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Regulation of anthocyanin metabolism in grape: Effect of light on teinturier cultivars

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**Regulation of anthocyanin metabolism in grape:
Effect of light on teinturier cultivars**

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Résumé

Les anthocyanes constituent une composante importante de la qualité des fruits rouges, particulièrement pour le raisin noir, et pour la couleur des vins qui en dérivent. La biosynthèse des anthocyanes est déterminée par des facteurs génétiques et affecté par des facteurs environnementaux, notamment la lumière. Pour la plupart des cépages, la pellicule des baies est le tissu principal ou exclusif accumulant les anthocyanes. Notre travail analyse les effets de la lumière et du génotype sur la biosynthèse des anthocyanes. Le matériel végétal que nous avons utilisé était constitué de cépages teinturiers, qui accumulent des anthocyanes dans la pellicule et dans la pulpe, et de populations hybrides. Dix-neuf anthocyanes mono-glycosylés ont été identifiés dans sept tissus colorés du cépage teinturier Yan-73 (*V. vinifera*). La composition et la concentration en anthocyanes varient selon les organes et le stade de développement. Les anthocyanes de la pellicule incluent principalement les dérivés de la malvidine, alors que les dérivés de la péonidine sont les plus abondants dans la pulpe. Les dérivés de malvidine et de péonidine prédominent dans le rachis, les pédicelles des baies, les limbes foliaires, les nervures et les pétioles, et dans l'écorce à la base du cep. Les concentrations des anthocyanes dans les pellicules, la pulpe, le rachis et les pédicelles augmentent rapidement à partir de la véraison, ou une semaine après la véraison. Elles sont élevées dans les limbes foliaires jeunes et sénescents, et faibles dans les feuilles en expansion et les feuilles adultes. Elles ne varient pas beaucoup au cours de la saison dans les nervures et les pétioles, ou dans l'écorce.

Les cépages ont pu être distingués selon leur réponse à des traitements d'exclusion de la lumière imposés de la nouaison à la maturité en entourant les grappes par des boîtes opaques. Le cépage non teinturier "Gamay" à peau rouge accumule très peu d'anthocyanes dans la pellicule en absence de lumière. Au contraire, les cultivars teinturiers, 'Yan-73' et 'Gamay Fréaux' (mutant teinturier de 'Gamay') accumulent des anthocyanes aussi bien dans la pellicule que dans la pulpe

et présentent une coloration sombre même en absence de lumière.

Les effets de la lumière solaire sur la biosynthèse des anthocyanes et sa régulation ont été étudiés dans différents tissus de 'Yan-73'. Des mesures de transmission montrent que le fait d'enfermer les grappes dans des boîtes opaques réduit sensiblement l'intensité de lumière qu'elles reçoivent: $\leq 0.25\%$ de la lumière incidente atteint la pellicule des baies et $< 0.05\%$ atteint la pulpe. La pulpe subit naturellement un tamisage croissant de la lumière par la pellicule durant la maturation. L'exclusion de la lumière réduit et retarde la biosynthèse des anthocyanes dans la pellicule et la pulpe pendant le développement de la baie, et ces deux tissus n'accumulent jamais suffisamment d'anthocyanes pour virer au rouge foncé. Aucune réduction de la concentration en anthocyanes n'a été observée dans les pédicelles et les rachis. L'exclusion de lumière diminue l'abondance des transcrits *VvUGFT*, *VvMybA1*, *VvMybA2*, et *VvMyc1* à la fois dans les pellicules et la pulpe et celle de *VvMycA1* dans la pulpe. L'absence de lumière influence de façon différentielle les différentes anthocyanes: elle diminue la proportion relative d'anthocyanes 3',4',5'-hydroxylés et augmente celle des anthocyanes 3',4'-hydroxylés dans la pellicule et la pulpe, ce qui correspond aux changements d'abondance du rapport des transcrits *VvF3'H/VvF3'5'H*.

La diminution de concentration des anthocyanes et l'altération de leur composition induite par l'absence de lumière dans la pellicule et la pulpe du 'Gamay Fréaux' sont corrélées avec les variations de transcrits relatifs à la biosynthèse et au transport des anthocyanes. Les sucres et la concentration en acide abscissique (ABA) ne sont que légèrement affectés par l'absence de lumière, et la lumière solaire peut donc affecter directement les changements de concentration et de composition en anthocyanes. L'analyse par composantes principales montre que les tissus (pellicule et pulpe), les cultivars ('Gamay' et 'Gamay Fréaux'), les conditions lumineuses (exposition ou exclusion de la lumière solaire) et les stades de développement de la baie peuvent être distingués par les concentrations en anthocyanes, en sucres et en ABA et par l'abondance des transcrits relatifs à la biosynthèse et au transport des anthocyanes.

Les concentrations et la composition en anthocyanes sont déterminés génétiquement. Le cépage Beifeng (*V. thunbergii* × *V. vinifera*) accumule des monoglucosides et des diglucosides dans la pellicule, mais pas dans la pulpe. La descendance du croisement de Beifeng et Yan-73 a été utilisée pour étudier le profil d'héritabilité de la teneur en anthocyanes de la pellicule et de la pulpe au cours de 3 années successives. Tous les descendants accumulent des anthocyanes dans la pellicule, alors qu'il y a une ségrégation (1:1) de la couleur de la pulpe. Ainsi, la coloration de la pulpe est contrôlée par une seule paire de gènes, et l'absence de couleur est récessive par rapport à la coloration rouge. Les concentrations en anthocyanes de la pellicule et de la pulpe des descendants présentent une distribution normale dissymétrique et la plupart des descendants ont une teneur faible en anthocyanes. Par conséquent, la teneur en anthocyanes est héritée de façon quantitative et contrôlée par de nombreux gènes mineurs. Tous les descendants accumulent des monoglucosides dans la pellicule et dans la pulpe, alors que l'absence/présence des diglucosides segrége avec un rapport de 1:1. Ainsi, la présence des diglucosides est contrôlée par une seule paire de gènes et la présence des diglucosides est dominante par rapport à l'absence. La présence des diglucosides dans la pulpe des baies résulte de la combinaison de gènes relatifs à la biosynthèse des diglucosides et à la biosynthèse tissu-spécifique des anthocyanes.

En conclusion, la biosynthèse des anthocyanes dans la pellicule et dans la pulpe est déterminée par des gènes majeurs et mineurs, et elle est affectée par la lumière qui régule directement leur biosynthèse et leur compartimentation de façon cultivar- et tissu-spécifique.

Mots-clés: teinturier; spécificité tissulaire; spécificité de cépage; lumière; anthocyanes; héritabilité

Abstract

Anthocyanins are an important component of red fruit quality, especially for grape berries, and for the color of the wines made from these berries. Anthocyanin biosynthesis is determined by genetic factors and affected by environmental factors, especially sunlight. For most grape cultivars, the berry skin is the main or only tissue accumulating anthocyanins. The present work investigates the effects of light and grape genotype on anthocyanin biosynthesis. We used teinturier grape cultivars (also called dyers, which synthesize anthocyanins in both skin and pulp) and its hybrid population as plant materials. Nineteen monoglucoside anthocyanins were identified in seven colored tissues of the teinturier cultivar Yan-73 (*V. vinifera*). Anthocyanin composition and concentration varied among grape organs and with developmental stage. Skin anthocyanins were mainly composed of malvidin derivatives, while peonidin derivatives were the most abundant anthocyanins in the pulp. Both malvidin and peonidin derivatives were predominant in rachis, berry pedicels, leaf lamina, vein and petioles, and living bark at the base of the shoot. The concentration of anthocyanins in berry skin, pulp, rachis and pedicels rapidly increased starting from veraison on, or one week after veraison. Anthocyanin concentrations were high in young and senescing leaf lamina and low in expanding and mature lamina. They did not vary much throughout the growing season in the leaf veins and petiole tissues, or in the bark.

Grape cultivars could be distinguished by their response to sunlight exclusion treatments imposed from fruit set to maturity by surrounding the clusters with opaque boxes. The red-skinned non-teinturier cultivar Gamay could barely accumulate anthocyanins in berry skin under sunlight exclusion. In contrast, teinturier cultivars, Yan-73 and Gamay Fréaux (teinturier mutant of Gamay) accumulated anthocyanins in both skin and pulp and showed dark color even under sunlight exclusion.

The effects of sunlight on anthocyanin biosynthesis and regulation were investigated in various tissues of Yan-73. Light transmission measurements showed that covering with opaque boxes substantially reduced light intensity around clusters:

Abstract

$\leq 0.25\%$ of incident light reached the berry skin and $< 0.05\%$ reached the pulp. The pulp naturally experiences increasing sunlight exclusion by skin during ripening. Sunlight exclusion reduced and delayed anthocyanin biosynthesis in skin and pulp during berry development, while both tissues nevertheless accumulated enough anthocyanins to turn dark red. No strong reduction of anthocyanin concentration was observed in pedicels or rachis. Sunlight exclusion decreased transcript abundance of *VvUFGT*, *VvMybA1*, *VvMybA2*, and *VvMyc1* in both skin and pulp and that of *VvMycA1* in pulp. Sunlight exclusion differentially influenced the anthocyanin components: it decreased the relative proportion of 3',4',5'-hydroxylated anthocyanins and increased that of 3',4'-hydroxylated anthocyanins in berry skin and pulp, which corresponded to the change in the ratio of *VvF3'H* to *VvF3'5'H* transcripts.

The decrease in anthocyanin concentration and alteration of their composition induced by sunlight exclusion in Gamay Fréaux berry skin and pulp correlated with the changes of gene transcripts related to anthocyanin biosynthesis and transport. Sugar and ABA concentrations were slightly affected by sunlight exclusion, and thus sunlight might directly mediate changes in anthocyanin concentration and composition. Principal component analysis showed that tissues (skin and pulp), cultivars ('Gamay' and 'Gamay Fréaux'), sunlight conditions (sunlight exposure and exclusion) and berry developmental stages could be distinguished by anthocyanin, sugar and abscisic acid (ABA) concentrations and by the abundance of transcripts related to anthocyanin biosynthesis and transport, and ABA biosynthesis and degradation.

Anthocyanin concentration and composition are genetically determined. Beifeng (*V. thunbergii* × *V. vinifera*) accumulates both monoglucosides and diglucosides in berry skin while it did not in the pulp. The cross progeny of Beifeng and Yan-73 was used to investigate anthocyanin inheritance pattern in berry skin and pulp in three successive years. All the progenies accumulated anthocyanins in the skin, while there was a segregation (ratio 1:1) of pulp color. Therefore, berry pulp color was controlled by a single pair of genes, and colorless is recessive to red. Anthocyanin concentrations in berry skin and pulp of cross progenies demonstrated skewed normal

distribution and most progenies had low anthocyanin concentration. Thus anthocyanin concentration was a quantitatively inherited that is controlled by multiple minor genes. All the progenies accumulated monoglucosides in berry skin and pulp, whereas the presence/absence of diglucosides segregated with a 1:1 ratio. Thus, the presence of diglucosides was controlled by a single gene pair and the presence of diglucosides was dominant to the absence. The presence of diglucosides in berry pulp resulted from a combination of genes related to diglucoside biosynthesis and tissue specific anthocyanin biosynthesis.

In conclusion, anthocyanin biosynthesis in berry skin and pulp was determined by major and minor genes, and was affected by light that directly regulates the anthocyanin biosynthesis and transport pathway in a cultivar- and tissue-specific manner.

Keywords: teinturier; tissue specific; cultivar specific; light; anthocyanin; inheritance

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Papers published and in preparation

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- Guan L**, Li JH, Fan PG, Chen S, Fang JB, Li SH, Wu BH (2012) Anthocyanin accumulation in various organs of a teinturier grape cultivar (*V. vinifera* L.) during growing season. *Amer. J. Enol. Viticul.* 63, 177-184.
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- Guan L**, Wu BH, Dai ZW, Hilbert G, Li SH, Delrot S. Influence of cultivar, tissue type and cluster shading on amino acid profiles of grape berries.

Chaper I. Anthocyanins in grapevine—composition, location and biosynthesis

Grape is the second largest fruit crop in the world after citrus fruits in term of the yield. According to the ‘Food and Agriculture Organization’ (FAO), 75,866 square kilometers are planted with vineyards and this figure increases by 2% each year. Approximately 71% of world grape production is used for wine, 27% as fresh fruit, and 2% as dried fruit (Wikipedia 2012).

Berry coloration is an important factor for market acceptance of table grape cultivars and red wine. Anthocyanin concentration and composition are responsible for the color of grape berries (Pomar *et al.* 2005; Liang *et al.* 2008). Anthocyanin biosynthesis and accumulation commence at veraison (beginning of ripening) via the phenylalanine pathway, and continue throughout the ripening process. Anthocyanin biosynthesis is determined by genetic factors (Kobayashi *et al.* 2004; Fournier-Level *et al.* 2009; Walker *et al.* 2007) and affected by environmental factors and viticulture practices (Hilbert *et al.* 2003; Mori *et al.* 2005; Castellarin *et al.* 2007a; Berli *et al.* 2010; Soubeyrand *et al.* 2014). Sunlight is the major environmental factor (Jackson and Lombard 1993). Meanwhile, anthocyanin biosynthesis is also regulated by plant hormones, such as abscisic acid (ABA) and ethylene (Wheeler *et al.* 2009, 2010; El-Kereamy *et al.* 2003). For most grape cultivars, berry skin is the main or only tissue to accumulate anthocyanins. However, teinturier grape cultivars (also called dyers) synthesize anthocyanins in both skin and pulp. This chapter aims to provide a comprehensive review of the regulation of anthocyanin biosynthesis and its response to environmental factors. Part of the chapter has been published in a review (Kuhn *et al.* 2013).

1.1 An introduction to anthocyanins

1.1.1 Anthocyanin structures, solubility and stability

Anthocyanidins are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Fig. 1-1). Among the 17 known naturally occurring anthocyanidins/aglycones, only six anthocyanidins are common in higher plants—pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp) (Table 1-1). Anthocyanidins are stabilized by modification of the aglycone forms (anthocyanidins) by glycosylation, with the C-3 position being the most frequently glycosylated, followed by C-5 and C-7. The most commonly sugars attached to the aglycone are glucose, rhamnose, xylose, lactose (Yonekura-Sakakibara *et al.* 2009). These sugars can be modified by acetyl, *p*-coumaryl, caffeoyl, and feruloyl for further stabilization. The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars, the position of sugar attachment, and the nature and number of aliphatic or aromatic acids. So far, it is estimated that more than 400 anthocyanins have been found in nature (Kong *et al.* 2003). Anthocyanins are easily soluble in polar solutions, such as water, methanol, and ethanol. They are normally extracted from plant materials by using methanol containing trace amounts of hydrochloric acid or formic acid.

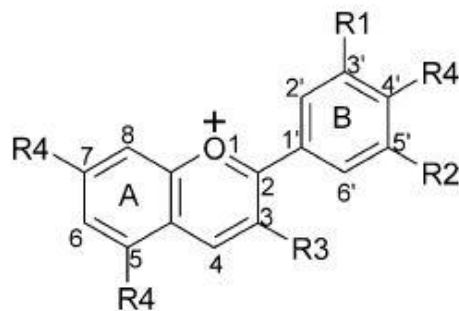


Figure 1-1 General structure of anthocyanidins (Kong *et al.* 2003).

Anthocyanin stability is influenced by various factors including pH, temperature, light, co-pigments, metal ions, oxygen, ascorbic acid, organic acids etc. (Giusti and

Wrolstad 2003). Increase in pH, temperature or light may degrade the anthocyanin molecule (Jenshi roobha *et al.* 2011). Treatment of anthocyanin pigments with H₂O₂ and ascorbic acid, degrades anthocyanins and results in color decrease (Nikkhah *et al.* 2010). The 525nm absorbance of peanut (*Arachis hypogaea*) skin pigment anthocyanin is higher with the addition of Cu²⁺ and Al³⁺, while the absorbance decreased with the addition of Mn²⁺ and Mg²⁺ (Wang *et al.* 2013). Organic acids such as caffeic, ferulic and *p*-coumaric acids may act as anthocyanin stabilizers in solution (Clemente and Galli 2013). The phenolic compounds are responsible for color stability during the aging of red wine (Pérez-Magariño and González-San José 2004).

Table 1-1 Commonly occurring anthocyanidins in higher plants (Kong *et al.* 2003).

Anthocyanidins	Substitution							color
	3	5	6	7	3'	4'	5'	
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H	orange
Cyanidin (Cy)	OH	OH	H	OH	OH	OH	H	orange-red
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH	red
Peonidin (Pn)	OH	OH	H	OH	OMe	OH	H	orange-red
Petunidin (Pt)	OH	OH	H	OH	OMe	OH	OH	red
Malvidin (Mv)	OH	OH	H	OH	OMe	OH	OMe	bluish-red

1.1.2 Anthocyanin composition depends on grape genotypes

Anthocyanins are frequently used as a fingerprint for cultivar or clonal recognition. Two clones of the same variety may present different anthocyanin contents (van Leeuwen *et al.* 2012). There are only 3-monoglucosides of five anthocyanidins, delphinidin, cyanidin, petunidin, peonidin, and malvidin in *V. vinifera*, whereas there are also much more abundant 3, 5-diglucosides of the five anthocyanidins in the other *Vitis* species and hybrids (Liang *et al.* 2008). Malvidin derivatives are the main anthocyanins in most *V. vinifera* cultivars (Liang *et al.* 2008), and they were suggested to derive from red varieties (Boss *et al.* 1996a; Mattivi *et al.* 2006). However, some pink sports derived from white varieties (Yakushiji *et al.*

2006), such as Muscat Rouge de Madere and the Gewuerztraminer in Mattivi *et al.* (2006) and Sultana and Chardonnay sport in Boss *et al.* (1996), had dominant cyanidin derivatives. Recently, trace amounts of pelargonidin derivatives were detected in *Vitis* (Castillo-Muñoz *et al.* 2009; He *et al.* 2010; Guan *et al.* 2012). Most anthocyanins do not combine with organic acids in *V. vinifera*. For example, Pinot Noir only produces non-acylated anthocyanins (Mazza *et al.* 1999). For those anthocyanins that combine with organic acids, *p*-coumaroyl derivatives are dominant (Núñez *et al.* 2004; Liang *et al.* 2008). Anthocyanin profiles are quite different among the 50 accessions from the “Misi’ón Biológica de Galicia” germplasm collection, and allows to distinguish and identify them (Pomar *et al.* 2005). Anthocyanin composition can be used as a variable for classification. Four different *Vitis vinifera* L. varieties (Cabernet Sauvignon, Merlot, Syrah and Monastrell) were differentiated by a model using the percentages of non-acylated anthocyanins, and of the peonidin and malvidin acetyl and coumaryl derivatives throughout three successive years as variables (Ortega-Regules *et al.* 2006). Mazzuca *et al.* (2005) distinguished American and European grapevines based on the identification of post-synthesis anthocyanidin modification. Therefore, grape cultivars are characteristic of anthocyanin composition, and provide a useful tool to differentiate the varietal origin of grapes and wines.

1.1.3 Time-dependent and spatial localization of anthocyanins in grape

Berry development is a complex process displaying a double sigmoid growth curve with three distinct phases (Figure 1-2), namely two periods of growth separated by a lag phase during which expansion slows and seeds mature (Conde *et al.* 2007). During the first phase, organic acids accumulate in the vacuoles, and tannins, hydroxycinnamates, and several phenolic compound precursors are synthesized. At the end of the lag phase, the short period known as veraison is characterized by the initiation of sugar accumulation and the rapid pigmentation of berries by anthocyanins in red grape varieties. High concentrations of glucose and fructose accumulate after veraison while organic acid levels decrease, and the berry softens (Zoccatelli *et al.* 2013). The acid to sugar ratio at harvest is important for the taste of

table grapes and for the sensory characteristics derived from wine grapes (Conde *et al.*, 2007). Towards the end of the third phase of berry development, many precursors of aroma and many aroma compounds (terpenes, norisoprenoids, esters, and thiols) are synthesized (Lund and Bohlmann 2006). The changes occurring from veraison onwards are accompanied by an increase in the content of abscisic acid (ABA), a hormone that promotes berry ripening (Wheeler *et al.* 2009). Other hormones and signals have also been shown to be involved in the control of berry development and ripening (ethylene, Chervin *et al.* 2004, 2008; steroids, Symons *et al.* 2006; ethylene and ABA, Sun *et al.* 2010).

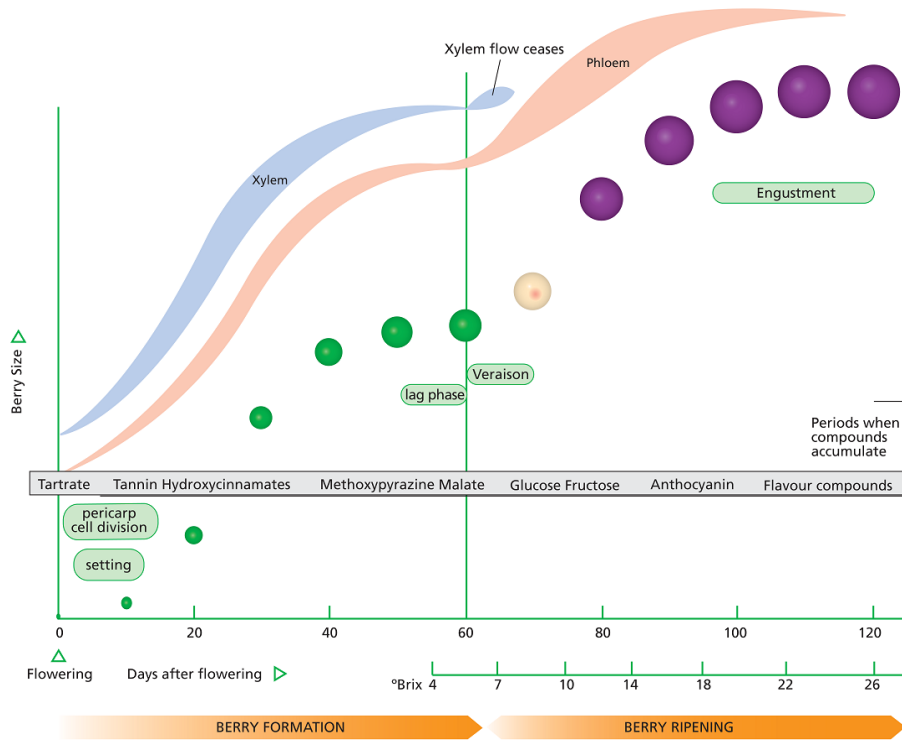


Figure 1-2 A typical double-sigmoid pattern of grape berry growth from flowering to maturity at 10-day intervals.

Relative size and color of berries, and major developmental events (green rounded boxes) were shown in the diagram. Also shown are the periods when compounds accumulate (the grey rectangle), the levels of juice °brix, and an indication of the rate of inflow of xylem and phloem vascular saps into the berry (Adapted from Kennedy *et al.* 2002).

The grape berry consists in the seeds and three tissue layers: the exocarp or skin, the mesocarp known as the pulp, and the endocarp, which directly surrounds the seed (Pratt 1971; Hardie *et al.* 1996). For most grape cultivars, berry skin is the main/unique organ accumulating anthocyanins. However, teinturier grape cultivars (also called dyers) synthesize anthocyanins not only in skin but also in pulp. Besides berry skin and pulp, some teinturier grape cultivars may accumulate anthocyanins in other organs, such as pedicels, rachis, leaves and stem epidermis (Jeong *et al.* 2006; Guan *et al.* 2012). Teinturier grapes are identified and their genetic relationship is explored (Santiago *et al.* 2008). Anthocyanin contents (Castillo-Muñoz *et al.* 2009), accumulation (Castellarin *et al.* 2011; Falginella *et al.* 2012), and the expression of anthocyanin biosynthesis genes (Castellarin *et al.* 2011) in berry skin and pulp are presently investigated. The most well-known teinturier grape cultivars are *V. vinifera* L. cv. Gamay Fréaux, Alicante Bouschet and its descendants. Teinturier cultivars generally have a much higher anthocyanin concentration per unit juice volume or fresh mass than non-teinturier cultivars (Ageorges *et al.* 2006; Balík and Kumšta 2008). Thus, teinturier grape cultivars are widely grown for juice and wine making. Furthermore, ‘Alicante Bouschet’ has been used to understand the ripening processes in different compartments of grape berry. Color development, sugar accumulation and acid loss begin in the flesh at the stylar end of the fruit, indicating that ripening in the grape berry originates in the stylar end flesh (Castellarin *et al.* 2011). Currently, cell lines derived from Gamay Fréaux and Bailey Alicante A are used to gain a better understanding of the regulation of anthocyanin biosynthesis in response to abiotic and biotic factors (Ananga *et al.* 2013).

Grapevine hairy roots (HRs) could store anthocyanins in the vacuoles and demonstrate very dense red coloration because of the constitutive ectopic expression of *VlmybA1-2*. The ectopic pigmentation is due to the activated constitutive expression of *UFGT* (UDP-glucose:flavonoid 3-O-glucosyltransferase), *GST* (Glutathione S-transferase), *OMT* (O-methyltransferase), and the vacuolar anthocyanin transport in grapevine (*anthoMATE*) (Cutanda-Perez *et al.* 2009). The HRs system provides a useful tool for anthocyanin biosynthesis and transport

investigation.

1.2 Anthocyanin biosynthesis and transport in grape cell

1.2.1 Anthocyanin biosynthesis

In grape cell, anthocyanins are synthesized at the cytosolic surface of the endoplasmic reticulum (ER) by a multienzyme complex via the flavonoid pathway (Boss *et al.* 1996b). Most genes encoding enzymes of the anthocyanin biosynthesis pathway in grapevine have been well identified. Genes encoding for the early step enzymes of biosynthetic pathway: chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone -hydroxylase (F3H) belong to multi-copied families with three copies of CHS and two copies of CHI and F3H being identified. Different copies of the shared genes either have temporally and spatially partitioned expression profiles, or coincide with the biosynthesis of a particular flavonoid (Jeong *et al.* 2008; Harris *et al.* 2013). The expression of flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-hydroxylase (*F3'H*) affects anthocyanin composition (Castellarin *et al.* 2006; Jeong *et al.* 2006). The prevalence of *F3'5'H* over *F3'H* would lead to more delphinidin, the precursor of petunidin and malvidin, and in contrast it would yield less cyanidin, the precursor of peonidin. The relative proportion of the two types of anthocyanins determines the color variation among red/purple/blue berry grape varieties and their corresponding wines and juices (Castellarin *et al.* 2006). *F3'5'HS* are present in highly redundant copy number. Spatiotemporal expression of *F3'5'H* increase the complexity and diversification of the fruit color phenotype among red grape varieties. Conversely, only two copies of the *F3'HS* are present in the grape genome, with one copy being expressed, and the other being transcriptionally silent (Falginella *et al.* 2010). The expression of the gene for UDPglucose: flavonoid 3-O-glucosyltransferase (*UFGT*) is critical for anthocyanin biosynthesis (Boss. *et al.* 1996; Zheng *et al.* 2013).

Although the early steps were clearly studied, much less is known about the subsequent acylation and methylation of anthocyanins (Grotewold, 2006). A model

was proposed for anthocyanin 3-glucoside sequential acylation and methylation in *Petunia* spp. (Figure 1-3), which was supported by the isolation of some of the genes (Brugliera *et al.* 1994; Kroon *et al.* 1994; Provenzano *et al.* 2014). Rhamnosylated anthocyanin3-glucosides in the petal were sequentially acylated by an AAT (anthocyanin acyltransferase), 5-glucosylated by a 5GT (5-glucosyltransferase), and then methylated in the 5' and/or 3' position by AMTs (anthocyanin methyltransferases) (Provenzano *et al.* 2014). In *V. vinifera*, some anthocyanin methyltransferases were identified. AOMT (anthocyanin O-methyltransferase) from Syrah methylates anthocyanins in the presence of divalent cations both *in vitro* and *in vivo* (Huguene *et al.* 2009). FAOMT (3',5'-O-methyltransferase) from Cabernet Sauvignon preferentially methylates anthocyanins and glycosylated flavonol *in vitro* in the presence of divalent cations (Lucker *et al.* 2010). The QTL colocalized with a cluster of O-methyltransferase coding genes explained 20% of the variance in the level of methylated anthocyanins (Fournier-Level *et al.* 2011). Among the gene cluster, *VvAOMT1*, *VvAOMT2* and *VvAOMT3* were identified. *VvAOMT1*, encodes a protein identical with the previous published AOMT (Huguene *et al.* 2009) and similar to FAOMT (Lucker *et al.* 2010); two SNPs of the newly characterized gene *VvAOMT2*, probably lead to *VvAOMT2* structure variation, associated with methylation level; *VvAOMT3*, was not expressed in mature berries (Fournier-Level *et al.* 2011). In contrast, anthocyanin acylation has not been investigated in detail and needs further studies. Although further stabilized by acylation and methylation, anthocyanins could be degraded by enzymatic oxidation or by further reaction with quinones (Sarni *et al.* 1995), which also needs further studies.

The expression of structural genes is controlled by several regulatory genes. The regulatory complex includes DNA-binding R2R3-MYB proteins, bHLH (basic helix-loop-helix, also known as MYC) proteins and WDR (tryptophan-aspartic acid repeat) proteins. Matus *et al.* (2008) described the genomic gene structures and classified 108 members of the grape R2R3 MYB gene subfamily regarding their similarity to the putative *Arabidopsis thaliana* orthologues. Several different MYB transcriptional factors were identified in grapevine. They were positive transcriptional

regulators involved in general flavonoid pathway (*VvMYB5a* and *VvMYB5b*, Deluc *et*

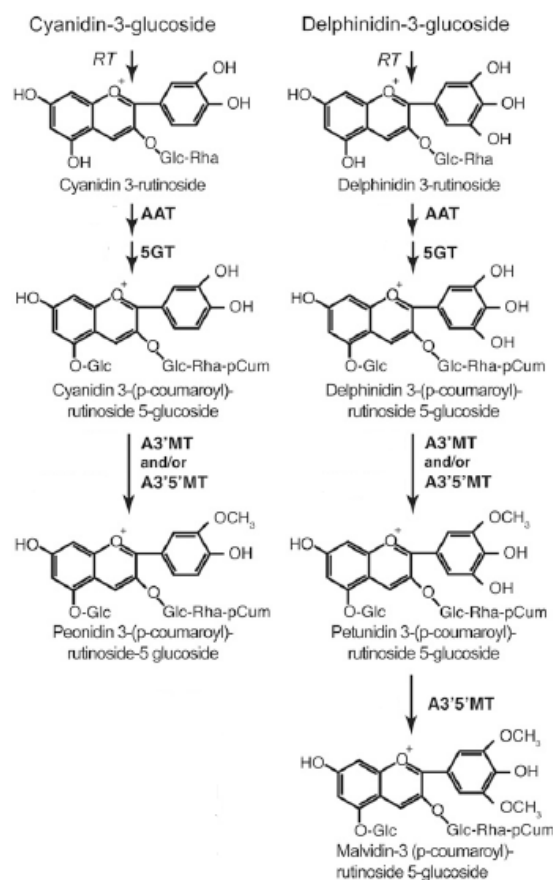


Figure 1-3 Diagram depicting anthocyanin modification (including glucosylation, acylation, and methylation) in *Petunia* spp.

AAT, anthocyanin acyltransferase; 5GT, 5-glucosyltransferase; A3'MT, 3' O-anthocyanin methyltransferases; A3'5'MT, 3',5' O-anthocyanin methyltransferases.

et al. 2006, 2008; see also Mahjoub *et al.* 2009), synthesis of tannins (*MYBPA1*, Bogs *et al.* 2007), flavonols (*MYBF1*, Czemplak *et al.* 2009) and anthocyanins (*VIMYBA1* and *VIMYBA2*, Kobayashi *et al.* 2004; *VvMyb5b*, Cavallini *et al.* 2014). *VvMYBC2-L1* was identified as a negative regulator of proanthocyanidin accumulation (Huang *et al.* 2014). Gret1, a Ty3–gypsy-type retro-transposon in the promoter region of *MYBA1*, is associated with white-fruited cultivars when present in a homozygous state (Kobayashi *et al.* 2004). Along more than 200 accessions of *V. vinifera*, the insertion

of *Gret1* in the promoter region of *VvMYBA1* is strongly associated with the white skinned phenotype, and additional polymorphisms in the gene were also tightly correlated with red or pink fruited phenotype (This *et al.* 2007). *VvWDR1* contributed positively to the anthocyanins accumulation when it was ectopically expressed in *A. thaliana* (Matus *et al.* 2010). *VvMYC1* is involved in the regulation of anthocyanins and tannins synthesis and its own feedback regulation (Hichri *et al.* 2010). AOMTs might be regulated in both transcriptional and post-transcriptional levels. The transient expression of *VvAOMT1* in tobacco (*Nicotiana benthamiana*) leaves where *PAP1* (production of anthocyanin pigment 1, a myb factor required for anthocyanin accumulation in Arabidopsis) was coexpressed leads to the accumulation of malvidin 3-rutinoside, while the expression of *PAP1* in the leaves only resulted in the accumulation of delphinidin 3-rutinoside (Hugueney *et al.* 2009). Fournier-Level *et al.* (2011) detected two QTLs explaining the variation in levels of anthocyanin methylation, one of which colocalized with the mybA gene cluster in LG 2 (linkage group 2) and explained 27% of variance. It is unclear whether myb factors regulate anthocyanin methylation directly at transcriptional level or indirectly by providing sufficient substrates. However, *VvAOMT2* was less influenced by mybA factor, while it was trans-regulated by protein structural variations that impacted catalytic efficiency (Fournier-Level *et al.* 2011). Overall, the regulation of anthocyanin subsequent modification, such as methylation and acylation, needs further studies.

1.2.2 Anthocyanin transport

In parallel with their biosynthesis in the cytosol, anthocyanins are rapidly transported into the vacuole for storage. Two major transport models have been proposed: membrane vesicle- and membrane transporter-mediated transport (Zhao and Dixon 2010).

Microscopic observations demonstrated the presence of spherical pigmented inclusions on the cytosolic side of the tonoplast and in vacuole, and they were identified as anthocyanin transport and storage complex separately (Conn *et al.* 2010; Gomez *et al.* 2011). In the cytosol, there are membrane covered anthocyanin bodies

called anthocyanoplasts. The vacuoles contain anthocyanic vacuolar inclusions (AVIs) that are non-membrane surrounded structures capable of concentrating anthocyanins by intravacuolar coalescence (Zhao and Dixon 2010). A tight timely correlation between AVI prevalence and anthocyanin content was found in a one year study on Gamay Freaux cell suspension cultures (Conn *et al.* 2010). AVIs purified from *V. vinifera* cell suspensions are dense, highly organic structures, with a lipid profiles similar to the tonoplast. They are also enriched in long-chain proanthocyanidins and exhibit a high affinity for acylated anthocyanins (Conn *et al.* 2003; Conn *et al.* 2008).

Concerning the tonoplast transporter-mediated model, two mechanisms have been identified in grapevine: primary transport mediated by ATP-binding cassette (ABC) transporters (Francisco *et al.* 2013) and secondary transport depending on the H⁺ gradient (Gomez *et al.* 2009). ABC transporters, in particular from the ABCC subfamily are involved in vacuolar flavonoid sequestration (Klein *et al.* 2006). ABCC1 mediates the transport of glycosylated anthocyanidins, and the transport depends on GSH (tripeptide glutathione) although there is no formation of an anthocyanin-GSH conjugate in grapevine. Multidrug And Toxic Extrusion (MATE) transporters have been identified as candidate secondary transporters for flavonoid/H⁺ exchange (Yazaki 2005). Two grapevine MATEs, AM1 and AM3, have been shown to transport specifically acylated anthocyanins in the presence of MgATP (Gomez *et al.* 2009). The AM transporters were distributed around the vacuole and closely associated with small vesicles in the epicarp cells of red berries (Gomez *et al.* 2011). In grape hair root cells where a gene encoding antisense *AM3* (HR-AM3AS) was overexpressed, anthocyanins accumulated in the vacuoles rather than in small vesicle-like structures surrounding the vacuoles (Gomez *et al.* 2011). The result indicated that other transporters besides AMs were involved in anthocyanin accumulation in the vacuole, which was substantiated by the previous studies (Gomes *et al.* 2009; Gomez *et al.* 2011; Francisco *et al.* 2013).

The different subcellular localizations between the GST protein and AMs suggest that they may be involved in different anthocyanin transport mechanisms (Gomez *et al.* 2011). GSTs might act as an escort protein/ligandin of anthocyanins,

delivering them to the transporter (Mueller *et al.* 2000, Gomez *et al.* 2011). The transcriptional profile of *VvGSTs* is positively correlated with key anthocyanin biosynthesis genes and anthocyanin accumulation in grape cell suspension and HRs system (Conn *et al.* 2008; Cutanda-Perez *et al.* 2009). Complementation experiment in maize Bronze-2-deficient corn kernels provided evidence for *VvGSTs*' function as anthocyanin transport proteins in grape cells (Conn *et al.* 2008).

Anthocyanin biosynthesis and its transcriptional regulation, and transport are summarized in Figure 1-4 according to Matus *et al.* (2009).

1.3 The factors affecting anthocyanin biosynthesis in grape

Berry skin color is determined by anthocyanin concentration and composition. Black and red cultivars accumulate anthocyanins in their skins, whereas 'white' cultivars do not accumulate them, displaying color variation between yellow-green. Anthocyanin concentration and composition in grape skins are determined by genetic factors and affected by environmental factors and viticultural practice.

1.3.1 Genetic inheritance

Reports on the genetics of grape skin color date back to 1960s. Barrit and Einset (1969) proposed a 'two gene pair model', suggesting that grape skin color was controlled by two pairs of genes: B (a dominant gene for black skin) is epistatic over R (a dominant gene for red skin). When both genes are recessive, the grape skin was 'white'. This was in accordance with He and Luo's (2004) results who evaluated berry skin color in 11 interspecific crosses between Chinese wild grapes and *V. vinifera*, five intraspecific crosses of *V. vinifera* and one intraspecific cross of *V. rotundifolia*. However, Goldy (1989) and Spiegel-Roy and Assaf (1980) reported that skin color was controlled by one gene pair, that is to say, color (including red and black) was dominant over 'white', with white being homozygous recessive. Shi *et al.*

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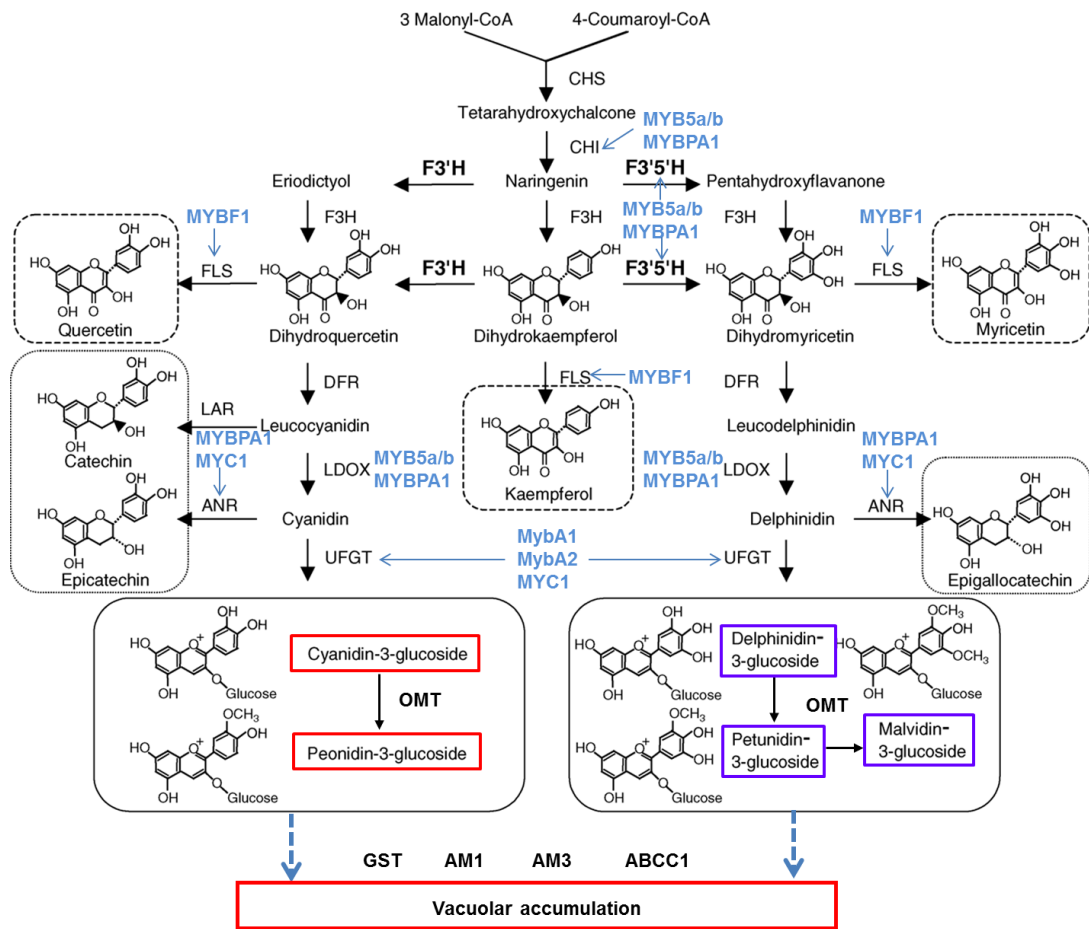


Figure 1-4 Simplified pathways of flavonoid biosynthesis and its regulation in grape (Matus *et al.* 2009).

CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanin dioxygenase; UFGT, UDPglucose: flavonoid 3-Oglucosyltransferase; OMT, O-methyltransferase; FLS, flavonol synthase; GST, glutathione-S-transferase; AM1/AM3, anthocyanin multidrug and toxic extrusion transporters, ABCC1; ATP, Binding Cassette transporter). Dotted blue arrows indicate anthocyanin transport by transporter-mediated model.

(1984) classified all kinds of grape skin colors into nine subgroups belonging to three groups: (1) black, including dark blue, dark red and dark brown; (2) purplish red, comprising purple, red and pink; (3) white, containing dark green, yellowish green

and cream color. Black and purplish red were dominant over white, with purplish red being heterozygotes and white being homozygous recessive. The study of anthocyanin concentrations in three cross combination progenies of common red maternal and three different white paternals, allowed Liang *et al.* (2009) to propose that the presence or absence of anthocyanins in grape skin is a quality character controlled by oligogenes of red maternal parent, while anthocyanin content was a quantitative character controlled by polygenes of both parents.

VvmybA1 is a major determinant of berry color variation (Lijavetzky *et al.* 2006). Somatic variation for berry skin color in the cultivar Italia is associated with the presence of a *Gret1* retrotransposon in the promoter region of *VvmybA1* (Figure 1-5, Kobayashi *et al.* 2004). In *V. vinifera*, two adjacent genes (*VvMYBA1* and *VvMYBA2*), inherited together regulate anthocyanin biosynthesis (Kobayashi *et al.* 2004; Walker *et al.* 2007). Thus, they can be regarded as a MYB haplotype and constitute the color locus.

A single quantitative trait locus (QTL) in linkage group 2 (LG2), flanking MYB haplotype, was responsible for the presence or absence of skin color (Fournier-Level *et al.* 2009). *VvMYBA1* and *VvMYBA2* have both functional and non-functional alleles: the original functional allele for *VvMYBA1* was named *VvMYBA1c*, whereas *VvMYBA1* with *Gret1* insertion in the promoter region was named *VvMYBA1a* (Figure 1-5 a); the original functional allele for *VvMYBA2* was named *VvMYBA2r*; whereas a point mutation in the *VvMYBA2* coding sequence resulting in a non-functional allele was named *VvMYBA2w* (Figure 1-5 b, Walker *et al.* 2007). Three common MYB haplotypes (Figure 1-6): A (HapA; *VvMYBA1a* and *VvMYBA2w*), C-N (HapCN; *VvMYBA1c* and *VvMYBA2r*), and C-Rs (HapC-Rs; *VvMYBA1c* and *VvMYBA2w*) tended to make grape skin presenting white, black and red color, respectively (Kobayashi *et al.* 2004; Lijavetzky *et al.* 2006; This *et al.* 2007; Azuma *et al.* 2008; Fournier-Level *et al.* 2009, 2010). In *V. labruscana*, two more functional MYB haplotypes have been proposed (Azuma *et al.* 2011). Red-skinned accessions tended to have E1 (Figure 1-6, HapE1; *VIMYBA1-2* and *VIMYBA1-3*), whereas black-skinned tended to have E2 (HapE2; *VIMYBA1-3* and *VIMYBA2*). Other detected QTLs in LG8

and LG14 may contribute the continuous distribution of anthocyanin concentration in berry skin (Ban *et al.* 2014).

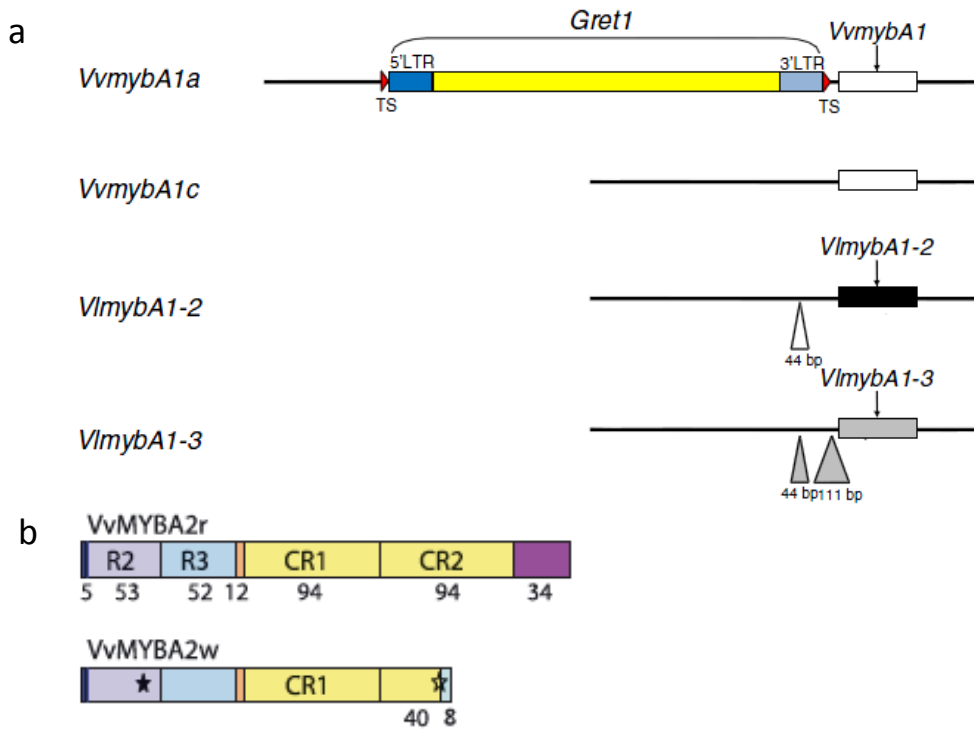


Figure 1-5 (a) Structures of *MybA* alleles isolated in *V. vinifera* and *V. labrusca* (Azuma *et al.* 2008). (b) Protein sequences of *VvMybA2* (Walker *et al.* 2007).

The insertion of retrotransposon *Gret 1* in the promoter region of functional allele *VvMybA1c* leads to the non-functional allele *VvMybA1a*. Coding regions with white, black and grey colors suggest that they have different nucleotide sequence. *VlMybA1-2* and *VlmybA1-3* contained insertions represented by triangles in the upstream part of the coding region. LTR represents long terminal repeats and TS represents a duplicated target site.

The functional protein *VvMybA2r* and the nonfunctional protein *VvMybA2w* are identical with the exception at the two starred positions. The first one leads to an amino acid substitution and the other to a frame shift resulting in a smaller protein. The numbers under the frame represents amino acid numbers in each domain. There are two identical C-terminal domain (CR) in *VvMybA2r*.

1.3.2 The effects of light on anthocyanin biosynthesis

The response of anthocyanin biosynthesis to light is complex. Out of 40 *V. vinifera* cultivars from which sunlight was excluded from clusters during fruit development, 38 cultivars colored similarly to the exposed berries except Tokay and Sulfanina Rose (Weaver and Mccune 1960). Similarly, anthocyanin concentration in berry skin showed no significant difference between sunlight exposure and exclusion in three successive years in Pinot noir (Cortell and Kennedy 2006) and in two out of three years in Shiraz (Downey *et al.* 2004). Conversely, light exclusion or leaf shading consistently and significantly decreased the berry anthocyanin concentration in cv. Cabernet Sauvignon (Koyama and Goto-Yamamoto 2008; Matus *et al.* 2009).

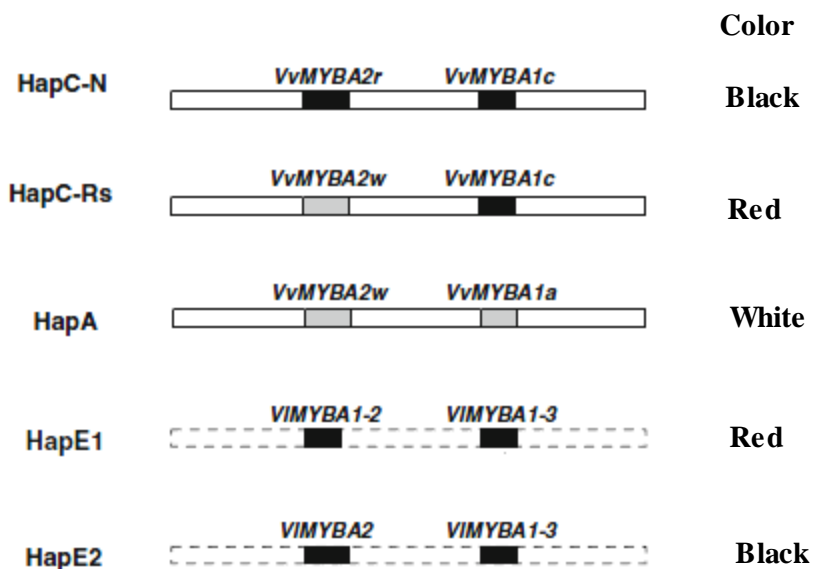


Figure 1-6 MYB Haplotypes (Azuma *et al.* 2011).

Gray and black color box indicate non-functional and functional gene, respectively. Dotted lines indicate that the exact positions of *VlmybA1-2*, *VlmybA1-3*, and *VlmybA2* within the color locus are unknown.

In detached cv. Pione berries maintained under light, anthocyanin content increased compared with those kept under darkness (Azuma *et al.* 2012). These different response patterns may reflect a genotype-dependent sensitivity to light. For example,

Zheng *et al.* (2013) compared two grape cultivars (Jingyan and Jingxiu), with the former accumulating considerable anthocyanins (although reduced compared with light-exposed berries) under light exclusion conditions while the later remained colorless (Figure 1-7a). However, Tarara *et al.* (2008) suggested that the effect of light on anthocyanin biosynthesis contributed more to anthocyanin composition than anthocyanin concentration. Artificial light exclusion achieved by surrounding 'Pinot noir' clusters with opaque boxes resulted in a decreased proportion of Dp, Pt, Mv and Cy derivatives and an increased proportion of Pn derivatives (Cortell and Kennedy 2006).

Light could regulate the expression of structural and regulatory genes related to anthocyanin biosynthesis, which may explain light-mediated changes of anthocyanin concentration. Light exclusion or leaf shading significantly decreased the expression of *VvUFGT*, *VvMybA1*, and *VvMybA2* in cv. Cabernet Sauvignon berry skin (Koyama and Goto-Yamamoto 2008; Matus *et al.* 2009). The expression of *VlmybA1-3*, *VlmybA* and *VlmybA1-2* was higher in detached cv. Pione berry skin maintained under light, while the expression of *Myb5a* and *Myb5b* under light was unchanged compared to darkness (Azuma *et al.* 2012). The contrasting anthocyanin accumulation observed in Jingxiu and Jingyan under sunlight exclusion is mainly due to *UFGT*, which is expressed in light-excluded Jingyan berries but not in Jingxiu berries (Zheng *et al.* 2013, Figure 1-7b). A further proteomic analysis conducted with skins of shaded Jingxiu berries showed that the amount of *UFGT* protein declined in shade conditions, while proteins related to energy production, glycolysis, and the tricarboxylic acid cycle were more abundant (Niu *et al.* 2013). All these results confirm that light is an important cue in the control of the anthocyanin biosynthesis.

1.3.3 Other factors

UV-B radiation, low temperature, moderate water deficiency, low N and low P induce anthocyanin biosynthesis in grape berry skin (Hilbert *et al.* 2003; Mori *et al.* 2005; Castellarin *et al.* 2007a; Berli *et al.* 2010; Soubeyrand *et al.* 2014). Plant hormones played important roles in anthocyanin biosynthesis. ABA and ethylene

promote anthocyanin biosynthesis (Wheeler *et al.* 2009, 2010; El-Kereamy *et al.* 2003), while auxin and cytokinins suppress it (Peppi and Fidelibus 2008; Ziliotto *et al.* 2012). Hormones may act as environmental mediators, and thus impact anthocyanin biosynthesis. For example, under water deficit and cold, ABA content increase in the berry (Yamane *et al.* 2006; Deluc *et al.* 2009). The observed changes in ABA content in response to those environmental cues could result in increased anthocyanin content.

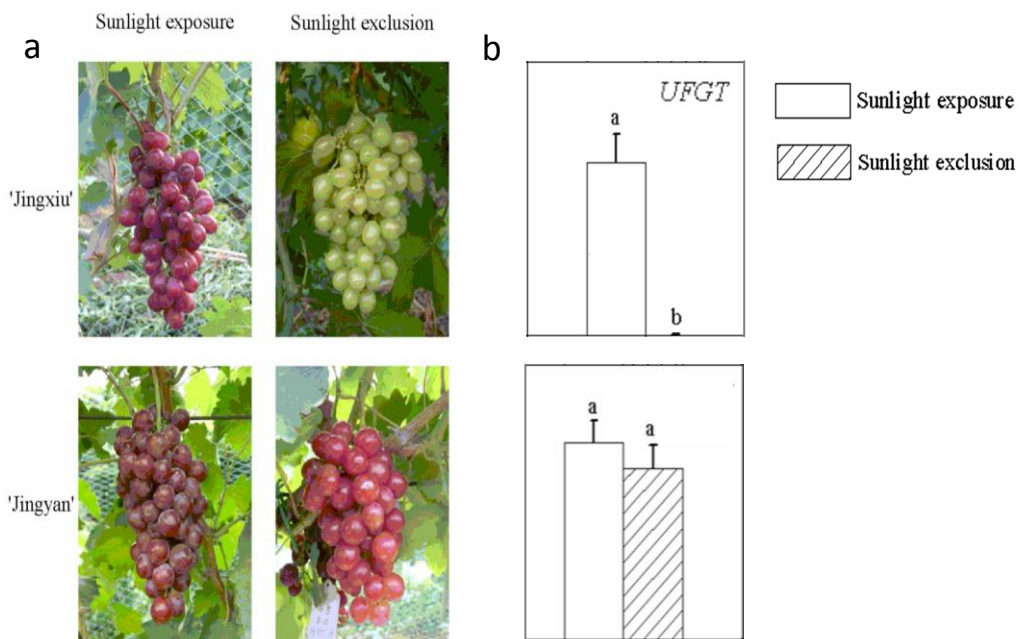


Figure 1-7 (a) Color of Jingxiu (upper) and Jingyan (lower) berry skin at maturity under sunlight exposure (left) and sunlight exclusion (right) from fruit set until maturity. (b) Relative expression of *UFGT* in berry skin of Jingxiu (upper) and Jingyan (lower) under sunlight exposure and sunlight exclusion from fruit set until maturity. Bars represent standard error of three biological replicates. Different letters indicate significant difference at $P < 0.05$. (Adapted from Zheng *et al.* 2013)

1.4 Objectives

Anthocyanins, responsible for grape skin color, play an important role in determining the market value of table grapes and the quality of red wine. Therefore, grape skin color is taken as a priority parameter during cultivar breeding. Berry skin

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color is determined by genetic factors and affected by environmental factors, with sunlight being the fundamental one. Teinturier grape cultivars accumulate anthocyanins in both berry skin and pulp, and are widely used in scientific research and in juice and wine making to give the product very dense color in China. Research on anthocyanin concentration and composition, accumulation profiles, light effects on anthocyanin biosynthesis would shed light on further study of anthocyanin biosynthesis and utilization of teinturier grape cultivars. Studies on anthocyanin inheritance would also bring useful information for grape breeding programmes.

Chapter II. Anthocyanin accumulation in various organs of a teinturier grapevine (*Vitis vinifera* L.) cultivar during the growing season

Article: Guan L, Li JH, Fan PG, Chen S, Fang JB, Li SH, Wu BH (2012) Anthocyanin accumulation in various organs of a teinturier cultivar (*Vitis vinifera* L.) during the growing season. *Am. J. Enol. Vitic.* **63**, 177-184.

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Abstract

The anthocyanin composition and concentration of various organs of teinturier grape Yan-73 were studied throughout the growing season. Nineteen anthocyanins were identified by HPLC-MS as monoglucosides and their derivatives. Anthocyanin composition and concentration varied among grape organs and with developmental stage. Skin anthocyanins were mainly composed of malvidin derivatives, while peonidin derivatives were the most dominant anthocyanins in the pulp. Both malvidin and peonidin derivatives were the major components in pedicels, rachis, leaf lamina, vein and pedicels, and living bark at the base of the shoot. Anthocyanins were very low before veraison, and then increased sharply at veraison in berry skin and pulp. Anthocyanins in pedicels and rachis also increased sharply, although their accumulation occurred later than in berry skin and pulp. Anthocyanins were high in young and senescing leaf lamina and low in expanding and mature lamina. Anthocyanins did not vary much in leaf vein and petiole tissue, or in bark, throughout the growing season.

Key words: teinturier grape, Yan-73, anthocyanin, organ specificity, accumulation

2.1 Introduction

In most grape cultivars, anthocyanins are synthesized and accumulated in berry skin, and therefore wine color essentially relies on the composition and concentration of anthocyanins in the skin. However, some grape germplasms, called teinturier cultivars (or dyers) accumulate anthocyanins in both skin and pulp. These cultivars have in general a much higher anthocyanin concentration per unit juice volume or fresh mass than non teinturier cultivars, and they are commonly used for blending to give a very dense color to red wines (Ageorges *et al.* 2006, Balik and Kumsta 2008, Kobayashi *et al.* 2005). Because the profile and concentration of anthocyanins in teinturier berries may significantly affect the color of the red wines that are produced from them, studies on their anthocyanin composition and concentration are interesting for winemaking and quality assessment.

Teinturier cultivars, such as Lacryma (Ageorges *et al.* 2006) and Neronet (Balik and Kumsta 2008) contain a higher level of anthocyanins in the skin than nonteinturier cultivars. Anthocyanin composition in the pulp of teinturier cultivars, such as Lacryma (Ageorges *et al.* 2006), Alicante Bouschet (Castillo-Munoz *et al.* 2009), and Yan-73 (He *et al.* 2010), is similar to that in the skin, with malvidin (Mv) dominating in the skin and peonidin (Pn) in the pulp. In addition to berry skin and pulp, teinturier cultivars accumulate anthocyanins in other organs such as leaves, tendrils, and shoots. Color determination of various organs of teinturier grape cultivars might result from tissue-specific expression of *VvmybA1*, a Myb-like transcriptional activator gene for anthocyanin synthesis (Jeong *et al.* 2006). Pigmented berry skin and pulp color might be related to the expression of structural genes of the anthocyanin synthesis pathway, such as UDP-glucose: flavonoid 3-*O*-glucosyltransferase (*UFGT*), chalcone synthase (isogene *CHS3*), glutathione S-transferase (*GST*), and caffeoyl methyl transferase (*CaOMT*) (Ageorges *et al.* 2006)—based on the association between gene expression and color evaluation of teinturier cultivars.

However, few studies detailed the composition and developmental changes of anthocyanins in various organs of teinturier cultivars. Therefore, it is unclear whether

other organs share the same anthocyanin composition and developmental changes as berry skin. The variety Yan-73 (*Vitis vinifera*, Muscat Hamburg × Alicante Bouschet) has been cultivated in China for over 50 years and is commonly used for wine blending. This teinturier cultivar can accumulate anthocyanins in different organs, including leaves, stems, tendrils, flower bracts, and berry pulp as well as berry skin.

In this study, the composition and concentration of anthocyanins in various organs of Yan-73 during development were investigated through HPLC-MS. The purpose was to identify possible developmental and organ-specific characteristics of anthocyanin accumulation during the growing season. This knowledge could provide a better understanding of the genetic basis underlying anthocyanin accumulation, and it may allow more efficient breeding strategies for higher levels of specific and/or total anthocyanins.

2.2 Material and methods

2.2.1 Plant materials

Eighteen-year-old Yan-73 grapevines, grown in the experimental vineyard of the Institute of Botany, Chinese Academy of Sciences, Beijing, were used in this study in 2010. The vines, trained to single-wire 1.7 m high fence trellis, were spaced 1.5 m apart within the row and 2.5 m apart between rows, with a north–south row orientation. Vines were maintained under routine cultivation, including irrigation, fertilization, soil management, pruning, and disease control. All shoots were tipped at the moment of fruit set, and the crop load was adjusted after fruit set to one cluster per one-year-old shoot.

2.2.2 Sampling

Clusters were randomly collected at 1- or 2-week intervals from 30 days after anthesis (30 DAA, 4 July) to 102 DAA (10 Sept). Veraison began about 65 DAA (4 Aug), and berry maturity was about 97 DAA (5 Sept). The maturity date was based on the seed color change to dark brown without senescence of berry tissue and on the

previous maturity date record. Three replicates were sampled, each consisting of two clusters. The clusters were taken to the laboratory immediately after harvest and were manually separated into berries, rachis, and pedicels (Figure 2-1). The berries were peeled with forceps to separate berry skin and pulp.

When three to four new leaves emerged (8 May), ~100 leaves of a similar size on the third node of selected shoots were tagged. The tagged leaves at the third node were sampled at young (11 May), expanding (20 May), mature (22 June), and senescing (5 Sept) stages. At the same time as leaf sampling, the shoots (nodes 1 through 3) bearing the leaves were collected. On each sampling date, three replicates each consisting of three leaves or three shoots were sampled. The leaves and shoot were taken to the laboratory immediately. Leaf lamina was separated from vein and petiole tissue, and living bark at the base of the shoot (hereinafter referred to as bark, see Figure 1-1) and xylem from the shoot were separated (Figure 2-1). Leaf vein and petiole tissues were combined. Thus, berry skin, pulp, rachis, pedicels, leaf lamina, vein and petioles, bark, and xylem were frozen separately in liquid nitrogen and stored at -40 °C for later anthocyanin analysis.

2.2.3 Extraction of anthocyanins.

The extraction of anthocyanins was performed as previously described (Liang *et al.* 2008). The samples were ground into powder in liquid nitrogen using an A 11 Basic Analytical Mill (IKA, Staufen, Germany). One g frozen powder was extracted in methanol solution (10 mL) containing 2% formic acid for 20 min with the aid of ultrasonic agitation, and then shaken in the dark at 4 °C at 120 rpm for 12 hr. The homogenate was centrifuged at $20,000 \times g$ for 10 min and the supernatant was collected. The residues were reextracted as above until they were colorless, and all the supernatants were pooled into a distilling flask. The extract was concentrated under vacuum at 39 °C using a rotary evaporator (RE-52AA, YaRong, Shanghai, China) until dryness. The dry residue was dissolved in 4 mL deionized water, and then ~1 mL was passed through a 0.22 µm Millipore filter for HPLC-ESI-MS/MS analysis.

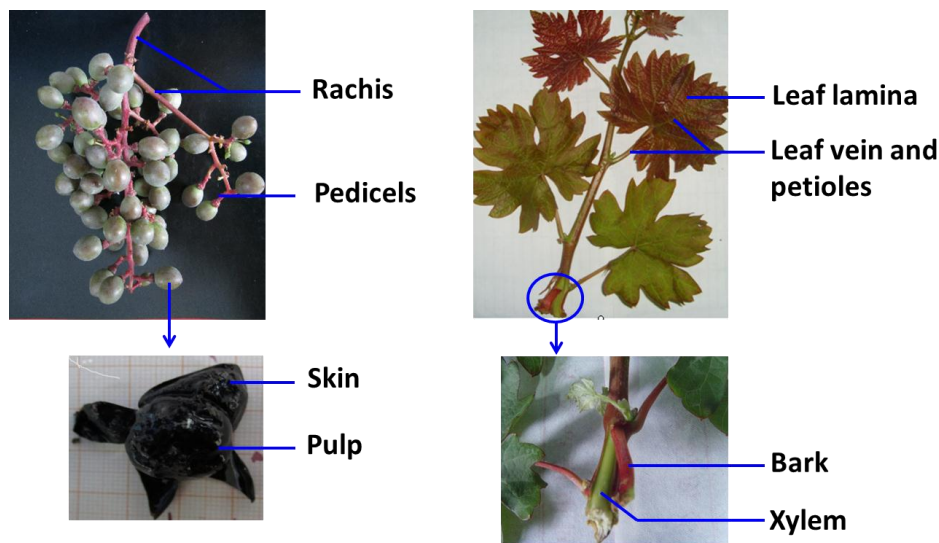


Figure 2-1 Eight sampled tissues of the teinturier cultivar Yan-73

2.2.4 Qualitative and quantitative analyses of anthocyanins.

High-performance liquid chromatography/triple quadruple tandem mass spectrometry (HPLC-MS/MS) (1290 series; Agilent, Palo Alto, CA) was used for anthocyanin identification. The Agilent 1290 Infinity HPLC pump, column oven, autosampler, and photodiode array detector (PAD) were coupled directly to the sprayer needle where ions were generated by electrospray ionization (ESI) in both positive and negative ionization modes. At the same time, anthocyanins were qualified using the Agilent 1290 Infinity HPLC system fitted with the PDA detector.

The same method was used by the two HPLC systems. The analytical method used a combination of two HPLC columns: a Kromasil-100 reversed-phase C18 column (5 μm particle sizes, 250 mm \times 4.6 mm i.d.; Tracer Analytica, Barcelona, Spain) and a Nova-Pak C18 guard precolumn (Waters, Milford, MA). All the sampled tissues were analyzed according to the following HPLC method. The solvents were (A) aqueous 2.5% formic acid and (B) acetonitrile containing 2.5% formic acid. The gradient was from 10 to 15.8% B for 7 min, from 15.8 to 17.3% B for 12 min, from 17.3 to 20% B for 3 min, from 20 to 22.3% B for 9 min, from 22.3 to 23% B for 9 min, isocratic 23% B for 20 min, and then returned to initial conditions for 4 min, at a

flow rate of 0.3 mL/min. Injection volumes were 5 μ L and the column temperature was set at 30 $^{\circ}$ C. Anthocyanins were detected by UV absorbance at 280 nm and 520 nm in order to obtain chromatograms.

HPLC-MS/MS was used to identify anthocyanins. The extracts were analyzed in positive ion ESI mode, as positive ion mode yielded more fragmentation information than negative ion mode (Farang *et al.* 2007). Nitrogen was used as the drying and the nebulizing gas and nebulizer pressure was 45 psi. The gas flow rate was 11 L/min at 350 $^{\circ}$ C. The capillary current flow was 7 μ A. The Delta EMV was 300 V. The spectra were recorded in positive ionization modes between m/z 100 and 1000.

Anthocyanidin-3-monoglucosides were quantified at 520 nm as malvidin-3,5-diglucoside chloride equivalents. The equation was A (area) = 18.5664793 Amt (amount, ng/ μ L) - 43.310899, with $r^2 = 0.9998$.

2.2.5 Chemicals.

The standard used for HPLC was malvidin-3,5-diglucoside chloride (Sigma, Castle Hill, NSW, Australia). All the solvents used for HPLC analysis, including acetonitrile and formic acid, were of HPLC grade (CNW, Dusseldorf, Germany). Deionized water was obtained from a Milli-Q Element water purification system (Millipore, Bedford, MA).

2.3 Results

2.3.1 Identification of anthocyanins

An HPLC chromatogram was obtained from berry pedicel extract, as the pedicel contained all the anthocyanins detected (Figure 2-2). A total of 19 peaks were detected and identified via HPLC-MS/MS based on the detected aglycones and MS/MS fragmentation profiles (Table 2-1). All anthocyanins identified were monoglucosides and their derivatives: malvidin (Mv), peonidin (Pn), petunidin (Pt), delphinidin (Dp), cyanidin (Cy), and pelargonidin (Pl). Mv, Pn, Pt, Dp, and Cy all included -3-O-glucosides and -3-O-(6-O-acetyl)-glucosides. Pt and Cy additionally

had -3-O-(6-O-coumaryl)-glucoside derivatives. Pn and Mv additionally had -3-O-(6-O-caffeoyl)-glucosides, -3-O-(cis-6-O-coumaryl)-glucosides, and -3-O-(trans-6-O-coumaryl)-glucosides. P1 only appeared as pelargonidin-3-O-glucoside.

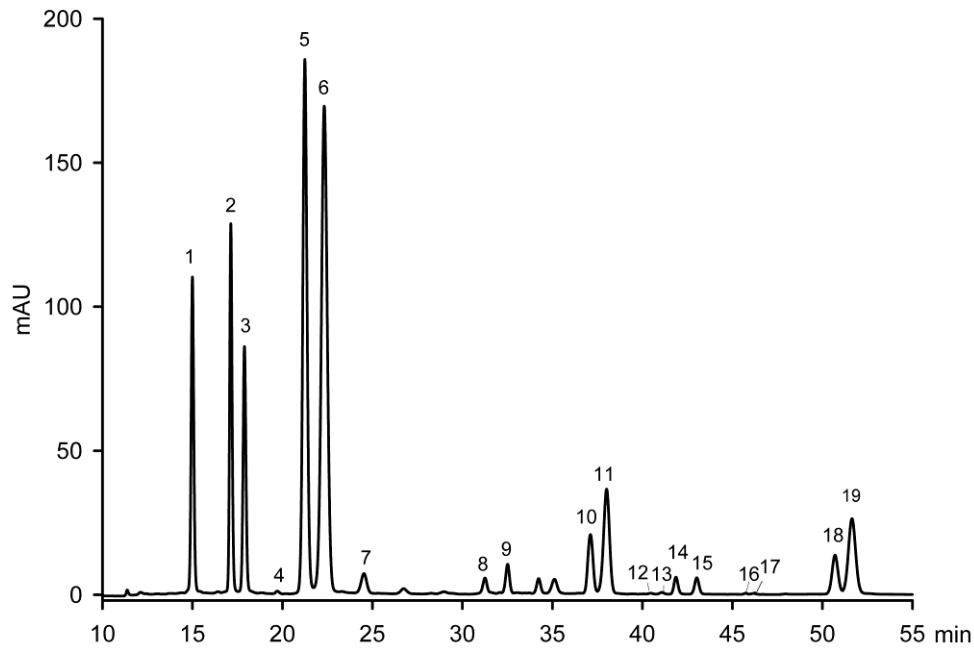


Figure 2-2 HPLC chromatogram at 520 nm of anthocyanins in berry pedicels of Yan-73 grape.

2.3.2 Composition of anthocyanins in grape organs.

The anthocyanin composition of mature berries and leaves differed by grape organ (Table 2-2). Berry skin contained 17 anthocyanins and Pn-3-O-(cis-6-O-coumaryl)-glucoside and Mv-3-O-(cis-6-O-coumaryl)-glucoside were not detectable. In addition to those two anthocyanins, Pn-3-O-(6-O-caffeoyl)-glucoside was also not detectable in berry pulp. Rachis contained all 19 anthocyanins, while pedicels lacked Pn-3-O-(cis-6-O-coumaryl)-glucoside. In leaf lamina, vein and petioles, and bark, 15, 11, and 16 anthocyanins were detected, respectively.

Anthocyanins in berry skin were mainly composed of Mv at berry maturity,

accounting for 57.6% of total anthocyanin (Table 2-2). Among Mv derivatives, Mv-3-O-glucoside was the predominant form, followed by Mv-3-O-(6-O-acetyl-glucoside). Pn, Pt, Dp, and Cy each accounted for 4.8 to 17.7% of total anthocyanin, and -3-O-glucoside was their main derivative. Berry pulp had much lower total anthocyanin (31.94 mg/100 g FW) than berry skin (701.68 mg/100 g FW) at berry maturity. Moreover, differing from berry skin, Pn was the most abundant anthocyanin in berry pulp (60.0%), with Pn-3-O-glucoside as a main derivative. Mv was the second most abundant anthocyanin and accounted for 25.7% of total anthocyanin. Pedicels contained much more total anthocyanin (139.35 mg/100 g FW) than rachis (11.61 mg/100 g FW). In pedicels and rachis, both Mv and Pn were the dominant anthocyanins, each accounting for 31.0 to 45.2%. Similar to berry skin and pulp, Mv- and Pn-3-O-glucoside were the main derivatives. Leaf lamina, leaf vein and petiole, and bark contained very low levels of anthocyanins (0.50 to 2.99 mg/100 g FW) when leaves matured (Table 2-2). Mv and Pn were still the main anthocyanins. In addition to -3-O-glucoside, Mv- and Pn-3-O-(trans-6-O-coumarylglucoside) contributed total anthocyanins, especially in vein and petiole and in bark.

In all organs, five components—Pt-O-(6-O-acetyl-glucoside), Dp-3-O-(6-O-acetyl-glucoside), Cy-3-O-(6-O-acetyl-glucoside), Pn-3-O-(6-O-caffeoyl-glucoside), and Pt-3-O-(6-O-coumaryl-glucoside)—were consistently less than 5.0% of total anthocyanin in various organs. Pl-3-O-glucoside was also detected in berry skin, pulp, pedicels, and pedicels in this study, although it was less than 1.44 mg/100 g FW (<1.4% total anthocyanin). In addition, trace amounts of Mv-, Pn-, Pt-, Dp-, and Cy-3-O-glucoside were detected in xylem, at 0.09 to 0.11 mg/100 g FW each, and their total concentration was ~0.6 mg/100 g FW (not shown).

2.3.3 *Developmental changes of anthocyanins in various grape organs.*

Anthocyanin components and concentrations in various organs varied along the season. Developmental variations of Mv, Pn, Pt, Dp, and Cy as well as total

anthocyanins in four berry tissues were shown in Figure 2-3. In berry skin and pulp, Mv, Pn, Pt, Dp, and Cy, as well as total anthocyanins, showed similar trends (Figure 2-3). Total anthocyanin was very low before veraison (DAA 65), <0.16 mg/100 g FW. Starting at veraison, total anthocyanin sharply increased until maturity (DAA 97) and still increased one week after maturity (DAA 102). In pedicels and rachis, Mv, Pn, Pt, Dp, Cy, and total anthocyanin concentrations slightly increased starting at veraison, but increased more sharply at DAA 80, later than in berry skin and pulp (Figure 2-3).

Table 2-1 Retention time, ultra violet and mass spectra data for the anthocyanins identified in various organs of Yan-73 grape.

Peak No.	Rt (min)	Molecular ion M ⁺ (m/z)	Fragmentions M ⁺ (m/z)	Absorbance maxima (nm)	Identity
1	15.242	465	465,303	523	Delphinidin-3- <i>O</i> -glucoside
2	17.306	449	449,287	516	Cyanidin 3- <i>O</i> -glucoside
3	18.127	479	479,317	527	Petunidin-3- <i>O</i> -glucoside
4	20.325	433	433,271	505	Pelargonidin-3- <i>O</i> -glucoside
5	21.360	463	463,301	517	Peonidin-3- <i>O</i> -glucoside
6	22.389	493	493,331	528	Malvidin-3- <i>O</i> -glucoside
7	25.888	507	507,465,303	528	Delphinidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
8	32.206	491	491,287	522	Cyanidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
9	33.234	521	521,317	530	Petunidin- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
10	37.769	505	505,463,301	519	Peonidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
11	38.391	535	535,493,331	528	Malvidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
12	41.683	625	625,463,301	521	Peonidin-3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)
13	42.304	655	655,493,331	534	Malvidin-3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)
14	43.373	595	595,449,287	522	Cyanidin-3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
15	44.635	625	625,479,317	531	Petunidin-3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
16	47.180	609	609,463,301	525	Peonidin-3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)
17	47.453	639	639,493,331	538	Malvidin-3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)
18	53.017	609	609,463,301	523	Peonidin-3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)
19	53.905	639	639,493,331	533	Malvidin-3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)

Anthocyanins in the leaf lamina varied with leaf age, and to a lesser degree in leaf vein and petioles and in bark (Figure 2-4). In the leaf lamina, Mv, Pn, Pt, Dp, Cy, and total anthocyanins showed similar variations; they were high in young leaf lamina,

tended to decrease in expanding and mature lamina, and dramatically increased to their highest concentration in senescing leaf lamina. In leaf vein and petioles, Pn, Pt, Dp, Cy, and total anthocyanin were low and did not show obvious differences during young, expanding, and mature leaf stages, and then increased at different rates at the senescing leaf stage. However, Mv in leaf vein and petioles tended to increase with leaf development, and then decreased at senescence. In general, Mv, Pn, Pt, Dp, Cy, and total anthocyanin concentration in the bark exhibited a different pattern than leaf development. Mv and Pn were the two dominant components, but had opposing trends. Mv increased and Pn decreased in bark from the young to mature leaf stage. Dp, Cy, and Pt were always less than 0.1 mg/100 g; Dp concentration was stable whereas Cy and Pt decreased and increased, respectively. Total anthocyanin in bark slightly increased from 2.3 to 3.0 mg/100 g FW.

2.4 Discussion

Anthocyanins in *Vitis vinifera* berries mainly consist of cyanidin-, delphinidin-, petunidin-, peonidin-, and malvidin-3-monoglucosides together with the corresponding acetyl, p-coumaroyl, and caffeoyl derivatives (Liang *et al.* 2009). Pelargonidin-3-O-glucoside has also been reported to occur in *V. vinifera* berries (Castillo-Munoz *et al.* 2009, He *et al.* 2010). Compared with an earlier study on Yan-73 (He *et al.* 2010), two additional anthocyanin components, Pn-3-O-(6-O-caffeoyl-glucoside) and Cy-3-O-(6-O-coumaryl-glucoside), were detected in skin, ranging from 0.45 to 2.95 mg/100 g FW (0.10–0.59% of total anthocyanin). Mv-3-O-(6-O-feruloyl-glucoside), Pn-3-O-(cis-6-O-coumaryl-glucoside) and Mv-3-O-(cis-6-O-coumaryl-glucoside) were not detected in skin in the current study, compared with 0.12 to 0.42 mg/100 g FW (0.10–0.35% of total anthocyanins) in the earlier study (He *et al.* 2010). In Yan-73 berry pulp, 16 anthocyanins were detected in the earlier study (He *et al.* 2010), with Pn-3-O-glucoside the most abundant anthocyanin, as in the current study. We detected 19 anthocyanins, among which Cy-3-O-(6-O-coumaryl-glucoside) and

Regulation of anthocyanin metabolism in grape: Effect of light on teinturier cultivars

Table 1-2 Anthocyanin concentration (mg/100 g FW) (mean \pm standard deviation) in berry tissues when berries matured (DAA 97), leaf tissues and living bark at the base of the shoot (bark) at mature leaf stage. Mv, Pn, Pt, Dp, Cy and Sum are malvidin, peonidin, petunidin, delphinidin, cyanidin and total anthocyanins, respectively.

Anthocyanins	Berry				Leaf		Bark
	skin	pulp	carpopodia	pedicel	lamina	vein and petioles	
Malvidin-3- <i>O</i> -glucoside	285.04 \pm 35.62	6.43 \pm 0.97	32.71 \pm 7.92	2.54 \pm 0.35	0.08 \pm 0.03	0.38 \pm 0.01	0.88 \pm 0.05
Malvidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	81.56 \pm 8.59	1.39 \pm 0.22	8.95 \pm 1.93	0.42 \pm 0.06	0.03 \pm 0.003	0.11 \pm 0.002	0.07 \pm 0.008
Malvidin-3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)	1.13 \pm 0.05	0.07 \pm 0.03	0.63 \pm 0.32	0.15 \pm 0.03	0.03 \pm 0.001	0.11 \pm 0.003	0.05 \pm 0.004
Malvidin-3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)	ND	ND	0.29 \pm 0.29	0.08 \pm 0.04	0.03 \pm 0.002	0.09 \pm 0.003	0.14 \pm 0.02
Malvidin-3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)	36.24 \pm 9.79	0.32 \pm 0.06	7.54 \pm 1.95	0.63 \pm 0.15	0.05 \pm 0.01	0.45 \pm 0.02	1.02 \pm 0.13
Mv	403.97 \pm 54.05	8.21 \pm 1.28	50.12 \pm 12.41	3.82 \pm 0.63	0.22 \pm 0.05	1.14 \pm 0.04	2.16 \pm 0.21
Peonidin-3- <i>O</i> -glucoside	90.82 \pm 17.15	17.04 \pm 0.68	33.10 \pm 1.53	4.18 \pm 0.45	0.07 \pm 0.02	0.10 \pm 0.002	0.13 \pm 0.01
Peonidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	23.23 \pm 3.93	1.75 \pm 0.16	5.36 \pm 0.47	0.42 \pm 0.01	0.03 \pm 0.002	0.06 \pm 0.0002	0.04 \pm 0.002
Peonidin-3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)	1.27 \pm 0.15	ND	0.59 \pm 0.30	0.10 \pm 0.001	0.02 \pm 0.008	0.05 \pm 0.0004	0.06 \pm 0.001
Peonidin-3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)	ND	ND	ND	0.07 \pm 0.04	0.03 \pm 0.001	ND	0.08 \pm 0.002
Peonidin-3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)	9.17 \pm 1.67	0.37 \pm 0.05	4.06 \pm 0.31	0.48 \pm 0.06	0.04 \pm 0.007	0.12 \pm 0.003	0.33 \pm 0.02
Pn	124.49 \pm 22.21	19.16 \pm 0.89	43.11 \pm 2.61	5.25 \pm 0.56	0.19 \pm 0.04	0.33 \pm 0.03	0.64 \pm 0.03
Petunidin-3- <i>O</i> -glucoside	74.60 \pm 9.36	0.86 \pm 0.11	8.88 \pm 1.46	0.31 \pm 0.02	0.03 \pm 0.001	0.06 \pm 0.0003	0.04 \pm 0.002
Petunidin- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	21.07 \pm 2.30	0.28 \pm 0.03	2.32 \pm 0.22	0.17 \pm 0.002	ND	ND	0.02 \pm 0.0001
Petunidin-3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)	5.97 \pm 0.63	0.11 \pm 0.003	1.64 \pm 0.17	0.10 \pm 0.05	0.02 \pm 0.01	0.06 \pm 0.0002	0.04 \pm 0.001
Pt	101.64 \pm 12.29	1.25 \pm 0.14	12.84 \pm 1.85	0.58 \pm 0.07	0.05 \pm 0.01	0.12 \pm 0.0002	0.10 \pm 0.01
Delphinidin-3- <i>O</i> -glucoside	9.97 \pm 0.61	0.83 \pm 0.12	10.63 \pm 1.54	0.35 \pm 0.02	0.02 \pm 0.01	0.05 \pm 0.0003	0.03 \pm 0.001
Delphinidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	23.37 \pm 1.40	0.24 \pm 0.06	2.45 \pm 0.24	0.14 \pm 0.002	ND	ND	ND
Dp	33.34 \pm 2.01	1.07 \pm 0.18	13.08 \pm 1.78	0.49 \pm 0.02	0.02 \pm 0.01	0.05 \pm 0.0003	0.03 \pm 0.001
Cyanidin-3- <i>O</i> -glucoside	25.31 \pm 4.74	1.57 \pm 0.11	15.28 \pm 1.05	0.93 \pm 0.14	0.01 \pm 0.01	ND	0.03 \pm 0.001
Cyanidin-3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	8.65 \pm 1.43	0.24 \pm 0.03	2.06 \pm 0.02	0.19 \pm 0.01	ND	ND	ND
Cyanidin-3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)	2.84 \pm 0.37	0.11 \pm 0.004	1.82 \pm 0.02	0.19 \pm 0.04	0.02 \pm 0.01	ND	0.03 \pm 0.0002
Cy	36.80 \pm 6.54	1.92 \pm 0.14	19.16 \pm 1.09	1.31 \pm 0.19	0.03 \pm 0.02	ND	0.06 \pm 0.001
Pelargonidin-3- <i>O</i> -glucoside	1.44 \pm 0.12	0.33 \pm 0.02	1.04 \pm 0.01	0.16 \pm 0.001	ND	ND	ND
Sum	701.68 \pm 97.22	31.94 \pm 2.65	139.35 \pm 19.75	11.61 \pm 1.47	0.50 \pm 0.13	1.64 \pm 0.08	2.99 \pm 0.25

Chapter II

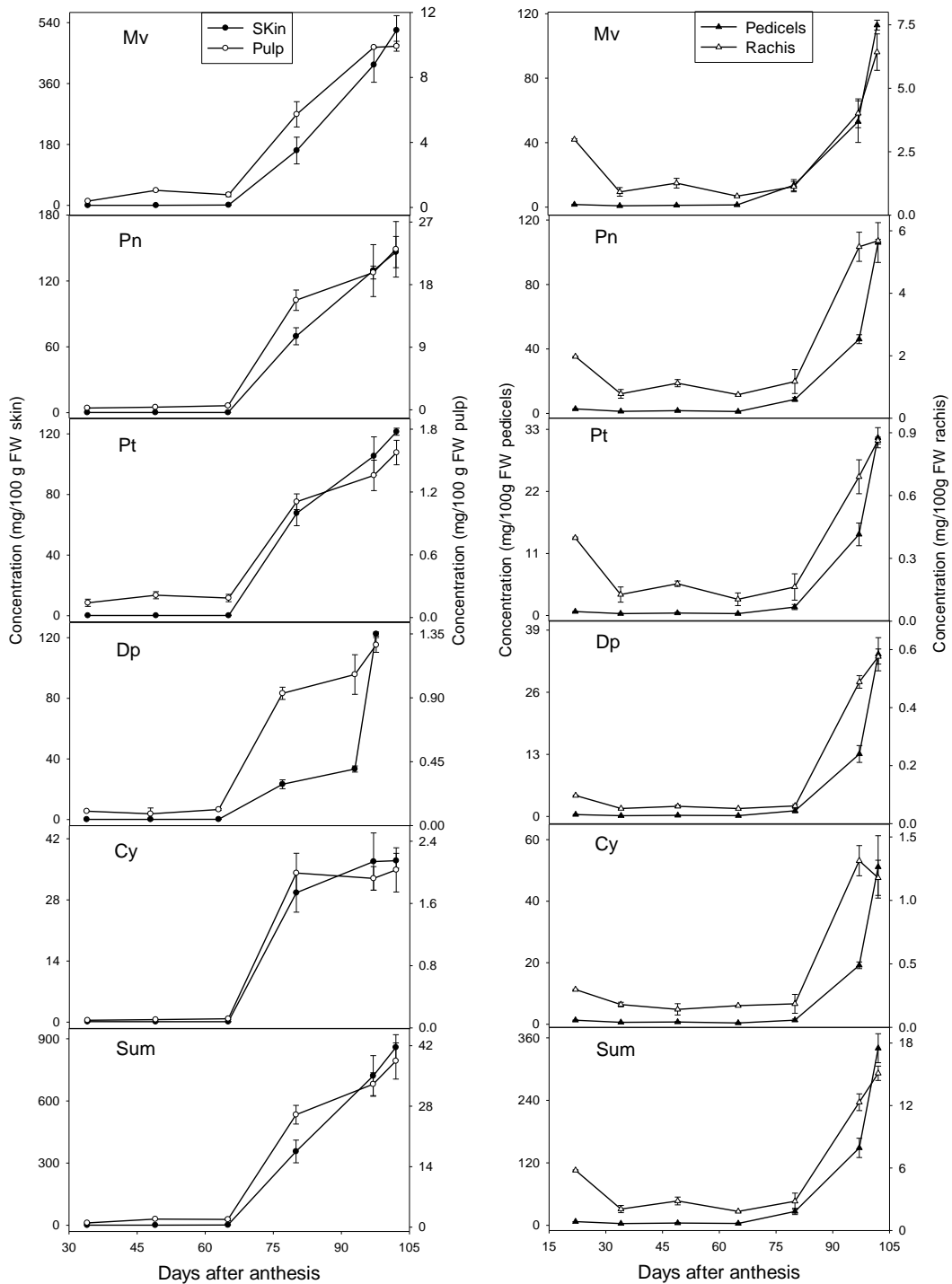


Figure 2-3 Dynamic changes of anthocyanins in Yan-73 grape skin and pulp (left), pedicels and rachis (right) throughout grape berry development. Mv, Pn, Pt, Dp, Cy and Sum are malvidin, peonidin, petunidin, delphinidin, cyanidin derivatives and total anthocyanins, respectively. Bars represent standard deviation of three biological replicates.

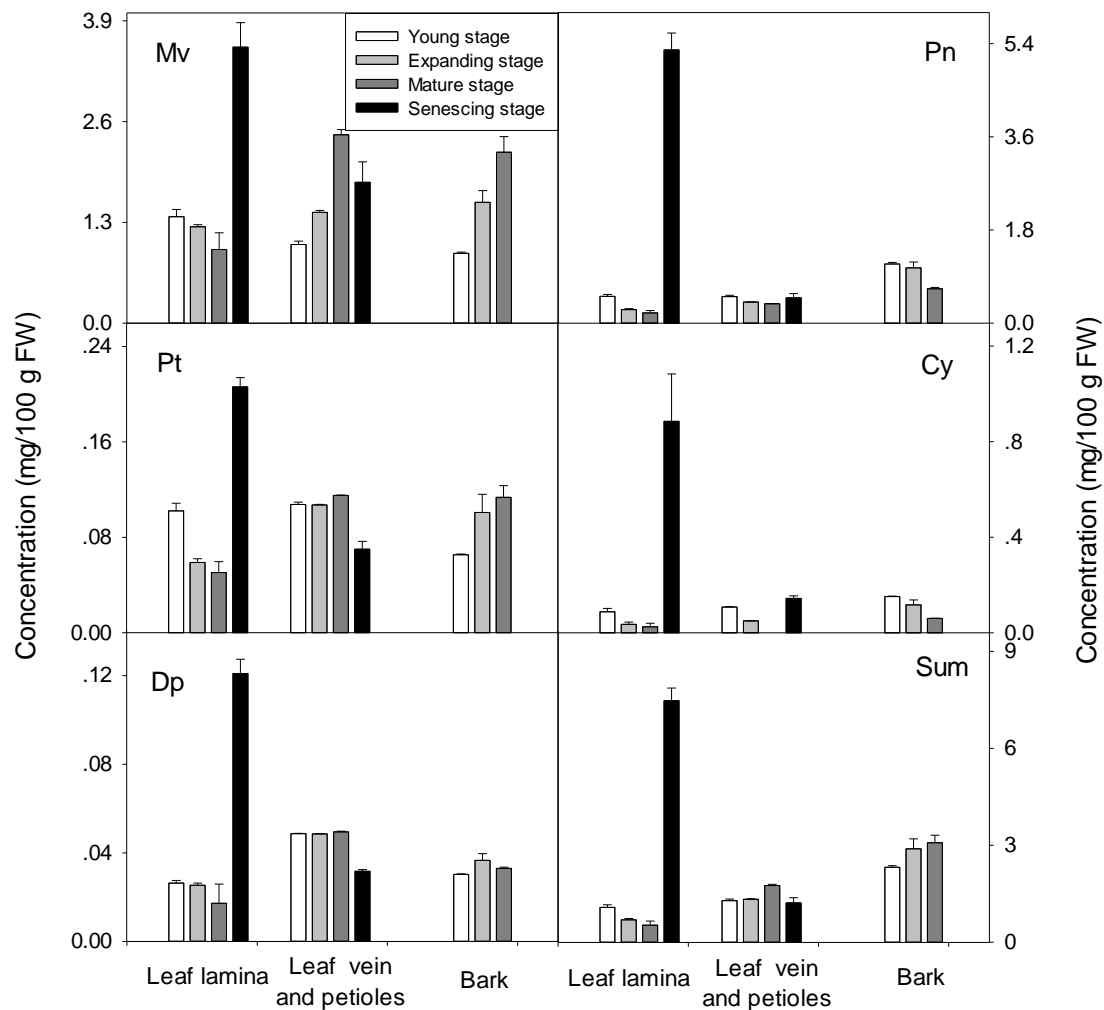


Figure 2-4 Anthocyanin concentrations in leaf lamina and leaf vein and petioles at young, expanding, mature, and senescing leaf stages and in living bark at the base of the shoot (bark) at the first three stages. Mv, Pn, Pt, Dp, Cy, and Sum are malvidin, peonidin, petunidin, delphinidin, cyanidin, and total anthocyanins, respectively. Bars represent standard deviation of three biological replicates.

Pn-3-O-(6-O-caffeoyl-glucoside) were newly detected, while Mv-3-O-(6-O-feruloyl-glucoside), Pn-3-O-(cis-6-O-coumarylglucoside) and Mv-3-O-(cis-6-O-coumaryl-glucoside) were not detected.

In Alicante Bouschet, the paternal parent of Yan-73, 20 anthocyanins in berry skin and 16 in berry pulp were previously identified (Castillo-Munoz *et al.* 2009) and

malvidin derivatives and peonidin derivatives were the main anthocyanins, respectively, in berry skin and pulp, as in the current study. Peonidin dihexoside, Dp-3-O-(6-O-coumaryl-glucoside), and Pl-3-O-(6-O-coumaryl-glucoside) were not detected in Yan-73 berry skin and pulp in the current study because of their trace amounts (0.12 to 1.72% of total anthocyanin). Pn-3-O-(6-Ocaffeoyl-glucoside) and Cy-3-O-(cis-6-O-coumaryl-glucoside) with trace amount (0.18–0.40% of total anthocyanin) in our study were not detectable in berry skin of Alicante Bouschet, while Mv-3-O-(6-O-caffeoyl-glucoside), Pt-O-(6-O-acetylglucoside), and Dp-3-O-(6-O-acetyl-glucoside) (0.22–0.88% of total anthocyanin) were not detectable in berry pulp (Castillo-Munoz *et al.* 2009). Therefore, Yan-73 seems to share a similar anthocyanin profile with its paternal parent Alicante Bouschet in both berry skin and pulp. Muscat Hamburg, the maternal parent of Yan-73, does not accumulate anthocyanins in pulp.

Anthocyanin composition in berry skin and in pulp was different. Pelargonidin-based, cyanidin-based (cyanidin and peonidin), and delphinidin-based (delphinidin, malvidin, and petunidin) anthocyanins are, respectively, orange-scarlet, red, and blue (Kong *et al.* 2003). The relative proportion of the three types of anthocyanins determines the color variation among red/purple/blue berry grape varieties and their corresponding wines and juices (Castellarin *et al.* 2006). Therefore, berry skin and pulp of Yan-73, primarily containing malvidin-derivatives and peonidin-derivatives, respectively, may contribute differently to wine color in wine blending.

To date, there has been no study of anthocyanin composition of pedicels, rachis, or bark. In grape leaves, Jeong *et al.* (2006) reported a higher total anthocyanin concentration in senescing Bailey Alicante A grape leaves than in young leaves, although anthocyanin composition was not analyzed. The present study showed that anthocyanin composition and concentrations differed among grape organs. In the teinturier cultivar Lacryma, the expression of *PAL* (encoding phenylalanine ammonia-lyase), *CHS3*, *F3H1* (encoding flavanone 3-hydroxylase), and *GST* was higher in berry pulp than in skin, while it was hardly detected in pulp of the non

teinturier cultivar Gamay (Ageorges *et al.* 2006). *VvmybA1* was expressed specifically in berry skin of non teinturier cultivars and in all pigmented organs of teinturier cultivars, including berry skin, pulp, and young and old leaves (Jeong *et al.* 2006). The results of these studies suggest that anthocyanin synthesis in pigmented organs of grape may be caused by the organ-specific expression of these structural and regulatory genes associated with anthocyanin biosynthesis, although that has yet to be confirmed.

As is well known, veraison is a key period for anthocyanin accumulation in berry skin (Boss *et al.* 1996b, Boss and Davies 2009). The present study showed that berry pulp had significant anthocyanin accumulation at the same time as berry skin, indicating that the initial appearance of color in both berry skin and pulp occurred at veraison. Moreover, our results showed that pecicels and rachis also had a sharp increase in anthocyanin accumulation, although it occurred later than veraison of berry skin and pulp. Anthocyanin accumulation in pecicels and rachis might be related to that in berry skin and pulp. Veraison might have an effect on physiological events and gene expression related to anthocyanin synthesis in pecicels and rachis. It will be necessary to study developmental variation of anthocyanins at shorter time intervals than in the current study to determine the role of veraison in anthocyanin accumulation of pecicels and rachis.

Variation in anthocyanin content along leaf development was most evident in the leaf lamina, and to a lesser degree in leaf vein and petiole tissue and in bark. Anthocyanins were high in young leaf lamina, decreased in expanding and mature leaf lamina, and increased in senescing leaf lamina. Young leaves of *Vitis vinifera* (Liakopoulos *et al.* 2006) and *Quercus coccifera* (Karageorgou and Manetas 2006) and senescing leaves of *Cornus stolonifera* (Feild *et al.* 2001) accumulated anthocyanins. In addition, higher total anthocyanin concentrations were observed in older grape leaves than in younger leaves (Jeong *et al.* 2006), consistent with the present results. Anthocyanins might play a photoprotective role. Young or senescing leaves were more susceptible to photoinhibition because of their immature or deteriorating photosynthetic apparatus. Expanding and mature leaves had a fully

developed functional photosynthetic apparatus and had increased photoprotection by biochemical mechanisms and therefore could endure high irradiances without photodamage (Liakopoulos *et al.* 2006). The role of anthocyanins in grape leaves, as well as in bark, whether for photoprotection or some other purpose, needs further study.

2.5 Conclusions

This study shows that anthocyanin composition and concentration differed among various grape organs and that not only berry skin but also berry pulp, rachis, and pedicels may have veraison period when anthocyanins accumulated rapidly. It would be interesting to further study the structural and regulatory gene expression to gain insight into anthocyanin synthesis in various organs. In addition, the possible veraison period in various organs needs to be further studied at biological and molecular levels.

2.6 Acknowledgments

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Chapter III. Regulation of anthocyanin biosynthesis in tissues of a teinturier grape cultivar under sunlight exclusion

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Abstract

Yan-73 (*Vitis vinifera*) is a teinturier grape cultivar, which accumulates anthocyanins in skin, pulp, pedicels, and rachis. The effects of sunlight on anthocyanin biosynthesis and regulation in various tissues of this teinturier cultivar were investigated. Light transmission measurements showed that covering with opaque boxes substantially reduced light intensity around clusters; $\leq 0.25\%$ of incident light reached the berry skin and $< 0.05\%$ reached the pulp. The pulp naturally experiences increasing sunlight exclusion by skin during ripening. Sunlight exclusion reduced and delayed anthocyanin biosynthesis in skin and pulp during berry development, while both tissues nevertheless accumulated enough anthocyanins to turn dark red. No strong reduction of anthocyanin concentration was observed in pedicels or rachis. Sunlight exclusion decreased transcript abundance of *VvUFGT*, *VvMybA1*, *VvMybA2*, and *VvMyc1* in both skin and pulp and also decreased transcript abundance of *VvMycA1* in pulp. Sunlight exclusion differently influenced

the anthocyanin components: it decreased the relative proportion of 3',4',5'-hydroxylated anthocyanins and increased that of 3',4'-hydroxylated anthocyanins in berry skin and pulp, which corresponded to the change in the ratio of $VvF3'H$ to $VvF3'5'H$. These results allow for insight on anthocyanin biosynthesis in various grape tissues in absence of sunlight.

Key words: teinturier, anthocyanin, sunlight exclusion, tissues, development

3.1 Introduction

Anthocyanins are a class of flavonoids responsible for the color of grape berries. Berry coloration is an important factor for market acceptance of table-grape cultivars and red wines. For most grape cultivars, berry skin is the main or only tissue to accumulate anthocyanins. However, teinturier grape cultivars (also called dyers) synthesize anthocyanins in both skin and pulp and are commonly used for blending to give an intense color to red wine (Ageorges *et al.* 2006, Jeong *et al.* 2006b). Much is known about the genetic relationships between teinturier varieties (Santiago *et al.* 2008) and about anthocyanin content (Castillo-Muñoz *et al.* 2009), seasonal accumulation profile (Guan *et al.* 2012), and biosynthesis related gene expression (Castellarin *et al.* 2011) in teinturier skin and pulp. Some teinturier grape cultivars may also accumulate anthocyanins in other tissues such as pedicels, rachis, leaf lamina and veins, petioles, living bark at the base of shoots, and seeds (Falginella *et al.* 2012, Guan *et al.* 2012, Jeong *et al.* 2006b).

Teinturier cultivars generally have a much higher anthocyanin concentration per unit juice volume or fresh mass than non-teinturier cultivars (Ageorges *et al.* 2006, Balík and Kumšta 2008), and among nine cultivars originating from south Moravia, the teinturier variety Neronet contained the highest concentration (2.15 to 4.49 g/kg fresh grapes) (Balík and Kumšta 2008). There are also notable differences in anthocyanin quantity and composition between berry skin and pulp of teinturier cultivars (Ageorges *et al.* 2006, Castillo-Muñoz *et al.* 2009, Falginella *et al.* 2012). We have previously shown that malvidin derivatives predominate in berry skin and

peonidin derivatives predominate in the pulp but that similar concentrations of malvidin and peonidin derivatives were found in pedicels, rachis, leaf lamina and veins, petioles, and living bark of Yan-73 (Guan *et al.* 2012).

Anthocyanin biosynthesis is widely studied in grape berry skin. It is primarily regulated at the transcription level of structural and regulatory genes. Among structural genes, the expression level of UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) is closely related to anthocyanin biosynthesis (Zheng *et al.* 2013), and the expression of flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-hydroxylase (*F3'H*) affects anthocyanin composition in berry skin (Castellarin *et al.* 2006, Jeong *et al.* 2006a). A prevalence of *F3'5'H* over *F3'H* would lead to greater production of delphinidin, the precursor of petunidin and malvidin, and would yield less cyanidin, the precursor of peonidin. The relative proportion of these two types of anthocyanins determines color variation among red/purple/blue grape varieties and their corresponding wines and juices (Castellarin *et al.* 2006). Three regulatory gene families encoding *R2R3-MYB*, basic helix-loop-helix (*bHLH*, also known as *MYC*), and tryptophan-aspartic acid repeat (*WDR*) proteins, which are involved in the control of anthocyanin biosynthesis in berry skin, have been extensively analyzed (Hichri *et al.* 2011). Gene expression in other tissues of teinturier cultivars has been lately studied. Color determination of various tissues of teinturier cultivars may be related to the expression of *UFGT*, chalcone synthase (*CHS*), glutathione *S*-transferase (*GST*), and caffeoyl methyl transferase (*CaOMT*) (Ageorges *et al.* 2006) and may result from tissue-specific expression of *VvMybA1* (Jeong *et al.* 2006b). In berries of the teinturier variety Alicante Bouschet (*Vitis vinifera*), anthocyanins initially appeared in the flesh near the stylar end, where localized transcription of *VvMybA* and *VvUFGT* can initially be detected (Castellarin *et al.* 2011). More recently, the expression of 41 genes related to anthocyanin biosynthesis and transport were characterized in the skin, pulp, and seed of Alicante Bouschet berries; expression varied with spatiotemporal distribution of anthocyanins and their chemical composition (Falginella *et al.* 2012).

Anthocyanin biosynthesis in grapes is controlled genetically but is also

influenced by environmental factors and viticultural practices. Sunlight is a fundamental requirement for color formation. Sunlight exclusion has been found to decrease the biosynthesis of anthocyanins (Cortell and Kennedy 2006, Jeong *et al.* 2004) and the expression of structural and regulatory genes *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, *UFGT*, and *MybA1* (Jeong *et al.* 2004) in grape berry skin. In Merlot grapes, light exposure contributed more to alterations of anthocyanin profiles in grape skin than to changes in total anthocyanin accumulation (Tarara *et al.* 2008). Most frequently, light exposure resulted in changes in the ratio of disubstituted/trisubstituted or acylated/nonacylated anthocyanins (Cortell and Kennedy 2006, Tarara *et al.* 2008). Differences between results may be related to specific cultivars and/or experimental designs, such as different developmental stages and the degree of sunlight exclusion. Out of 40 *V. vinifera* cultivars from which sunlight was excluded from clusters during fruit development, Tokay and Sulfanina Rose berries remained green, while those of the other 38 cultivars colored similarly to the exposed berries (Weaver and Mccune 1960). Similarly, anthocyanin accumulation showed no significant difference between sunlight exposed and excluded berry clusters in Pinot noir (Cortell and Kennedy 2006) and in two out of three years in Shiraz (Downey *et al.* 2004). In the red grape cultivars Jingxiu and Jingyan, anthocyanin biosynthesis in skin was closely linked to sunlight exposure in Jingxiu whereas it was relatively sunlight independent in Jingyan (Zheng *et al.* 2013). Thus, under sunlight exclusion from fruit set until maturity, Jingxiu berry skin failed to color and the two anthocyanin biosynthesis genes, *VvUFGT* and *VvMybA1*, were not expressed, while Jingyan skin developed color and the transcripts of these two genes were at high levels. However, the effect of sunlight exclusion on anthocyanin biosynthesis and regulation in berry skin, pulp, pedicels, and rachis of teinturier grapes has not yet been investigated.

The aim of this study was to gain insight into the regulation of anthocyanin accumulation in response to sunlight exclusion in various tissues. To this end, sunlight was excluded from clusters from fruit set until one week after maturity for the teinturier cultivar Yan-73 in two successive years. Anthocyanin composition and

concentration in berry skin, pulp, pedicels, and rachis was measured using HPLC, and the expression of some structural and regulatory genes in skin and pulp during berry development was monitored via real-time PCR.

3.2 Materials and Methods

3.2.1 Plant materials

Eighteen-year-old teinturier cultivar ‘Yan-73’ (*V. vinifera*, Muscat Hamburg × Alicante Bouschet) vines grown in the experimental vineyard of the Institute of Botany, Chinese Academy of Sciences, Beijing (lat. 39°90’N; long. 116°30’E) were used in this study. The vines were trained to a fan-shape trellis with single trunk, allowing for ease in burial during winter. The vines had a height of 1.7 m, spaced 1.5 m apart within the row and 2.5 m apart between rows. Rows were oriented north-south. The vines were maintained under routine viticulture conditions for irrigation, fertilization, soil management, pruning, and disease control.

3.2.2 Treatment

Over two successive seasons (2010 and 2011), the shoots of ~50 vines on three rows were tipped and the crop load was adjusted to one cluster per shoot at 9 days after anthesis (DAA). Opaque boxes were applied to 66 randomly selected clusters until one week after maturity (sunlight exclusion treatment), and the same number of randomly selected clusters were tagged as controls (sunlight exposure treatment). The boxes used were designed following Downey *et al.* (2004), with minor modifications, to eliminate sunlight while maximizing airflow and minimizing the impact on temperature. Boxes were white on the outside and black on the inside and measured approximately 400 x 200 x 150 mm, which was sufficient for cluster development.

3.2.3 Temperature and light transmission measurements

During the experimental period of 2011, the temperatures inside and outside boxes were monitored with temperature and humidity data loggers (ZDR-20h, Zeda,

Hangzhou, China). The box paper material and fresh berry skin at different developmental stages (36, 55, 69, and 91 DAA in 2011) were cut into oblong shape to fit the side of the quartz cuvette facing the light beam. Light transmission through the box material and fresh berry skin was then measured every 50 nm from ~300 to 1100 nm using a UV/Vis spectrophotometer (Specord 200, AnalytikJena, Jena, Germany). Furthermore, a spectrum transmission meter (LS108, Linshang, Shenzhen, China) was used to measure light transmission with light sources (1.5 mm diam.) covered by the box material and fresh berry skin. Light sources included ultraviolet (UV, median wavelength 365 nm), visible (380–760 nm with $\pm 2\%$ accuracy), and infrared (IR, median wavelength 950 nm). Eight replicates were performed for light transmission measurements. In 2011, a QRT1 Quantitherm light meter (Hansatech Instruments, Norfolk, UK), measuring the level of photosynthetically active radiation (PAR, 400–700 nm), was placed inside a box with a sunlight-excluded cluster.

3.2.4 Sampling

From 30 DAA to one week after maturity, the clusters from both treatments were collected every second week in 2010 and at weekly intervals in 2011. Veraison was determined as the time when the berries began to soften and ~50% of the berries started to change color (Negri *et al.* 2008). The maturity date was estimated by assessing physical properties of the berries, the ease of removal of berries from pedicels without berry tissue shriveling resulting from loss of water, and the change of seeds from bright green to tan-brown or woodier (Bisson 2001). The estimation of the maturity date also referred to previous records. Berries were harvested by a single person to maintain consistency of maturity grade. Veraison and maturity dates were ~65 and 97 DAA in 2010, and 55 and 91 DAA in 2011, respectively. On each date, three replicates for each treatment were sampled and each replicate consisted of three clusters. Clusters were manually separated into pedicels, rachis, and berries, and then berry skin was immediately peeled with forceps in the field. Berry skin, pulp, pedicels, and rachis were separately frozen in liquid nitrogen. They were stored at $-40\text{ }^{\circ}\text{C}$ for anthocyanin analysis in both years. In 2011, some berry skin and pulp was stored at

-80 °C for RNA extraction and some at -40 °C for analysis of soluble sugars and organic acids.

3.2.5 Analysis of anthocyanins, soluble sugars, and organic acids

Anthocyanin extraction and analysis was conducted following Guan *et al.* (2012). Berry pulp was powdered in liquid nitrogen using an A11 basic analytical mill (IKA, Staufen, Germany). A 1 g portion of the resultant powder was mixed for 2 h with 6 mL ultrapure water, and then centrifuged at 20,000 x g for 10 min at 4 °C. The supernatant was recovered and filtered through a C18 cartridge (WAT020515; Waters, Milford, MA) to eliminate any interfering apolar chains, then through a 0.45 mm Sep-Pak filter (TR200102; Jasco, Nantes, France) to eliminate large particles. The extract was stored at - 40 °C for soluble sugars and organic acid measurements. Sugars were measured by injecting a 10 µL aliquot of the extract into a Waters Sugar-Pak I column (10 µm particle sizes, 6.5 x 300 mm ID) at 80 °C. Eluted peaks were detected with a refractive index detector (RI-1530; Jasco, Tokyo, Japan). Ultrapure water (Milli-Q Element water purification system; Millipore, Bedford, MA) was used as the mobile phase with a flow rate of 0.6 mL/min. Organic acids were measured by injecting a 10 µL aliquot of the extract into an ODS C18 column (5 µm particle size, 4.6 x 250 mm ID; Beckman, Fullerton, CA) at a flow rate of 0.8 mL/min using 0.02 M K₂HPO₄ (pH 2.4) as solvent. Organic acids were detected at a wavelength of 210 nm. The eluted peaks were detected with a PDA detector (Dionex, Sunnyvale, CA). Quantification of individual sugars and acids was done by comparison with the peak area of standards (Sigma Aldrich, St. Louis, MO).

3.2.6 Gene expression

Total RNA was isolated from berry skin and pulp by using a Universal Plant Total RNA Extraction Kit (Biotek, Beijing, China) and then treated with RQ1-DNase I (Promega, Madison, WI) to remove DNA contamination. First-strand cDNA was synthesized from 1 µg DNase-treated total RNA using M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo (dT) primers

according to the manufacturer's instructions. Relative transcript quantification of genes related to anthocyanin biosynthesis was done using 10x diluted cDNA, gene-specific primers (Table 3-1) and SYBR Green RealMasterMix (Tiangen Biotech, Beijing, China) via a Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Thermal cycling conditions were 94 °C for 2 min followed by 94 °C for 10 sec, 58 °C for 18 sec, and 68 °C for 20 sec, for 40 cycles. Dissociation curves for each amplicon were then analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60 to 95 °C. No evidence for primer dimers or other nonspecific product formation was detected for any of the primer pairs used. Gene transcripts were quantified by comparing the *Ct* of the target gene with that of *Actin* (EC969944). Experiments were performed with three biological replicates and three technical replicates. Reaction specificities were tested with melting gradient dissociation curves, electrophoresis gels, and cloning and sequencing of each PCR product. RT-PCR efficiencies and *Ct* values were calculated using LinRegPCR ver. 12.X (Heart Failure Research Center, Amsterdam, Netherlands). Normalized relative quantities were obtained using the $2^{-\Delta\Delta C_t}$ method, with the normalized relative quantities of sunlight-exposed skin/pulp on the first measurement date as 1 (Schmittgen and Livak 2008).

3.2.7 Statistical analysis

Relative gene expression was expressed as mean and standard error calculated over the three biological replicates. Significance of differences in sugars, acids, anthocyanin concentrations and relative gene expression between treatments on each sampling date was tested with *t* tests using SPSS ver. 16.0 (IBM, Chicago, IL).

3.3 Results

3.3.1 Sunlight exclusion without modification of berry temperature

The difference between temperatures inside and outside boxes ranged from 0.1 to 1.7 °C under ambient canopy temperatures ranging from 17 to 35 °C (Figure 3-1).

Visible and UV light have wavelengths ranging from 380 to ~760 nm and 100 to 400 nm, respectively (UV-A: 400–315 nm; UV-B: 315–280 nm; UV-C: 280–100 nm). UV-C rays are blocked by the ozone layer and do not reach the earth. Light transmission through the box material was measured as <0.01% in the wavelength range 350 to 1100 nm (UV-A, visible, and infrared light) by UV/vis spectrophotometer and as 0% by spectrum transmission meter (data not shown). The level of PAR inside boxes was <0.25% of that outside, ranging from 1000 to 2000 mmol/m²/sec during the experimental period (data not shown). These results indicated the boxes excluded sunlight efficiently without affecting berry temperature.

Table 3-1 Forward (F) and reverse (R) primers, and expected amplicon sizes of structural and regulatory genes involved in anthocyanin biosynthesis.

<i>Gene ID</i>		Sequence of forward (5'-3') and reverse primers (5'-3')	Amplicon length (bp)
<i>VvF3'H</i>	F	5'-TGGGCTGA CCCTACAACG5-3'	200
	R	5'-TCTTAACCAATGGGGCAAAGA-3'	
<i>VvF3'5'H</i>	F	5'-AGAATGGGAATA GTGCTGGT-3'	135
	R	5'-CCGAAAGA GAAACTGCCTT-3'	
<i>VvUFGT</i>	F	5'-GGGATGGTAATGGCTGTG-3'	148
	R	5'-GGGTGGA GA GTGA GTTA GGC-3'	
<i>VvMybA1</i>	F	5'-GAGA GTTTGCATTA GACGA GG-3'	83
	R	5'-CCTACCCGCAATCAA GGAC-3'	
<i>VvMybA2</i>	F	5'-TTATCGCAA GCCTCAG-3'	92
	R	5'-AATCACCCCTCA CCTCC-3'	
<i>VvMyb5a</i>	F	5'-CAATGAGAATTGGCAAA GCG-3'	144
	R	5'-GCAGCAGGTTCCCA GACA G-3'	
<i>VvMybPA1</i>	F	5'-TTTGGGAAATCGGTGGTC-3'	198
	R	5'-GACATGGA GATTAAGGA GGTGA-3'	
<i>VvMycA1</i>	F	5'-ATGATATAGAGGGGATGA GTGA-3'	148
	R	5'-CTTGGGAAGCA CCTCCATTA-3'	
<i>VvMyc1</i>	F	5'-CAGGGAA GGGCTGTTGC-3'	192
	R	5'-CACTGGGGTATTATTTGGTTTATT-3'	
<i>VvWDR1</i>	F	5'-GGTGAAGCA GGGGTTTTTCG-3'	162
	R	5'-TGGGTCCA GCAA GCGTAG-3'	
<i>VvWDR2</i>	F	5'-CATTCCCGACAA GGA CTGC-3'	229
	R	5'-CACGA GCCGAACAA GAGG-3'	

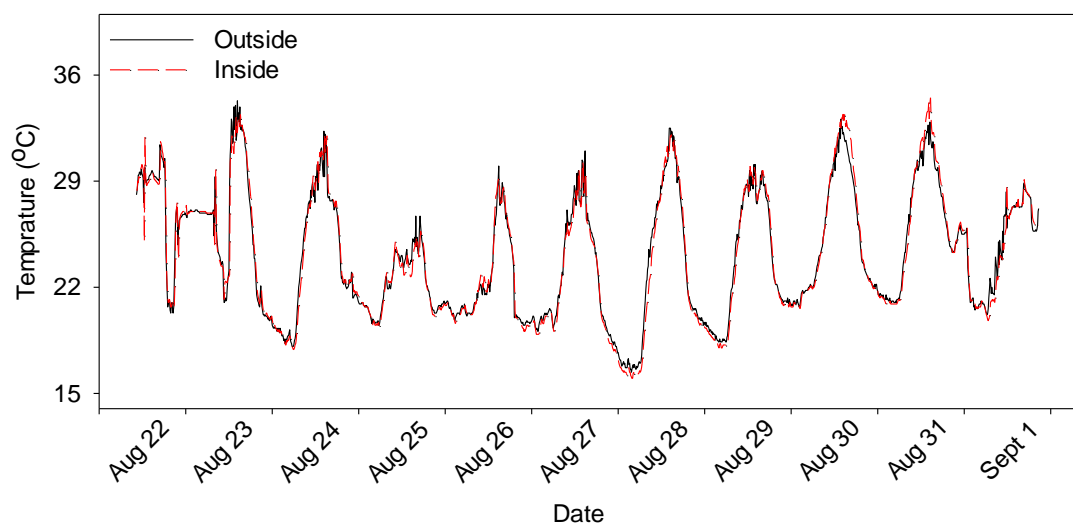


Figure 3-1 Temperatures inside and outside the box monitored with a temperature and humidity data logger during the nine successive days of the study.

3.3.2 Berry pulp and sunlight exclusion during ripening

Light transmission of sunlight-exposed berry skin (Figure 3-2) increased with wavelength but decreased with berry development for all wavelengths. UV/vis spectrophotometer measurement showed that light transmission for wavelengths 300 to 1100 nm was 0.09 to 27.7% at 36 DAA (green-red), 0.04 to 23.3% at 55 DAA (red-purple, veraison), 0.03 to 19.0% at 61 DAA (purple), and 0.02 to 14.1% at 91 DAA (black, maturity). Based on spectrum transmission meter measurements, light transmission of UV and visible light decreased from 4.3% (36 DAA) to 0% (69–91 DAA). For infrared light, transmission decreased from 10.5% at 36 DAA to 7.0% at 91 DAA. Both types of measurement indicated that, under natural growing conditions (sunlight exposure), berry pulp received progressively less sunlight during berry development.

3.3.3 Sugars and organic acids

Fructose and glucose were the main sugars in the berries, and sucrose concentration was consistently <1.75 mg/g fresh weight (FW) during berry development (data not shown). Fructose and glucose concentrations exhibited similar

trends and rapidly accumulated around veraison. However, this rapid accumulation occurred approximately one week later in sunlight-excluded berries (55 DAA) than in sunlight-exposed berries (48 DAA) (Figure 3-3). Generally, sugar concentrations did not differ significantly between sunlight-exposed and sunlight-excluded berries at most sampling dates, although they were significantly higher in the former at 55, 62, and 85 DAA and lower at 91 DAA.

Malic acid and tartaric acid were the main organic acids in the berries. Tartaric acid decreased and then increased slightly near maturity (Figure 3-3). Malic acid increased slightly and then decreased from veraison until one week after maturity. Tartaric and malic acid were generally lower in sunlight-excluded berries before veraison but did not differ significantly between two treatments near maturity.

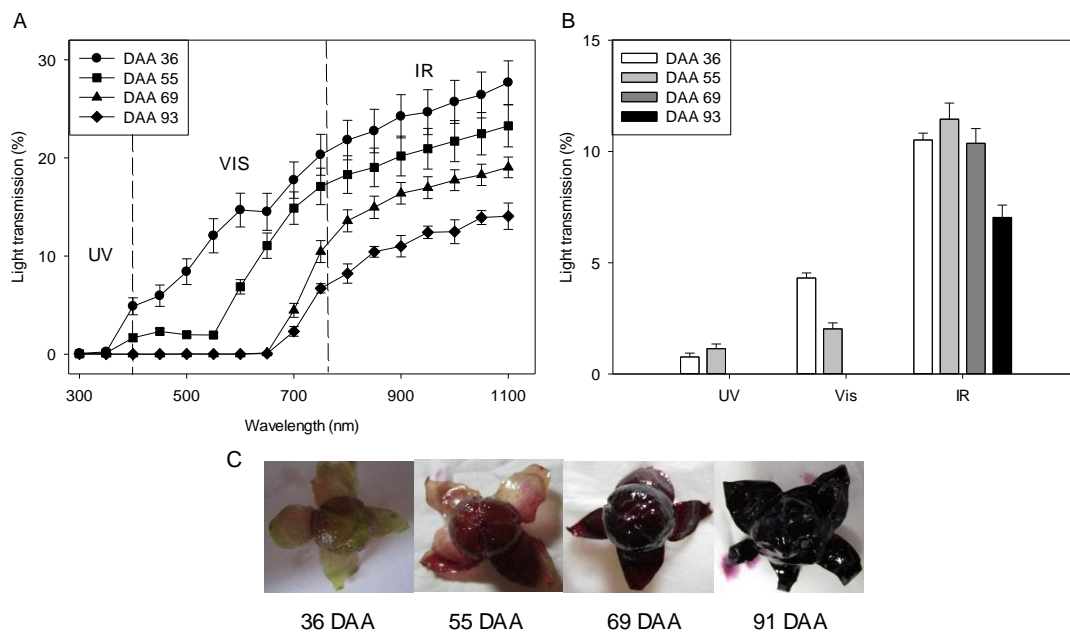


Figure 3-2 Light transmission through Yan-73 grape skin was determined by a photometer (A) and a Spectrum Transmission Meter (B). Berries at the four different ages, measured in days after anthesis (DAA), are shown at the bottom of the figure (C).

3.3.4 Anthocyanin accumulation

A total of 19 anthocyanins were identified in berry skin, pulp, pedicels, and rachis using HPLC/triple quadruple tandem mass spectrometry (HPLC/ MS-MS)

performed throughout the developmental period (Table 3-2). All anthocyanins identified were monoglucosides, including malvidin, peonidin, petunidin, delphinidin, cyanidin and pelargonidin derivatives (Mv, Pn, Pt, Dp, Cy, and Pl, respectively), with

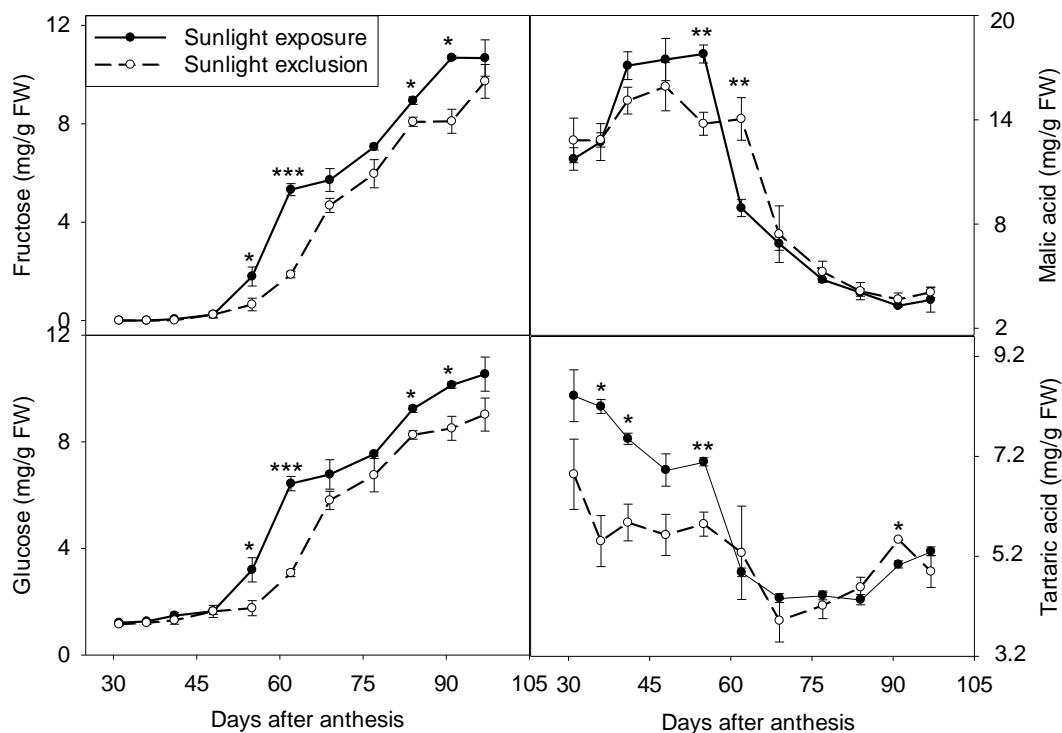


Figure 3-3 Seasonal changes in sugar and organic acid concentration (mean \pm SE) in Yan-73 grape berries. “FW” stands for “fresh weight”. Statistical significance of differences between treatments is indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

-3-*O*-glucoside as the main derivative. There were wide variations in concentrations of individual anthocyanins among tissues and across season; for example, Mv- and Pn-3-*O*-(6-*O*-caffeoyl-glucoside) and Cy-3-*O*-(6-*O*-acetyl-glucoside) were not detected in 2011. However, the overall anthocyanin profile was relatively conserved for each tissue across years. Mv and Pn derivatives consistently represented a large proportion of total anthocyanins, accounting for a maximum of 64.5 to 67.8% in 2010 and 80.2 to 80.7% in 2011 (Table 3-2). Proportions of Dp, Cy, and Pt derivatives were 0.9 to 20.2%, 0 to 47.2%, and 1.7 to 22.4%, respectively. Pl only appeared as pelargonidin-3-*O*-glucoside, ranging from 0% to 2.4%. Patterns of accumulation of

Mv, Pn, Dp, Cy, and Pt derivatives were similar to those of total anthocyanin for both treatments. For the analysis of seasonal variations and the effect of sunlight exclusion in four tissues, only total anthocyanin concentration is therefore presented (Figure 3-4).

In both sunlight-exposed and sunlight-excluded berry skin, total anthocyanin was less than 0.19 mg/g before veraison, although significant differences were observed between treatments in 2011 (Figure 3-4). Total anthocyanin concentration increased sharply at veraison (65 DAA in 2010 and 55 DAA in 2011) in sunlight-exposed skin and at 65 DAA (2010) and 62 DAA (2011) in sunlight-excluded skin. Total anthocyanin accumulation, as well as that of individual anthocyanins, was lower in sunlight-excluded than in sunlight-exposed skin for both years. At maturity, total anthocyanin concentration in sunlight-excluded skin was 24.5% (2010) and 14.7% (2011) of that of sunlight-exposed skin (Table 3-3). Although these concentrations were low (1.66 mg/g in 2010 and 2.67 mg/g in 2011), sunlight-excluded skins still attained a deep color. Skin anthocyanins were mainly composed of Mv (47.5–62.7%) and Pn (16.7–37.4 %) derivatives. At maturity, the proportion of Mv derivatives was significantly reduced under sunlight exclusion and that of Pn derivatives was enhanced (Table 3-3).

Seasonal changes in total anthocyanin concentrations were similar in both sunlight-exposed and sunlight-excluded berry pulp. The sharp increase in total anthocyanins in skin also occurred in pulp at veraison, although it occurred later in sunlight-excluded pulp in 2011. The reduction in total anthocyanins in pulp under sunlight exclusion was less marked than in skin. At maturity, total anthocyanin concentration in sunlight-excluded pulp was ~32.3% (2010) to 103.2% (2011) of that in sunlight-exposed pulp. As in berry skin, proportions of Mv and Pn derivatives were significantly reduced and enhanced, respectively, but Pn derivatives comprised the predominant anthocyanin in pulp (60.3–80.2%).

Total anthocyanin accumulation in pedicels and rachis showed a similar pattern (Figure 3-4), although the rapid accumulation phase started later than in berry skin and pulp. Until this phase began, there was generally no significant difference

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between treatments, except in anthocyanin accumulation in pedicels in 2011. Near maturity and one week after maturity, sunlight exclusion did not significantly affect total anthocyanin concentration in 2010, with the exception of a reduction in pedicels at one week after maturity. However, in 2011, total anthocyanin in pedicels and rachis significantly increased under sunlight exclusion (Figure 3-4). At maturity, both Mv and Pn derivatives were predominant in pedicels and rachis, and in general sunlight exclusion did not significantly influence the proportions of anthocyanin components (Table 3-3).

Table 3-2 Relative content of the 19 identified anthocyanins (expressed as % of total anthocyanins) in the four tissues throughout the developmental stage in 2011 and 2010. Mv, Pn, Pt, Dp, and Cy represent malvidin, peonidin, petunidin, delphinidin and cyanidin derivatives respectively.

Peak order	t _R (min)	Identity	Range (%)	
			2010	2011
		Mv	8.3–64.5	6.3–67.8
6	22.389	Malvidin 3- <i>O</i> -glucoside	4.5–45.4	5.5–47.6
11	38.391	Malvidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	0–19.1	0–21.3
13	42.304	Malvidin 3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)	0–6.32	ND
17	47.453	Malvidin 3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)	0–5.8	0–5.8
19	53.905	Malvidin 3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)	0–22.8	0–9.2
		Pn	12.9–80.7	10.6–80.2
5	21.360	Peonidin 3- <i>O</i> -glucoside	4.3–70.7	4.2–74.0
10	37.769	Peonidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	0–14.6	0–11.8
12	41.683	Peonidin 3- <i>O</i> -(6- <i>O</i> -caffeoyl-glucoside)	0–4.2	ND
16	47.180	Peonidin 3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)	0–5.9	0–5.4
18	53.017	Peonidin 3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)	0–27.8	0–16.9
		Dp	1.4–20.2	0.9–19.3
1	15.242	Delphinidin 3- <i>O</i> -glucoside	0.9–10.4	0.9–20.2
7	25.888	Delphinidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	0–4.9	0–5.9
		Cy	0–24.4	1.8–47.2
2	17.306	Cyanidin 3- <i>O</i> -glucoside	1.0–23.7	0–17.6
8	32.206	Cyanidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	0–3.1	ND
14	43.373	Cyanidin 3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)	0–11.1	0–14.3
		Pt	1.8–22.4	1.7–20.6
3	18.127	Petunidin 3- <i>O</i> -glucoside	1.0–25.0	0.9–20.1
9	33.234	Petunidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)	0–4.5	0–14.7
15	44.635	Petunidin 3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)	0–4.9	0–6.3
4	20.325	Pelargonidin 3- <i>O</i> -glucoside	0–2.4	0–1.8

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Table 3-3 Mv,Pn, Dp, Pt, Cy derivatives and total anthocyanin concentration (mean \pm standard error) and relative content of total anthocyanins (%) in four berry tissues harvested in 2010 (97 DAA) and 2011 (91 DAA). Different letters between light condition indicate significant differences ($P < 0.05$).

	Year	Skin		Pulp		Rachis		Berry pedicel	
		Exposure	Exclusion	Exposure	Exclusion	Exposure	Exclusion	Exposure	Exclusion
Concentration (mg/g)									
Mv	2010	4.04 \pm 0.50a	0.79 \pm 0.09b	0.08 \pm 0.01a	0.02 \pm 0.00b	0.50 \pm 0.12a	0.40 \pm 0.11b	0.04 \pm 0.01a	3.3 \pm 1.4b
	2011	11.37 \pm 1.10a	1.35 \pm 0.18b	0.56 \pm 0.01a	0.35 \pm 0.03b	3.17 \pm 0.06a	3.00 \pm 0.08a	0.13 \pm 0.01b	25.4 \pm 0.5a
Pn	2010	1.25 \pm 0.23a	0.62 \pm 0.06a	0.19 \pm 0.01a	0.17 \pm 0.01a	0.43 \pm 0.03a	0.34 \pm 0.02a	0.05 \pm 0.00a	5.8 \pm 3.0a
	2011	3.02 \pm 0.35a	0.57 \pm 0.04b	1.16 \pm 0.03b	1.40 \pm 0.06a	2.50 \pm 0.05b	3.86 \pm 0.08a	0.16 \pm 0.01b	35.4 \pm 1.9a
Dp	2010	0.31 \pm 0.04a	0.04 \pm 0.01b	0.01 \pm 0.00a	0.003 \pm 0.00b	0.04 \pm 0.00a	0.03 \pm 0.01b	0.01 \pm 0.00a	0.01 \pm 0.00b
	2011	1.59 \pm 0.16a	0.31 \pm 0.02b	0.05 \pm 0.01a	0.03 \pm 0.00b	0.66 \pm 0.03b	1.15 \pm 0.03a	0.03 \pm 0.00b	0.07 \pm 0.00a
Cy	2010	0.14 \pm 0.02a	0.04 \pm 0.00b	0.02 \pm 0.00a	0.02 \pm 0.00b	0.06 \pm 0.00a	0.04 \pm 0.00a	0.02 \pm 0.00b	0.03 \pm 0.02a
	2011	0.34 \pm 0.05a	0.11 \pm 0.01b	0.06 \pm 0.00b	0.10 \pm 0.01a	1.09 \pm 0.02b	2.04 \pm 0.03a	0.05 \pm 0.00b	0.14 \pm 0.01a
Pt	2010	1.02 \pm 0.12a	0.15 \pm 0.02b	0.01 \pm 0.00a	0.004 \pm 0.00b	0.13 \pm 0.02a	0.09 \pm 0.02b	0.01 \pm 0.00a	0.01 \pm 0.00a
	2011	1.60 \pm 0.16a	0.31 \pm 0.02b	0.04 \pm 0.00b	0.05 \pm 0.00a	0.61 \pm 0.04a	0.94 \pm 0.03a	0.03 \pm 0.00b	0.06 \pm 0.00a
Sum	2010	6.77 \pm 0.91a	1.66 \pm 0.17b	0.32 \pm 0.03a	0.21 \pm 0.01b	1.16 \pm 0.17a	0.90 \pm 0.15b	0.13 \pm 0.01a	0.13 \pm 0.07a
	2011	18.13 \pm 3.45a	2.67 \pm 0.27b	1.84 \pm 0.04b	1.93 \pm 0.09a	8.09 \pm 0.15b	11.13 \pm 0.24a	0.41 \pm 0.03b	0.88 \pm 0.04a
Relative content (%)									
Mv	2010	59.86 \pm 0.92a	47.47 \pm 0.98b	25.38 \pm 2.84a	8.39 \pm 0.80b	29.59 \pm 4.57a	26.42 \pm 4.23a	41.62 \pm 6.23a	38.06 \pm 7.07a
	2011	62.70 \pm 0.42a	50.15 \pm 1.51b	29.90 \pm 0.89a	18.15 \pm 0.52b	39.13 \pm 0.41a	26.95 \pm 0.21b	31.09 \pm 0.69a	28.97 \pm 0.97a
Pn	2010	18.12 \pm 1.38b	37.43 \pm 0.62a	60.35 \pm 2.91b	80.21 \pm 2.19a	40.63 \pm 2.93a	43.19 \pm 2.44a	38.17 \pm 4.70a	41.69 \pm 4.91a
	2011	16.71 \pm 1.03b	21.32 \pm 0.53a	61.90 \pm 0.87b	72.65 \pm 0.63a	30.84 \pm 0.27b	34.70 \pm 0.11a	39.47 \pm 0.40a	40.03 \pm 0.31a
Dp	2010	4.63 \pm 0.14a	2.65 \pm 0.49b	3.30 \pm 0.24a	1.43 \pm 0.40b	6.54 \pm 0.25a	5.11 \pm 1.23a	3.16 \pm 0.17a	2.98 \pm 0.30a
	2011	8.76 \pm 0.33b	11.81 \pm 0.53a	2.48 \pm 0.29a	1.38 \pm 0.08b	8.19 \pm 0.10b	10.34 \pm 0.06a	7.30 \pm 0.15a	7.45 \pm 0.08a
Cy	2010	2.10 \pm 0.08b	2.68 \pm 0.13a	6.04 \pm 0.39b	6.98 \pm 0.87a	17.52 \pm 2.59a	18.38 \pm 2.53a	5.07 \pm 1.15a	5.53 \pm 1.45a
	2011	1.89 \pm 0.04b	4.04 \pm 0.12a	2.94 \pm 0.10b	5.10 \pm 0.10a	13.41 \pm 0.17	18.33 \pm 0.18a	12.73 \pm 0.26b	15.64 \pm 0.78a
Pt	2010	15.08 \pm 0.39a	9.01 \pm 0.44b	3.88 \pm 0.20a	1.86 \pm 0.25b	4.46 \pm 0.62a	5.31 \pm 0.81a	11.04 \pm 0.44a	10.03 \pm 0.31a
	2011	8.82 \pm 0.27b	11.67 \pm 0.40a	2.23 \pm 0.14a	2.33 \pm 0.09a	7.50 \pm 0.50a	8.5 \pm 0.04a	6.85 \pm 0.07a	6.52 \pm 0.09b

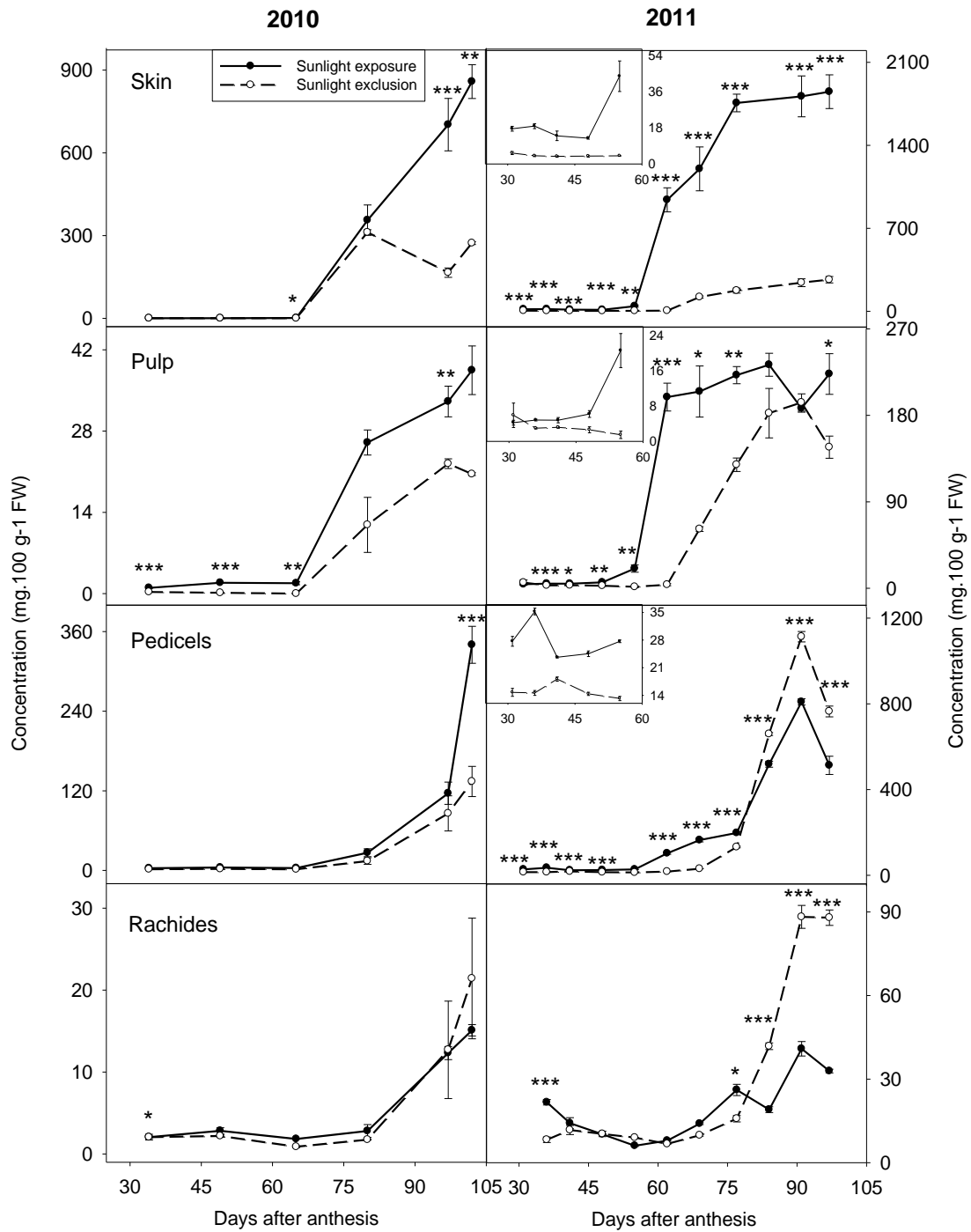


Figure 3-4 Seasonal changes in total anthocyanin concentrations (mean \pm SE) in four tissues of the Yan-73 cultivar in 2010 (left) and 2011 (right). *, **, and *** indicate statistical significance of differences between sunlight exposure and exclusion at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Inserts show low concentration values using a greater y-axis scale.

3.3.5 Expression of genes in anthocyanin biosynthesis

In sunlight-exposed berry skin, *VvUFGT* expression was low at the beginning of berry development, increased dramatically from 41 DAA until veraison (~55 DAA), and decreased temporarily near maturity (Figure 3-5). In sunlight-excluded skin, *VvUFGT* expression exhibited a similar pattern, but the sharp increase was delayed (65 DAA) and peaked later, about three weeks postveraison. In pulp from both treatments, *VvUFGT* expression increased and then decreased until one week after maturity, but maximal expression was much greater and occurred earlier in sunlight-exposed pulp.

There was no significant difference in *VvF3'H* expression between treatments for either berry skin or pulp around veraison. In contrast, expression of *VvF3'5'H* in skin and pulp was reduced under sunlight exclusion, except for an increase at 77 DAA in skin and no difference at 69 DAA in pulp.

VvMybA1 expression exhibited patterns resembling that of *VvUFGT* in berry skin and pulp, gradually increasing and then decreasing with different peaking times (Figure 3-6). *VvMybA1* expression was significantly higher in sunlight-exposed than in sunlight-excluded skin and pulp, except that there was no difference at maturity or one week after maturity. Generally, the expression levels of *VvMybA2* and *VvMycI* decreased in sunlight-excluded berries in both skin and pulp around veraison, although expression levels were less decreased than *VvMybA1*. In skin and pulp, *VvMyb5a*, *VvMybPA1*, *VvWDR1*, and *VvWDR2* generally had similar trends, and their transcript abundance around veraison did not significantly differ between treatments. *VvMycA1*, however, had different expression patterns in skin and pulp. In skin, *VvMycA1* transcript abundance generally did not differ significantly between treatments except at 77 DAA, when it was greater in sunlight-exposed skin. In sunlight-exposed pulp, *VvMycA1* transcripts were generally more abundant, except at 79 DAA, when levels were approximately the same.

3.3.6 Anthocyanin composition and *VvF3'H/VvF3'5'H* expression

Under sunlight exposure, grape skin anthocyanins mainly comprised Mv

(59.9–62.7%) and Pn (16.7–18.1%) derivatives, although Pn derivative was the most abundant anthocyanin in berry pulp (60.3–61.9%) at maturity. Under sunlight exclusion, the proportion of Mv derivatives was significantly reduced, but that of Pn derivatives was greater at maturity both in skin and pulp (Table 1). The ratio of 3',4'-hydroxylated (Cy and Pn derivatives) to 3',4',5'-hydroxylated (Dp, Pt, and Mv derivatives) anthocyanins in skin and pulp was compared between treatments during the berry developmental period (Figure 3-7). The ratios were significantly higher in sunlight-excluded skin and pulp, and sunlight-excluded pulp had the highest ratio. The changes in transcription levels of *VvF3'H* and *VvF3'5'H* around veraison were consistent with the accumulation pattern of the anthocyanins. The ratio of transcription levels of *VvF3'H* to *VvF3'5'H* was generally higher in sunlight-excluded skin and pulp and generally higher in pulp than in skin.

3.4 Discussion

3.4.1 Effect of sunlight on anthocyanin accumulation

In the study here, opaque boxes were used to cover clusters of the teinturier grape Yan-73, with the clusters remaining enclosed from fruit set until one week after maturity. Under sunlight exposure, the Yan-73 berry skin filtered greater amounts of sunlight as the berry developed and the skin color deepened, so that the berry pulp received increasingly less sunlight than the skin, which may partly explain the lower anthocyanin concentrations in pulp than in skin. Covering with opaque boxes substantially reduced light intensity around clusters; no more than 0.25% of incident light reached the skin and even less (<0.05%) reached the pulp. Thus, under sunlight exclusion, both skin and pulp developed in almost complete absence of sunlight throughout the developmental period. Typically, ~5 °C of day and/or night temperature difference can influence anthocyanin concentration and coloration in grape berry skin (Spayd *et al.* 2002, Tarara *et al.* 2008). In Grenache clusters growing under conditions with a difference of 3 to 4 °C, similar anthocyanin levels were reached (Bergqvist *et al.* 2001). In the study here, the temperature difference between

the inside and outside of the box was minimal (0.1–1.7 °C) and not likely to greatly affect anthocyanin biosynthesis.

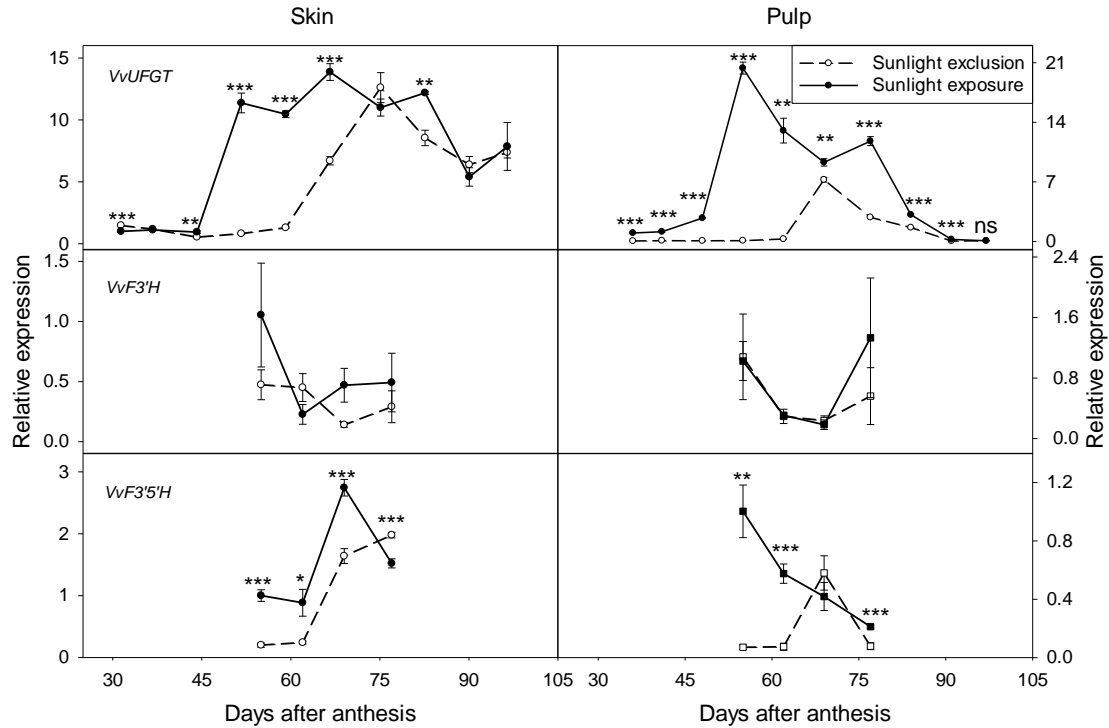


Figure 3-5 Expression of three anthocyanin biosynthesis genes in Yan-73 skin (left) and pulp (right) during berry development in 2011. *, **, and *** indicate statistical significance of differences between sunlight exposure and exclusion at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Anthocyanin accumulation during berry development results from complex interactions between environmental and developmental factors, including sugars (Vitrac *et al.* 2000). Many authors have reported a promoting effect of sugars on anthocyanin accumulation in various plant species. In grapevines, sucrose caused upregulation of anthocyanin accumulation in cell suspensions (Larronde *et al.* 1998). Of eight tested sugars, anthocyanin accumulation was enhanced by glucose, sucrose, and fructose but anthocyanin composition was not affected (Mori and Sakurai 2006). The effect of sugars on anthocyanin accumulation apparently correlates with their effect on the transcripts of genes involved in this pathway. Several genes, including

PAL, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *FLS*, *DFR*, *LDOX*, and *UFGT*, and transcription factors MYB75/ PAPP1 were regulated by sucrose in *Arabidopsis* (Solfanelli *et al.* 2006, Teng *et al.* 2005). In the present study, at 55 and 62 DAA (around veraison) and 85 DAA (one week before maturity) sugar concentrations were significantly lower in sunlight-excluded berries but were significantly higher at 91 DAA (maturity date). With these exceptions, sugar concentrations did not significantly differ between treatments. Given the well-documented regulation of anthocyanin synthesis by sugars (Larronde *et al.* 1998, Mori and Sakurai 2006), it is possible that the lower level of sugars at these sampling dates (55, 62, and 85 DAA) might result in lower anthocyanin concentrations in sunlight-excluded skin and pulp. This conclusion, however, requires further investigation.

Sunlight exclusion from Yan-73 berry clusters had different effects on anthocyanin accumulation in the four tissues studied. It significantly reduced anthocyanin biosynthesis in berry skin and pulp, and although it had no effect on pedicels and rachis in the first season, it increased anthocyanin accumulation in the second season. However, all tissues (skin, pulp, pedicels, and rachis) still accumulated sufficient anthocyanins to change color under sunlight exclusion. Although other studies have found that berry skin can accumulate anthocyanins under sunlight exclusion/shading (Tarara *et al.* 2008, Weaver and Mccune 1960), they did not explicitly discuss the opacity coefficient around the clusters during the experimental period. Many factors, such as the developmental stage during which berries are shaded and the duration, type, and degree of shading, differ between studies and prevent useful comparisons. In the present study, we applied sunlight exclusion strictly from fruit set until maturity so that berries received a very low level of incident light (<0.25% for skin and <0.05% for pulp). Under such conditions, the red grape Jingxiu failed to color while Jingyan still developed red color (Zheng *et al.* 2013). Thus, anthocyanin biosynthesis in Yan-73, similar to Jingyan, was less influenced by sunlight exclusion than in grape cultivars that do not develop color in the absence of sunlight (Jingxiu).

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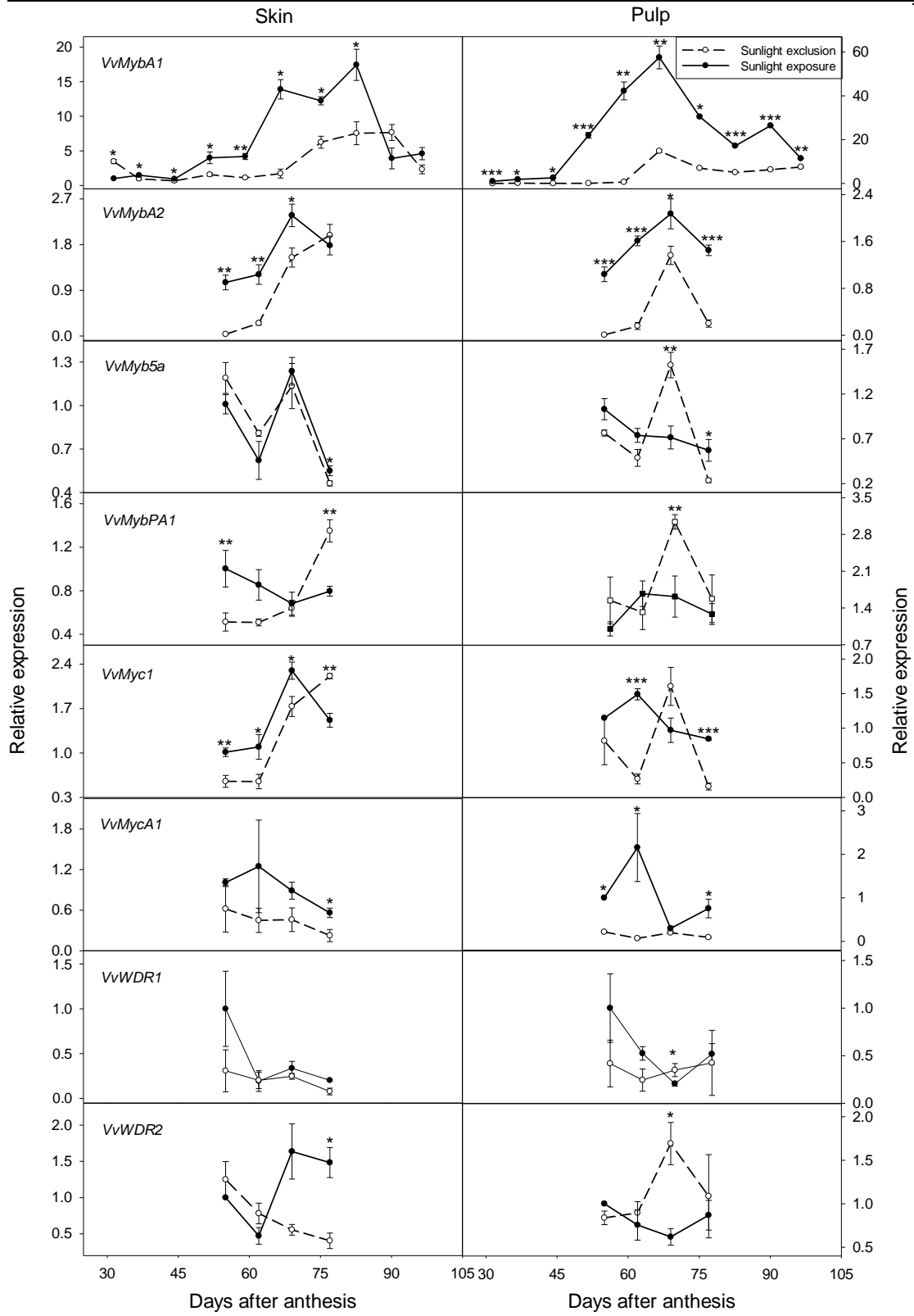


Figure 3-6 Expression of anthocyanin regulatory genes in ‘Yan-73’ skin (left) and pulp (right) during berry development in 2011. *, **, and *** indicate statistical significance of differences between sunlight exposure and exclusion at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.4.2 Effect of sunlight on gene expression

Although under sunlight exclusion *VvUFGT* expression was obviously reduced, it was still expressed in Yan-73 skin and pulp, which was consistent with anthocyanin accumulation. *UFGT* has been reported to be the critical gene for the coloration of grape berry skin (Zheng *et al.* 2013), as well as in other tissues, such as pulp, young and old leaves, and stem epidermis of Bailey Alicante A (Jeong *et al.* 2006b). Its transcription can be decreased by shading in Cabernet Sauvignon grape skin (Jeong *et al.* 2004) and Lambrusco f.f. grape seedlings (Sparvoli *et al.* 1994). In a recent study on two red grape cultivars, darkness repressed *UFGT* in Jingxiu skin but not in Jingyan skin, which resulted in no color in the former and red color in the latter (Zheng *et al.* 2013).

In grape berry skin, *MybA1* is directly involved in the regulation of *UFGT* (Kobayashi *et al.* 2002) and is thought to be the key regulatory gene in the anthocyanin biosynthesis pathway (Azuma *et al.* 2008). Color determination of various organs of teinturier grape cultivars resulted from tissue-specific expression of *MybA1* (Jeong *et al.* 2006b). *MybA1* is expressed in the berry skin of non-teinturier grapes, such as Cabernet Sauvignon, Dornfelder, Muscat Hamburg, and Muscat Bailey A, but it is not expressed in berry pulp, young leaves, tendrils, or stem epidermis. However, *MybA1* was transcribed in the colored organs, such as berry pulp, young leaves, tendrils, and stem epidermis of the teinturier varieties Bailey Alicant A (Jeong *et al.* 2006b) and Alicante Bouschet (Falginella *et al.* 2012). *VvMybA2* is also involved in color regulation (Walker *et al.* 2007), and *Myc1* is a part of the transcriptional cascade controlling anthocyanin and condensed tannin biosynthesis in grapevines (Hichri *et al.* 2010). Among the regulatory genes studied, *MybA1* expression corresponded well with the developmental pattern of *UFGT* expression and was significantly reduced in sunlight-excluded Yan-73 skin and pulp. In addition, transcription levels of *VvMybA2* and *VvMyc1* were decreased under sunlight

exclusion in skin and pulp, although not as strongly as *VvMybA1*. This indicated that, among the regulatory genes measured, *VvMybA1* together with *VvMybA2* and *VvMyc1* likely had a key role in color formation in both skin and pulp under sunlight exclusion.

Other regulatory genes had little effect on anthocyanin biosynthesis under sunlight exclusion, perhaps explained by their regulation of a broader range of pathways and genes. These were transcriptional regulators involved in general flavonoid pathways (*VvMYB5a*; Deluc *et al.* 2008) and the biosynthesis of condensed tannins (*VvMYBPA1*, Bogs *et al.* 2007; *VvMycA1*, Matus *et al.* 2010) and anthocyanins (*VvWDR1* and *VvMycA1*, Matus *et al.* 2010). The function of *VvWDR2* was not determined by ectopic expression (Matus *et al.* 2010). *VvMYB5a*, *VvMYBPA1*, *VvWDR1*, and *VvWDR2* generally did not show significant differences between sunlight exposure and exclusion treatments around veraison, with the exception that *VvMYB5a*, *VvMYBPA1*, and *VvWDR2* were induced by sunlight exclusion at 70 DAA. This finding indicated that the expression of the four genes in skin and pulp may be little affected by sunlight exclusion. Expression of *VvMycA1* responded differently to sunlight exclusion in skin and pulp: it was not affected in skin (similar to *VvMYB5a*, *VvMYBPA1*, *VvWDR1*, and *VvWDR2*) but it decreased in pulp. *VvMycA1* may therefore also contribute to a lower level of anthocyanin biosynthesis in the pulp, as do *VvMybA1*, *VvMybA2*, and *VvMyc1*.

3.4.3 *VvF3'H/VvF3'5'H* expression related to anthocyanin composition

Flavonoid 3'-hydroxylase (*F3'H*) and flavonoid 3',5'-hydroxylase (*F3'5'H*) are involved in the biosynthetic pathway of cyanidin- and delphinidin-based anthocyanins, respectively. In the grapevine, transcription levels of *VvF3'H* and *VvF3'5'H* were consistent with the anthocyanin composition of berry skin (Castellarin *et al.* 2006, Falginella *et al.* 2012); the flower, stem, tendril, and seed, which accumulated a higher level of *VvF3'H* mRNA than *VvF3'5'H* mRNA, had higher concentrations of 3',4'-hydroxylated flavonoids, such as anthocyanins (Jeong *et al.* 2006a). Ectopic expression of *VvF3'H* and *VvF3'5'H* genes not only changed the

anthocyanin composition and concentration but also altered flower color, as was observed through ectopic expression of *VvF3'H* and *VvF3'5'H* in petunia (Bogs *et al.* 2006). In the present study, sunlight exclusion did not significantly affect *VvF3'H* expression, which controls the synthesis of cyanidin and peonidin, but it did reduce *VvF3'5'H* expression, which controls the synthesis of delphinidin, petunidin, and malvidin. It was consistent with findings that bunch shading before the onset of Cabernet Sauvignon berry ripening decreased the proportion of 3',4',5'-hydroxylated anthocyanins and the expression of *VvF3'5'H* (Koyama and Goto-Yamamoto 2008).

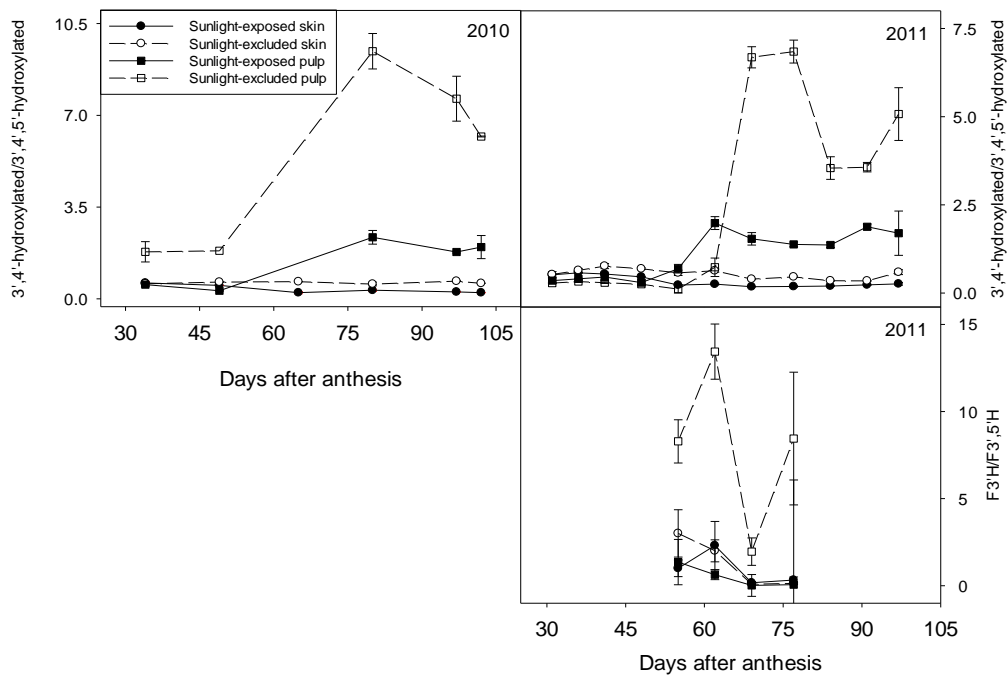


Figure 3-7 The ratio of 3',4'-hydroxylated and 3',4',5'-hydroxylated anthocyanins (2010 and 2011) and the ratio of transcript level of *VvF3'H* and *VvF3'5'H* in 2011. Ratio of *VvF3'H* to *VvF3'5'H* expression is expressed relative to that in sunlight-exposed berries at the first sampling date.

This may help explain the lower proportion of Mv derivatives and higher proportion of Pn derivatives in sunlight-excluded Yan-73 berry skin and pulp. Moreover, the ratio of 3',4'-hydroxylated to 3',4',5'-hydroxylated anthocyanins correlated closely with the ratio of transcript levels of *VvF3'H* to *VvF3'5'H*. When the ratio of *VvF3'H/F3'5'H* was increased by sunlight exclusion, the ratio of 3',4'-hydroxylated/3',4',5'-hydroxylated anthocyanins was higher in sunlight-excluded

skin and pulp, resulting in a higher proportion of Pn derivatives. In addition, the ratio of 3',4'-hydroxylated/3',4',5'-hydroxylated anthocyanins, together with the ratio of transcript levels of *VvF3'H* to *VvF3'5'H*, increased in both skin and pulp during the progression of ripening.

3.5 Conclusions

Both berry skin and pulp could accumulate anthocyanins under sunlight exclusion, even though the accumulation level was reduced (to a less extent in pulp). The anthocyanin accumulation in pedicels and rachis seemed to be little influenced by sunlight exclusion. The decreased anthocyanin concentrations in sunlight-excluded skin and pulp were in accordance with the lower transcript levels of *VvUFGT*, *VvMybA1*, *VvMybA2*, and *VvMyc1* and, additionally, the lower level of *VvMycA1* in sunlight-excluded pulp. The ratio of 3',4'-hydroxylated/3',4',5'-hydroxylated anthocyanins was correlated with the transcript ratio of *VvF3'H*/*VvF3'5'H*.

3.6 Acknowledgments

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Chapter IV. Anthocyanin biosynthesis is differentially regulated by light in the skin and flesh of white-fleshed and teinturier grape berries

4.1 Introduction

Anthocyanins are a class of flavonoids responsible for a wide range of colors in many plant organs, including grape berries. The color of red grape berries is an important factor for the market acceptance of table grapes and plays a key role in determining wine color. The anthocyanins present in red grapes are derived mainly from five anthocyanidins: cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv), which exhibit different patterns of hydroxylation, methylation, and acylation (Mattivi *et al.* 2006; Mazza 1995; Figure 1-1). The relative anthocyanin composition of berries has an impact on the color hue and color stability of resultant wines (Cortell and Kennedy 2006; Mazza 1995; Ristic *et al.* 2007; Rustioni *et al.* 2013). For example, anthocyanin color progressively changes from red to blue with the increasing number of free hydroxyl groups on the phenolic B ring (He *et al.* 2010a; Sarni *et al.* 1995). Therefore, a better understanding of the regulation of anthocyanin accumulation and composition is both of scientific and economical importance.

The quantity and composition of anthocyanins in grape are strongly determined by genotypes (Fournier-Level *et al.* 2009, 2010, 2011) and also depend on various environmental factors (Downey *et al.* 2006; Kuhn *et al.* 2014). In most grape cultivars, the trihydroxylated malvidin 3-glucoside is the predominant anthocyanin, while in a limited number of cultivars dihydroxylated peonidin 3-glucoside accumulates predominantly (Castellarin and Di Gaspero 2007; Falginella *et al.* 2010; Jeong *et al.* 2006; Mattivi *et al.* 2006). The anthocyanin biosynthesis in the two type of cultivars seems to have different sensitivities in response to light (Zoratti *et al.* 2014). Weaver and Mccune (1960) found that only cv. Tokay and Sultanina Rose out

of 40 tested grape cultivars failed to form anthocyanins in light exclusion conditions, while the rest showed the same color as in the light exposed berries. Kiewer (1977) postulated that the cultivars rich in dihydroxylated anthocyanins (Emperor with 60-90% Pn-derivatives; Tokay with 80-90% Cy-derivatives) might be most sensitive to light. In agreement with this hypothesis, a recent study showed that cv. Jingxiu (containing ~50% Pn- and Cy-derivates) was unable to accumulate anthocyanin when the berries were not exposed to light (Zheng *et al.* 2013). However, not all the cultivars that are rich in dihydroxylated anthocyanins react in this way, because the total anthocyanin contents of cv. Nebbiolo (~60% Pn- and Cy-derivates), Crimson seedless (~70% Pn- and Cy-derivates), Reliance (~85% Pn- and Cy-derivates), and Jingyan (~90% Pn- and Cy-derivates) were either unchanged or only partially reduced by light exclusion (Chorti *et al.* 2010; Human and Bindon 2008; Zheng *et al.* 2013). On the other hand, no cultivar rich in trihydroxylated anthocyanins has been reported to fail to form anthocyanins in the light exclusion and, in fact, the total anthocyanin content of these cultivars were either maintained (Cortell and Kennedy 2006; Downey *et al.* 2004; Ristic *et al.* 2007) or only partially decreased by light exclusion (Jeong *et al.* 2004; Koyama and Goto-Yamamoto 2008; Spayd *et al.* 2002). Therefore, the richness in dihydroxylated anthocyanins may be necessary but it is not a sufficient marker of the inability of a grape cultivar to accumulate anthocyanins under light exclusion. Despite these diverse responses of the total anthocyanin content to light exclusion, the spectrum of anthocyanin composition changes rather consistently, with an increased proportion of the dihydroxylated anthocyanins under light exclusion for most of the studied cultivars (Downey *et al.* 2006). However, the mechanisms underlying the cultivar-specific sensitivity to light and the alteration of anthocyanin composition in response to light are less known.

Anthocyanin biosynthesis is mainly regulated at transcriptional level by transcription factors, which respond to environmental and biological factors such as light, sugars, and plant hormones. Sunlight is a fundamental requirement for color formation (Jackson and Lombard 1993). Sunlight exclusion decreases the biosynthesis of anthocyanins in grape berry skin (Cortell and Kennedy 2006; Jeong *et al.*

al. 2004; Matus *et al.* 2008), as a result of lower expression of structural (*CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, *UFGT*) and regulatory genes (*MybA1*) in berry skin (Azuma *et al.* 2012; Guan *et al.* 2014; Jeong *et al.* 2004). In addition to these genes, whether the transport of anthocyanins from their site of synthesis (cytosol) to the storage organelle (vacuole) is also affected by light exclusion has not yet been investigated.

Sugars have been reported to accelerate pigmentation in *Arabidopsis* (Solfanelli *et al.* 2006; Teng *et al.* 2005), grape cell suspension cultures (Larronde *et al.* 1998; Vitrac *et al.* 2000), and *in vitro* cultured grape berries (Dai *et al.* 2014; Gambetta *et al.* 2010; Hiratsuka *et al.* 2001; Roubelakis-Angelakis and Kliewer 1986). In *Arabidopsis*, this sugar effect correlates with modified transcript levels of structural genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *FLS*, *DFR*, *LDOX*, *UFGT*), and transcription factors (MYB75/PAP1; Teng *et al.*, 2005; Solfanelli *et al.*, 2006). Treatments by exogenous abscisic acid (ABA) caused an increase in total anthocyanin content in grape berry skin, which was related to the increased expression levels of *CHI* and *MybA1* (Gambetta *et al.* 2010; Hiratsuka *et al.* 2001; Jeong *et al.* 2004). However, whether light affects anthocyanin biosynthesis directly or through the interrelationship with sugars and ABA remains to be investigated in grape.

Teinturier grapes may provide a valuable system to investigate these questions. Unlike most grape cultivars that only accumulate anthocyanins in the skin, teinturier grape cultivars also accumulate anthocyanins in the flesh, with concentration and composition that differ from that found in the skin. The anthocyanins found in the flesh of teinturier grapes are usually lower in total content and contain more dihydroxylated anthocyanins than those present in the skin (Ageorges *et al.* 2006; Castillo-Muñoz *et al.* 2009; Falginella *et al.* 2012; Guan *et al.* 2012; He *et al.* 2010b). The skin and flesh in a teinturier berry may therefore mimic two types of cultivars with distinct anthocyanin compositions. Moreover, during berry ripening, the light transmission through the skin decreases as a result of skin coloration (Guan *et al.* 2014), and this results in natural shading of the flesh. As a reduced light often causes an increase in the proportion of dihydroxylated anthocyanins (Downey *et al.* 2006), it is worth clarifying whether this self-shading imposed by the skin is responsible for

the higher proportion of dihydroxylated anthocyanins in the flesh than in the skin. The most recognized teinturier grape cultivars are *Vitis vinifera* L. cv. Alicante Bouschet (and its descendants) and Gamay Fréaux. Cell suspensions of these cultivars have been extensively used in the search for production of natural colorants (Ananga *et al.* 2013). Recently, cv. Alicante Bouschet was used to investigate the initiation of ripening and anthocyanin accumulation under natural growth conditions (Castellarin *et al.* 2011; Falginella *et al.* 2012), providing novel insights into their spatial and temporal regulations. We previously investigated the light effect on anthocyanin biosynthesis in a teinturier cultivar Yan-73 (*V. vinifera*, Muscat Hamburg × Alicante Bouschet), and found that the expression of *UFGT*, *MybA1*, *MybA2*, and *Myc1* were decreased by light exclusion in both berry skin and flesh (Guan *et al.* 2014). Despite extensive application as cell suspensions (Conn *et al.* 2008; Conn *et al.* 2010; Cormier *et al.* 1990; Do and Cormier 1991; Saigne-Soulard *et al.* 2006), the developmental profiles and organ-specific biosynthesis of anthocyanins in the berries of Gamay Fréaux have not been well studied, and their tissue-specific responses to light exclusion are still an open question.

In the present study, we compared the responses of anthocyanin biosynthesis to light exclusion in the skin of cv. Gamay (white-fleshed) and the skin and flesh of its teinturier mutant cv. Gamay Fréaux. The concentration and composition of anthocyanins and sugars, together with ABA and its catabolites in berry skin and flesh were quantified. The expression of genes related to anthocyanin biosynthesis, transcriptional regulation, and transport was also monitored in skin and flesh during berry development. By integrating metabolite profiling and gene expression, the study provides comprehensive insights into the tissue-specific regulation of light in anthocyanin accumulation in grape berry.

4.2 Materials and methods

4.2.1 Plant materials

All experiments were conducted in year 2012 in Bordeaux, France (44°47'N,

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0°34'W) with red wine grape (*Vitis Vinifera* L.) cv. Gamay (white-fleshed) and Gamay Fréaux (a mutant with colored flesh originated from Gamay, Figure 4-1) from a germplasm collection vineyard growing in level ground with no slope or geo-spatial variations. The field-grown grapevines were 23- year old, spur pruned, with a density of 1.6 m between rows and 1 m between plants. Vineyard management followed the local standards. Eighteen vines of each cultivar were chosen and two clusters with similar size from two adjacent shoots of each vine were tagged. Opaque boxes were applied to one of the two tagged clusters of each vine from 33 days after flowering (DAF) until maturity for light-exclusion treatment, and the other clusters were exposed under natural light conditions as the control. The boxes used in this experiment, described in Guan *et al.* (2014), excludes almost all light with only minimal effects on other microclimate parameters (temperature and humidity).



Figure 4-1 Accumulation of anthocyanins in berries of cv. Gamay (left) and Gamay Fréaux (right). Gamay accumulates anthocyanins exclusively in the skin after veraison (lower panel); Gamay Fréaux accumulates anthocyanins in the pulp even before veraison (upper panel), while it accumulates anthocyanins in the skin only after veraison.

Light-exposed or shaded clusters of both cultivars were sampled weekly from 40 DAF (one week after treatment) until maturity. Three clusters (replicates) from three vines were sampled for each treatment at each sampling date. Berries were weighed, deseeded, separated into skin and flesh, and the skin and flesh were immediately frozen in liquid nitrogen. The samples were ground into powder in liquid nitrogen using a ball grinder MM200 (Retsch, Haan, Germany), and stored at -80°C for later

analysis.

4.2.2 Sugar and ABA analysis

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). ABA and its catabolites (phaseic acid, dihydrophaseic acid, and abscisic acid glucose ester) were determined by using LC-MS/MS (Agilent 6410) as described in Speirs *et al.* (2013).

4.2.3 Anthocyanin analysis

Anthocyanins were extracted from 50 mg freeze-dried ground powder, 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 was analyzed as in Dai *et al.* (2014) with a HPLC system consisting of a P680 pump, ASI-100TTM autosampler and UVD 340U UV–vis detector. Separation was conducted on a reversed-phase Ultrasphere ODS column 250 mm \times 4.6 mm, 5 μm particle sizes fitted with an Ultrasphere C18 guard column 45 mm \times 4.6 mm purchased from Beckman Coulter Inc. (Fullerton, USA). 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 obtained on the commercial standard.

4.2.4 Gene expression quantification

Total RNA from the ground sample was isolated according to (Reid *et al.* 2006). RNA isolation was followed by DNase I treatment (Turbo DNA-freeTM kit, Ambion, Austin, TX, USA). Afterward, total RNA was quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, DE). First-strand cDNA was synthesized from 2 μg purified RNA using Superscript III enzyme (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA obtained was

diluted 10-fold in distilled water for later analysis. Primer pairs amplifying a GAPDH gene and spanning one intron were used to check the absence of genomic DNA contamination with normal PCR.

qPCR expression analysis was carried out using the CFX96 Real-Time PCR Detection system (Bio-Rad). Reaction mixes (10 μ L) included 5 μ L of iQTM SYBR Green Supermix (Bio-Rad), 2 μ L of diluted cDNA, and 0.2 μ M of each primer. Specific annealing of the oligonucleotides was verified by dissociation kinetics at the end of each PCR run. The efficiency of each primer pair was quantified using a PCR product serial dilution. All biological samples were assayed in triplicate. The genes coding *VvEF1* and *VvGAPDH* were used as internal standards and for normalizing the expression. Expression levels were calculated based on the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) with the flesh of Gamay Fréaux at the first sampling date as a reference sample. All primer sequences are listed in Table 4-1.

4.2.5 Statistical analysis

Data were analyzed with multivariate analysis methods using R software (R Development Core Team 2010). Differences between genotypes and light conditions for a given sampling date were analyzed by a two-way ANOVA followed with a Tukey multiple comparison test at $P < 0.05$. Principal component analysis (PCA) was conducted on the mean-centered and scaled data in order to investigate the discriminations of skin and flesh of different cultivars and different light conditions by their profiles of metabolites and transcripts. Correlation network analysis was conducted in R software and was visualized with Cytoscape 3.0.1 (Cline *et al.* 2007). Significant correlations were considered only when an adjusted P value was less than 0.05 after Benjamini and Hochberg (1995) false discovery rate correction (namely, a correlation coefficient > 0.62 and an unadjusted P value $< 10^{-5}$).

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Table 4-1 List of primer sequences used in qPCR analysis

<i>Gene/Copy</i>	sequence of forward (5'-3') and reverse @primers	References for primer
<i>CHS2</i>	F 5'-GTTCTGTTTGGATTGGACCAG-3'	Belhadj <i>et al.</i> , 2008
	R 5'-GTGAGTCGATTGTGTAGCAAGG-3'	
<i>CHI</i>	F 5'-AGGAGTTAGCGGATTCGGTTGAC-3'	
	R 5'-AAGGCAACACAATTCTCTGACACC-3'	
<i>F3'5'Hi</i>	F 5'-GCCAGAGACCACTCGATTAC-3'	Falginella <i>et al.</i> , 2010
	R 5'-ACCCAGATTTTCTGGACGTG-3'	
<i>F3'5'Ha</i>	F 5'-GGCGGAAGGTTTCCTTGAT-3'	Falginella <i>et al.</i> , 2010
	R 5'-GCACGTTGATCTCGGTGAG-3'	
<i>F3'5'Hf</i>	F 5'-AAGAAGTTCGACAAGTTATGACAAG-3'	Ali <i>et al.</i> , 2011
	R 5'-AGCGCCTTAATGTTGGTAAGG-3'	
<i>F3'5'Hg</i>	F 5'-AACGTCCCTAAAATGTACTCAACC-3'	Falginella <i>et al.</i> , 2010
	R 5'-GAACCTTCCTCGTGTCTCAAA-3'	
<i>F3'5'Hh</i>	F 5'-CGGGCTTCCCAGACATT-3'	Falginella <i>et al.</i> , 2010
	R 5'-GCAGGAACAGACACTTCATCG-3'	
<i>F3'5'Hj</i>	F 5'-CAGAAAATCTGGGTTTCCCTTT-3'	Falginella <i>et al.</i> , 2010
	R 5'-TTCGAGCCATGCTTGAGTTA-3'	
<i>F3'5'Hi</i>	F 5'-GGCTTGTAGGTATGGAAGTTTTT-3'	Falginella <i>et al.</i> , 2010
	R 5'-GTAGTCATGGGCCATCAAGG-3'	
<i>F3'5'Hm, -n</i>	F 5'-CACGCTGAGTCTAGTGTCTCC-3'	Falginella <i>et al.</i> , 2010
	R 5'-GGATCATGTGATTGGAAGGAA-3'	
<i>F3'5'Ho</i>	F 5'-CCATCTTATGCCGGAACAG-3'	Falginella <i>et al.</i> , 2010
	R 5'-CGAAGATTTCGCACCAGAG-3'	
<i>F3'5'Hp</i>	F 5'-TTCCAGTCAAATGAATGTACCAG-3'	Falginella <i>et al.</i> , 2010
	R 5'-TTGAGCGAAAAGAATGCAAG-3'	
<i>F3'5'H1a</i>	F 5'-GAAGTTCGACTGGTTATTAACAAAGAT-3'	Falginella <i>et al.</i> , 2010
	R 5'-AGGAGGAGTGCTTTAATGTTGGTA-3'	
<i>DFR</i>	F 5'-GAAACCTGTAGATGGCAGGA-3'	Jeong <i>et al.</i> , 2004
	R 5'-GGCCAAATCAAACCTACCAGA-3'	
<i>UFGT</i>	F 5'-GGGATGGTAATGGCTGTGG-3'	Azuma <i>et al.</i> , 2012
	R 5'-ACATGGGTGGAGAGTGAGTT-3'	
<i>AOMT</i>	F 5'-CTCTGCAGGCGCCTCTATTA-3'	Cutanda-Perez <i>et al.</i> ,
	R 5'-CCCAAAACAGAGTCTGGACA-3'	
<i>AMI</i>	F 5'-TGCTTTTGTGATTTTGTAGAGG-3'	Gemoz <i>et al.</i> , 2009

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	R 5'-CCCTTCCCCGATTGAGAGTA-3'	
<i>AM3</i>	F 5'-GCAAACAACAGAGAGGATGC-3'	Gemoz <i>et al.</i> , 2009
	R 5'-AGACCTCGACAATGATCTTAC-3'	
<i>GST</i>	F 5'-ACTTGGTGAAGGAAGCTGGA-3'	Cutanda-Perez <i>et al.</i> ,
	R 5'-TTGGAAAGGTGCATACATGG-3'	
<i>MybA1</i>	F 5'-TAGTCACCACTTCAAAAAGG-3'	Jeong <i>et al.</i> , 2004
	R 5'-GAATGTGTTTGGGGTTTATC-3'	
<i>Myb5a</i>	F 5'-GTGCAGCAGCCATCTAATGTG-3'	Matus <i>et al.</i> , 2009
	R 5'-GCAGCAGGTTCCCAGACAGT-3'	
<i>Myb5b</i>	F 5'-GGTGTCTTTAATTTGGCTTCA-3'	Deluc <i>et al.</i> , 2009
	R 5'-CACAACAACACAACCACATACA-3'	
<i>MybpA1</i>	F 5'-AGATCAACTGGTTATGCTTGCT-3'	Bogs <i>et al.</i> , 2007
	R 5'-AACACAAATGTACATCGCACAC-3'	
<i>NCED1</i>	F 5'-GAGACCCCAACTCTGGCAGG-3'	Wheeler <i>et al.</i> , 2009
	R 5'-AAGGTGCCGTGGAATCCATAG-3'	
<i>NCED2</i>	F 5'-AGTTCCATACGGGTTTCATGGG-3'	Wheeler <i>et al.</i> , 2009
	R 5'-CCATTTTCCAAATCCAGGGTGT-3'	
<i>EF1γ</i>	F 5'-CAAGAGAAACCATCCCTAGCTG-3'	Hichri <i>et al.</i> , 2010
	R 5'-TCAATCTGTCTAGGAAAGGAAG-3'	
<i>ACT</i>	F 5'-CTTGCATCCCTCAGCACCTT-3'	Reid <i>et al.</i> , 2006
	R 5'-TCCTGTGGACAATGGATGGA-3'	
<i>CytoB5</i>	F 5'-ACAAGGAAACAAAGACCAAAGA-3'	Bogs <i>et al.</i> , 2006
	R 5'-GAACATACAATGCAGCAAGAAA-3'	
<i>Hyd2</i>	F 5'-CTCTCAGTGCCACCAATCAA-3'	Speirs <i>et al.</i> , 2013
	R 5'-TCAGCTTGCAATCTTTCTGG-3'	

4.3 Results

4.3.1 Berry size and sugar accumulation

The different effects of light on berry weight of the cultivars studied are shown in Figure 4-2. Under light-exposed conditions, Gamay Fréaux and Gamay had similar skin and flesh weights along berry development (Figure 4-2a and b). Light exclusion markedly reduced flesh weight of Gamay Fréaux but had less effect on the flesh

weight of Gamay berries (Figure 4-2b).

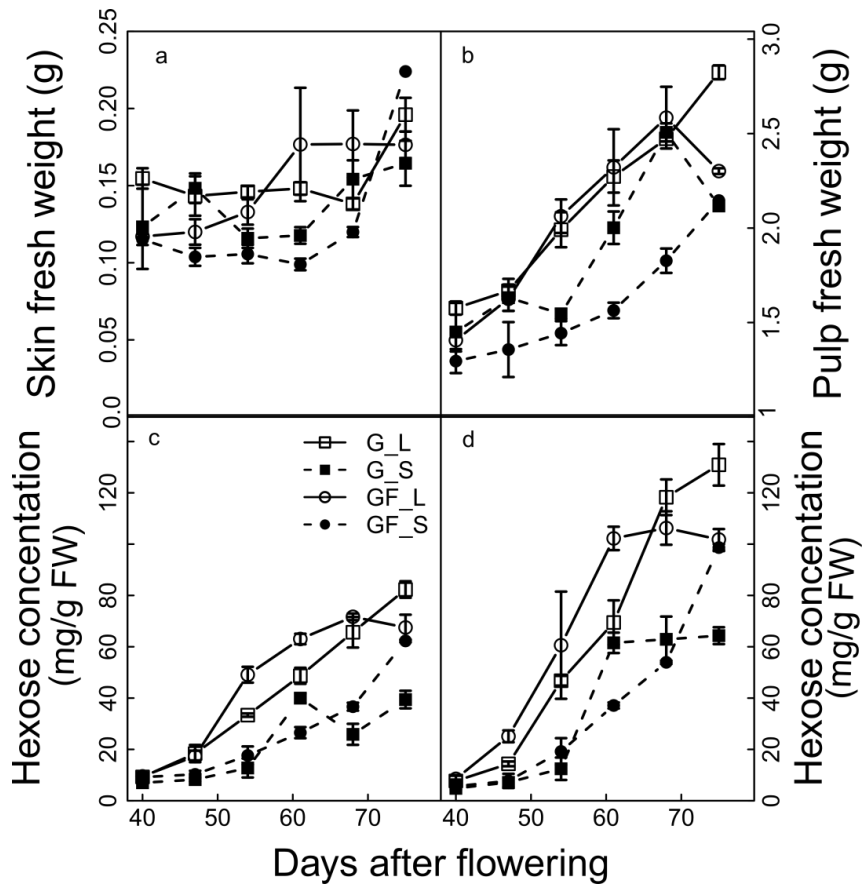


Figure 4-2 Fresh weight (a and b) and hexose concentration (c and d) in the skin (a,c) and pulp (b,d) of cv. Gamay (G) and Gamay Fr éaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions. Data are means \pm SE of three biological replicates.

The time-course of sugar accumulation was different between cultivars and was modified by light conditions (Figure 4-2c and d). Under light-exposed condition, hexose (glucose + fructose) accumulated faster and reached the plateau earlier in Gamay Fr éaux than in Gamay in both skin and flesh. However, the longer duration of sugar accumulation in the flesh of Gamay compensated for its lower accumulation rate, resulting in a higher hexose concentration (130.9 mg/g FW) at maturity than in Gamay Fr éaux (101.8 mg/g FW). Light-exclusion delayed the onset of sugar accumulation by one week in both cultivars compared to the light-exposed berries. However, light exclusion induced different effects on the kinetics of sugar

accumulation, leading to significant lower hexose concentrations at maturity in light-excluded Gamay (64.3 and 39.4 mg/g FW for flesh and skin, respectively), but similar concentration in Gamay Fréaux (98.7 and 67.5 mg/g FW for flesh and skin, respectively), compared to light-exposed berries.

4.3.2 Anthocyanin concentration and composition

There was a significant difference in the level of total anthocyanins as well as substantial differences in anthocyanin composition between the light-exposed and -excluded berries for both cultivars (Figure 4-3). Under light-exposed conditions, total anthocyanins increased rapidly between two and three weeks after veraison, and then stayed at a steady level in berry skins of Gamay and Gamay Fréaux as well as in berry flesh of Gamay Fréaux (Figure 4-3a and b). At maturity, the skins of Gamay Fréaux had the highest concentration of anthocyanins (1306.2 mg/100 gFW), followed by the skins of Gamay (939.3 mg/100 gFW), and both values were significantly higher than anthocyanin concentration in the flesh of Gamay Fréaux (48 mg/100 g FW). Light-exclusion strongly decreased anthocyanin accumulation in berry skin and flesh, which both had significantly lower total anthocyanin concentrations from veraison to maturity. At maturity, total anthocyanin concentration in light-excluded skin of Gamay only accounted for 14.1% of the light-exposed ones. For the teinturier cultivar Gamay Fréaux, there was a 51.7% and 52.3% reduction in total anthocyanins in light-excluded berry skin and flesh, respectively.

While the total anthocyanin concentration was lower in light-excluded berries, the decrease in 3',4',5'-trihydroxylated anthocyanins (Dp-, Pt-, and Mv-derivatives) was greater than that of 3',4'-dihydroxylated (Cy- and Pn-derivatives) anthocyanins (Figure 4-3c-f). At maturity, the decrease in trihydroxylated anthocyanin concentration was 90.6%, 68.3%, and 87.6% in light-excluded Gamay skin, Gamay Fréaux skin and flesh compared to the light-exposed ones. On the other hand, a lower decrease in dihydroxylated anthocyanin concentration was observed in light-excluded Gamay skin, Gamay Fréaux skin and flesh (73.4%, 21.7%, and 36.5%, respectively) compared to the light-exposed ones. Thus, the ratio of dihydroxylated to

trihydroxylated anthocyanins in light-excluded colored tissues was increased (Figure 4-3g and h). Interestingly, the ratio increased with berry development regardless of cultivar, tissue, and light conditions. Despite this modification, the flesh of Gamay Fréaux accumulated predominately dihydroxylated anthocyanins under both light-exposed and -excluded conditions. In contrast, the skins of Gamay and Gamay Fréaux accumulated predominantly trihydroxylated anthocyanins under light, but dihydroxylated ones under light-exclusion.

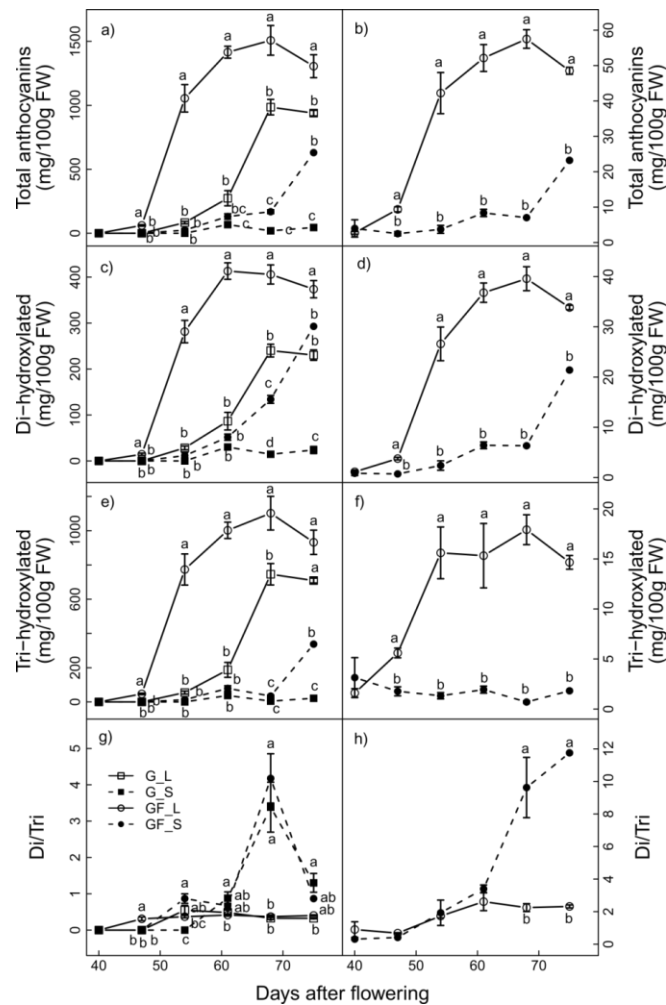


Figure 4-3 Total anthocyanin concentration and anthocyanin composition in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fréaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions. Different letters between genotype and light condition combinations at a given sampling date indicate significant differences ($P < 0.05$).

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In addition to the modification in hydroxylation, light-exclusion also influenced the acylation and methylation of anthocyanins (Figure 4-4). Light-exclusion significantly increased the proportion of acylated anthocyanins in the skin of Gamay and the flesh of Gamay Fréaux, but had minor effect in the skin of Gamay Fréaux. Similarly, light-exclusion only increased the proportion of methylated anthocyanins in the skin of Gamay compared to light conditions. However, the composition of methylated anthocyanins was modified in all the three colored tissues, with increased 3'-methylated anthocyanins at the expense of 3'5'-methylated ones under light-exclusion.

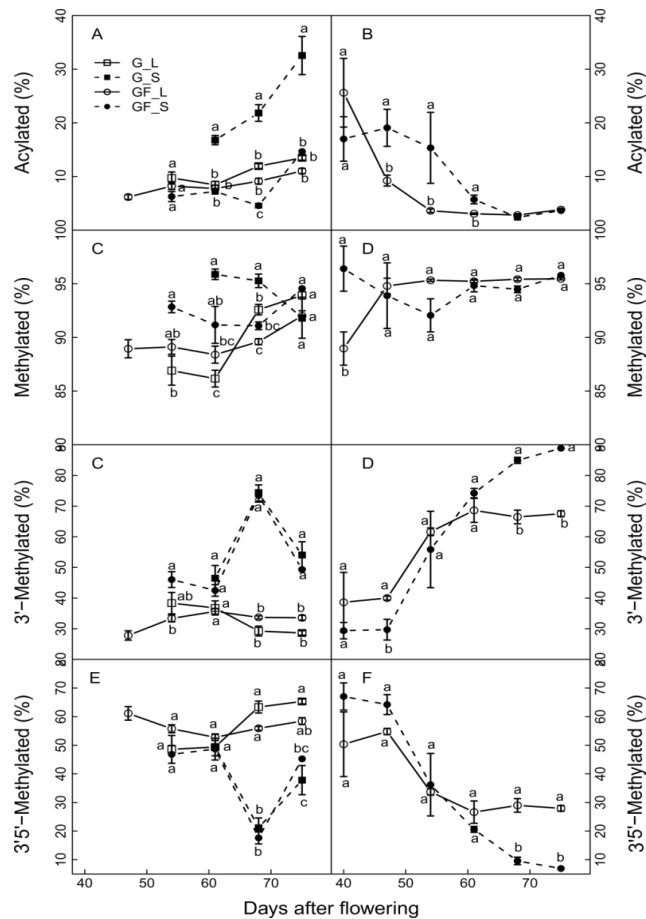


Figure 4-4 Modifications in anthocyanin acylation (a,b) and methylation (c,d,e,f,g,h) in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fréaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions. Different letters between genotype and light condition combinations at a given sampling date indicate significant differences ($P < 0.05$).

4.3.3 Transcripts abundance of structural genes of anthocyanin biosynthesis

Transcript levels of structural genes involved in anthocyanin biosynthesis and their responses to light conditions were cultivar and tissue dependent (Figure 4-5 and Figure 4-6). Six out of the seven analyzed genes showed a higher expression level in the skin of Gamay Fréaux than that of Gamay under light-exposed condition, except *AOMT* that had similar expression levels in the skins of both cultivars. In the skin of Gamay Fréaux and Gamay, the expressions of *CHS2*, *CHI*, *F3'5'H*, and *AOMT* were all significantly decreased by light-exclusion (Figure 4-5a, b, g, and c). However, the expression of *F3'H*, *DFR*, and *UFGT* was reduced by light-exclusion only in the skin of Gamay Fréaux and was not affected in the skin of Gamay (Figure 5e and i).

Because Gamay Fréaux was the only cultivar developing color in its flesh, transcript levels were only determined in the flesh of this cultivar (right panels of Figure 4-5 and Figure 4-6). Under light-exposed condition, flesh had lower expression levels in *CHI*, *F3'5'H*, *UFGT*, and *AOMT*, higher expression levels in *F3'H* and *DFR*, and comparable levels of *CHS*, comparing with the skin of Gamay Fréaux. Moreover, the developmental expression profiles in flesh exhibited patterns that differed from skin under light-exposed conditions. The most contrasting pattern was observed for *F3'5'H* (Figure 4-5h), whose transcripts decreased continually over the studied periods in flesh, while they showed a S-shape in the skin. In fact, *F3'5'H* belongs to a multi-copy gene family, among which at least 8 members have been identified (Falginella *et al.* 2010). A preliminary PCR analysis showed that there were only 5 genes expressed in our samples (data not shown), and all the five continuously declines in the flesh of Gamay Fréaux (Figure 4-7). *AOMT* also showed tissue-specific expression profile, which started to decrease as early as 47 DAF in flesh (Figure 4-6d), while it still increased linearly in the skin (Figure 4-6c). To a lesser extent, *CHS*, *CHI*, *F3'H*, *DFR* transcript abundance decreased sharply from 61DAF to maturity in light-exposed flesh, while they remained constant or decreased slightly in the skin (left panels of Figure 4-5). As observed in the skin, light-exclusion down-regulated all the genes studied in the flesh during the early developmental stages (before 61DAF). Thereafter, the extent of decrease was diminished and the

expression reached a similar level between light-excluded and -exposed berries.

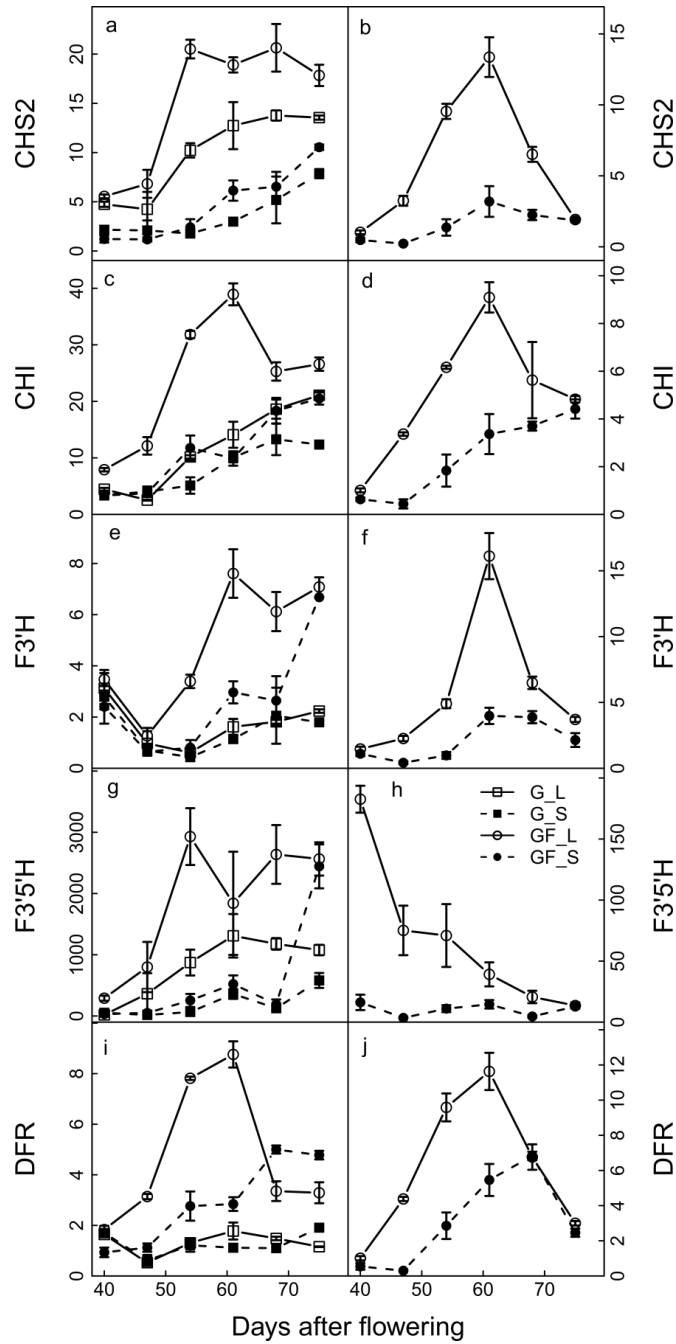


Figure 4-5 Relative expression levels of structural genes involved in anthocyanin biosynthesis in the skin (left panel) and pulp (right panel) of cv. Gamay and Gamay Fr éaux berries over development under light-exposed (L) or -exclusion (S) conditions.

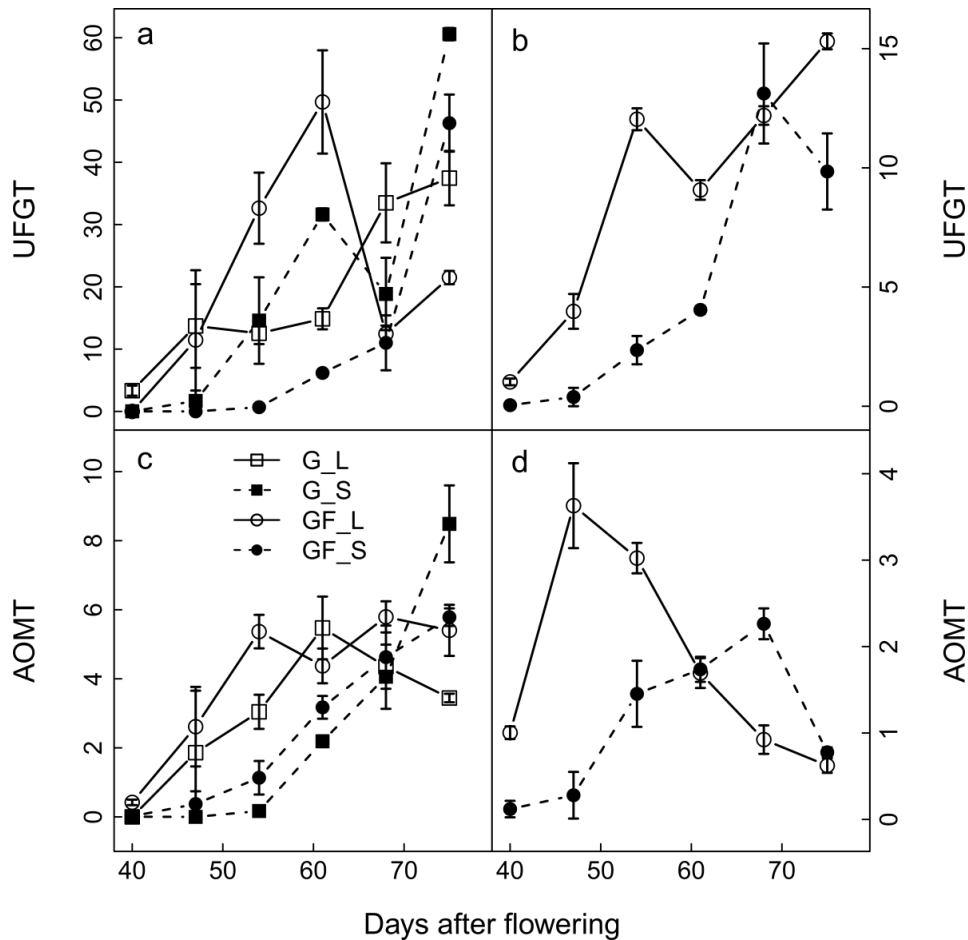


Figure 4-6 Relative expression levels of *UFGT* (a and b) and *AOMT* (c and d) in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fréaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions.

4.3.4 Transcripts abundance of regulatory genes of anthocyanin biosynthesis

Transcript abundance of regulatory genes that modulate the expression of the structural genes studied in the skin and flesh was affected by light-exclusion (Figure 4-8). Transcripts of *MybA1*, *Myb5b*, *MybPA1* and *CytoB5* were less abundant in both skin and flesh of Gamay Fréaux under light-exclusion than under light-exposure. Under light-exclusion, the transcript levels of *Myb5a* were not modified in the skin (Figure 4-8c) but they increased in the flesh of Gamay Fréaux (Figure 4-8d). Light had similar effects on transcript abundance of the regulatory genes in Gamay skin, with transcriptional level of *MybA1*, *Myb5b*, and *CytoB5* being decreased while that

of *Myb5a* and *MybPA1* were not affected. Regardless of light conditions, the flesh consistently had lower levels of transcripts than that of skin.

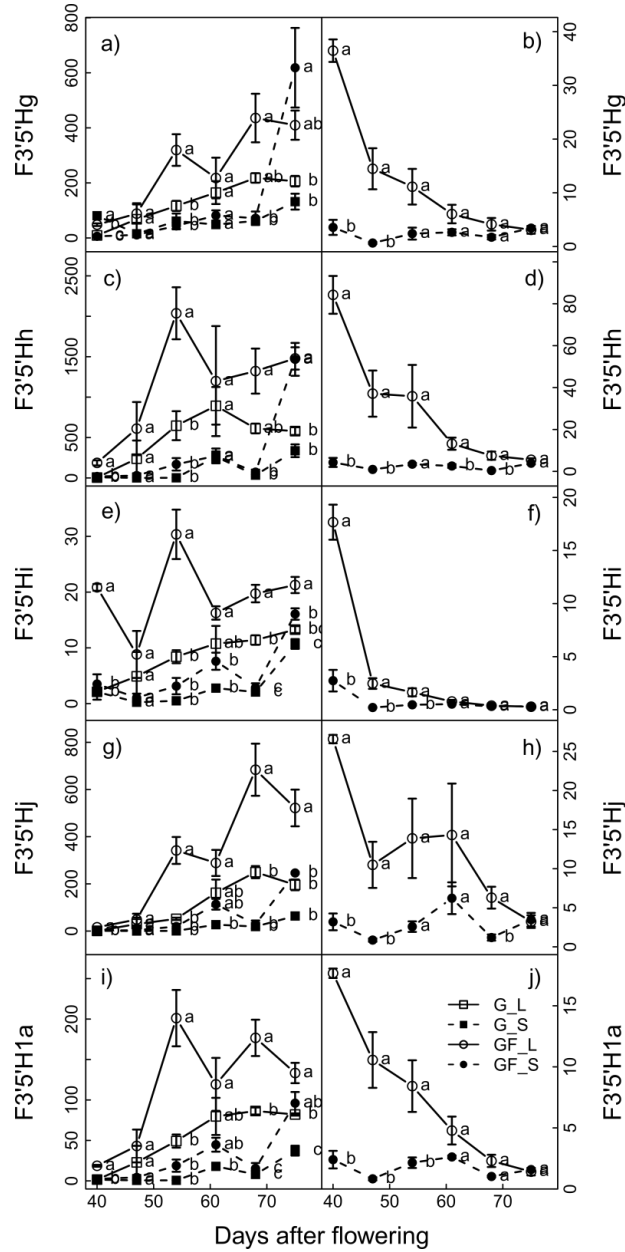


Figure 4-7 Effects of light exclusion on the relative expression levels of different gene copies of *F3'5'H* in the skin (left panel) and pulp (right panel) of cv. Gamay and Gamay Fr aux berries. Data are means \pm SE for three biological replicates. The genotypes and light conditions are abbreviated as G for Gamay, GF for Gamay Fr aux, L for light-exposed, and S for light-exclusion. Different letters between genotype and light condition combinations at a given sampling date indicate significant differences ($P < 0.05$).

4.3.5 Transcripts abundance of genes encoding anthocyanin transporters

Expression of genes encoding anthocyanin transporters was also affected by light conditions and some responded reversely between skin and flesh (Figure 4-9). Light-exclusion decreased the expression level of *AM3* and *GST* in the skin of Gamay Fréaux and Gamay and in the flesh of Gamay Fréaux compared to light-exposed berries. However, light-exclusion down-regulated *AMI* in the skin of Gamay Fréaux and Gamay but upregulated it in the flesh of Gamay Fréaux (Figure 4-9a and b). Regardless of light conditions, expression of *AMI* exhibited a bell shape while *AM3* and *GST* showed an S shape in the skin of both cultivars. In contrast to the skin, the gene expression patterns found in the flesh exhibited two distinct temporal profiles, including a continuous decrease (*AMI*) and an S shape (*AM3* and *GST*).

4.3.6 ABA accumulation and genes of ABA biosynthesis

The concentration of free ABA in the skin and flesh of both cultivars was significantly decreased by light exclusion from 40 to 54 DAF, after which the difference diminished (Figure 4-10a and b). In parallel with ABA concentration, light-exclusion also down-regulated *NCED1* and *NCED2* in the skin of both cultivars (Figure 4-10c and e). Moreover, light-exclusion also decreased the concentration of major ABA catabolites, including ABA glucose ester (ABAGE), phaseic acid (PA), and dihydrophaseic acid (DPA), and the levels of *Hyd2* transcripts that code for an enzyme involved in ABA catabolism (Figure 4-11).

However, light-exclusion had different effects in flesh and skin. *NCED1* was slightly decreased by light-exclusion in the flesh of Gamay, while its accumulation in the flesh of Gamay Fréaux was delayed significantly, peaking at 54DAF in light-exposed flesh, but at 68 DAF in light-excluded flesh (Figure 4-10d). On the other hand, a reverse effect of light-exclusion was observed on *NCED2*, which was up-regulated in the flesh but down-regulated by light-exclusion in the skin of both cultivars (Figure 4-10e and f). It is worth to note that the developmental profiles of free ABA and both *NCEDs* showed high similarity between the two cultivars under both light conditions.

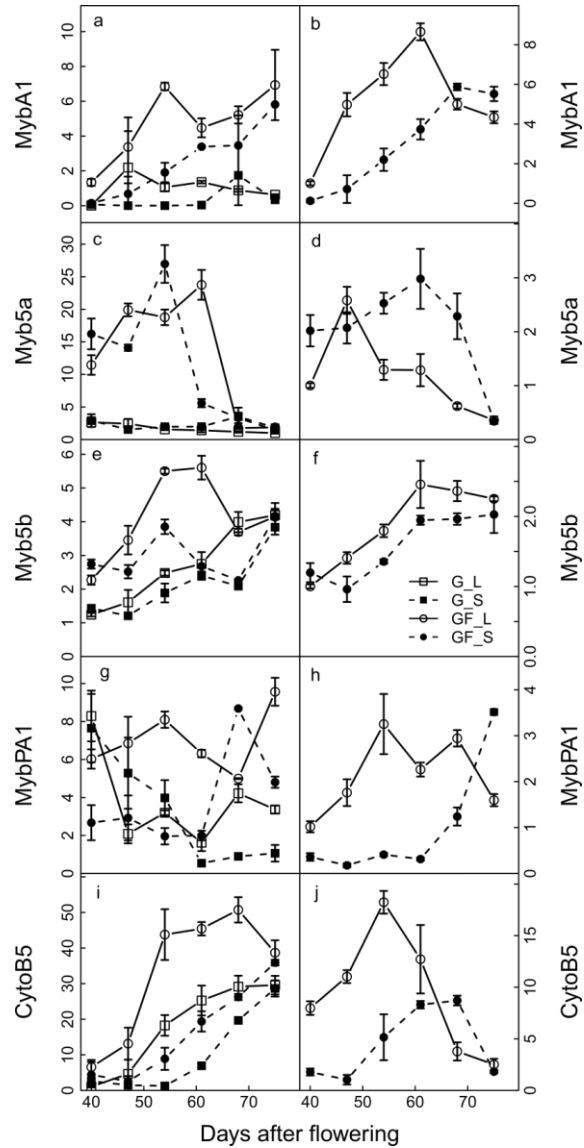


Figure 4-8 Relative expression levels of regulatory genes involved in anthocyanin biosynthesis in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fr éaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions.

4.3.7 Multivariate analysis to assess cultivar and tissue similarity and specificity

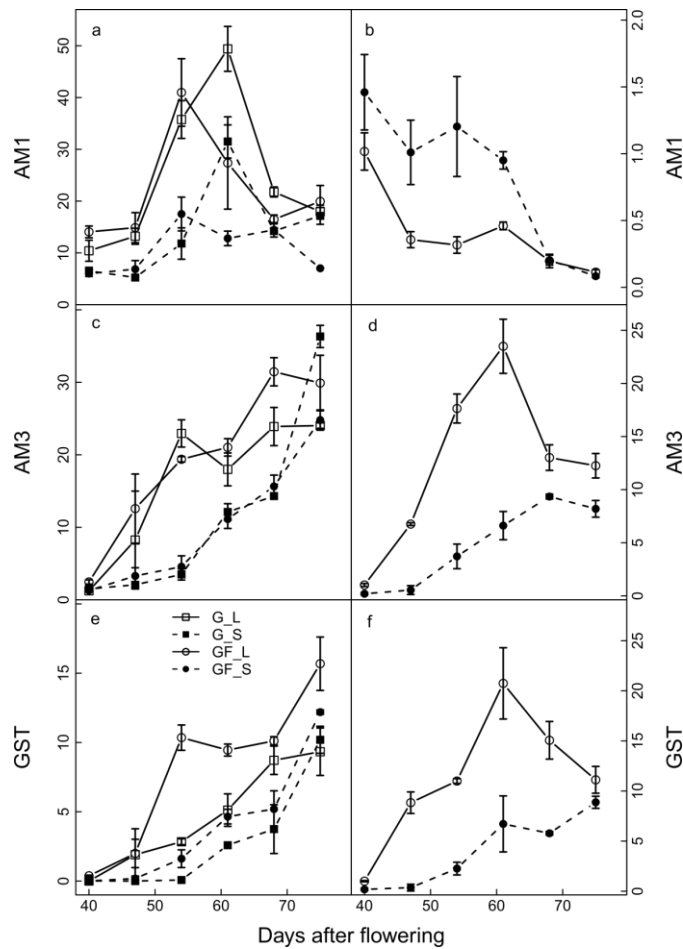


Figure 4-9 Relative expression levels of genes involved in anthocyanin transport in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fréaux (GF) berries over development under light-exposed (L) or –exclusion (S) conditions.

In addition to the point-by-point analysis, principal component analysis (PCA) and correlation network analysis were conducted to gain an integrative view of the spatialtemporal anthocyanin accumulation and gene expressions under both light conditions (Figure 4-12 and Figure 4-13). PCA readily discriminated the flesh of Gamay Fréaux from the skin of both cultivars by the first two PCs, which explained 63.7% of the total variance (Figure 4-12a). This discrimination between flesh and skin was largely determined by the relative composition between dihydroxylated and trihydroxylated anthocyanins (Di/Tri) and the ratio of gene expression *F3'H/F3'5'H* (Figure 4-12b and Figure 4-14). In addition, the expression of *NCED2*, *AMI* and

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Myb5a and the concentration of DPA also contributed to separate the flesh with skin (Figure 4-12b) in PCA. To a less extent, PCA also discriminated different developmental stages and the two light conditions (Figure 4-12a). PC1 separated the flesh of different light conditions and the skin of different development stages, based mainly on changes in total anthocyanin concentration and the transcript levels of *F3'5'H*, *CHI*, *Myb5b*, *CytoB5*, and *AOMT*. PC2 resolved the flesh of different ripening stages and the skin of different light conditions, related mainly to differences in *DFR*, *F3'H*, *MybA1*, *GST* transcript abundances, and the ratio *F3'H/F3'5'H* (Figure 4-12b).

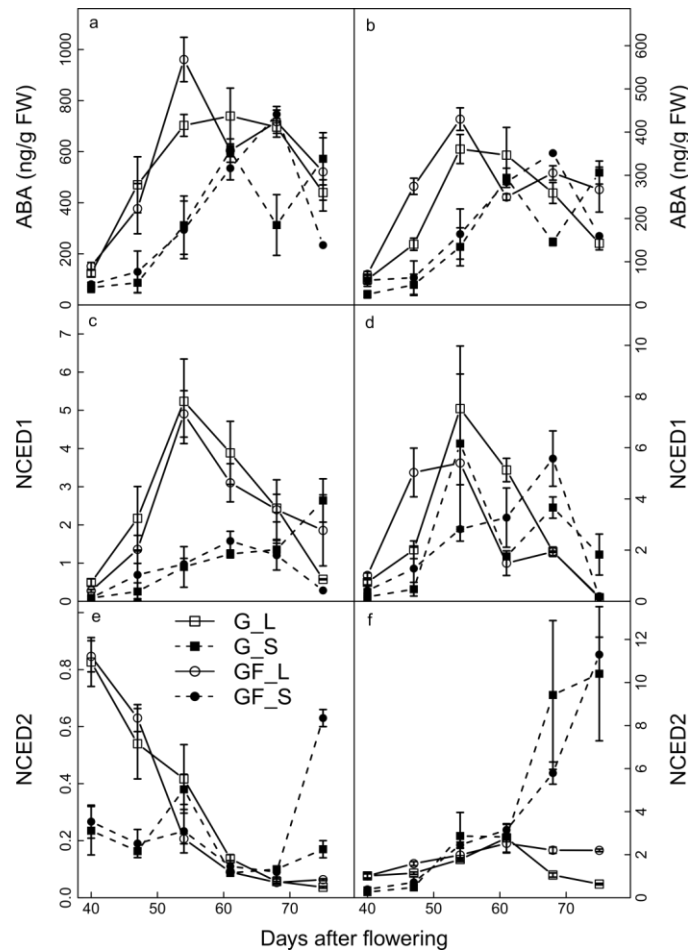


Figure 4-10 Free ABA concentration (a and b) and relative expression levels of genes involved in ABA biosynthesis (c, d, e, and f) in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fréaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions.

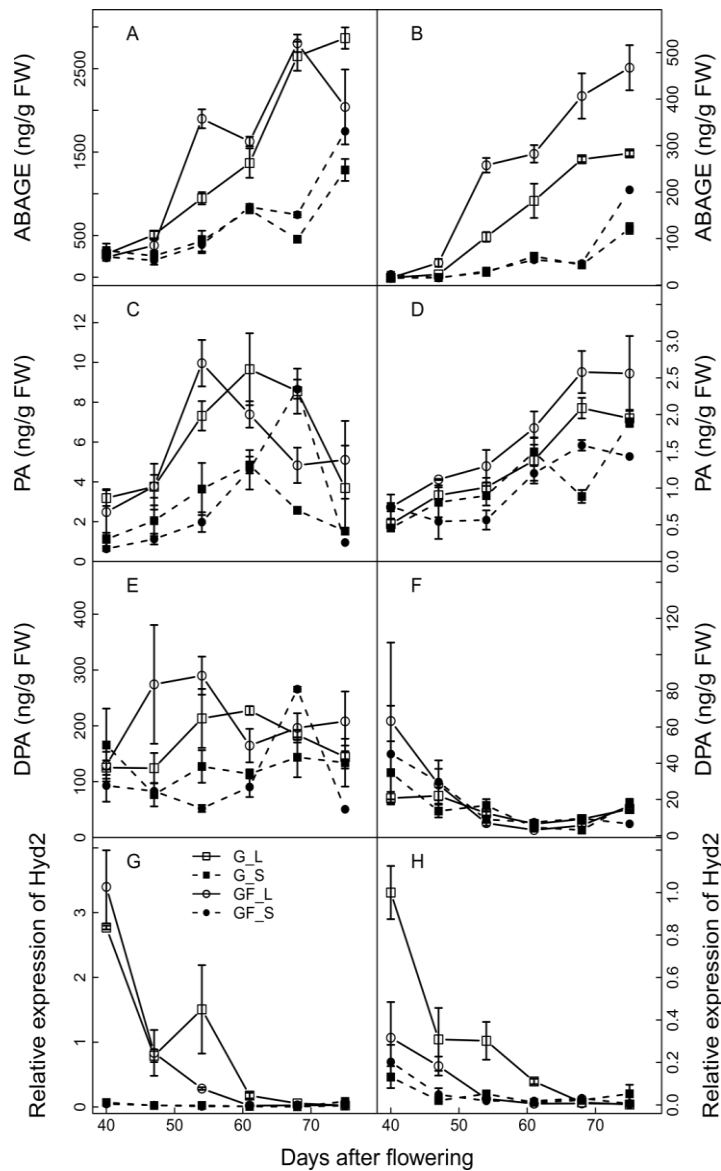


Figure 4-11 Concentrations of ABA catabolites (a-f) and relative expression levels of genes involved in ABA catabolism (g and h) in the skin (left panel) and pulp (right panel) of cv. Gamay (G) and Gamay Fr éaux (GF) berries over development under light-exposed (L) or -exclusion (S) conditions.

Correlation networks among metabolites and transcripts were constructed for each cultivar and tissue combination, namely the skin of Gamay (Figure 4-13a), and the skin (Figure 4-13b) and flesh (Figure 4-13c) of Gamay Fr éaux. There was similar number of nodes (metabolites and transcripts) in the three networks (32 for Gamay

skin, 34 for Gamay Fréaux skin, and 35 for Gamay Fréaux flesh). However, the connectivity between nodes were different, with 236 for Gamay Fréaux skin > 184 for Gamay skin > 142 for Gamay Fréaux flesh. In each network, there was a major module accompanied by several minor modules. The major module included total anthocyanins and structural and regulatory genes involved in anthocyanin biosynthesis. Minor modules were mainly related to ABA biosynthesis and catabolism, the ratio between *F3'H/F3'5'Gs*, and the expression between different *F3'5'H* copies. Network comparison was used to further explore the similarity and specificity among the three colored tissues. It revealed that there were 26 nodes with 60 linkages common in the three colored tissues (Figure 4-13, Figure 4-15 and Figure 4-16d). Among them, several unprecedented links were observed, such as ABA with *AOMT*, *CytoB5* with *AOMT*, and *CytoB5* with *CHS2* (Figure 4-13 and Figure 4-16d).

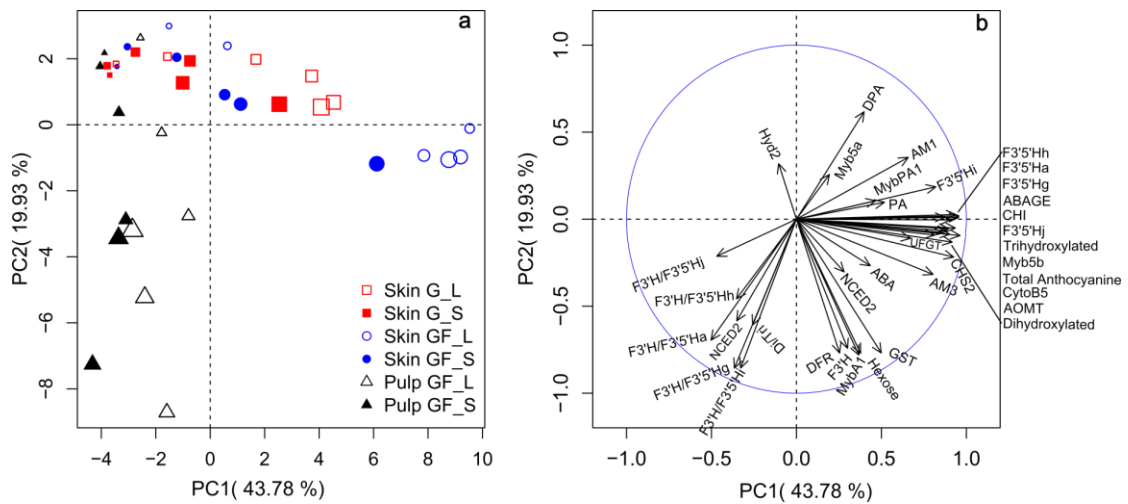


Figure 4-12 Principal component analysis of the skin and pulp of cv. Gamay and Gamay Fréaux berries over development under light-exposed or -exclusion conditions. The genotypes and light conditions are abbreviated as G for Gamay, GF for Gamay Fréaux, L for light-exposed, and S for light-exclusion. Panel a) illustrates the discriminations of cultivars, treatment, and development stages and the symbol size represents berry size; Panel b) illustrates the loading plots of metabolites and transcripts for the first two principle components (PC1 and PC2).

Pairwise network comparison showed that the skin of Gamay and Gamay Fréaux had the highest number of common links (147), while the flesh of Gamay Fréaux was

more identical to the skin of Gamay Fréaux (94 common links) than to the skin of Gamay (65 common links, Figure 4-16). On the other hand, network comparison also revealed that 30.3% of the significant links in flesh of Gamay Fréaux were specific to this tissue, while there were only 23.3% and 17.4% tissue-specific links in the skin of Gamay Fréaux and Gamay, respectively (Figure 4-15 and Figure 4-17).

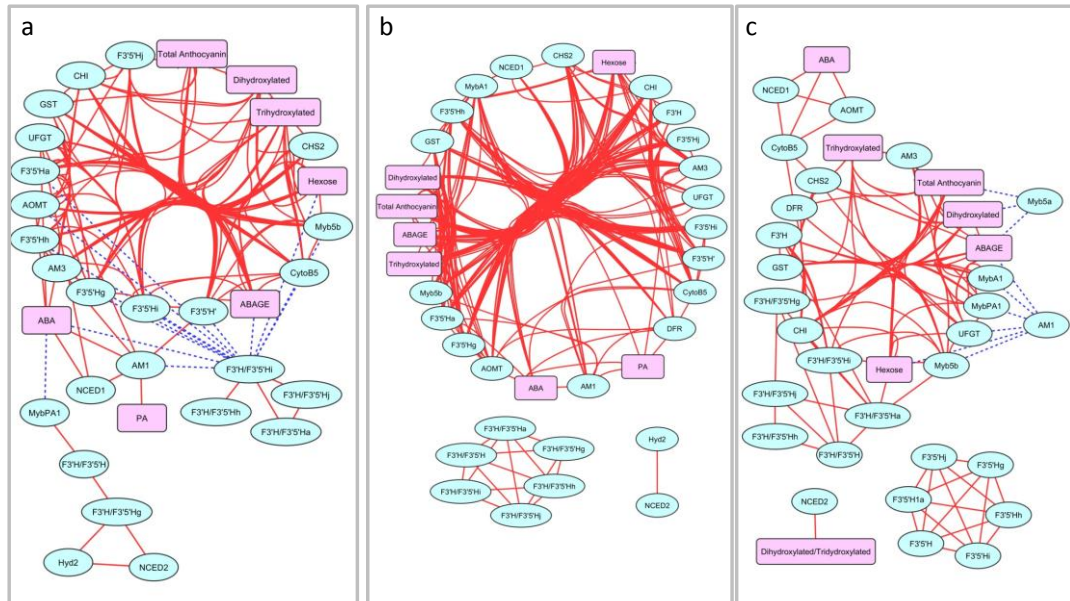


Figure 4-13 Correlation networks of metabolites and transcripts in the skin of cv. Gamay (a), the skin of cv. Gamay Fréaux (b), and the pulp of cv. Gamay Fréaux (c) grown under light-exposed or -exclusion conditions. Metabolites are assigned in rectangle and transcripts in ellipse. Solid lines indicate a significant positive correlation, while dashed lines indicate a significant negative correlation.

4.4 Discussion

Metabolite and transcript profiling was integrated to study the effect of light on anthocyanin accumulation and composition in three colored grape tissues, including the skin of cv. Gamay, the skin and flesh of cv. Gamay Fréaux (a teinturier mutant of Gamay). The potential involvement of sugar and ABA in light induced modifications in anthocyanin was also investigated. The results showed that light significantly reduced anthocyanin concentration and profoundly modified anthocyanin

composition. These modifications were associated with changes in expression of structural and regulatory genes of anthocyanin biosynthesis pathway, and genes of anthocyanin transport, in a cultivar- and tissue-specific manner. Sugar and ABA accumulation were also affected by light-exclusion, but they seem to play a limited role in mediating the light effect on anthocyanin biosynthesis.

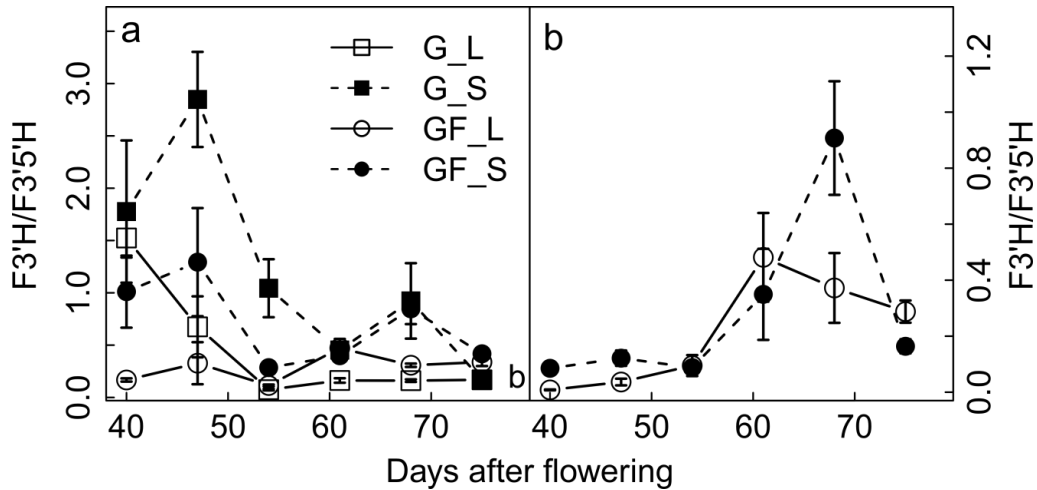


Figure 4-14 Effects of light exclusion on the ratios of the relative expression levels between *F3'H* and *F3'5'H* in the skin (a) and pulp (b) of cv. Gamay and Gamay Fréaux berries. Data are means \pm SE for three biological replicates. The genotypes and light conditions are abbreviated as G for Gamay, GF for Gamay Fréaux, L for light-exposed, and S for light-exclusion.

4.4.1 Comparison of Gamay Fréaux and Gamay under light-exposed growth condition

The mutation in Gamay Fréaux not only leads to anthocyanin accumulation in its flesh, but also significantly increases anthocyanin concentration in its skin compared to its wild counterpart Gamay under natural growth conditions (Figure 4-3). The latter difference is a result of higher expression in Gamay Fréaux than in Gamay of all the genes studied (except *AOMT*) that are involved in anthocyanin biosynthesis and regulation (Figure 4-5, Figure 4-6, and Figure 4-8). The transcripts of *GST*, which plays a role in anthocyanin transport from cytosol to vacuole (Conn *et al.* 2008; Gomez *et al.* 2011), were also more abundant in the skin of Gamay Fréaux than in the skin of Gamay (Figure 4-9e). Moreover, the expressions of these genes were tightly

coordinated along berry development, as revealed by the highly connected correlation networks among them (Figure 4-13). This confirms that the pathway is developmentally regulated as a module (Boss *et al.* 1996b). Despite the difference in concentration, the skin of both cultivars had similar anthocyanin compositions in terms of hydroxylation (Figure 4-3g), acylation and methylation (Figure 4-4). The identical hydroxylation pattern found over berry development imost likely results from the similar ratio of the relative expression of *F3'H* and *F3'5'H* in both cultivars (Figure 4-14), as suggested in previous investigations (Bogs *et al.* 2006; Castellarin and Di Gaspero 2007; Falginella *et al.* 2012; Jeong *et al.* 2006). The similar expression profile of *AOMT* could be responsible for, at least in part, the identical methylation pattern (Fournier-Level *et al.* 2011; Hugueney *et al.* 2009). In addition, the similar expression of *AMI* and *AM3*, which mediate the specific transport of acylated anthocyanins (Gomez *et al.* 2011; Gomez *et al.* 2009), may play a role in determining the proportion of acylated anthocyanins.

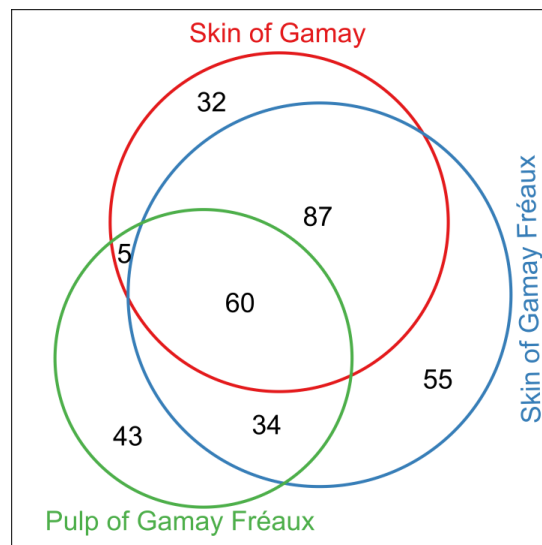


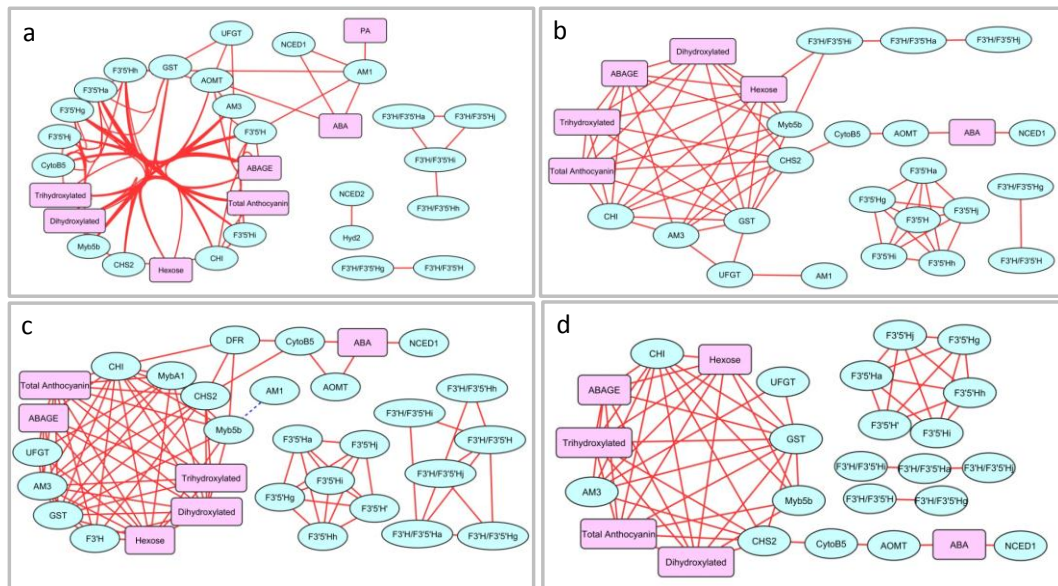
Figure 4-15 Venn diagram summaries of the specific and common significant correlations (as illustrated in Figure 4-13, Figure 4-15 and Figure 4-16) in the skin and pulp of cv. Gamay and Gamay Fréaux berries over development under light-exposed or -exclusion conditions.

The anthocyanin concentration is much lower in the flesh of Gamay Fréaux than in the skin the skin of both cultivars (Figure 4-3), most likely as a result of the lower

expression of *F3'5'H* and *UFGT* (Figure 4-5 and 4-6). However, anthocyanins constitutively accumulate from fruit-set to maturity (Figure 4-1) and reached a concentration of 2.7 mg/100 gFW one week before veraison (Figure 4-3). This is different from another commonly used teinturier cultivar Alicante Bouschet, which does not accumulate anthocyanins in flesh before veraison (Castellarin *et al.* 2011). Despite the differences in the onset of coloration, the flesh of Gamay Fréaux showed a similar anthocyanin composition as in the flesh of other teinturier cultivars (e.g. Alicante Bouschet and its descendants), accumulating predominately dihydroxylated Cy- and Pn-derivatives (Ageorges *et al.* 2006; Castillo-Muñoz *et al.* 2009; Falginella *et al.* 2012; Guan *et al.* 2012; He *et al.* 2010b). Similarly, the commonly used cell suspensions of Gamay Fréaux accumulate mainly the di-hydroxylated anthocyanins (Conn *et al.* 2008; Conn *et al.* 2010; Cormier *et al.* 1990; Do and Cormier 1991; Saigne-Soulard *et al.* 2006), and, should therefore be considered as a representative model system for berry flesh rather than skin.

In parallel with its distinct temporal profile of anthocyanin accumulation, the flesh of Gamay Fréaux also showed different gene expression profiles under light-exposed conditions in comparison with the skin of both cultivars (Figure 4-5, 4-6, 4-8, 4-9 and 4-10). The most distinct profile was observed for *F3'5'H*, which decreased continuously in the flesh of Gamay Fréaux (Figure 4-5h and Figure 4-7) while it increased from veraison and reached a plateau at maturity in the skin of both cultivars (Figure 4-5g). We then investigated the potential regulatory genes that might be involved in regulating *F3'5'H* expression in flesh of Gamay Fréaux (Figure 4-8). De Vetten *et al.* (1999) observed that a cytochrome b5 can regulate the activity of *F3'5'H* in *Petunia*, and this was later confirmed by Bogs *et al.* (2006), showing that the putative cytochrome b5 (*CytoB5*) is expressed concomitantly with *F3'5'H* in the skin of Shiraz berry. Our results in skin (Figure 4-8) supported the observation of Bogs *et al.* (2006); however, the *CytoB5* in flesh (Figure 4-8) exhibited a more asynchronous pattern of expression than *F3'5'H*, indicating a different regulation mode in the flesh. In addition, none of the other four regulatory genes (including *MybA1*, *Myb5a*, *Myb5b*, and *MybPA1*) showed a tightly correlated expression with

F3'5'H in flesh of Gamay Fr éaux. These results indicate that either the expression of F3'5'H in flesh of Gamay Fr éaux is not regulated at transcriptional level, or that other



unknown transcriptional factors are yet to be discovered.

Figure 4-16 Correlation networks of metabolites and transcripts shared between the skin of cv. Gamay and of cv. Gamay Fr éaux (a), between the skin of cv. Gamay and the pulp of cv. Gamay Fr éaux (b), between the skin and pulp of cv. Gamay Fr éaux (c), and between the three combinations of cultivar and tissues (d) grown under light-exposed or -exclusion conditions. Metabolites are assigned in rectangle and transcripts in ellipse. Solid lines indicate a significant positive correlation, while dashed lines indicate a significant negative correlation.

4.4.2 Light-exclusion reduces anthocyanin concentration and increases the ratio between dihydroxylated and trihydroxylated anthocyanins

Light-exclusion induced a large decrease in total anthocyanin concentrations for the three colored tissues (Figure 4-3), in agreement with many previous studies (Azuma *et al.* 2012; Guan *et al.* 2014; Jeong *et al.* 2004; Koyama and Goto-Yamamoto 2008; Spayd *et al.* 2002). However, several authors also reported no modification in total anthocyanin concentration by light-exclusion (Cortell and Kennedy 2006; Downey *et al.* 2004; Ristic *et al.* 2007). These differences may reflect the cultivar sensitivity to light-exclusion (Downey *et al.* 2004; Zheng *et al.* 2013).

di-hydroxylated anthocyanins in all the three colored tissues (Figure 4-3), supporting previous reports (Azuma *et al.* 2012; Downey *et al.* 2004; Guan *et al.* 2014). A detailed analysis of the temporal profile revealed that light-exclusion suppressed the biosynthesis of tri-hydroxylated anthocyanins by ~80%, but reduced the di-hydroxylated anthocyanins only by ~44%. These results suggest that the two metabolic branches leading to the biosynthesis of tri- and di-hydroxylated anthocyanins have different sensitivities to light-exclusion. Interestingly, distinct sensitivities of the two branches were also reported in response to other environmental factors. For example, a high temperature decreased the proportion of dihydroxylated anthocyanins (Mori *et al.* 2007; Tarara *et al.* 2008) with a reduced total anthocyanin concentration, and water stress also decreased the proportion of dihydroxylated anthocyanins, but with an increased total anthocyanin concentration (Castellarin *et al.* 2007a; Castellarin *et al.* 2007b). Similarly, Kovicich *et al.* (2014) observed that different environmental conditions triggered distinct sets of anthocyanins in *Arabidopsis*, providing 'fingerprints' that reflect the physiological status of the plants. Uncovering the underlying mechanisms will enable us to tune finely the anthocyanin composition by combining different environment factors.

4.4.3 Coordinated reduction in structural, regulatory and transporter genes by light-exclusion is responsible for the reduced total anthocyanin concentration

Gene expression was quantified to investigate the regulation of the anthocyanin response to light-exclusion in each colored tissue (Figure 4-5, 4-6, 4-8 and 4-9). Seven structural genes, three transport genes, and four regulatory genes were all significantly down-regulated by light-exclusion in the skins of Gamay Fréaux. This coordinated reduction is in line with the decreased total anthocyanin concentration by light-exclusion in the skin of this cultivar. Although previous studies also observed down-regulation of structural and regulatory genes by light-exclusion (Guan *et al.* 2014; Jeong *et al.* 2004), we further illustrated that the transporters for anthocyanin storage were also reduced in concert, which underlines the importance of coordinated regulation to optimize the accumulation efficiency of the pathway. A similar

down-regulation of the genes studied was observed in the skin of Gamay, except that *F3'H*, *DFR*, and *UFGT* were not affected (Figure 4-5 and 4-6), highlighting the different regulation modes between the two cultivars. Surprisingly, *MYB5a* was the only gene studied that was not significantly affected by light-exclusion in the skins of the two cultivars, as observed by Guan *et al.* (2014). *MYB5a* was initially identified as a regulatory gene involved in the control of different branches of the phenylpropanoid pathway in grapevine, including anthocyanins (Deluc *et al.* 2006). More recently, (Cavallini *et al.* 2014) showed that *MYB5a* has a more limited influence on the expression of genes directly related to anthocyanin biosynthesis.. This may explain why it is not responding to light-exclusion.

The flesh of Gamay Fréaux showed a different and less coordinated response to light-exclusion than skins (Figure 4-5, 4-6, 4-8, 4-9 and Figure 4-13). Among the 15 genes assayed, twelve were down-regulated by light-exclusion with different magnitudes, with *F3'5'H* being the most sensitive as it was completely repressed by light-exclusion. These down-regulations are most likely responsible for the observed decrease in total anthocyanin concentration in the flesh. In contrast, *MYB5a* and *AMI* were up-regulated. The up-regulation of *MYB5a* may control other pathways than anthocyanin biosynthesis, as recently shown (Cavallini *et al.* 2014). Interestingly, the up-regulation of *AMI* was significantly and positively correlated with the increased proportion of acylated anthocyanins in the flesh under light-exclusion ($r = 0.67$, $P < 0.0001$, Figure 4-4). *AMI* and *AM3* are both MATE-type proteins transporting acylated anthocyanins (Gomez *et al.* 2009). Despite their overlapping transport functions, the expression of *AM3* is more tightly correlated with skin acylated anthocyanins than that of *AMI* (Gomez *et al.* 2009). Moreover, ectopic expression of *MYBA1* in grapevine only induced the expression of *AM3* but not that of *AMI*, indicating that *AMI* may be regulated by other transcriptional factors (Cutanda-Perez *et al.* 2009; Gomez *et al.* 2009). Our results provide novel evidence that *AMI* and *AM3* are under different regulation within the flesh and between the flesh and skin. In this context, Gamay Fréaux may serve as a valuable model system to further study the tissue-specific regulation of anthocyanin transport.

4.4.4 Different sensitivities of *F3'H* and *F3'5'H* to light-exclusion result in fine-tuning the anthocyanin hydroxylation pattern

Anthocyanin hydroxylation is known to be mediated by *F3'H* and *F3'5'H*, which lead to the biosynthesis of the 3'4'-dihydroxylated and 3'4'5'-trihydroxylated anthocyanins respectively in plants, (Tanaka and Brugliera 2013), including grape berry (Bogs *et al.* 2006). The relative expression levels of *F3'H* and *F3'5'H* largely determine the hydroxylation patterns of anthocyanins in different genotypes or tissues and under different growth conditions (Bogs *et al.* 2006; Castellarin and Di Gaspero 2007; Falginella *et al.* 2012; Jeong *et al.* 2006). Our results show that the two genes have at least two modes of response to light-exclusion in the three colored tissues. In the skin of Gamay, light-exclusion only reduced the expression of *F3'5'H* but not *F3'H*, resulting in a higher ratio of *F3'H/F3'5'H* and consequently a higher ratio of dihydroxylated/trihydroxylated anthocyanins. This is in agreement with our previous results on the teinturier cultivar Yan-73 (a progeny of Muscat Hamburg × Alicante Bouschet) (Guan *et al.* 2014). *F3'H* transcript abundance is relatively stable under various conditions, since water stress did not affect *F3'H* either, while it up-regulated *F3'5'H* in Merlot (Castellarin *et al.* 2007b) and in Cabernet Sauvignon (Castellarin *et al.* 2007a), resulting in a lower ratio of dihydroxylated/trihydroxylated anthocyanins. However, in the skin and flesh of Gamay Fr éaux, both *F3'H* and *F3'5'H* were down-regulated by light-exclusion, with *F3'H* less reduced than *F3'5'H*, which therefore increased the dihydroxylated/trihydroxylated anthocyanins ratio in both tissues. This is in agreement with the observations made for shaded Cabernet Sauvignon (Koyama and Goto-Yamamoto 2008). The two response modes observed here highlight the complex nature of regulation for the hydroxylation pattern of anthocyanins as a function of genotype and environmental factors.

Our previous results in Yan-73 showed that the light transmission through the skin decreased with the accumulation of anthocyanins, and hence the colored skin increases the shading effect on the berry flesh (Guan *et al.* 2014). The present results and other previous studies consistently show that light-exclusion can alter the balance between di- and tri-hydroxylated anthocyanin, by favoring the accumulation of the

dihydroxylated anthocyanins. This prompted us to test further the hypothesis that the skin-shading on flesh is responsible, to some extent, for the enrichment of dihydroxylated anthocyanins in the flesh. Several lines of evidence support this hypothesis: 1) *F3'5'H* is very sensitive to light-exclusion in the flesh, and its gradual decrease in flesh under light-exposed conditions parallels the increase of anthocyanin concentration in skin; 2) the accumulation of trihydroxylated anthocyanins stopped (around 54 d after flowering) once the skin accumulated considerable amount of anthocyanins under light-exposed condition, while the less light-sensitive dihydroxylated anthocyanins continued to accumulate in the flesh; 3) the ratio of di-/tri-hydroxylated anthocyanins in the flesh increased along berry development; 3) many structural genes showed a sharp decrease from 61 d after flowering, when skin anthocyanins reached a high level. These facts strongly suggest that the skin-shading effect plays a significant role in impacting anthocyanin biosynthesis in the flesh.

4.4.5 Sugar and ABA play a limited role in regulating anthocyanin synthesis between cultivars

High sugar and ABA are known to promote anthocyanin accumulation in grape (Hiratsuka *et al.* 2001; Vitrac *et al.* 2000). Interestingly, sugars and ABA concentration in both skin and flesh showed a good correlation in both cultivars under light-exposed conditions (Figure 4-2c, 4-2d, 4-10a, 4-10b), indicating that the mutation in Gamay Fréaux does not affect the accumulation of sugar and ABA. Moreover, this excludes that sugar and ABA control the higher skin anthocyanin concentration observed in Gamay Fréaux compared to Gamay and the presence of color in the flesh of Gamay Fréaux. Sugar and ABA were both reduced by light-exclusion in the three colored tissues, but this reduction seems to be only concurrences little with the reduction in anthocyanin. In fact, there was no marked difference between the skins of Gamay and Gamay Fréaux for the concentrations of hexose and ABA under light-exclusion, while the total anthocyanins were quite different. An experiment combing light-exclusion, and ABA and sugar feeding with *in vitro* cultured berry may further clarify the role of sugar and ABA in the modification

of anthocyanin pools induced by light-exclusion (Azuma *et al.* 2012; Dai *et al.* 2014). Moreover, the strong correlation between ABA and AOMT transcript abundance found in all the three colored tissues under both light conditions provides a relevant basis for further functional analysis.

4.5 Conclusions

Light was artificially excluded from cv. Gamay (white-fleshed) and Gamay Fréaux (teinturier mutant from Gamay) from fruit set until one week after maturity. Anthocyanins were quantified in parallel with profiling of genes involved in anthocyanin biosynthesis, regulation, and transport. Results showed that light exclusion reduced the total anthocyanins, most severely in the skin of Gamay and to a lesser extent in the flesh and skin of Gamay Fréaux, in comparison with light-exposed berries. Coordinated reduction in structural, regulatory and transporter genes by light-exclusion is responsible for the reduced total anthocyanin concentration in a cultivar- and tissue-specific manner. Moreover, light-exclusion increased the ratio of dihydroxylated to trihydroxylated anthocyanins in the three colored tissues. This results from different sensitivities of *F3'H* and *F3'5'H* to light-exclusion: only *F3'5'H* was reduced by light-exclusion with *F3'H* unaffected in the skin of Gamay, while both *F3'5'H* and *F3'H* were repressed with *F3'5'H* more reduced than *F3'H* in the skin and flesh of Gamay Fréaux. Sugar and ABA accumulation were also affected by light-exclusion, however, they seem to play a limited role in mediating light effect on anthocyanin biosynthesis. Overall, this study provides novel insights into the light regulation of anthocyanin biosynthesis in berry skin and flesh at physiological and transcript levels.

Chapter V. Genetic inheritance of anthocyanin in grape skin and pulp

5.1 Introduction

Grapevine is the second largest fruit crop in the world after citrus fruits (FAO). Most of grape production is used for wine (71%), while the rest is consumed as fresh fruit (27%) and dried as raisins (2%). The high economical value of grape products has stimulated great amount of research on improving grape cultivars with better fruit quality and accommodating to various environments and uses.

Vitis vinifera, mainly located in the Mediterranean regions with cool and wet winters, is widely grown because of its high fruit quality (Owens 2008). Although it is susceptible to pathogen infection, the traditional varieties as scions grafted to rootstocks with pathogen tolerance or resistance allowed the European countries to continue to cultivate their traditional cultivars (Alleweldt and Possingham 1988; Owens 2008). Many *Vitis* species were used to breeding the disease resistant rootstocks (Reisch et al. 2012). However, new grape cultivars need to be developed when *V. vinifera* was introduced into regions where it could not survive or susceptible to pathogens (Alleweldt and Possingham 1988; Owens 2008; Sabbatini and Howell 2014). For example, since the winter is very cold and the summer very wet in China, *V. vinifera* either enables to survive or demonstrates low yield and fruit quality (Lin 2007; Jiang *et al.* 2010). Even when the plants are buried by soil in winter, frequent cold damage to grapevines of *V. vinifera* occurs; while in summer, great rainfall may lead to low sugar and anthocyanin content and easily infected by pathogens.

Chemical mutagens or irradiation did not prove efficient for producing improved cultivars, partially because it is difficult to separate mutagenized cell layers from the rest of the tissues (Alleweldt and Possingham 1988). Only a few tetraploids arisen spontaneously or induced by colchicine were of commercial value since most of them were less fruitful (Gargiulo 1957, 1960). Interspecific hybrids were used to develop

successfully new cultivars which are better adapted to local environments and to local consumers' preference (Mitani *et al.* 2010; Sabbatini and Howell 2014). Therefore, crossbreeding is so far the most efficient approach of cultivar breeding (Owens 2008). Since grapevine is highly heterozygous, it is important to investigate the inheritance pattern of desirable traits when choosing the parents in crossbreeding. Berry skin color plays an important role in determining the market value of table grapes and the quality of red wine. Therefore, it is constantly taken as a major parameter during cultivar breeding. The inheritance pattern of anthocyanins, which is responsible for berry coloration, need to be investigated.

The black, red or white color of grape skin is controlled by one or two gene pair (s) (Barrit and Einset 1969; Spiegel-Roy and Assaf 1980; Goldy 1989; Shi *et al.* 1984). MYB haplotype (*MYBA1* and *MYBA2*) regulates anthocyanin biosynthesis and constitutes the color locus (Kobayashi *et al.* 2004; Walker *et al.* 2007; Ban *et al.* 2014). *VvmybA1* is a major determinant of berry color variation (Lijavetzky *et al.* 2006). A single quantitative trait locus (QTL) in linkage group 2 (LG2), flanking MYB haplotype, was responsible for the presence or absence of skin color (Fournier-Level *et al.* 2009). Recently, Liang *et al.* (2009) proposed that the presence or absence of anthocyanins in grape skin was a quality character controlled by oligogenes of red skinned maternal, and anthocyanins content was a quantitative character controlled by polygenes of both red- and white-skinned parents. Studies on inheritance of anthocyanin content in berry skin of tetraploid and diploid cross populations suggested that total anthocyanin content increased with the ploid level (Liang *et al.* 2012). Currently, there is very little information available on anthocyanin inheritance in grape pulp. Luo *et al.* (2004) suggested that grape juice color was controlled by more than one gene pair with red juice being dominant over white via segregations of 10 interspecific combinations between *V. vinifera* and Chinese wild grape cultivars.

The objective of the present study was to investigate the anthocyanin inheritance pattern in berry skin and pulp, which may be useful to improve grape cultivars by crossbreeding. To this end, anthocyanin inheritance in four berry tissues of the

interspecific F1 hybrids between Beifeng and Yan-73 was studied: the segregation ratios of the presence/absence of anthocyanins in berry skin, pulp, rachis and pedicels were recorded; meanwhile, distribution of anthocyanin concentrations in berry skin and pulp were also shown.

5.2 Material and methods

5.2.1 Plant materials

The F1 hybrids between Beifeng (B) and Yan-73 (Y), grown in the experimental vineyard of the Institute of Botany, Chinese Academy of Sciences, Beijing, were studied in three successive years, 2011-2013. Beifeng (*V. thunbergii* × *V. vinifera*) is a red-skinned grape cultivar, while Yan-73 (*V. vinifera*, Muscat Hamburg × Alicante Bouschet) is a teinturier cultivar with red skin and pulp. Each individual of the cross progeny on its own root were planted randomly in 2006. The field-grown grapevines were trained to a fan-shape trellis, with a density of 2.5 m between rows and 1 m between plants. Vines were maintained under routine management, including irrigation, fertilization, soil management, pruning, and disease control.

The coloration of berry skin, pulp, rachis and pedicels of each clone bearing fruits was recorded at maturity. The berry maturity for each individual was estimated as described in 3.2.4 of Chapter III. Since the number of progenies bearing fruits varied with years, 244, 340 and 220 of 492 F1 progenies were recorded in 2011, 2012, and 2013, separately. Three replicates, each consisting of ~50 randomly harvested matured berries from one previously recorded vine, were sampled for each individual. The recorded progenies bearing a few or unhealthy berries were not sampled. In that case, 225 out of 244, 212 out of 340 and 220 progenies were sampled in 2011, 2012, and 2013, separately. A sum of 212 progenies was common in the three years. The samples were taken to the laboratory immediately after harvest, washed with deionized water and dried with gauze. Berry skin was peeled with forceps. Berry skin and pulp were frozen in liquid nitrogen, and stored at -40 °C for subsequent anthocyanin analysis.

5.2.2 Anthocyanin extraction

Anthocyanin extraction was conducted as described in 2.2.3 in chapter II.

5.2.3 Anthocyanin analysis

Anthocyanin identification and concentration determination were made by HPLC/MS according to 2.2.4 in chapter II.

5.2.4 Statistical analysis

The significance of the difference between the predicted and the observed segregation ratio was tested via chi-square test. Distribution of anthocyanin concentration for the cross combination was displayed in boxplots (SigmaPlot 12.5, SPSS, USA.). In the box plot the central rectangle spanned the first quartile to the third quartile (the interquartile range or IQR). A segment inside the rectangle showed the median and whiskers above and below the box showed the locations of the minimum and maximum. Suspected outliers were plotted as individual points. They are either $1.5 \times \text{IQR}$ or more above the third quartile or $1.5 \times \text{IQR}$ or more below the first quartile.

5.3 Results

5.3.1 Genotype variation of berry color in F1 hybrid population

Segregation of colored and white pulp in three successive years was shown in Table 5-1. The number of offsprings with white berry pulp accounted for 49.4-54.1%, and offsprings with colored pulp accounted for 45.9-50.6%. The offsprings with white and colored pulp was consistent with the predicted ratio 1:1 via the chi-square test. All of the offsprings had colored berry skin. Most offsprings had white rachis and berry pedicels except that several colored slightly at the berry end of berry pedicels (data not shown).

5.3.2 Identification of anthocyanins

A total of 28 anthocyanins were identified via HPLC-MS/MS based on the

detected molecular ions, aglycone ions, and fragmentation ions (Table 5-2). The identified anthocyanins included monoglucosides and diglucosides and their acylated derivatives of delphinidin (Dp), petunidin (Pt), malvidin (Mv), cyanidin (Cy) and peonidin (Pn). In more detail, they all included 3-O-glucosides and 3-O-(6-O-coumaryl)-glucosides and 3,5-O-diglucosides. Pt and Cy additionally had -3-O-(6-O-coumaryl)-glucoside derivatives. Cy, Pt, Mv 3-O-glucosides and Pt 3,5-O-diglucoside additionally had acetylated derivatives. In addition, pelargonidin 3-O-glucoside was also detected.

Table 5-1 Ratio of white- and red-fleshed offspring in Beifeng (B)× Yan-73 (Y) cross progeny populations in three successive years.

Year	Colored	White	Predicted Ratio	<i>P</i> value
2011	117	127	1:1	0.651
2012	172	168	1:1	0.878
2013	101	119	1:1	0.390

There is no significant difference between the predicted and observed ratio when $P > 0.05$.

5.3.3 Distribution of anthocyanin concentrations in grape skin and pulp of the F1 hybrid population

Dp, Pt, Mv, Cy, Pn derivatives and total anthocyanin concentrations in Yan-73 berry skin were higher than those in Beifeng. Anthocyanin concentrations in berry skin of the F1 hybrid population exhibited continuous variation with a distribution skewed to the low values (Figure 5-1). However, a few progenies contained a considerably higher concentration of anthocyanins in three successive years. The highest total anthocyanin concentration of the progenies were 2.5~6 times higher than in Yan-73 in the three years. The median concentration of Dp, Pt, Mv, Cy derivatives and total anthocyanins were higher than Beifeng and lower than Yan-73 in three successive years. The median concentration of Pn derivatives was slightly higher than Yan-73 in 2011, while it is higher than Beifeng and lower than Yan-73 in 2012 and 2013.

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Table 5-2 Retention time, ultra violet and mass spectra data for the anthocyanins identified by chromatography and mass spectrometry.

Peak	t _R	Molecular ion	Fragment ions	Absorbance	Identity
1	11.018	627	627,465,303	522	Delphinidin 3,5- <i>O</i> -glucoside
2	13.496	611	611,449,287	514	Cyanidin 3,5- <i>O</i> -glucoside
3	14.376	641	641,479,317	523	Petunidin 3,5- <i>O</i> -glucoside
4	15.834	465	465,303	527	Delphinidin 3- <i>O</i> -glucoside
5	18.098	625	625,463,301	518	Peonidin 3,5- <i>O</i> -glucoside
6	18.682	655	655,493,331		Malvidin 3,5- <i>O</i> -glucoside
7	19.629	449	449,287	526	Cyanidin 3- <i>O</i> -glucoside
8	21.456	479	479,317	519	Petunidin 3- <i>O</i> -glucoside
9	23.861	683	683,521,479,317	527	Petunidin 3,5- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
10	24.369	433	433,271	505	Pelargonidin 3- <i>O</i> -glucoside
11	26.051	463	463,301	527	Peonidin 3- <i>O</i> -glucoside
12	27.508	493	493,331	518	Malvidin 3- <i>O</i> -glucoside
13	29.040	697	697,535,493,331	528	Malvidin 3,5- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
14	33.641	773	773,611,465,303	530	Delphinidin 3,5- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
15	35.099	491	491,449,287	520	Cyanidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
16	36.556	521	521,479,317	529	Petunidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
17	38.235	757	757,595,449,287	522	Cyanidin 3,5- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
18	38.672	787	787,625,479,317	530	Petunidin 3,5- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
19	39.491	801	801,639,493,331		Malvidin 3,5- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
20	42.689	611	611,465,303	530	Delphinidin 3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
21	44.294	535	535,493,331	530	Malvidin 3- <i>O</i> -(6- <i>O</i> -acetyl-glucoside)
22	45.530	771	771,609,463,301	530	Peonidin 3,5- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
23	49.984	625	625,479,317	531	Cyanidin 3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
24	51.811	595	595,449,287	529	Petunidin 3- <i>O</i> -(6- <i>O</i> -coumaryl-glucoside)
25	60.384	609	609,463,301	525	Peonidin 3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)
26	61.369	639	639,493,331	538	Malvidin 3- <i>O</i> -(cis-6- <i>O</i> -coumaryl-glucoside)
27	63.017	609	609,463,301	523	Peonidin 3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)
28	63.905	639	639,493,331	533	Malvidin 3- <i>O</i> -(trans-6- <i>O</i> -coumaryl-glucoside)

Chapter V

Table 5-3 Anthocyanin concentration values including parent values, mid-parental value (\bar{x}), and mean progeny value in Beifeng (B) × Yan-73 (Y) progeny skin and pulp in three successive years. Dp, Pt, Mv, Cy and Pn represent all the derivatives of delphinidin, petunidin, malvidin, cyanidin and peonidin, respectively. The sum represents the total anthocyanin concentration.

Traits	Years	Skin				Pulp	
		Beifeng	Yan-73	\bar{p} (mg/g)	\bar{x} (mg/g)	Yan-73	\bar{x} (mg/g)
Dp	2013	0.66	3.04	1.85	2.97	0.22	0.12
	2012	1.21	2.70	1.96	3.57	0.16	0.14
	2011	0.94	1.83	1.38	3.44	0.15	0.20
Pt	2013	0.88	2.72	1.80	2.65	0.09	0.10
	2012	0.89	2.67	1.78	2.72	0.07	0.12
	2011	0.89	1.43	1.16	3.27	0.05	0.22
Mv	2013	4.22	22.34	13.28	10.99	0.95	0.46
	2012	6.78	16.38	11.58	9.71	0.62	0.51
	2011	5.50	11.48	8.49	13.04	0.57	0.90
Cy	2013	0.09	0.80	0.45	0.65	0.08	0.06
	2012	0.18	1.35	0.76	0.68	0.09	0.06
	2011	0.14	0.79	0.46	0.71	0.07	0.13
Pn	2013	0.12	4.34	2.23	1.76	1.03	0.49
	2012	0.58	5.17	2.87	1.95	0.89	0.57
	2011	0.35	2.51	1.43	3.78	1.06	1.18
Sum	2013	5.97	33.30	19.63	19.03	2.38	1.23
	2012	9.65	28.25	18.95	18.67	1.84	1.42
	2011	7.81	18.04	12.92	25.94	1.90	2.66

Anthocyanin concentration in Yan-73 berry pulp ranged from 1.84 to 2.38 mg/g in three successive years (Table 5-3). However, ‘Beifeng’ could not accumulate anthocyanins in berry pulp and was a white-fleshed cultivar. About half of the F1 hybrids accumulated anthocyanins in berry pulp. Therefore, distribution of anthocyanin concentrations only in the offsprings with colored pulp was studied in three successive years (Figure 5-2). Anthocyanin concentrations in berry pulp showed continuous variation with a nearly normal distribution. Berry pulp of some offsprings had extraordinary high anthocyanin concentration.

5.3.4 Genetic inheritance of monoglucosides and diglucosides in grape skin and pulp

Monoglucoside and diglucoside concentrations in berry skin of Beifeng were respectively 2.67-4.82 and 3.29-4.82 mg/g in three successive years, while ‘Beifeng’ pulp did not contain either monoglucosides or diglucosides (Table 5-4). Berry skin and pulp of Yan-73 only contained monoglucosides, and their concentrations were 18.01-33.15 and 1.83-2.37 mg/g respectively (Table 5-4).

Table 5-4 The numbers of progenies in which monoglucosides/diglucosides was present /absent in the skin/pulp of Beifeng (B) × Yan-73 (Y) population in three successive years. Monoglucoside and diglucoside concentration value including parent values, mid-parental value (\bar{x}), range and mean progeny value were also present in the table.

Traits	Tissues	Year	Parents		\bar{p}	\bar{x}	Range	Presence	Absence	Predicted ratio
			B	Y						
Mono-glucosides	skin	2013	2.6	33.1	17.9	13.7	0.31-121.2	225	0	
		2012	4.8	28.2	16.5	13.3	0.39-88.7	212	0	
		2011	3.7	18.0	10.8	17.2	0.01-108.2	220	0	
Di-glucosides	pulp	2013	0	2.37	/	0.85	0.13-2.5	102	0	
		2012	0	1.83	/	0.93	0.01-4.2	108	0	
		2011	0	1.90	/	1.78	0.004-5.6	98	0	
Di-glucosides	skin	2013	3.2	0	1.65	5.21	0.00-38.56	122	103	1:1
		2012	4.8	0	2.41	5.30	0.00-42.15	121	91	1:1
		2011	4.0	0	2.03	6.93	0.00-48.72	106	114	1:1
Di-glucosides	pulp	2013	0	0	/	0.38	0.00-1.69	55	47	1:1
		2012	0	0	/	0.48	0.00-2.16	68	40	1:1
		2011	0	0	/	0.82	0.00-5.08	52	46	1:1

The segregation ratios of present or absent in progenies have no significant difference with the predicted ratio via the χ^2 test, and their range and \bar{x} were based on the progenies in which monoglucoside or diglucoside were present. ‘/’, unable to calculate mid-parental value since the volatile was not detectable in one of the parents.

All the F1 hybrids had monoglucosides in colored skin and pulp. However, 48.1-57.1% of total progenies contained diglucosides in their skin, while progenies containing diglucosides in their pulp accounted for 53.1-63.0% of colored-fleshed progenies. The diglucosides segregated into presence or absence with a theoretical ratio of 1:1 in three successive years via the chi-square test both in berry skin and pulp.

In berry skin and pulp of progenies where they were present, monoglucoside and diglucoside concentration exhibited continuous genotypic variation as quantitative traits with distribution skewed towards low values (Figure 5-3, Figure 5-4). The median and average monoglucoside concentration in berry skin of progenies was higher than monoglucoside concentration in Beifeng but lower than Yan-73 (Figure 5-3, Table 5-4). In the pulp of progenies, the median and average monoglucoside concentration was lower than Yan-73 (Figure 5-3, Table 5-4). The median and average diglucoside concentration of progenies was lower than that in Beifeng (Figure 5-4, Table 5-4).

5.4 Discussion

In this study, all the F1 hybrids between Beifeng and Yan-73 presented colored skin, and their anthocyanin concentrations showed continuous variation (Figure 5-1). Therefore, anthocyanin concentration was inherited quantitatively. According to the previous studies, the presence or absence of anthocyanins in berry skin was controlled by a single gene pair (Spiegel-Roy and Assaf 1980; Goldy *et al.* 1989). These authors suggested that berry skin color was controlled both by major gene and minor polygenes: the major gene controlled the presence or absence of skin color, and the minor polygenes controlled the richness of color. The results were in accordance with the previous work of Liang *et al.* (2009), who studied inheritance patterns for table grape anthocyanins in three half-sib crosses.

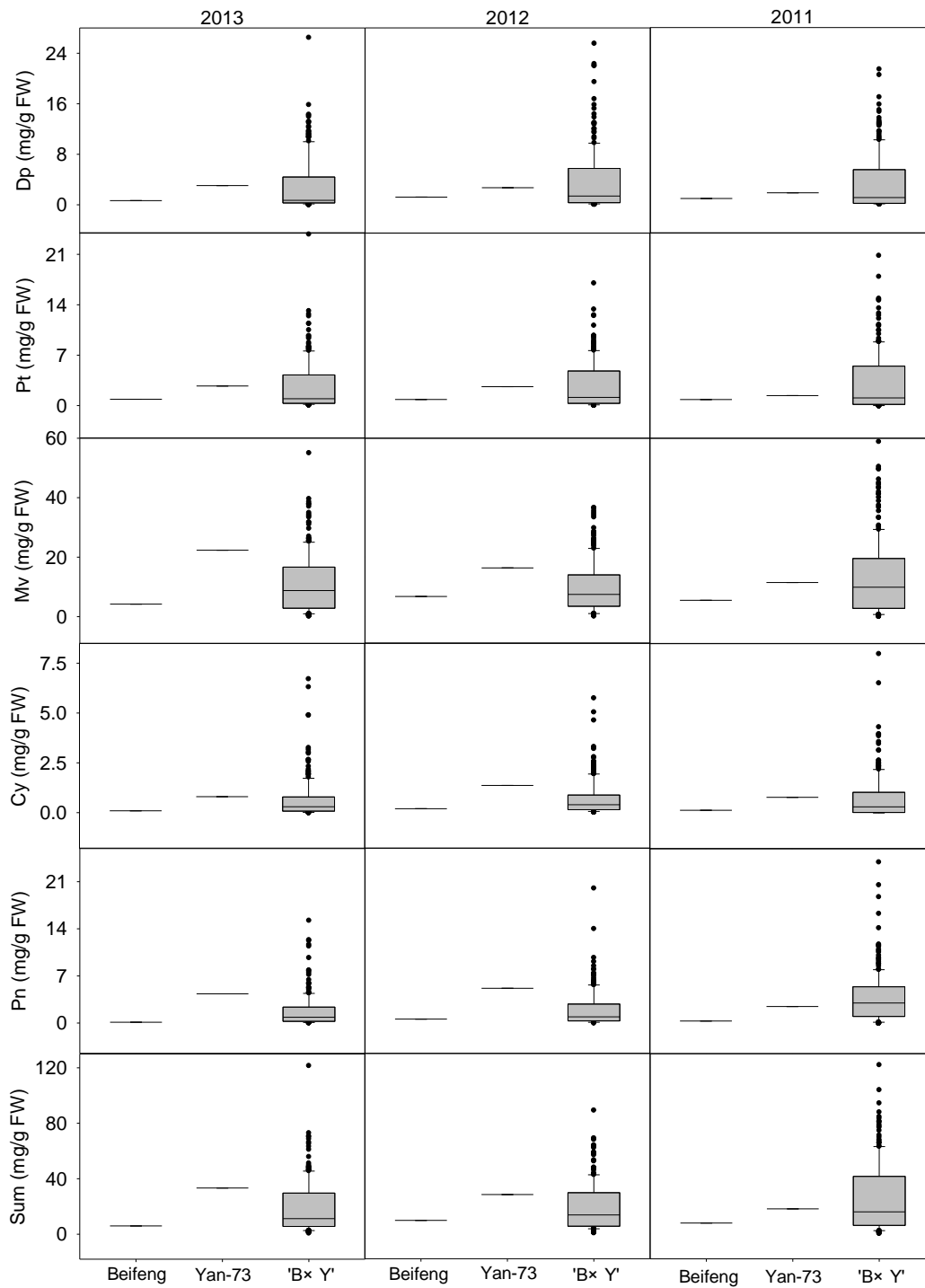


Figure 5-1 Range and distribution of skin anthocyanin concentrations in Beifeng (B) × Yan-73(Y) progeny in three successive years.

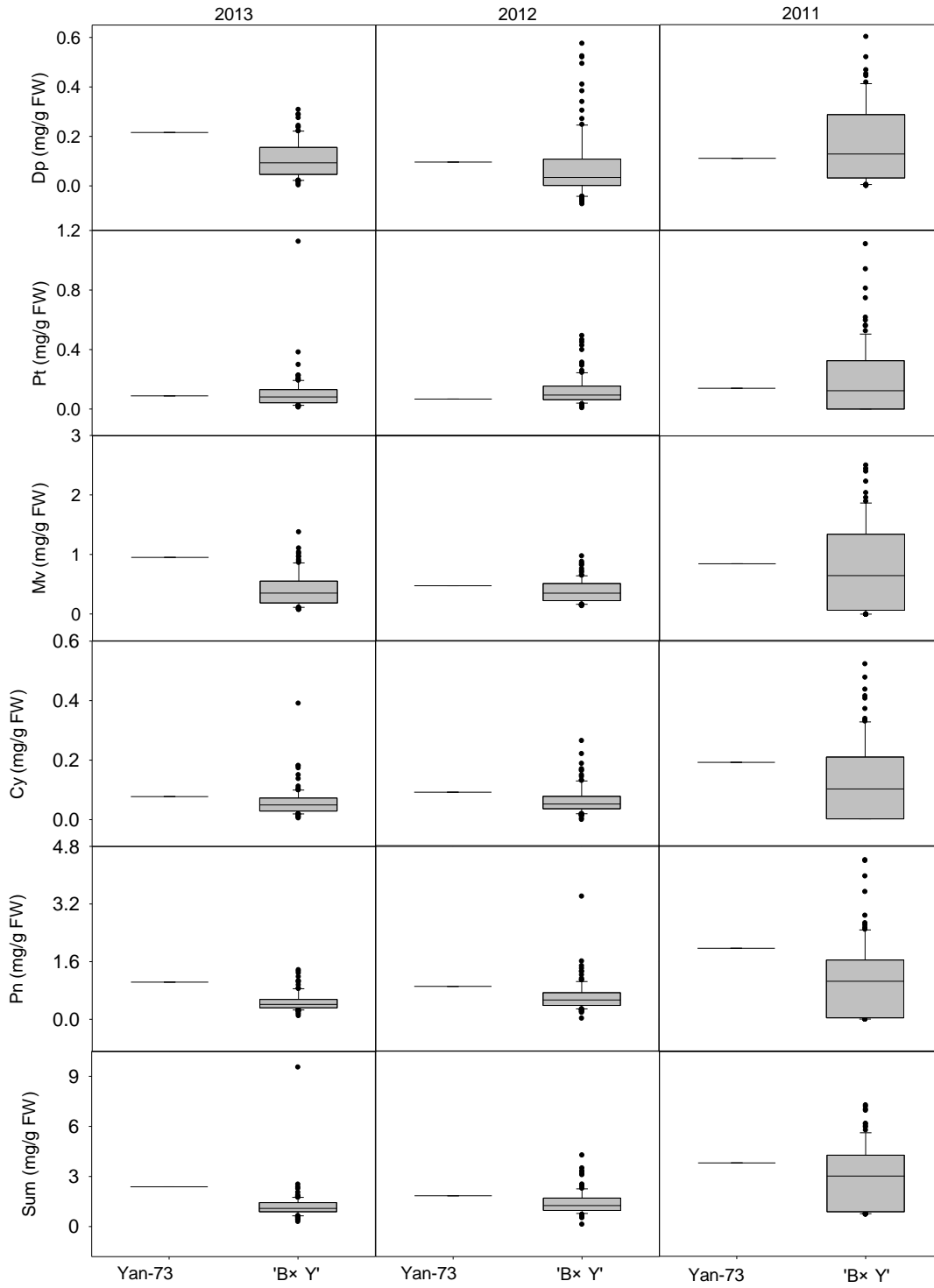


Figure 5-2 Range and distribution of pulp anthocyanin concentrations in Beifeng (B) × Yan-73 (Y) progeny in three successive years.

The difference in berry pulp color of parents resulted in the pulp color segregation in Beifeng and Yan-73 hybrid population. The segregation ratio was 1:1 via chi-square test in three successive years (Table 5-1). The results were in agreement with the 'one gene pair model' proposed by Spiegel-Roy and Assaf (1980), and thus the presence or absence of color in berry pulp was a qualitative trait and controlled by single pair of genes. Anthocyanin concentration in berry pulp of progeny was a quantitative trait and controlled by polygenes, which was the same with berry skin.

Beifeng and most grape cultivars were only colored in berry skin, whereas other tissues (berry pulp, pedicels and rachis) could not color. The fact that tissues other than berry skin could not accumulate anthocyanins throughout berry development, may be due to the tissue specific expression of gene controlling anthocyanin biosynthesis only in berry skin. However, Yan-73 accumulated anthocyanins in berry skin, pulp, rachis and pedicels, and rapid accumulation started at veraison or about one week after veraison (Figure 5-3). This suggests that multiple colored tissues of 'Yan-73' grape may result from the loss of function of tissue specific genes, which led to anthocyanin biosynthesis in berry skin, pulp, rachis and pedicels. The F1 hybrids between 'Beifeng' and Yan-73 showed single gene pair controlled inheritance of the presence and absence of color in berry pulp, and the presence was dominant over the absence. The 1:1 segregation ratio suggested that the gene controlling the presence of color in berry pulp was heterozygous, and it may be related to tissue specific of anthocyanin biosynthesis. Unlike what is observed for the pulp, most progenies could not accumulate anthocyanins in berry pedicels and rachis, which indicates that anthocyanin biosynthesis in both tissues is controlled by more than one gene pair and that the absence of color was dominant over the presence.

Beifeng, the hybrid between *V. thunbergii* and *V. vinifera*, contained both anthocyanidin monoglucosides and diglucosides in berry skin; however, 'Yan-73' (*V. vinifera*) only accumulated monoglucosides in berry skin and pulp. The presence or the absence of diglucosides in the F1 population showed 1:1 segregation ratio in both skin and pulp via chi-square test (Table 5-5), and thereafter it may be controlled by

one single gene pair. Contrary to *V. vinifera*, *V. thunbergii* could biosynthesize anthocyanidin diglucosides in berry skin (Liang *et al.* 2009). The gene controlling diglucoside biosynthesis in Beifeng and its F1 descendants was passed down from *V. thunbergii*, and it is heterozygous in Beifeng. The progeny which could biosynthesize diglucosides in berry pulp may be inherited from both loss of function of tissue specific expression gene in Yan-73 and the gene controlling diglucoside biosynthesis in Beifeng.

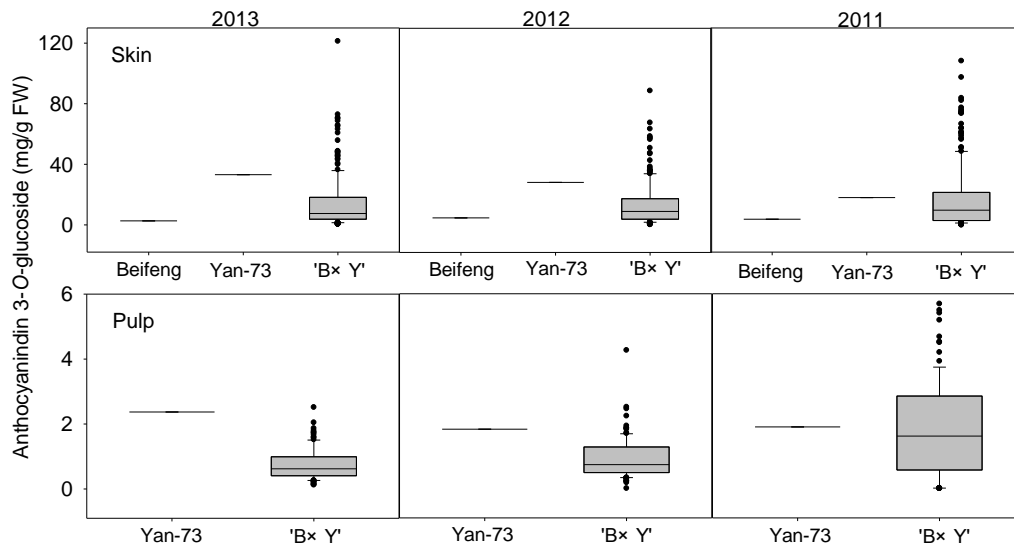


Figure 5-3 Range and distribution of anthocyanidin monoglucoside concentrations in skin and pulp of Beifeng (B) × Yan-73 (Y) progenies in the three successive years.

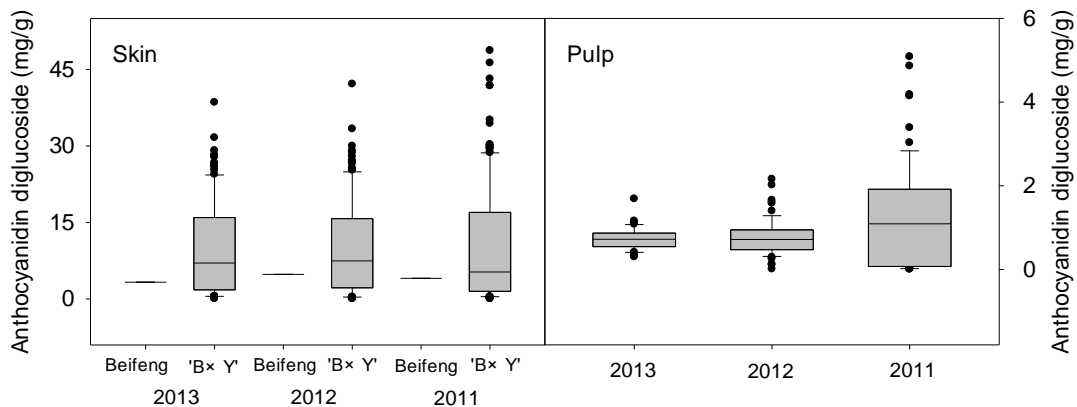


Figure 5-4 Range and distribution of anthocyanidin diglucoside concentrations in skin and pulp of Beifeng (B) × Yan-73 (Y) progenies in the three successive years.

The distribution of Dp, Pt, Mv, Cy, Pn and total anthocyanin concentration in berry skin and pulp of F1 population was skewed to the low values. However, a few progenies had extraordinarily high anthocyanin concentration (Figure 5-1, Figure 5-2). Therefore, improvement of grape cultivars for high anthocyanin concentration is possible by crossbreeding.

5.5 Conclusions

All the F1 hybrids between Yan-73 and Beifeng presented colored skin, while there was a segregation in pulp color (ratio 1:1). Therefore, berry pulp color was controlled by a single pair of genes, which might be inherited from Yan-73 which lost the function of gene responsible for tissue specific anthocyanin biosynthesis. Anthocyanin concentrations in berry skin and pulp of progenies showed skewed normal distribution and therefore anthocyanin concentration was a quantitative inheritance and controlled by multiple minor genes. All the progenies accumulated monoglucosides in berry skin and pulp, whereas there were segregations of the presence/absence of diglucosides (ratio 1:1). Thus, the presence of diglucosides was controlled by single gene pair and the presence was dominant to the absence. The presence of diglucosides in berry pulp resulted from the combinatory effect of genes related to diglucoside biosynthesis inherited from Beifeng and loss of function of tissue specific anthocyanin biosynthesis inherited from Yan-73.

Chapter VI. Conclusions

Combining the use of several grapevine genotypes and different light exposure treatments allowed us to improve significantly our knowledge of anthocyanin biosynthesis, regulation and tissue compartmentation. Our most significant findings are summarized below.

1. Anthocyanin composition varied among grape organs. Anthocyanins in the teinturier cultivar ‘Yan-73’ berry skin were mainly composed of malvidin derivatives, while peonidin derivatives were the most dominant anthocyanins in berry pulp. Both malvidin and peonidin derivatives were predominant components in rachis, pedicels, leaf lamina, vein and petioles, and living bark at the base of the shoot.

2. Anthocyanin concentration varied by developmental stage. Berry skin, together with berry pulp, rachis and pedicels had a veraison period when anthocyanins accumulated rapidly.

3. Anthocyanin biosynthesis in different grape cultivars may present opposite sensitivity to light. Yan-73 grape berry skin and pulp deeply colored under sunlight exclusion, which might result from the expression of *VvUFGT* and *VvMybA1*. Red-skinned non-teinturier cultivar Gamay and its bud teinturier mutation Gamay Fréaux presented opposite response to light: the skin of Gamay berries barely colored, while berries of Gamay Fréaux, similar with Yan-73, deeply colored their skin and pulp under sunlight exclusion.

4. Light exclusion decreased anthocyanin concentration in berry skin and pulp, with the extent of decline in berry pulp was less than that in berry skin. Anthocyanin concentration was slightly affected by sunlight exclusion in berry pedicels and rachis. Therefore, grape tissues responded differently to sunlight on anthocyanin biosynthesis: Berry skin was most sensitive to light, followed by berry pulp, with berry pedicels and rachis being the least. The low anthocyanin in berry skin and pulp under sunlight exclusion might result from the lower expression of *VvUFGT*, *VvMybA1*, *VvMybA2*, and *VvMyc1*.

5. Sunlight exclusion increased the ratio of 3',4'-hydroxylated to 3',4',5'-hydroxylated anthocyanin concentration in berry skin and pulp of Yan-73 and Gamay Fréaux, which was closely related to the increased expression ratio of *VvF3'H/VvF3'5'H*.

6. The decreased anthocyanin concentration and the composition shift under sunlight exclusion in Gamay Fréaux berry skin and pulp correlated with transcript changes related to genes mediating anthocyanin biosynthesis and transport. Sugar and ABA concentrations were slightly affected by sunlight exclusion, and thus sunlight might directly mediate anthocyanin concentration and composition changes.

7. Light exclusion reduces anthocyanin concentration and modifies anthocyanin composition by altering the expression of genes involved in anthocyanin biosynthesis, regulation, and transport, in a cultivar- and tissue-specific manner.

8. The color of berry skin and pulp was controlled both by major genes and minor polygenes: the major gene controlled the presence or absence of color, with colorless being recessive, and the minor polygenes controlled the richness of color. The presence or absence of diglucosides was controlled by single gene pair, with the presence being dominant. The presence of diglucosides in berry pulp resulted from a combination whereby genes related to diglucoside biosynthesis passed down from Beifeng and tissue specific anthocyanin biosynthesis passed down from Yan-73. The presence of diglucosides in berry skin was passed down from Beifeng.

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