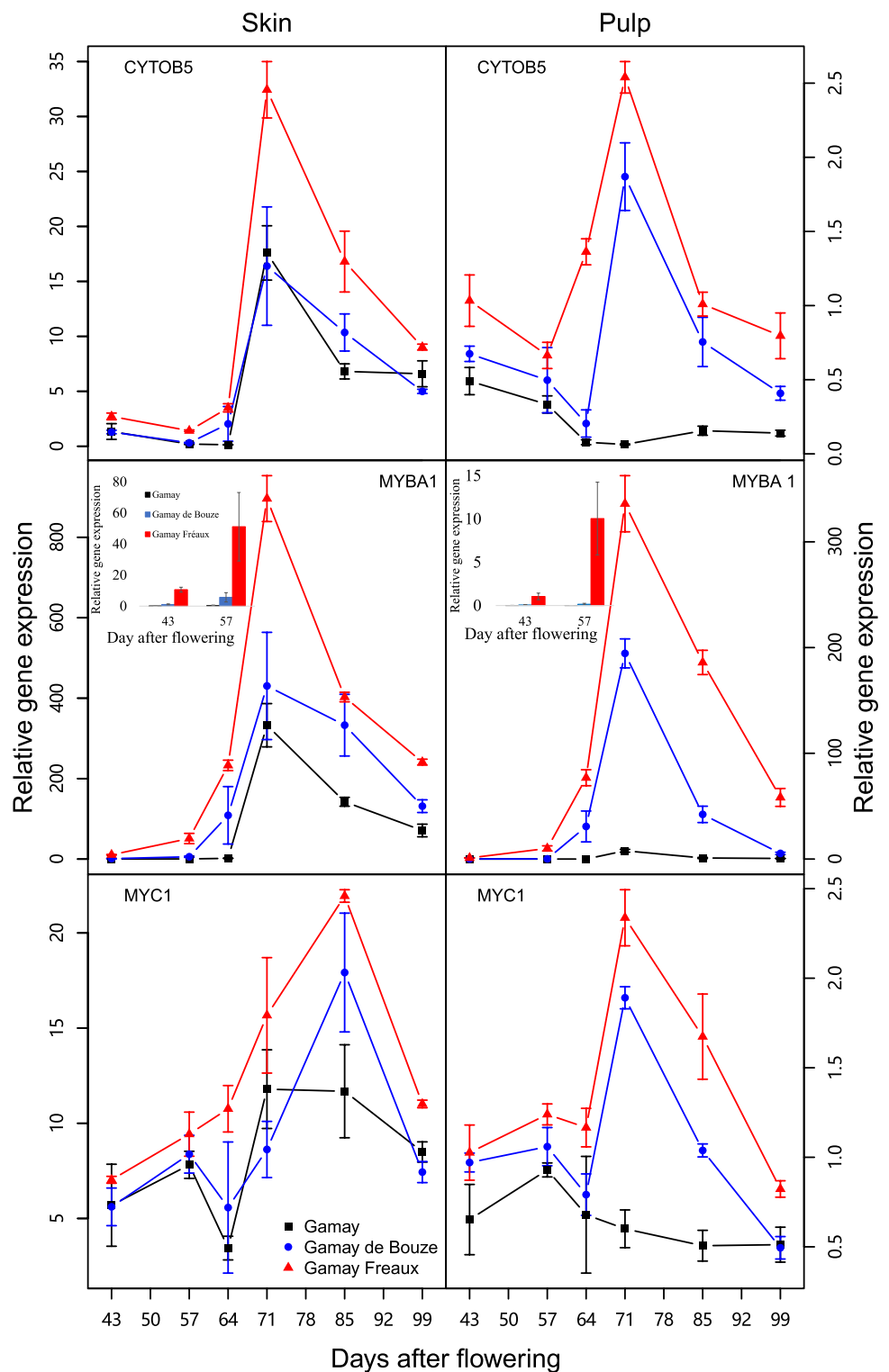
Fig. 6 Relative expression of
structural genes involved in
anthocyanin biosynthesis in the
skin (left panel) and pulp (right
panel) of cvs. 'Gamay', 'Gamay
de Bouze' and 'Gamay Fréaux'.
Values are the mean ± SE of
three biological replicates

2010). The differences in anthocyanins between tissues and among the three cultivars can be attributed to the differences in the transcript abundances of both structural and regulatory genes involved in anthocyanin biosynthesis pathway. However, additional regulatory processes could account for the timing of anthocyanin accumulation in the pulp before veraison (e.g. higher hierarchy transcriptional regulators, acting upstream of MYBA1).

MYBA6 and *MYBA7*, two regulatory genes responsible for anthocyanin accumulation in grape vegetative organs, were downregulated throughout berry ripening (Matus et al. 2017). As expected, their expression in the pulp of

Fig. 8 Distinct spatio-temporal accumulation of anthocyanin in the skin (left panel) and pulp (right panel) of the three inspected cultivars of in this study. a, Illustration shown the color change of skin (outer circle) and pulp (inner circle) of the three grape cultivars at pre- and post-veraison. Veraison was abbreviated as V; b, Heatmap shown the metabolic and transcript change in the skin and pulp of the three cultivars at 43 DAF (pre-veraison), 71 DAF (veraison) and 85 DAF (post-veraison)
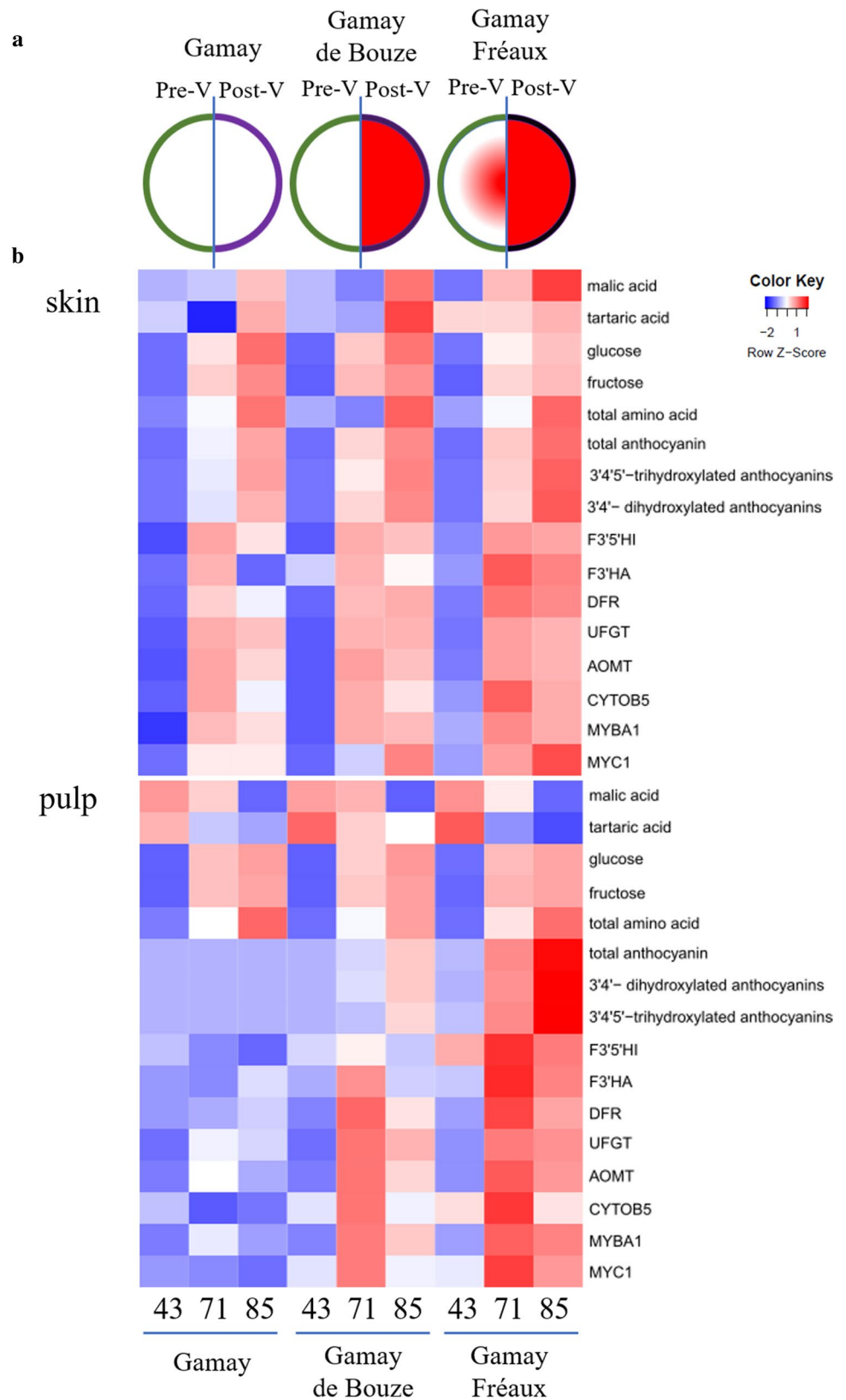
'Gamay' and 'Gamay Fréaux' did not show significant differences (Fig. S4), suggesting that these *MYBA* paralogs may not be the causal of the anthocyanin accumulation in the pulp of 'Gamay Fréaux'. It is worth to pinpoint that anthocyanin synthesis in pulp was not the only alteration in the two 'Gamay' mutants. Anthocyanin accumulation was also largely enhanced in the skin, indicating the variations related to anthocyanin biosynthesis occurred both in the skin and pulp tissues. Recently, a 408 bp duplication in the promoter of *MYBA1* located in the apical meristem L2 layer of bud sports was found to be responsible for pulp anthocyanin biosynthesis in many teinturier cultivars. Moreover, the number of duplications of the 408 bp sequence exhibits positive correlation with the transcript abundance of anthocyanin related genes and anthocyanin concentration (Röckel et al. 2020). The authors identified several MYB binding sites in these duplications and suggested them as potential MYBA1 positive auto-regulatory loops. However, the 408 bp duplication was not detected in the 'Gamay de Bouze' and the authors did not investigate 'Gamay Fréaux', indicating there might be others, yet uncovered, mechanisms responsible for anthocyanin biosynthesis in the pulp of 'Gamay de Bouze' and 'Gamay Fréaux'. Layer specific analysis of the color locus and whole-genome sequencing are required to identify the causal variants.

Further inspection of anthocyanin composition and abundance in berry skin found that peonidin-3-glucoside and malvidin-3-glucoside increased until maturity, after which were kept stable. However, the three other individual anthocyanins (delphinidin-3-glucoside, cyanidin-3-glucoside and petunidin-3-glucoside) began to decrease at 1 week after veraison. A similar trend was also found in cv 'Tempranillo' (Esteban et al. 2001) and 'Crimson seedless' (Singh Brar et al. 2008). This decrease may be due to continued methylation of cyanidin-3-glucoside, delphinidin-3-glucoside and petunidin-3-glucoside into more stable pigments, including peonidin-3-glucoside and malvidin-3-glucoside by AOMT. The developmental expression profile of *AOMT* is in agreement with this assumption. Unlike in the skin, all individual anthocyanins remained at high level without clear decrease around maturity in the pulp of 'Gamay Fréaux' and 'Gamay de Bouze'. This suggests that the pulp has a dissimilar temporal regulation mechanism for anthocyanin accumulation compared to skin.

The transcript abundance variations of anthocyanin biosynthetic related genes supported the finding that anthocyanin being accumulated continues throughout ripening. The transcript levels of all the five structural genes analyzed (*F3'5'HI*, *F3'HA*, *DFR*, *UFGT* and *AOMT*) was in line with the rate of anthocyanin accumulation. In the pulp of 'Gamay' (the white-fleshed cultivar), the expression of *UFGT*, a specific and critical enzyme for anthocyanin accumulation was barely detected (Ono et al. 2010). However,

F3'5'HI, *F3'HA* and *DFR* transcripts were detectable in the non-colored pulp, even if little or no anthocyanins accumulated (Boss et al. 1996). This is because these three enzymes are involved not only in the anthocyanin biosynthesis but also in other phenolic compounds pathway such as proanthocyanidins (Holton and Cornish 1995; Zhang et al. 2008).

In the pulp of pre-veraison 'Gamay Fréaux', and in the pulp of 'Gamay de Bouze' at the very beginning of veraison, the ratio of di/trihydroxylated anthocyanins was less than one, as in the skin. With the color accumulation in their skin, this ratio in the pulp became more than one. This reflects well the color changes during ripening, with pink-reddish around veraison and becoming more and more dark bluish towards maturity. This color change in skin can provide a natural sunlight protection for the pulp. *F3'5'HI* expression in the pulp decreased from the beginning of skin coloring in Gamay Fréaux and increase of *F3'5'HI* transcript abundance was much smaller in the pulp than in the skin of 'Gamay de Bouze'. Sunlight exclusion negatively affected the transcript level of *F3'5'H*, which controls the synthesis of trihydroxylated anthocyanins for the pulp (Guan et al. 2015). This result is consistent with the finding of Guan et al. (2015) observing that sunlight exclusion did not significantly affected *F3'H* transcripts, which controls the synthesis of cyanidin and peonidin derivatives, but it reduced *F3'5'H* transcript abundance in 'Gamay Fréaux'. Moreover, Koyama et al. (2012) found that bunch shading before the onset of ripening in 'Cabernet Sauvignon' decreased the proportion of trihydroxylated anthocyanins and the amount of *F3'5'H* transcripts. Taken together we propose that a self-shading mediated down-regulation of *F3'5'H* may explain, at least partly, why there was a lower proportion of malvidin derivatives and a higher proportion of peonidin derivatives in the pulp of the two teinturier cultivars.

Anthocyanin biosynthesis is controlled by a MBW complex, composed of DNA-binding R2R3-MYB proteins, bHLH (basic helix-loop-helix, also known as MYC) proteins, and WDR (tryptophan-aspartic acid repeat) proteins (Kuhn et al. 2013). In this study, expression patterns of *MYBA1* were consistent with *UFGT* transcript levels in both skin and pulp. In previous work, the *MYBA1* transcripts amounts paralleled *UFGT* transcripts and anthocyanin biosynthesis, as expected for a positive regulatory gene (Kobayashi et al. 2002). We also observed that *MYC1* transcript level positively correlated with anthocyanin accumulation. *MYC1* was reported to be part of the transcriptional cascade controlling not only anthocyanin but also proanthocyanidin biosynthesis in grapevine(Hichri et al. 2010). This can explain why *MYC1* was expressed in white flesh. *CYTOB5* transcript abundance was correlated with transcript levels of both *F3'HA* and *F3'5'HI* transcripts according to Bogs et al. (2006). Here, we found that *CYTOB5* expression was more relevant with *F3'HA* transcript levels, while its expression

was not correlated with *F3'5'HI* transcripts in pulp, indicating potential tissue-specific function of *CYTOB5*.

Uncoupled accumulation of sugar and anthocyanins in Gamay Fréaux

Growing evidences suggest that sugar can not only provide carbon skeleton for synthesizing various metabolites but also act as signaling molecules and possess hormone-like signaling functions in plants (Durán-Soria et al. 2020). A positive relationship between sugar accumulation and anthocyanin biosynthesis has been reported in several plants, and functional analysis established that sugar plays both metabolic and signaling roles in promoting anthocyanin biosynthesis (Durán-Soria et al. 2020). In grape berry cell suspensions, including 'Gamay Fréaux' and 'Barbera', sugar supplied in the culture medium enhanced anthocyanin accumulation by regulating the expression of anthocyanin biosynthetic related genes (Larronde et al. 1998; Vitrac et al. 2000; Ferri et al. 2011). Sugar-induced anthocyanin biosynthesis was also reported in Arabidopsis, strawberry and apple (Solfanelli et al. 2006; Jia et al. 2013; Liu et al. 2017). In the current study, anthocyanin concentrations was positively correlated with sugar concentrations from 29 to 85 DAF for 'Gamay' and 'Gamay de Bouze', in agreement with previous report (Gonzalez-SanJose and Diez 1992). However, 'Gamay Fréaux', which accumulated the highest concentration of anthocyanins in the skin, only half the concentration of sugars compared to 'Gamay' and 'Gamay de Bouze'. This seems to be inconsistent with the aforementioned reports. Moreover, anthocyanin accumulation occurred much earlier than sugar increases in the pulp of 'Gamay Fréaux', suggesting that anthocyanin and sugar accumulations are uncoupled in this particular tissue and cultivar. This strongly implies that the relationship between sugar and anthocyanin accumulation is more complex than expected. The uncoupling of sugar and anthocyanin accumulation has been reported under specific circumstances (Hiratsuka et al. 2001; Sadras and Moran 2012). For example, carbon starvation induced greater reduction in anthocyanin than sugar content both in grape berry and kiwifruit, because carbon is preferentially allocated to sugar accumulation rather than anthocyanin biosynthesis under carbon starvation (Bobeica et al. 2015; Nardozza et al. 2019). The anthocyanin pathway may serve as a carbon sink competing with sugar accumulation for carbon allocation at the organ and cell level (Arnold et al. 2004; Soubeyrand et al. 2018). Consequently, when the anthocyanin biosynthesis machinery is strongly up-regulated, as in the skin of 'Gamay Fréaux', it probably competes for carbon at the expense of sugar accumulation. More detailed investigations are required to elucidate this hypothesis. In addition, the correlation between sugar and anthocyanins was also altered when berry reached overripe phase with clear berry dehydration, as observed for Gamay de Bouze and Gamay Fréaux from 85 to 99 DAF. During the overripe phase, sugar import generally ceased in parallel with a decreased expression of sugar transporters, so the changes in sugar concentration is a result of berry dehydration (Terrier et al., 2005). This may explain the continued increase in berry sugar content in Gamay Fréaux and Gamay de Bouze from 85 to 99 DAF (Fig. 2), when both cultivars showed clear overripe dehydration (Fig. S2). Berry dehydration will also influence other metabolites, including anthocyanins. Although there was a clear increase of anthocyanins in the pulp of Gamay Fréaux, pulp anthocyanin concentration in Gamay de Bouze remained rather stable from 85 to 99 DAF (Fig. 2), indicating complex balances between berry dehydration and anthocyanin degradation during overripe. Therefore, caution should be paid when explore the correlation between sugar and anthocyanins during the overripe phase.

Conclusion

We characterized the differences between grape cv. 'Gamay' and its two teinturier mutants, 'Gamay Fréaux' and 'Gamay de Bouze' with comprehensive metabolic and transcript analyses. The results show that the pulp of 'Gamay Fréaux' and 'Gamay de Bouze' exhibited distinct temporal patterns of anthocyanin accumulation, with the former starting to accumulate as early as fruit set while the latter starting to accumulate only at the onset of ripening. These differences could be attributed to the expression of structural and regulatory genes involved in the flavonoid biosynthesis pathway. In addition, sugars were also influenced by the variant, with the skin of 'Gamay Fréaux' accumulating less sugar than those of 'Gamay' and 'Gamay de Bouze'. The difference in sugar content likely results from a metabolic shift caused by the alternative pathway of anthocyanin biosynthesis. In contrast, organic acids and free amino acids were hardly influenced among the three cultivars. This study provides a comprehensive view of metabolic changes in grape somatic mutants and the three isogenic genotypes can serve as ideal materials to uncover the mechanisms underlying the linkage between sugar and anthocyanin biosynthesis.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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