Combining leaf-to-fruit ratio manipulations with exogenous abscisic acid applications to adjust sugar and anthocyanin concentrations in grape berry

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TITRE: Effets d'une combinaison de manipulation du rapport surface foliaire/charge en fruits avec une application d'acide abscissique sur les teneurs en sucres et en anthocyanes dans les baies de raisins.

Résumé: Le changement climatique en cours entraîne une augmentation de la fréquence et de l'intensité des événements météorologiques extrêmes, affectant la composition des raisins. Les températures élevées pendant la maturation augmentent la teneur en sucre du moût et réduisent les teneurs en acides organiques et en anthocyanes, modifiant qualité et typicité du vin. Les pratiques telles que les manipulations du rapport feuilles/fruits combinées à des applications d'hormones végétales peuvent potentiellement être utilisées pour adapter et maintenir la durabilité de la production viticole. Dans ce but, il est nécessaire de mieux comprendre les liens physiologiques entre accumulation de sucres et synthèse d'anthocyanes au cours de la maturation des baies. Des boutures fructifères de vigne (cv. Cabernet sauvignon) ont été cultivées en serre et différents rapports feuilles/fruits (2 à 12 feuilles par grappe) ont été appliqués et combinés à la pulvérisation d'acide abscissique (ABA) sur les baies au stade de pré-*véraison*. Pour étudier les mécanismes sous-jacents à l'effet de la manipulation du rapport feuille-fruit et de l'application d'ABA sur la composition des baies, l'expression de gènes liés au métabolisme des sucres, des anthocyanes et de l'ABA a été étudiée sur ce matériel. Afin de réaliser une analyse fine de l'interaction potentielle sucres/signalisation par l'ABA sur la biosynthèse des anthocyanes, des expériences de culture de baies *in vitro* ont également été menées. Les baies ont été cultivées 2-3 semaines sur un milieu solide supplémenté avec différents niveaux de sucre (0%, 2%, 8%), combinés avec de l'ABA (200 µM), un inhibiteur de biosynthèse d'ABA, ou des inhibiteurs d'hexokinase. Les résultats confirment que la réduction du rapport feuille/fruit a un effet significatif sur la composition des baies: réduction de la teneur en sucres et en anthocyanes, augmentation des concentrations en acides organiques totaux, modification de la composition en acides aminés libres. L'application d'ABA augmente les teneurs en sucres et en anthocyanes, et restaure donc partiellement le couplage entre accumulation de sucre et d'anthocyanes, sous de faibles ratios feuille-fruit, sans affecter les ratios acides aminés/sucres/acides organiques. Plusieurs gènes de la voie de biosynthèse des anthocyanes (*CHS2*, *CHS3*, *CHI*, *F3H*, *DFR*, *LODX*, *UFGT*, *MybA1*, *MybA2*) sont réprimés en situation de faible rapport feuille/fruit ; alors qu'une partie d'entre eux sont au contraire induits (*CHI*, *F3H*, *F3'5'H*, *LODX*, *UFGT*, *MybA1*, *MybA2*) après un traitement ABA. L'ABA a en revanche peu d'effet sur les gènes liés à l'accumulation des sucres; de même que le rapport feuille/fruit sur les gènes de biosynthèse de l'ABA. Les expériences de culture *in vitro* de baies ont montré que l'ABA et les concentrations élevées de glucose induisent de manière synergique la biosynthèse des anthocyanes pendant la maturation. L'ABA et le glucose ont diminué la concentration en phénylalanine dans les baies en cours de maturation, très probablement en raison de leur rôle dans l'accroissement de la synthèse des anthocyanes, et une analyse ANOVA croisée à deux facteurs indique une interaction significative entre les niveaux de sucre et d'ABA et l'accumulation d'anthocyanes. En conclusion, nos travaux ont montré que l'ABA et la signalisation par les sucres interagissent pour réguler l'expression des gènes de biosynthèse des anthocyanes et augmenter l'accumulation d'anthocyanes pendant la maturation des baies. L'application d'ABA a conduit à l'augmentation du rapport anthocyanes/sucres à la récolte, en conditions de faible rapport feuilles/fruits. La combinaison de la manipulation du rapport feuille/fruit avec des applications d'ABA permet donc de réduire la concentration en sucres à la récolte, tout en maintenant les concentrations en anthocyanes, offrant ainsi un moyen potentiel d'atténuer partiellement les effets négatifs des températures élevées liées aux modifications climatiques en cours.

Mots clés: Baie de raisin, composition, changement climatique, acide abscissique, sucres, anthocyanes.

TITLE: Combining leaf-to-fruit ratio manipulations with exogenous abscisic acid applications to adjust sugar and anthocyanin concentrations in grape berry.

Abstract: Global climate change is increasing in frequency and intensity, significantly affecting vine physiology and grape berry composition at harvest. Elevated temperatures during the growing season increase must sugar content, while reducing organic acid and anthocyanin levels, modifying wine quality and typicality. Viticultural practices such as leaf-to-fruit ratio manipulations combined with applications of plant hormones can potentially be used to adapt to climate change and maintain the sustainability of wine production. To optimize adaptation strategies, we need to gain better insights into the links between sugar and anthocyanin accumulations during berry development and ripening. Fruiting-cuttings made up of one vertical shoot with one grape cluster of cv. Cabernet Sauvignon were grown between 2013 and 2018 in a naturally lighted and semi-controlled greenhouse. Various leaf-to-fruit ratios (2 to12 leaves per cluster) were applied, and combined with spraying abscisic acid (ABA, 400 $mg.L^{-1}$) on berries at the pre-*véraison* stage. To explore the biological mechanisms underlying the effect of leaf-to-fruit ratio manipulation and application of exogenous ABA on berry composition, transcript abundance of genes related to sugar, anthocyanins and ABA metabolism were studied in this material. To further analyze the potential interplay between sugar and ABA signaling on anthocyanins biosynthesis, *in vitro* berry culture experiments were also conducted. Berries were cultured 2-3 weeks on solid medium supplemented with different sugar levels (0%, 2%, 8%) combined with ABA (200 µM), ABA biosynthesis inhibitor (NDGA), or hexokinase inhibitors (NAG, GDH). The results confirmed that reducing the leaf-to-fruit ratio had a significant effect on berry composition: reduction of sugar and anthocyanin content, slight increase in total organic acids and modification of the free amino acids composition. Exogenous ABA application increased sugar and anthocyanin concentrations, and partially restored the coupling between sugar and anthocyanin accumulations under low leaf-to-fruit ratios, without affecting the amino acids/sugar/organic acids ratios. Transcript abundance analysis revealed that several anthocyanin biosynthetic genes (*CHS2*, *CHS3*, *CHI*, *F3H*, *DFR*, *LODX*, *UFGT*, *MybA1* and *MybA2*) were decreased under low leaf-to-fruit ratio, whereas some genes (*CHI*, *F3H*, *F3'5'H*, *LODX*, *UFGT*, *MybA1* and *MybA2*) were up-regulated after exogenous ABA treatment. Exogenous ABA had little effect on the transcript abundance of sugar accumulation related genes, and leaf-to-fruit ratio also had little effect on ABA biosynthetic genes. Berry *in vitro* culture experiments showed that ABA and elevated concentrations of glucose synergistically induced anthocyanins biosynthesis during ripening. Both ABA and glucose decreased Phenylalanine concentration in ripening berries, most probably due to their role in promoting anthocyanins synthesis, and two-way crossed ANOVA analysis indicate a significant interaction between sugar and ABA levels and anthocyanin accumulations in berries. To conclude, our results showed that ABA and sugar signaling synergistically interact to regulate the expression of anthocyanin biosynthetic genes and increase anthocyanin accumulations during berry ripening. Thus, exogenous ABA application was able to increase the ratio of anthocyanins to sugar under low leaf-to-fruit ratio at harvest, and combining leaf-to-fruit ratio manipulation with exogenous ABA applications may offer a fine-tuned way to reduce sugar concentration, while maintaining anthocyanin concentrations in grape berry, potentially offering a way to partially alleviate climate change related high temperature effects.

Keywords: Grape berry, composition, climate change, abscisic acid, sugar, anthocyanins

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Abbreviation

4CL	4-coumaroyl-coa synthase	His	Histidine
ABA	Abscisic acid	HXK	Hexokinase
ABA-GE	ABA-glucose ester	Ileu	Iso-leucine
ACT	Anthocyanin acyltransferase	L	Leaves
Ala	Alanine	L/F	Leaf to fruit ratio
ANOVA	Analysis of variance	L-IdnDH	L-idonate dehydrogenase
ANS	Anthocyanidin synthase	LDOX	Leucoanthocyanidin dioxygenase
AOMT	Flavonoid 3',5'-methyltransferase	Leu	Leucine
Arg	Arginine	Lys	Lysine
Asn	Asparagine	NAG	N-acetyl-glucosamine
Asp	Aspartate	MAPK	Mitogen activated protein kinase
C4H	Cinnamate-4-hydroxylase	MBP	Myelin basic protein
CHI	Chalcone isomerase	Met	Methionine
CHS	Chalcone synthase	Mv	Malvidin
CT	Control	OMT	O-methyltransferase
Cy	Cyanidin	PA	Phaseic acid
Cys	Cysteine	PAL	Phenylalanine ammonia-lyase
DAF	Days after flowering	PCR	Polymerase chain reaction
DFR	Dihydroflavonol 4-reductase	Phe	Phenylalanine
DGH	D-(+)-Glucosamine hydrochloride	Pn	Peonidin
Dp	Delphinidin	Pro	Proline
DPA	Dihydrophaseic acid	Pt	Petunidin
F3H	Flavanone 3β-hydroxylase	RT-qPCR	Real-time polymerase chain reaction
F3'H	Flavonoid 3'-hydroxylase	SE	Standard error
F3'5'H	Flavonoid 3',5'-hydroxylase	Ser	Serine
FLS	Flavonol synthase	Thr	Threonine
FW	Fresh weight	Tyr	Tyrosine
GABA	γ-Aminobutyric acid	TSS	Total soluble solids
Glu	Glutamate	UFGT	Flavonoid glucosyltransferase
Gln	Glutamine	UDPG	Flavonoid-3-O-glucosyltransferase
Gly	Glycine	Val	Valine
GST	Glutathione-S-transferase	WAT	Weeks after treatment

Chapter 1 Introduction

1.1 The grapevine fruit development and ripening

Grapevine (*Vitis vinifera* L.) is one of the most important economic fruit crops worldwide, with 7.8 million ha producing 77.8 million of tons of grapes (source: OIV report 2019, http://oiv.int/public/medias/6782/oiv-2019-statistical-report-on-world-vitiviniculture.pdf).
The majority of this production (57%) is used for wine making, but the berries are also used to produce fresh fruit, juice, raisin, and other products. Grape is a non-climacteric fleshy fruit, whose development has been divided into three phases: two successive sigmoidal growth periods separated by a lag phase (Figure 1.1, Kennedy, 2002, Serrano et al., 2017, Monselise, 2018). The first phase (also called green or herbaceous phase) is characterized by a rapid berry growth phase immediately after fruit set, lasting from 45 to 60 days after flowering (Monselise, 2018). During the first growth period, the berry is formed, and grows both through rapid cell division and cell enlargement (Dokoozlian, 2000). Several metabolites begin to accumulate such as organic acids, several amino acids, tannins and some aroma compounds such as methoxypyrazines (Kennedy, 2002). The second phase, the lag phase, is a transition stage which is marked by the change of skin colour and berry softening (Serrano et al., 2017). Within this phase, called *véraison*, several important physiological and biochemical changes are initiated, began with the softening of berry, decreases in turgor and increases in ABA, followed by the sugar accumulation, a decreases in organic acid content and the synthesis of anthocyanins (Castellarin et al., 2016). *Véraison* marks the onset of the third phase, the ripening phase, involving the rapid accumulation of sugar and polyphenols, turgor continuous reduction and berry enlargement, a decrease in acidity, as well as aroma and aroma precursors synthesis (Serrano et al., 2017).

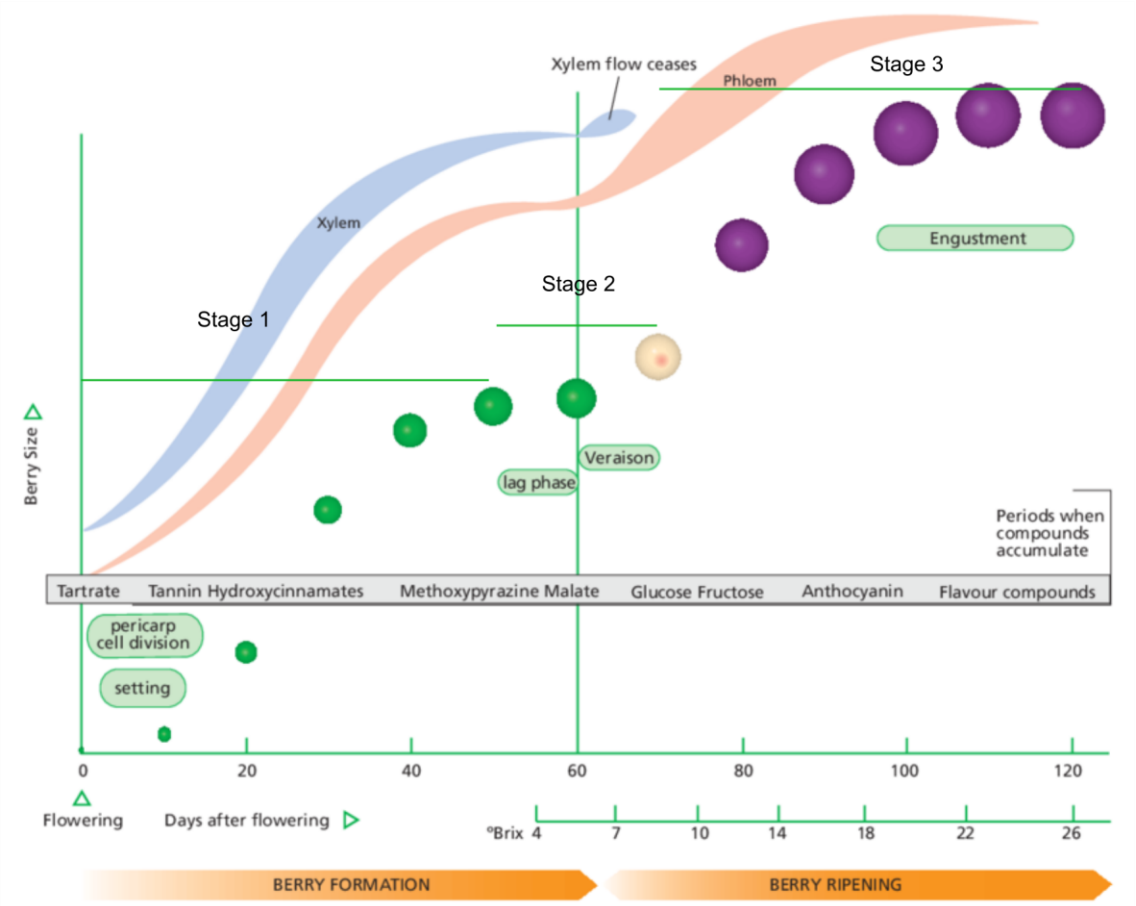

Fig. 1 The development events of grape berry(adapted from Kennedy, 2002).The diagram shows the relative size and color of berries at 10-day intervals after flowering passing through major developmental events (rounded boxes), the periods when compounds accumulate, the levels of juice brix, and an indication of the rate of inflow of xylem and phloem vascular saps into the berry.

1.1.1 Sugars accumulation during berry development and maturation

The sugar content of the berry remains low in the pre-*véraison* stage and begins to increase in an almost linear fashion at the third stage of berry development (Robinson and DAVIES, 2000). At maturity, the sugar content in the vacuoles of pulp cells accounts for 65–91% of the berry dry weight (Agasse et al., 2009). Sugar is produced through photosynthesis in the mature leaves and transported via phloem to berries in the form of sucrose (Figure 1.2, Swanson and El-Shishiny, 1958, Agasse et al., 2009). Sucrose/H^+ transporters (SUC/SUT)

and Sugars Will Eventually be Exported Transporter (SWEET) efflux proteins are involved in this long-distance translocation proceed (Zhang et al., 2019). Once transported into ripening berries, the sucrose is unloaded from phloem in berry and transported to recipient cells through apoplasmic or symplasmic pathway. During the first or the early second stage, sucrose is unloaded from phloem mostly by symplasmic pathway into sink cells; but it changes to the apoplasmic pathway in later stage (post-*véraison*) in order to adapt to higher sugar content in berry cells (Zhang et al., 2006). Along this process, sucrose is taken up into sink cells transported by disaccharide transporters or hydrolysed into glucose and fructose by cell wall invertase, which are then transported by monosaccharide transporters (MSTs), and finally stored in the vacuoles (Davies and Robinson, 1996, Zhang et al., 2019). The ratio of glucose-to-fructose varies with different developmental stages of berry as well as varieties (Kliewer, 1967, Varandas et al., 2004). Usually, in the first stage, glucose is the predominant sugar, then the ratio of glucose-to-fructose drops to stabilize around 1 at maturity (Varandas et al., 2004), and in overripe berries, the concentration of fructose is more than that of glucose. The glucose-to-fructose ratio shows some variation in different grape varieties at maturity. For example, Chardonnay and Pinot blanc are rich in fructose, while Chenin blanc and Zinfandel have more glucose (Kliewer, 1967).

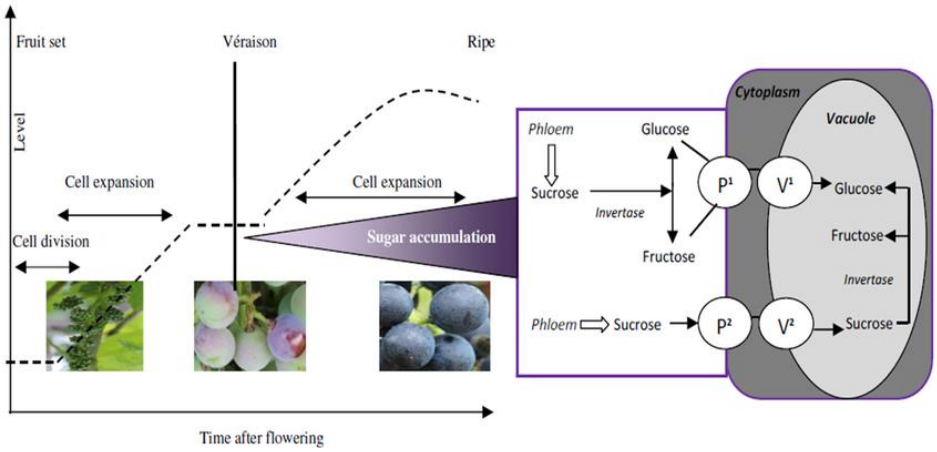

Fig.2 Schematic representation of grape berry development and sugar uptake. P1 and V1, Hexose transporters; P2 and V2, sucrose transporters (Jordão et al., 2015).

According to the above, sugar accumulation involves various membrane transporters during berry development. The uploading and entry of sugars into cells has been extensively studied

on a molecular and physiological basis and several key sugar transporters have been identified (Fig.3) (Figure 1.3, Afoufa-Bastien et al., 2010). Four sucrose transporters (VvSUC11/VvSUT1, VvSUC12, VvSUC27 and VvSUT2) likely to be located on the plasma membrane have been shown to be associated with sugar accumulation during berry ripening development. The expressions of *VvSUC11* and *VvSUC12* genes were up-regulated in berries when hexose accumulation started in the vacuole and have been functionally characterized as proton-sucrose symporters by heterologous expression in yeast; while the expression of *VvSUC27* gene was higher during the first stage but declined during ripening (Davies et al., 1999, Manning et al., 2001). *VvSUT2*, whose sequence is 59% similar to that of *VvSUC27*, is poorly expressed in the berries (Afoufa-Bastien et al., 2010). Forty-six sugar transporters of the SWEET family may perform sugar transport in both directions across the membrane (Lecourieux et al., 2014). Among them, *VvSWEET4*, *VvSWEET7*, *VvSWEET10*, *VvSWEET11*, *VvSWEET15*, and *VvSWEET17d* were shown to be highly expressed in berries after *véraison* by Chong et al. (2014). Plasma membrane-located *VvSWEET10* transporter was found to be strongly expressed at *véraison* in berries, and its overexpression increased the glucose, fructose and total sugar levels in transgenic grapevine cells (Zhang et al., 2019). Moreover, *VvSWEET10* and *VvSWEET15* were found to be differentially expressed among different cultivars: *VvSWEET10* was highly expressed at post-*véraison* in the Cabernet Sauvignon and Riesling berries, while *VvSWEET15* was highly expressed in the Petit Manseng berries during ripening (Ren et al., 2020). Twenty putative hexose/H+ co-transporters (*VvHTs*) have been identified in grapevine, and 9 of them are reported to be potentially involved, to various extends, in hexose accumulation during berry ripening (Afoufa-Bastien et al., 2010). Of these *VvHT*s, different expression patterns of *VvHT1* were found during berry development, in addition to the generally found high expression at anthesis (Deluc et al., 2007, Hayes et al., 2007). Fillion et al. (1999) also detected another peak about 5 weeks after *véraison*, while Afoufa-Bastien et al. (2010) found *VvHT1* was poorly expressed in berry. As *VvHT1* is likely to be co-regulated by both sugar and ABA (Vignault et al., 2005), the different expression patterns may be due to the concentrations of sugar and ABA in berry. *VvHT2* seem to be localized in the tonoplast (Lecourieux et al., 2014), and it is highly expressed during the third stage in berry (Fillion et al., 1999, Hayes et al., 2007). *VvHT3* (also named *VvHT7*) showed

high expression levels in early developmental stages (pre-*véraison*), then decreased around *véraison* and increased again during the ripening phase in the work of Hayes et al. (2007), but conversely showed a continuous decrease from *véraison* onwards according to Ren et al. (2020). *VvHT4* and *VvHT5* (plasma membrane located) were poorly expressed in berries, but have high affinity for glucose; and might involve into responses to environmental stresses rather than in berry ripening (Afoufa-Bastien et al., 2010). *VvHT6* (also identified as *VvTMT1*) is localized in the tonoplast of grape mesocarp cells and its expression decreases with berry development (Zeng et al., 2011). *VvHT11* was found to be weakly constitutively expressed during all phases of berry development, whereas *VvHT12* and *VvHT13* transcripts were barely detectable (Afoufa-Bastien et al., 2010).

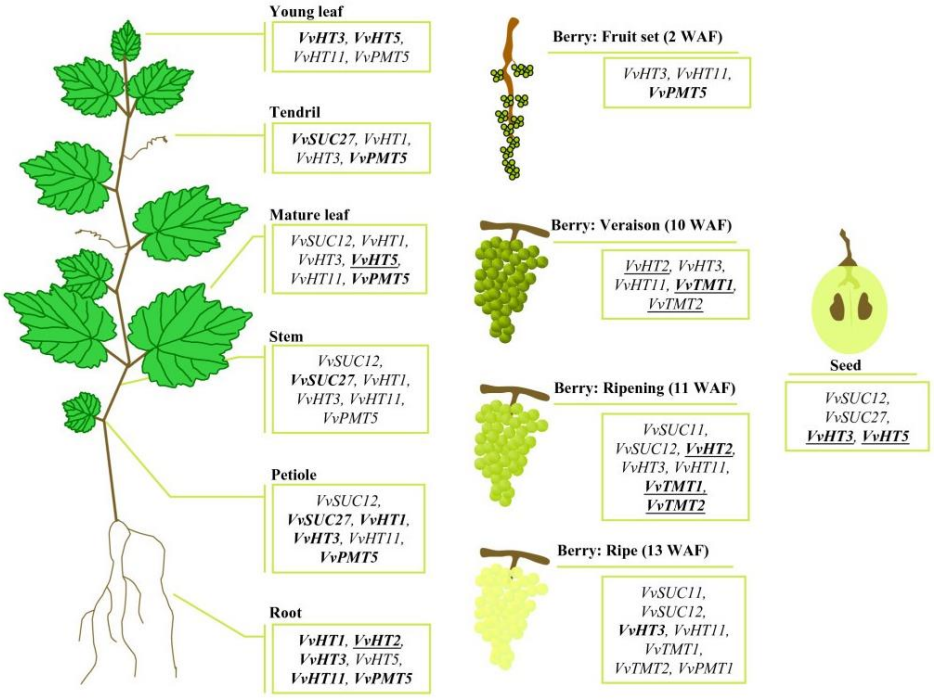

Fig.3 Schematic representation of the expression of *Vitis vinifera* sugar transporter genes during berry development (Afoufa-Bastien et al., 2010). Genes indicated in bold are the most expressed in the indicated organ. Underlined genes are those showing a preferential expression or being induced during the development of the considered organ.

1.1.2 Anthocyanins biosynthesis

Anthocyanins, the members of flavonoid compounds responsible for the colour of ripe red grapes, began to be synthesized and accumulated in berry skin at the onset of ripening (i.e. *véraison*), and their concentration increases until ripeness (Boss et al., 1996). As shown in Fig.4, anthocyanins structure consists in an anthocyanidin aglycon that is bound to one or more sugar moieties (Benmeziane et al., 2016). In grape, there are five anthocyanidins, namely cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt), and malvidin (Mv) (Benmeziane et al., 2016). Berry anthocyanin composition vary according to genotypes. Malvidin derivatives are dominant in various of wine grape (*Vitis vinifera* L.), while the derivatives of peonidin are dominant in table grape, which can be interspecific hybrids (Liang et al., 2008). It is related to the colour of the berries of the different cultivars. Delphinidins

and its methylated derivatives, petunidins and malvidins, are responsible for dark bluish and purple colours; whereas cyanidins and pelargonidins show bright-red colours (Jaakola, 2013).

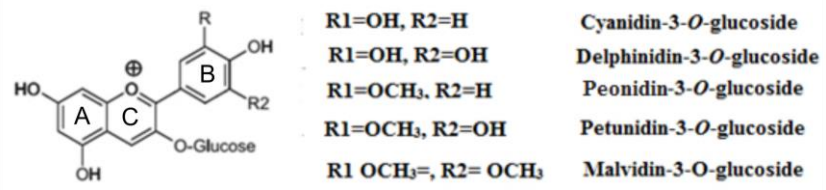

Fig.4 Chemical structure of anthocyanins with A, B and C rings in grape (adapted from Benmeziane et al., 2016)

The anthocyanins biosynthesis pathway in grape is nowadays rather well characterized, with most of its enzymatic steps characterized, and most of its structural and regulatory genes identified (Figure 1.4, He et al., 2010). Anthocyanins are synthesized through the flavonoid pathway, which begins with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA via chalcone synthase (CHS) to form naringenin chalcone, and then the pathway splits into side branches leading to different classes of flavonoids, including anthocyanins and stilbenes (Jaakola, 2013). In grape, there are at least three genes encoding CHS, *Chs1*, *Chs2* and *Chs3*, with different transcriptional regulations. *Chs1* and *Chs2* are mostly expressed in the leaves and berry skin of a white and red cultivars, while *Chs3* mainly in the berry skin of red cultivars (Goto-Yamamoto et al., 2002). Then, chalcone isomerase (or named chalcone-flavanone isomerase, CHI) converts naringin chalcone to its isomer naringenin flavanone, stereospecifically (He et al., 2010). Flavonoid 3'-hydroxylase (F3'H) or flavonoid 3'5'-hydroxylase (F3'5'H) further hydroxylates the B ring of the naringenin flavanone to produce eriodictyol or pentahydroxy-flavanone, respectively. These three (*2S*)-flavanones are then substrates for the flavanone 3-hydroxylase (F3H) to produce the corresponding dihydroflavonols, which decide the type and colour of the anthocyanins that are eventually synthesized (He et al., 2010). After these modifications, the dihydroflavonol 4-reductase (DFR) reduces these dihydroflavonols to the corresponding leucoanthocyanidins, who will to be further oxidized to their corresponding coloured anthocyanidins by the catalysis of leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/ANS), with the help of ferrous iron as co-factors. Anthocyanidins are *O*-glycosylated by the UDP-glucose:flavonoid-*O*-glycosyltransferase (UFGT) and further modified by *O*-

methyltransferases (OMTs) to form *O*-methylated anthocyanins such as malvidin, peonidin and petunidin. Finally, *O*-methylated anthocyanins can be even further modified by anthocyanin acyltransferases (ACT) to produce the most stable acylated anthocyanins (He et al., 2010). After biosynthesis, anthocyanins are transported into skin cell vacuoles for storage, glutathione-*S*-transferase (GST) being a key player in this transportation process (Zhao, 2015).

The expressions of the anthocyanin biosynthetic genes were detected during berry development in numerous studies, validating the pathway presented Fig.5. In the skin of *Vitis vinifera* L. cv Shiraz grape, *VvCHS*, *VvCHI*, *VvF3H*, *VvDFR* and *VvLDOX* transcripts were detected up to 4 weeks after flowering, followed by a reduction of their abundance around 6 to 8 weeks after fruit set, before a sharp re-increase, together with *VvUFGT* transcripts, at 10 weeks after flowering when the anthocyanin synthesis began (Boss et al., 1996). In the red-skinned cultivar 'Merlot', *VvF3'H* was expressed at very early stage but decreased 8-10 weeks after flowering, then followed by an increase at *véraison*, while *F3'5'H* was weakly expressed at pre-*véraison* developmental stages but increased strongly from *véraison* onwards (Castellarin et al., 2006). The predominance of tri- over di-substituted anthocyanins (and vice versa) is associated to *VvF3'5'H*-to-*VvF3'H* transcript levels ratios (Castellarin et al., 2006, De Lorenzis et al., 2016). In Corvina grape, the expression of genes *CHI1*, *F3'H*, *F3'5'H*, *F3H1*, *DFR* and *LDOX* showed their highest levels at 10 days before *véraison*, earlier than the *UFGT* up-regulation (De Lorenzis et al., 2016). Anthocyanin biosynthetic gene transcription levels are usually strongly correlated to the total anthocyanin content and to the relative proportions of the different anthocyanins, and a harvesting delay led to the production of more complex and stable anthocyanins derivatives (De Lorenzis et al., 2016).

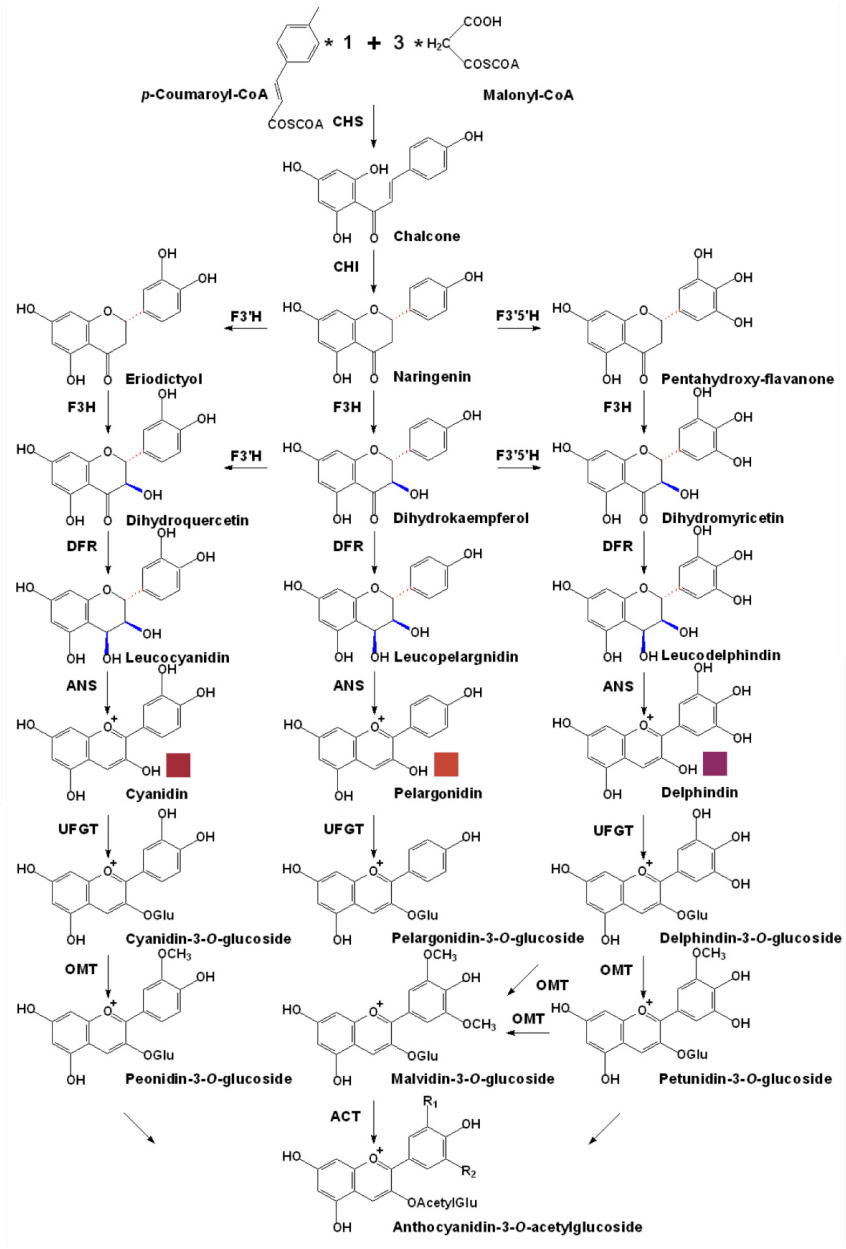

Fig.5 Biosynthetic pathways of anthocyanins in grape (He et al., 2010). CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3β-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid glucosyltransferase; OMT, O-methyltransferase; ACT, anthocyanin acyltransferase.

1.1.3 Organic acids metabolism during berry development and ripening

Second to sugar, organic acids are the most abundant soluble components present in grape berry (Dharmadhikari, 1994). The ratio of sugar to organic acids is an important ripening index for deciding the harvest date (i.e. the so-called "technical maturity"). The main organic acids found in grapes are tartaric and malic acids, accounting for more than 90% of the total organic acids, the 10% remaining being citric, succinic acid and oxalic acids (Dharmadhikari, 1994, Yinshan et al., 2017). About 50% of organic acids in green berries are accumulated through photosynthesis (Conde et al., 2007). The concentrations of both acids increase during the green phase of berry growth and reach their peaks just prior to *véraison*, and then gradually decrease during the third stage, most particularly malic acid, tartaric acid being fairly stable. From *véraison* onwards, malic acid levels reduce greatly, which leads the ratio of tartaric-to-malic acid to increase until ripeness (Dharmadhikari, 1994).

Tartaric acid biosynthesis starts with L-ascorbic acid. L-idonate dehydrogenase (L-IdnDH) catalyses the conversion of L-idonate to 5-keto D-gluconic acid, which is then cleaved between the position C4/C5 to produce tartaric acid and a two-carbon compound (Figrue 1.5, Conde et al., 2007). The expression of *IdnDH* was detected since flowering and increased in young green fruits, then decreased at the beginning of véraison (10% ripe berries) (Cholet et al., 2016). However, the content of tartaric acid in berries may be also related to other, yet unknown enzymes activities and/or other metabolic processes since the content of IdnDH protein exhibited little variation during berry development (Cholet et al., 2016). Thus, more detailed studies of this pathway are needed to further decipher the tartaric acid biosynthetic pathway in grape.

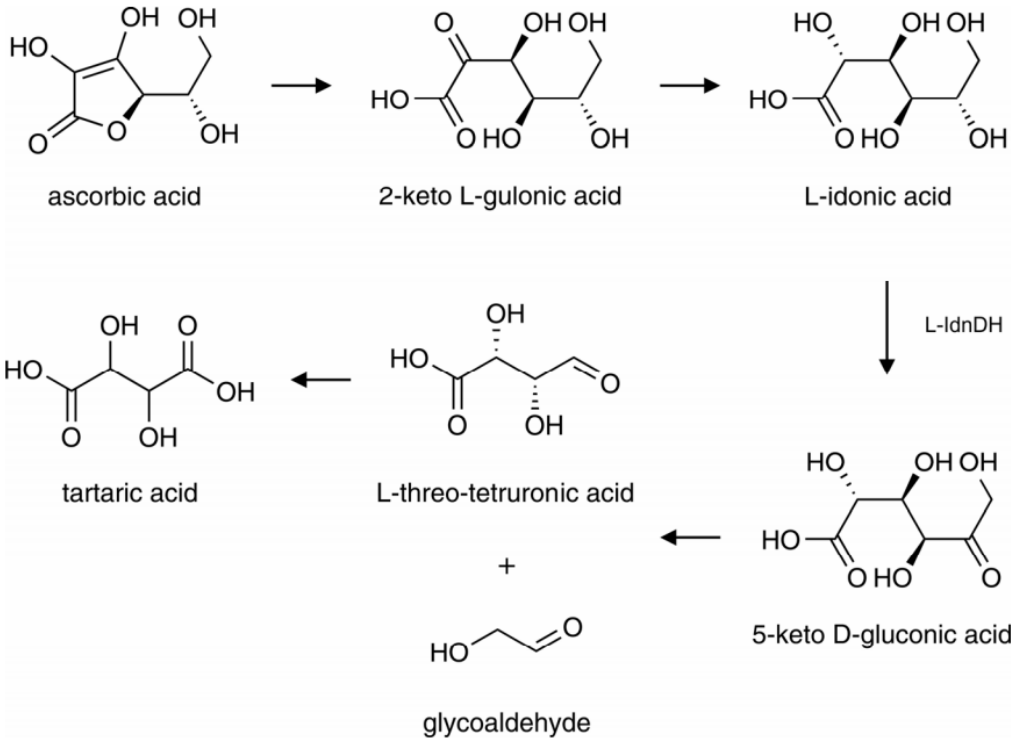

Fig.6 Proposed pathway for tartaric acid synthesis in grape berry (Conde et al., 2007). L-IdnDH, L-idonate dehydrogenase.

Malic acid is mostly synthesised in grape berries from phosphoenolpyruvate via the phosphoenolpyruvate carboxylase that irreversibly catalyses the production of oxaloacetate, that is then reduced to malate by a cytosolic malate dehydrogenase (Conde et al., 2007). The decrease of malic acid after the onset of ripening (at *véraison*) is associated with the decline of the activity of the key enzyme of the synthesis pathway (phosphoenolpyruvate carboxylase), and the activation of three distinct malate catabolic pathways. Firstly, malic acid can be converted to sugars through gluconeogenesis, as attested by the large increase of the phosphoenolpyruvate carboxykinase activity (Hawker, 1969, Famiani et al., 2018). Secondly, malic acid can be also used as a substrate for respiration, which may occur more in early ripening (immediately after the onset of *véraison*), since high NADP-malic enzyme activity is detected at that stage (Ruffner et al., 1984, Famiani et al., 2000). Indeed, NADP-malic enzyme is considered to play a key role to regulate malate breakdown by catalysing the oxidative decarboxylation of malate to pyruvate and CO_2 in the cytoplasm or the mitochondria. Finally, another possible pathway is the mitochondrial malate dehydrogenase

that convert malate to oxaloacetate, which is afterwards degraded into pyruvate and CO_2 by the mitochondrial NAD-malic enzyme (Yinshan et al., 2017).

1.1.4 Amino acid accumulation

Amino acids play vital roles in plant metabolism, being the primary products of inorganic nitrogen assimilation and precursors of proteins and nucleic acids (Stewart and Larher, 1980). In grapes, they also play key roles in berry oenological potential, for they are precursors of many quality-related secondary metabolites, and because must free amino acid content are important for correct yeast fermentation initiation. Phenylalanine (Phe) is the substrate of phenylalanine ammonia-lyase (PAL), which is the entry step of the penylpropanoid metabolism, which feeds the polyphenol biosynthesis pathway (Kataoka et al., 1983). γ-aminobutyric acid (GABA) is a non-proteinaceous, four carbon amino acid that is reported to be involved in multiple processes in plant, including the carbon flux into the TCA cycle, pH regulation, plant development and insect defence signalling (reviewed by Shelp et al., 1999, Hildebrandt et al., 2015). Besides, GABA might play a role in the regulation of malate metabolism via the connection with the TCA cycle (Han et al., 2018b). Among amino acids, the most easily available nitrogen source in musts for yeast growth are alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, threonine and tyrosine. Conversely glycine, lysine and proline are poorer nitrogen sources for yeasts (Bell and Henschke, 2005). Additionally, amino acids are also important in oenology since they influence the fermentation of musts and play a role in the formation of some aromatic substances (Bouard et al., 2000). Isoleucine, leucine, phenylalanine and valine are precursors of higher alcohols and esters, which contribute to desirable aromas in wines (Garde-Cerdán and Ancín-Azpilicueta, 2008).

The content of total free amino acids increases in berry during the ripening process, and individual amino acid showed distinct accumulation dynamics during grape berry development (Kliewer, 1968, Hernández-Orte et al., 1999, Wang et al., 2017a). Proline and arginine are predominant in the berries of most vine making-oriented varieties, at harvest (Kliewer, 1968, Bouard et al., 2000, Miele et al., 2000, Stines et al., 2000). The accumulation

of proline occurred late in ripening, around four weeks post-*véraison*, and increased during later ripening and fruit senescence; while arginine accumulation began before *véraison* and increased rapidly during ripening before decreasing as the berry continued to develop (Kliewer, 1968, Stines et al., 2000). In *V. vinifera* L. cv. Campbell, arginine and asparagine are the most abundant amino acids in young berries and alanine and glutamine in mature berries, respectively; whereas lysine, tyrosine, arginine, ornithine and phenylalanine increased significantly at very early stage, followed by a sharp decrease until the beginning of the *véraison,* and remained at a low level until harvest (Wang et al., 2017a). Other amino acids fluctuate during grape berry development, but all showed a turning point at the beginning of the *véraison* (Wang et al., 2017a). In Trincadeira cultivar, contents in most of free amino acids including valine, proline, and γ-amino butyric acid (GABA) were increased while glutamate decreased during ripening in post-*véraison* stages (Fortes et al., 2011).

1.1.5 ABA accumulation

The plant hormone ABA is recognized as the key signal triggering the onset of berry ripening (Gagné et al., 2006, Wheeler et al., 2009, Gambetta et al., 2010, Koyama et al., 2010, Pilati et al., 2017). Prior studies reported that the application of exogenous ABA on berry at around *véraison* was able to improve anthocyanin content in berry (Peppi et al., 2006, Zhu et al., 2016, Koyama et al., 2018, Shahab et al., 2020), whereas the application of the inhibitor of ABA synthesis nordihydroguaiaretic acid (NDGA) resulted in the opposite tendency (Jia et al., 2018, Guo et al., 2020, Yu et al., 2020). Moreover, exogenous ABA sprayed on the whole vine increased berry sugar concentration by improving sugar transport (Murcia et al., 2016, Murcia et al., 2018). Free ABA levels decline from the fruit setting until *véraison*, and most studies have shown that free ABA levels increase markedly at about the time of *véraison*, followed by another decrease until maturity (Kondo and Kawai, 1998, Wheeler et al., 2009, Villalobos-González et al., 2016, Coelho et al., 2019). Castellarin et al. (2016) further found, by the precise sampling of individual berries, that the accumulation of ABA in parallel with berry softening happened before sugar and anthocyanins accumulations, and prior to the significant expression of the 9-cis-epoxycarotenoid dioxygenases (NCEDs, a key enzyme in ABA synthesis pathway). This inconsistency between the increase of ABA level and the

expression of NCEDs suggested a yet uncharted complexity of berry ABA sources, while bound froms of ABA can release free ABA (Dietz et al., 2000, Lee et al., 2006, Xu et al., 2012, Hussain et al., 2020). Besides, both exogenous ABA (Wheeler et al., 2009, Pilati et al., 2017) and ethylene (Sun et al., 2010) are able to regulate the expression of NCED to trigger ABA biosynthesis, which makes it more difficult to analyse the source of berry ABA. The initial increases in ABA at the onset of *véraison* may be due to the remobilization of the bound ABA pools or import from the leaves (Castellarin et al., 2016). However, the later accumulation of ABA is at least partly due to the neo-synthesis in berries, according to the up-regulated expression of NCED reported during *véraison* (Sun et al., 2010, Pilati et al., 2017) and the inhibitory effect of an application of NDGA on anthocyanin accumulation and berry ripening dynamics (Jia et al., 2018, Guo et al., 2020, Yu et al., 2020).

1.2 Climate change and viticulture

1.2.1 The impact of climate change on berry metabolism

The quality of berry at harvest greatly determines the quality of the wine. Optimal grape maturity is essential for wine quality, but is becoming more and more difficult to reach in the context of global climate change, which is modifying vine physiology and especially the biochemical composition of grape berries at harvest (Drappier et al., 2019). Global climate change is intensifying, and the occurrence of regional extreme weather events is increasing, both in frequency and intensity, while the yield and quality of grapes are very sensitive to climate and weather (Jones and Webb, 2010). The major threats from climate change for maintenance of optimal grape quality and current vineyard geographical distribution are the elevated temperature, modified rainfall patterns and incoming radiations, in particular UV-Bs (Martínez-Lüscher, 2014, Martínez-Lüscher et al., 2016, Van Leeuwen and Darriet, 2016). Among these abiotic factors, temperature is the most critical one influencing grapevine phenology, altering grape ripening dynamics and ultimately grape composition at harvest (Drappier et al., 2019).

1.2.1.1 Effects of temperature rising on primary metabolites in berry

The general trends of the impact of mean temperatures elevation observed in past decades already impacted grapevine phenology and altered berry composition at harvest. Twenty years ago, Jones and Davis (Jones and Davis, 2000) analysed datasets collected from 1952 to 1997 in the Bordeaux area and demonstrated that Merlot and Cabernet Sauvignon varieties tended to produce berries with higher sugar-to-acids ratios, while climate tended to become warmer. The same year, Schultz et al. (Schultz, 2000) predicted that changes in the climate of the European viticultural regions in the coming decades (post year 2050) could potentially have a serious impact on wine quality by affecting the composition of the berries at harvest. Changes observed over recent years confirmed these predictions. Through an analysis of the climate and grape phenological data series from 1972 to 2003 in the Alsace region (France), Duchene and Schneider (Duchêne and Schneider, 2005) found that the developmental stages of *Vitis vinifera cv.* Riesling occurred earlier with the increase in mean temperature, which

led the harvest dates to occurs earlier in the season. In particular, the results showed that potential alcohol levels at harvest rose by an average of 0.08% per year, meaning that the sugar content in berry also increased by year, causing the technological ripeness of the grapes (based on the sugar-to-total acidity ratio) to be reached earlier. Another analysis of climatic data between 1950 and 2006 in France indicated that the mean annual temperatures increased 1.3 °C in this period, while the mean potential evapotranspiration increased by 900 mm per year since 1999. This resulted in sugar concentrations increased leading to harvest dates already advanced by up to three weeks in French Mediterranean Hérault vineyard (Laget et al., 2008). Mean data from Languedoc (France) shows that over a 35-year time span, alcohol in wine increased from 11% to 14%, pH from 3.50 to 3.75 and total acidity decreased from 6.0 to 4.5 $g.L^{-1}$ (van Leeuwen et al., 2019). Recently, a report analysed the effect of the trend of climate change between 1953 and 2013 in Italy on grape Romagna Sangiovese production area potential alcohol concentration. The results showed that the increase of potential alcohol level was largely (but not totally) explained by climatic factors during that period (Teslić et al., 2018). Similar impacts of climate change trends were also found on berry compositions in the southern hemisphere production areas. The researchers found that the date of technological ripeness (21.8°Brix) advanced at rates between-0.5 and -3.1 days/year between 1993 and 2006, and that the concentration of sugar in the berries of Cabernet Sauvignon and Shiraz still increased up to ~0.3° Brix/year at harvest in Australia (Petrie and Sadras, 2008). Based on these reports, it is concluded that increasing temperatures already had a notable effect on berry primary metabolism, leading to a general increase in sugar concentrations and lower acidity accompanied by hastened berry ripening rates, which would further result in the alcohol content in wines to increase, affecting their balance (Tilloy et al., 2014, Jordão et al., 2015). In particular, the sensory properties of wines may be affected, breaking the balance responsible of wine typicity and typicality (King et al., 2013).

1.2.1.2 Effects of temperature rising on secondary metabolites in berry

There are no long-term observational datasets to show that secondary metabolites have been affecting climate changes in the last decades. However, a large body of comparisons studies

between different vineyards with different climates, and different climate change simulation experiments in the field or in greenhouses showed that elevated temperature conditions reduced total anthocyanin content in grape berries and alter the anthocyanins profiles at harvest (Kliewer, 1970, Buttrose et al., 1971, Kliewer and Torres, 1972, Coombe, 1987, Mori et al., 2007, Sadras and Moran, 2012, Fernandes de Oliveira et al., 2015, Martínez de Toda and Balda, 2015, Martínez-Lüscher et al., 2016, de Rosas et al., 2017). During the 2010-2012 period, total anthocyanins content of *Vitis vinifera* L. "Maturana Tinta de Navarrete" berries grown in warmer (up to +1.3°C) vineyards decreased, as well as the ratio of anthocyanins-to-sugars (Martínez de Toda and Balda, 2015). After a 2-3°C temperature raise under field-crop conditions, total anthocyanins content decreased by 28-41% and the relative proportion of acylated anthocyanins increased in cultivars of "Malbec" and "Bonarda" in Argentina (de Rosas et al., 2017). Additionally, high temperatures were found to also affect amino acids and aroma compounds in berries at harvest. By raising the temperature of the fruit to the expected climate conditions of the mid-21st century (c.a. +1.5°C in average) in vineyards using polycarbonate screens, anthocyanins, amino acids (especially alanine, serine, and phenylalanine), and IBMP (2-methoxy-3-isobutylpyrazine) concentrations were reduced; while total tannin content was increased before *véraison* followed by a decrease thereafter in Cabernet-Sauvignon berries (Wu et al., 2019). Conversely, UV-B exposure, which is linked to temperature by the fact that they both come through global solar radiation intercept by berry surface, increased the concentrations of anthocyanins in skin and GABA in pulp; while reducing some individual amino acids, such as threonine, isoleucine, methionine, serine and glycine, partially alleviating the effect of temperature (Martínez-Lüscher et al., 2014). However, after ten combinations of temperature and solar radiation exposure experiments, Tarara et al. (2008) demonstrated that temperature is among the strongest environmental determinant of anthocyanin profile in the berry skins, above a potentially low threshold of exposure to solar radiation.

The molecular mechanism by which high temperature affects the accumulation of anthocyanins in berries has been largely studied. The expression of anthocyanin biosynthetic genes is strongly affected by temperature, with high temperature causing a decrease in the transcript levels of the genes. Mori et al. (2005b) found that high night temperatures inhibited the expression of *CHS*, *F3H*, *DFR*, *LDOX* and *UFGT* genes at *véraison*, but also an increase

in UFGT activity after *véraison*. Yamane et al. (2006) reported that high temperatures reduced the expression of *VvmybA1*, one of key transcription factors that positively regulate *UFGT* gene expression in Japanese red table grape Aki of Queen. These authors also hypothesized that a decrease in ABA content of berries grown under high temperature was responsible for the reduction of *VvmybA1* transcript levels. De Rosas et al. (2017) found that the expression of *VvmybA1* and *UFGT* genes was correlated with anthocyanin accumulation in half-ripe and harvest stage berries; and that the acyltransferase gene *Vv3AT* transcript levels were increased and associated with higher anthocyanin acylation in berries cultivated under high temperature. Using ^{13}C-labelling experiments, Mori et al. (2007) showed that the content of total anthocyanins was markedly reduced compared to berries grown under lower temperatures. Furthermore, using genome-wide transcriptomic analysis, Lecourieux et al. (2017) showed that numerous genes potentially related to anthocyanin degradation increased their transcript levels in response to high temperature. Thus, it appears that high temperatures reduce anthocyanin contents by both inhibiting anthocyanins biosynthesis and accelerating their degradation processes.

In summary, under the warming trend the increase of berry sugar content and the advance of technological ripeness, as well as the decrease of anthocyanin accumulation, are likely to result in the decoupling of technological and phenolic ripeness, as predicted by Sadras and Moran (Figure 1.7, 2012).

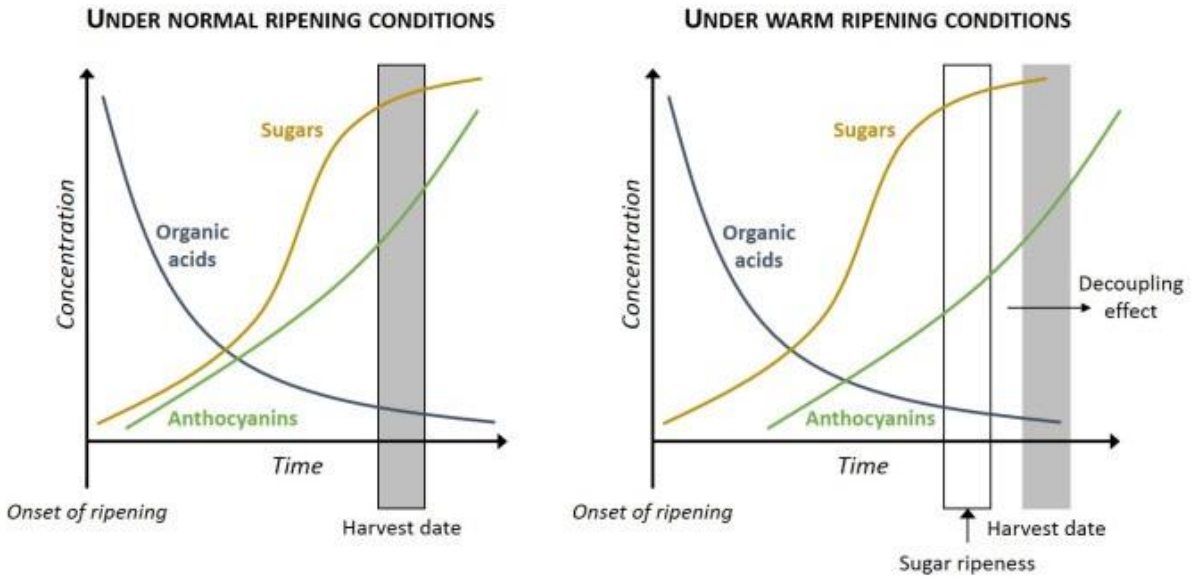

Fig.7 Schematic representation of the hypothesis of decoupling of sugar (a.k.a. technological) and phenolic ripeness under warming temperature. Under normal (left) climate conditions, sugar and phenolic ripeness are occurring at the same time, at harvest date. Under warmer (i.e. future climate) ripening conditions, the sugar ripeness occurs earlier in the season and is not synchronized anymore with phenolic ripeness and optimal harvest date. (source: viticulturallogbook, https://viticulturallogbook.wordpress.com).

1.2.2 Adaption strategies to climate change-induced berry composition alterations

Optimal grape maturity is the right balance between technological ripeness (sugar-to-total acidity ratio above a threshold, for example 50 for Cabernet Sauvignon in Bordeaux area) and phenolic and aromatic ripeness (when anthocyanin are concentrated enough in skins, tannins soften enough and aromatic potential optimal) (Conde et al., 2007). These different maturities are the results of both primary and secondary metabolic pathways regulations that must be synchronized and coordinated to result in berries with high oenological potential. As the mean temperatures continue to rise, the differential responses of sugars, organic acids anthocyanins, tannins, aroma and aroma precursors metabolic pathways to temperatures will result in technological ripeness to be achieved earlier in the season, while the phenolic and aromatic ripeness will be delayed, an uncoupling effect described by Sadras and Moran

(2012). In that case, in order to achieve a decent phenolic and aromatic maturity, the berries will have to be harvested later than the optimal technological ripeness, which will further lead to produce unbalanced wines with a higher alcohol content. Conversely, harvesting berries on their optimal to technological ripeness will lower wine alcohol content but the degree of phenolic and aromatic maturity may not reach the standards of high-quality wines. There are several possible approaches to control the wine alcohol content, like using techniques to remove or lower the wine alcohol content, adaptive evolution of the wine yeast to reduce the alcohol yield or through viticultural interventions and decision-making to decrease the berry sugar concentrations, the latter approach being the most promising looking (Novello and De Palma, 2013, Tilloy et al., 2014, Jordão et al., 2015).

Currently, three main types of viticultural strategies are considered possible to resolve this contradiction: changes in the cultivation areas, changes in the viticultural techniques and practices and changes in plant material to use climate change better adapted genotypes (Duchêne, 2016). New vineyards can be established in areas of high latitude with cooler climate (Mozell and Thach, 2014), but this is not acceptable for most of current grape growers. In the long term, modifications in plant material should be considered as a priority taking into account the economic costs and environmental friendliness (Duchêne, 2016). Thus, the most useful strategy for current grape growers is to modify their viticultural practices, for example by modifying vine canopy management and by implementing irrigation strategies. Indeed, viticultural practices can be altered to try to mitigate the negative effects of the uncoupling between technological ripeness and phenolic ripeness, based on two principles: firstly, reducing or slowing down the input of sugar (limiting carbon source) to the ripening berries to delay sugar ripeness; and secondly enhancing the concentration of phenolic compounds to hasten phenolic ripeness.

1.2.2.1 Viticultural practices aiming to reduce carbon source: canopy management

Mature leaves are the main source of carbohydrates produced through photosynthesis and transported via the phloem to berries during ripening (Kliewer and Dokoozolian, 2005), even though in early developmental stages young bunches can also make a significant contribution to carbon assimilation (Vaillant-Gaveau et al., 2011). Hence, the sugar content in beries is largely determined by the total leaf area and the photosynthesis efficiency (Kliewer and Dokoozlian, 2005, Novello and De Palma, 2013). In general, a leaf area-to-fruit ratio around 0.8 to 1.2 m^2/kg is able to produce the optimal level of total soluble solids, berry weight and berry coloration at harvest in vineyard conditions (Kliewer and Dokoozlian, 2005). To cope with the decoupling of anthocyanins and sugars accumulations under high temperature, one strategy is to control carbon source by reducing leaf-to-fruit ratio appropriately to delay berry ripening and slow down sugar accumulation rate (Martínez de Toda et al., 2014). Shoot trimming and leaves removal are the cultural practices to reduce leaf-to-fruit ratio of vines, and the application of anti-transpirant sprays can envisioned to decrease photosynthesis activity by reducing gas-exchange (Palliotti et al., 2013a).

As shoot trimming promotes lateral shoots growth below the cutting point, which can then become net exporters of carbohydrates as soon as they have two fully expanded leaves (Vasconcelos and Castagnoli, 2000), the timing and degree of shoot trimming will highly influence the effect on delaying of sugar accumulation. An early shoot trimming experiment carried out on Pinot noir at bloom improved fruit set by 25% with the Brix index increasing (Vasconcelos and Castagnoli, 2000). However, shoot trimmings at a 50% or 100% level pre-anthesis and post-anthesis stages or 50% at both times reduced fruit set and increased sugar concentration in field-grown “Barbera” and “Trebbiano” cultivars (Poni et al., 2004). In another experiment, three different shoot trimming treatments were done during a 3-year period: 1) a control with untrimmed vines; 2) vines with one trimming after fruit set; and 3) vines with two trimmings, one after fruit set and another one at *véraison* (Martínez de Toda and Balda, 2013). The results of this experiment showed that by harvesting at the same date, both trimming treatments delayed the *véraison* date by 18-20 days and decreased soluble solids by 12-14%, but the double trimming (leaf-to-fruit ratio less than 0.5 m^2/kg) led to a

greater reduction in total anthocyanin content compared to single trimming treatment (Martínez de Toda and Balda, 2013). Interestingly, applying the same above-mentioned treatment but harvesting berries at the same sugar concentrations, induced a delay in berry development that led to an increase in anthocyanin concentrations, which partially restored the anthocyanins-to-sugars ratio (Martínez de Toda et al., 2014). Finally, a severe shoot trimming at fruit set was found to reduce sugars levels at harvest, without affecting the accumulation of anthocyanins in Merlot (Herrera et al., 2015). Hence, it looks like that shoot trimming at fruit set stage, associated with a leaf-to-fruit ratio kept above 0.5 m^2/kg and a harvest based on sugar concentrations constitute one of the optimum practices for harvesting high quality berries.

Leaf removal is another commonly used canopy management practice to improve berry quality. Leaf removal in different parts of vines would have different influence on berry composition. After *véraison*, basal leaves are no longer the main source of photosynthetic assimilates (Poni et al., 1994), so the removal of the medial and apical part of the canopy around *véraison* got favoured in recent years as a field practice (Zhang et al., 2017). By removing 35% leaf area apical to the bunch zone using a leaf-plucking machine, when berry sugar content was approximately 16–17°Brix, the reach of the optimal TSS level of Sangiovese berries was delayed by 2 weeks, without significantly changing neither the concentration of total phenolic compounds in the grapes nor the chemical and chromatic characteristics of the wines (Palliotti et al., 2013b). Similarly, Poni et al. (2013) found that leaf removal above the bunch zone at pre-*véraison* and post-*véraison* developmental stages delayed technological ripeness without affecting colour and phenolics. However, Bobeica et al. (2015) reported that carbon source limitation induced by severe leaf removal at one week before *véraison* in Cabernet Sauvignon and Sangiovese fruiting-cuttings caused a significant accumulation of anthocyanins and sugars in berries, but the impact on anthocyanins accumulation being even greater (Bobeica et al., 2015). In addition, it was shown that the effect of leaf removal at *véraison* on berry composition also depends on the cultivar (Lanari et al., 2012, Gutiérrez-Gamboa et al., 2019). Removal of the most functional leaves on the upper part of the canopy of vertical shoot positioned medium-vigour ‘Sangiovese’ and

'Montepulciano' grapevines at post-*véraison* reduced juice soluble solids, anthocyanins and polyphenols concentration at harvest in 'Montepulciano' grapevines, but not in 'Sangiovese'(Lanari et al., 2012). As the leaf-to-fruit ratio decreased, the accumulation of soluble solids was delayed in "Sauvignon blanc", "Cabernet Sauvignon", and "Syrah" cultivars, but not in "Carmenère", and the leaf-to-fruit ratio value to reach an optimum content of soluble solids was different among these cultivars (Gutiérrez-Gamboa et al., 2019). This means that we still need to deepen our understanding of the relationship between primary metabolites (sugar, organic acids) and polyphenol accumulation in response to leaf removal around *véraison*, in order to find the optimal trade-off points for synchronizing phenolic ripeness with sugar ripeness in grape.

Carbon source limitation might also be achieved through reducing photosynthesis efficiency. The anti-transpirant di-1-*p*-menthene was found to reduce gas-exchange in grapevine, and it can slow down sugar accumulation in berries and reduced TSS by 1.2°Brix at harvest, without affecting organic acids, pH, and phenolic richness when applied at post-*véraison* on field-grown Sangiovese grape leaves (Palliotti et al., 2013a). However, it also did decrease by 15 to 20% anthocyanins skin content, unbalancing the sugar-to-anthocyanin ratio. Thus, it appears that this practice is not suitable for the cultivation of red grapes.

1.2.2.2 Viticultural practices aiming to enhance phenolic compounds in berries

According to previous studies, several practices are in theory able to alleviate the decoupling between the technical (sugar-to-acidity ratio) ripeness and phenolic ripeness by enhancing the pace of the accumulation of phenolic compounds, especially anthocyanins. Different irrigation strategies may be an option to improve the concentrations of berry phenolic substances. Matthews and Anderson (1988) reported that water deficits imposed both before and after *véraison* increased the concentrations of phenolics in juice and skin extracts and anthocyanins in skin. Also, water deficit before *véraison* significantly reduced malate concentrations and water deficit after *véraison* increased proline concentration (Matthews and

Anderson, 1988). Ojeda et al. (2002) found that water deficit between anthesis and *véraison* promotes biosynthesis of flavonols but reduces total tannin content; and water deficit at post-*véraison* stages increased biosynthesis of proanthocyanins and anthocyanins. Another study in Spain found that spring water deficit strategy was more effective than that after *véraison* in reducing berry growth leading to more concentrated berries in terms of anthocyanins, while water deficit after *véraison* impaired berry sugar accumulation might due to the detrimental effect of water stress on leaf photosynthetic rate (Yeves et al., 2011). Additionally, these authors also found that water deficits reduced berry size and increased the ratio of skin to pulp especially when occurring during early developmental stages (Triolo et al., 2019).

Ultraviolet-B (UV-B) and ABA were also reported to increase grape anthocyanin concentrations (Alonso et al., 2016). Removal of basal leaves at 5 weeks before *véraison* is beneficial to improve UV-B radiation exposures and change the microclimate around the fruiting zones, increasing total phenolic compounds substantially in skin in Sauvignon blanc grapes (Gregan et al., 2012). Martínez-Lüscher et al. (2016) found that increasing UV-B alleviates the decoupling of anthocyanins and sugars by slowing berry development and up-regulating flavonol and anthocyanin biosynthesis in *Vitis vinifera* cv. Tempranillo fruit-bearing cuttings. Another study in a high-altitude vineyard showed that reduction of UV-B delayed berry ripening, whereas ultraviolet-B and exogenously applied ABA combined treatments reduced sugars per berry and increased anthocyanins accumulation by hastening the onset of ripening (Berli et al., 2011).

These practices aiming to enhance phenolic substances accumulation might relate to ABA synthesis and signalling during berry ripening and development, as ABA biosynthesis in grapevine is induced by water deficit in leaves and berries, but also by high UV-B doses (Alonso et al., 2016). ABA is recognized as an important hormone for ripening inception and colour development in berries (Pilati et al., 2017). Exogenous ABA application on berries has been shown to stimulate anthocyanin accumulation, and the effects of sprayed ABA depend on the developmental stage at which it is applied, the doses and frequency of application as well as cultivar genotypes (Koyama et al., 2018). The authors reported that two applications of a 400 $mg.L^{-1}$ ABA spray at 7 and 21 days after *véraison* was more efficient than a 200

mg.L^{-1} one to increase the total anthocyanin content of *Vitis vinifera* × *Vitis labrusca* berry skin at harvest (Koyama et al., 2018). Peppi et al. found that 300 mg.L^{-1} of ABA applied at *véraison* was more efficient to improve "Flame seedless" skin anthocyanin concentrations than other doses (0, 75 and 1000 mg.L^{-1}) or ethephon applications (Peppi et al., 2006). In another study, 4 doses of exogenous ABA spraying (0, 200, 400, 600 mg.L^{-1}) at *véraison* in 'Merlot' and 'Cabernet Sauvignon' showed that ABA improved phenolic contents (mainly anthocyanins and flavonols) of the grape skins, but no distinct relationships were observed between ABA concentrations and wine phenolic characteristics, maybe because of differential extractability of the various polyphenols during winemaking (Zhu et al., 2016).

In conclusion, the choice of an appropriate strategy is determined by vineyard location and environment and different wine making requirements. Manipulating vineyard conditions and viticultural practices to alter the anthocyanin profile of the berry may become a useful approach to manage and maintain grape optimal phenolic composition, in spite of on-going climate changes. Meanwhile, these strategies mainly regulate the accumulation of sugar and anthocyanins by modifying the leaf-to-fruit ratio or ABA level in berry. Hence, Finding the best-suited adaptation strategies will however require deepening our understanding of the physiological mechanisms and regulations underlying the coordination of the primary metabolites and polyphenol accumulation in response to the change of leaf-to-fruit ratio and berry ABA levels. In particular, deciphering the complex interplay occurring between sugar accumulation, anthocyanin biosynthesis and ABA signalling during berry ripening appears to be of uttermost importance.

1.3 Interplay between sugar, accumulation, anthocyanins biosynthesis and ABA signalling

A number of studies focused on how to restore the coupling of the sugar ripeness and phenolic ripeness, aiming at alleviating the decoupling of sugar and anthocyanins accumulation dynamics in red grapes. Most of them found that leaf-to-fruit ratio manipulations aimed at delaying berry ripening could be an efficient solution to this problem. However, the underlying mechanisms of the relationship between primary metabolites accumulation and grape polyphenol composition in response to leaf-to-fruit ratio manipulations are still far to be fully understood. The practices aiming to enhance phenolic substances accumulation might relate to ABA synthesis and flavonoid biosynthetic pathway regulation during berry ripening and development, which is still in the need of an in-depth study about the underlying mechanisms. In order to find the best trade-off points for synchronizing sugar ripening and phenological ripeness of grape, we need to further investigate the relationship between sugar accumulation and anthocyanins biosynthesis and the role of ABA signalling during berry ripening and development.

1.3.1 Sugars as metabolites and signalling molecules for anthocyanins synthesis

Sugars are important substrates for the biosynthesis of anthocyanins biosynthesis, and the anthocyanin content in grape berry is often positively correlated with the sugar level, especially in the skin (González-SanJosé and Diez, 1992, Liang et al., 2011). A reduction in the whole vine photosynthesis of potted grapevines at pre-*véraison*, achieved by reduction in ambient CO_2, led to the rate of sugar accumulation and the absolute amount of sugar in the berries to decrease, which caused the accumulation of anthocyanins to decrease at the same rate (Smith et al., 2019). Sugar availability was confirmed to influence the synthesis of anthocyanins by numerous studies.

Recently, the mechanism of anthocyanin synthesis in response to low carbon supply has been investigated in kiwifruit, and trehalose-6-phosphate and the active repressor *MYB27* are key elements in the regulation of anthocyanin synthesis under this condition (Nardozza et al., 2020). Trehalose-6-phosphate and SnRK1 kinases were considered as central mediator of energy signalling acting opposite roles in response to carbohydrate levels in plant (Gazzarrini and Tsai, 2014), and MdSnRK1.1 was also proved to involve into the sucrose-induced anthocyanin pathway in apple interacting with MdJAZ18 (Liu et al., 2017).

Sugars can also act as signalling molecules whose transduction pathways may influence anthocyanins biosynthesis (Figure 1.8, Hara et al., 2003, Hu et al., 2016, Durán-Soria et al., 2020). After measuring berry sugar content at the onset of anthocyanin synthesis in eighteen red grape varieties, Garcia et al. (2017) found that this value is fairly stable over the year for a given cultivar, and in fact much more stable than the calendar date at which anthocyanin biosynthesis initiates. This suggests that sugar content may be one of the signals triggering the onset of anthocyanins synthesis in grape berry. Using an innovative *in vitro* cultured berry model, Dai et al. (2014) reported that high concentration of sugar can increase the concentration of anthocyanin and hastens its synthesis, while the concentration of its precursor, Phe, was decreased by the high sugar supply. This suggested that the rate of induction of anthocyanins biosynthesis by sugar levels was higher than that of Phe accumulation. This implies that sugar not only provide carbon skeletons via the phenylalanine metabolic pathway for anthocyanins synthesis, but also might act as signalling molecules to promote anthocyanins synthesis. Application of 10% sucrose at 5 days after the beginning of *véraison* and 90 mM sucrose at 13 days after *véraison* on the bunches of Crimson Seedless didn't modify the concentration of total soluble solids but increase total anthocyanins at harvest (Ferrara et al., 2015, Olivares et al., 2017), also supporting that sucrose potentially acted as a signalling molecule to induce anthocyanins accumulation. Moreover, Vitrac et al. (2000) reported that sugar-induced anthocyanin biosynthesis may be mediated by Ca^{2+}-calmodulin dependant kinases and hexokinases (HXK). Indeed, Zheng et al. (2009) showed that the glucose analog 3-*O*-methylglucose, which is transported into cells but not phosphorylated by HXK activity, has no effect on anthocyanin production in sliced grape berries, while mannose which was phosphorylated by hexokinase but are poorly metabolized, was able to induce the anthocyanin accumulation and *F3H* expression. Moreover,

mannoheptulose, a specific inhibitor of hexokinase, could reduce the accumulation of anthocyanins. Furthermore, it was confirmed that in apple, the glucose sensor HXK1 promotes anthocyanin biosynthesis by phosphorylating and stabilizing the transcription factor MdbHLH3 which bounds to the promoters of *DFR*, *UFGT* and *Myb1* genes under low temperature to increase anthocyanin accumulation (Xie et al., 2012, Hu et al., 2016). Furthermore, the expressions of *VvF3H*, *VvDFR* and *VvANS* (also known as *VvLDOX*) genes were also demonstrated to be induced by sucrose in *vitis vinifera* cells (Gollop et al., 2001, Gollop et al., 2002), and *MYB75/PAP1* was found to be an essential factor for the sucrose-mediated expression of the *DFR* gene in Arabidopsis (Teng et al., 2005).

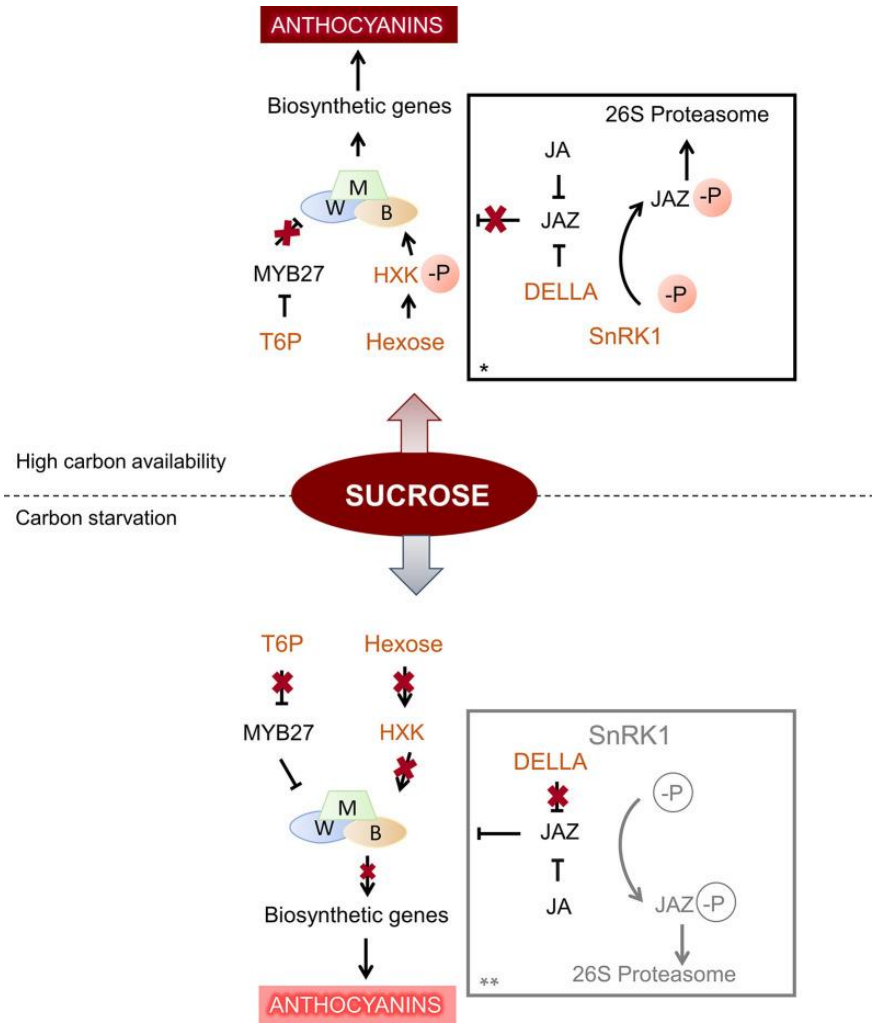

Fig.8 Proposed pathway for sugar-induced anthocyanins synthesis in plant(adapted from Durán-Soria et al., 2020). Components of sugar signaling cascade are shown in orange. When there is a high supply of sucrose, hexokinase (HXK) is phosphorylated and activates *bHLH3* (B) transcription factor, part of the MBW complex, which promotes anthocyanin synthesis by its action upon downstream biosynthetic genes. In addition, high levels of trehalose-6-phosphate (T6P) represses *MYB27*, a general repressor of anthocyanin synthesis, leading to increased anthocyanin content showed in darker hues. DELLA protein degradation is also repressed under sucrose treatment, and favors anthocyanin accumulation by sequestering JAZ repressors. Under carbon starvation, HXK is no more phosphorylated, and thus does not activate *bHLH3* (B). In addition, low T6P level removes the repression upon *MYB27*, resulting in lower anthocyanin accumulation, represented in paler tone. * Under a specific range of sucrose (under sucrose oversupply, this process is inhibited, possibly by T6P repression over SnRK1), SnRK1 induces the phosphorylation of JAZ18, promoting its degradation through the 26 proteasome. JAZ18 repression upon *bHLH3* (B) is removed, allowing the

MBW complex to activate downstream biosynthetic genes for anthocyanin accumulation. ** No data are available about SnRK1 effect under sucrose starvation (shown in gray).

1.3.2 Abscisic acid induction of anthocyanins biosynthesis

ABA has been proposed as an important hormone triggering the onset of the ripening process in grapes (Fortes et al., 2015, Villalobos-Gonzalez et al., 2016, Pilati et al., 2017). By a two-week temperature treatment on potted vines of Aki Queen (*Vitis labrusca* x *V. vinifera*), Yamane et al. (2006) suggested that temperature affects the expression of *VvmybA1* to regulate the structural genes of the flavonoid biosynthetic pathway via modulating endogenous ABA levels, thus affected the accumulation of anthocyanins in berry. Moreover, Lecourieux et al. (2017) reported that early berry heat exposure (during herbaceous stage) in cultivar Cabernet Sauvignon significantly affects the expression of genes involved in ABA metabolism and signalling. Besides temperature, water deficit and high UV-B doses induction of anthocyanins biosynthesis may also be related to ABA biosynthesis in grapevine (Alonso et al., 2016). Hence, the effect of climate change on endogenous ABA level should be partly responsible for the change of anthocyanin concentration.

Several studies have shown that exogenous application of ABA can significantly increase the expressions of genes involved in anthocyanin biosynthesis (Fig.9). Exogenously applied ABA at pre-*véraison* accelerated sugar and anthocyanins accumulation from *véraison* onwards, and up-regulated the expression of anthocyanins biosynthesis related genes, including *VvDFR*, *VvANS*, *VvUFGT* and *VvMybA1* (Villalobos-Gonzalez et al., 2016). ABA applications after *véraison* also increased the gene expression of *VvCHI*, *VvF3H*, *VvDFR*, and *VvUFGT* structural genes as well as of the *VvMYBA1* and *VvMYBA2* transcription factors and resulted in a higher accumulation of total anthocyanins in the skin of berries (Koyama et al., 2018). A putative ABA receptor gene encoding the magnesium chelatase H subunit named FaCHLH/ABAR was shown to play an important role in ABA-induced anthocyanins synthesis in strawberry, since exogenous ABA cannot reverse the colourless phenotype of FaCHLH/ABAR-down-regulated RNAi fruit (Jia et al., 2011). Moreover, the application of

exogenous ABA or Abscinazole (a specific inhibitor of CYP707A that causes endogenous ABA levels to rise) increased the expression levels of *VvUFGT* and *VvMYBA2* as well as the accumulation of anthocyanin in 'Kyoho' grape berries (Jia et al., 2018). However, Saito et al. (2018) reported different genes expression profiles in grapes after ABA and Abz application in a genome-wide transcriptomic analysis, which strongly suggests that exogenous ABA and endogenous ABA may affect anthocyanin biosynthesis genes through two not entirely identical mechanisms.

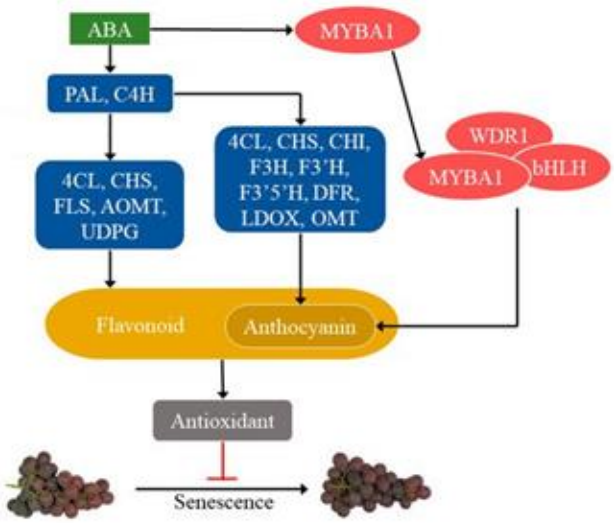

Fig.9 Proposed pathway for ABA-induced anthocyanin synthesis in grape (adapted fromYang et al., 2020). PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA synthase; CHS, chalcone synthase; CHI, chalcone isomerase; FLS, flavonol synthase; F3H, flavanone 3β-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; AOMT, flavonoid 3',5'-methyltransferase; UDPG, flavonoid-3-O-glucosyltransferase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, flavonoid glucosyltransferase; OMT, O-methyltransferase.

1.3.3 Crosstalk between abscisic acid and sugar signalling in anthocyanins biosynthesis induction

As presented in Section 1.3.1 and Section 1.3.2, during grape berry development both sugars and ABA regulate anthocyanins biosynthesis process, and some structural genes in the anthocyanins biosynthetic pathway responded to the applications of both molecules. In

Arabidopsis thaliana, Li et al. (2006) found that 14% of the ABA up-regulated genes were induced in response to glucose as well, and 37 genes were repressed by both ABA and glucose, including two genes encoding putative sugar transporters. This demonstrated that the expression of many genes is co-regulated by both sugar and ABA. The knowledge of the mechanisms underlying these interactions between sugars and ABA signalling pathways remains incomplete, and there are three possibilities for ABA and sugar to co-regulate anthocyanins biosynthesis during berry development.

The first one is ABA to promote sugar accumulation, which in turn would induce anthocyanins biosynthesis. Several reports support that hypothesis. Çakir et al. (2003) observed an ASR (ABA-, stress-, and ripening-induced) protein, VvMSA, which can bind to the promoter of the monosaccharide transporter gene *VvHT1*, is induced by sucrose treatment in grape cell suspension, an effect strongly enhanced by ABA. Lecourieux et al. (2010) identified a glycogen synthase kinase type 3 protein kinase gene called *VvSK1,* which expression was up-regulated both by sugars and ABA in grapevine cell suspensions, and overexpression of *VvSK1* up-regulated monosaccharide transporters (*VvHT3*, *VvHT4*, *VvHT5*, and *VvHT6*), resulting in an increase in sugars uptake. Moreno et al. (2011) demonstrated that foliar applications of ABA on grape plants promoted carbon allocation towards berries, which resulted in the monosaccharide levels to dramatically increase in ripe fruits. Murcia et al. (2016) found that ABA improved long-distance sugar transport by enhancing phloem area and promoting the expression of *VvHT2* and *VvHT6* in berry, at *véraison*. Later on, they reported that ABA may improve phloem apoplastic unloading via increasing the expression of *VvHT2*, *VvHT3* and *VvHT6*, favouring sucrose transport and hexoses accumulation in berries at the onset of ripening (Murcia et al., 2018). Additionally, although it is known that high concentrations of glucose repressed the expression level of cell wall invertase (*cwINV*) through a hexokinase-dependant phosphorylation reaction, ABA was shown to increase the expression level of *cwINV* and block the repression induced by glucose (Wang et al., 2017b). Taken together, this means that ABA decreases the concentration of glucose in berry phloem apoplast and induced a decrease of osmotic and turgor pressures via improving

monosaccharide transport from apoplast to berry cells, by increasing the expression of monosaccharide transporter genes. In summary, ABA can increase berry sugar content by improving the expressions of sugar transporters and cell wall invertase in berry.

The second possibility is sugar inducing ABA accumulation to finally induce anthocyanins biosynthesis. In *A. thaliana*, glucose insensitive mutant *gin5* was affected in the HXK-mediated signal transduction pathway, but not on genes regulated by the HXK-independent pathway. The accumulation of ABA was reduced in *gin5* mutant plants, and exogenous ABA can restore normal glucose responses. These information suggest that HXK1 acts upstream of ABA in glucose signalling pathway (Arenas-Huertero et al., 2000). Jia et al. (2011) observed that exogenous sugars, particularly sucrose, can significantly increase ABA levels in strawberry. Moreover, Luo et al. (2019) further found out this effect of exogenous sucrose on ABA level may occur through significantly enhancing the expression of *FaBG* (coding a ß-glucosidase) which was shown to catalyse the release of free ABA from ABA-GE.

The third possibility is ABA and sugar signalling pathways interacting to induce the biosynthesis of anthocyanins (Fig.10). SnRK1 (sucrose non-fermenting-related kinase-1), the homologue of adenosine monophosphate-activated protein kinase (AMPK) from mammals and SNF1 protein from yeast, has been implicated in the regulation of carbon metabolism and energy status (Baena-González et al., 2007). High sugar might inhibit SnRK1 activity via T6P in Arabidopsis (Gazzarrini and Tsai, 2014). Overexpression of *AtSnRK1.1* (*KIN10*) gene blocked sucrose-induce accumulation of anthocyanin under a sucrose concentration of 3%, and repressed the expression of *MYB75/PAP1* which is a key transcription factor for anthocyanin biosynthesis in Arabidopsis (Baena-González et al., 2007). Interestingly, *MYB75/PAP1* is essential for the sucrose-mediated expression of the *DFR* gene in Arabidopsis, and *MYB75/PAP1* is also a putative early DELLA target who act as a key positive regulators in the sucrose signalling pathway controlling anthocyanin biosynthesis and growth (Li et al., 2014). All together, this suggests that high sugar might repress SnRK1 activity to induce anthocyanin biosynthesis. However, in apple, MdSnRK1.1 interacts with and phosphorylates MdJAZ18, which acts as a repressor in the jasmonate acid (JA) signaling pathway, promotes its 26S proteasome-mediated degradation and releases MdbHLH3, which up-regulates the expressions of genes in flavonoid pathway, and ultimately promotes

anthocyanin and proanthocyanidin accumulation (Liu et al., 2017). This finding suggested that SnRK1 can induce anthocyanins biosynthesis. These findings were conflicting with respect to the effect of SnRK1 on anthocyanin synthesis, and the inhibition or induction effect of SnRK1 on anthocyanin synthesis may depend on the expression level of its downstream proteins (i.e. MYB75/PAP1 and MdJAZ18), which was consistent with the function of SnRK1 in maintain energy homeostasis. Moreover, ABA promotes degradation of non-phosphorylated SnRK1 (inactive) in wheat (Coello et al., 2012). Rodrigues et al. (2013) reported that PP2Cs, a protein phosphatase, negatively regulate ABA signalling by interacting with the SnRK1 catalytic subunit, causing its dephosphorylation and inactivation, supporting the fact that ABA activate SnRK1 signalling in Arabidopsis. Hence, ABA and sugar might also interact to induce SnRK1 to affect anthocyanin biosynthesis. Nevertheless, the idea that SnRK1 playing a key role in ABA and sugar signalling cross-talk in grapes is highly tempting.

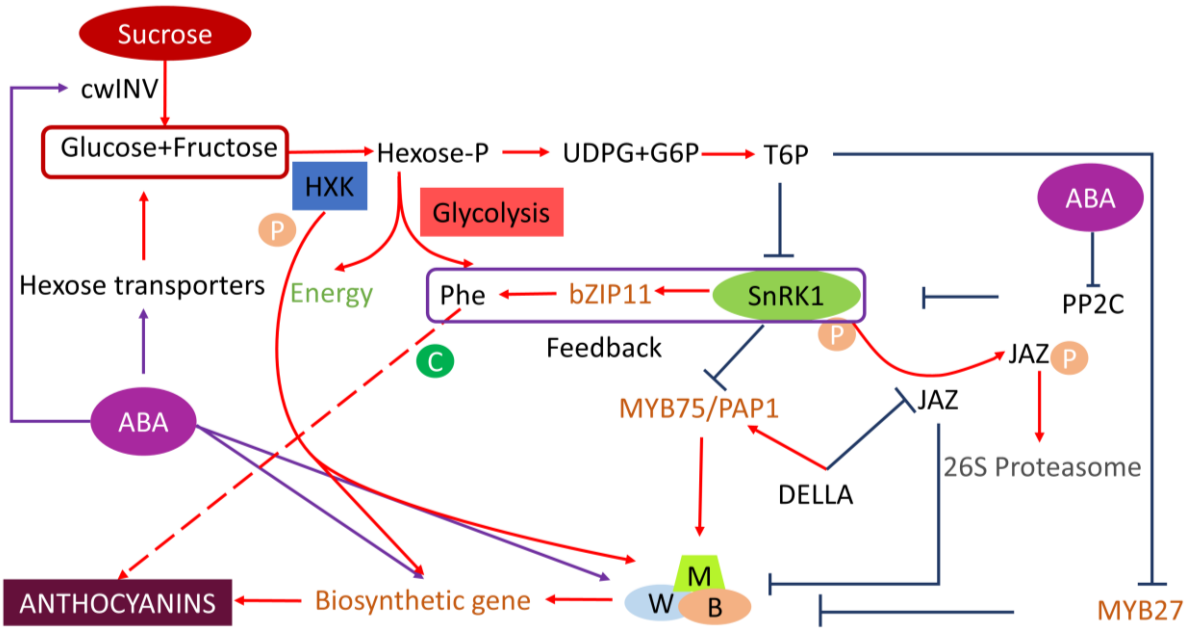

Fig.10 Proposed signalling pathways for anthocyanins synthesis induction by sugar and ABA showing the potential cross-talk points based on previous studies

1.4 Objectives of this work

To face the coming challenges imposed by climate change on grape berry quality and viticultural industry, more knowledge about the effect of viticultural practices on berry composition and the regulation mechanisms of metabolite accumulation in grape is needed to choose and optimize potential adaptive strategies. As viticultural practices aiming to reduce berry sugar at harvest (thus delaying technological ripeness) are related to the modification of leaf-to-fruit ratio, and viticultural practices aiming to improve berry colour are related to the change of ABA level in berry, we mainly investigated the effect and regulation mechanism of leaf-to-fruit ratio manipulation and exogenous ABA application on berry composition, and the relationship between sugar, ABA and anthocyanins in berry. Specifically, the objective was divided into the following partial objectives:

Objective 1, the effect of leaf-to-fruit ratio manipulation on reducing berry sugar and its influence on other components in berry at maturity, including organic acids, free amino acids and anthocyanins. (Chapter 2)

Objective 2, the effect of exogenous ABA application on berry sugar and anthocyanins, and the effect of exogenous ABA in combination with leaf-to-fruit ratio manipulation on berry composition at maturity. (Chapter 2)

Objective 3, the relationship between sugar, ABA and anthocyanins in response to leaf-to-fruit ratio manipulation and exogenous ABA application over fruit development at both metabolite and transcript levels. (Chapter 3)

Objective 4, the relationship between sugar and ABA in inducing anthocyanins in berry at *véraison* and the possible interaction between ABA and sugar. (Chapter 4)

Chapter 2 Differential response of the accumulation of primary and secondary metabolites to leaf-to-fruit ratio and exogenous abscisic acid

Foreword

Leaf-to-fruit ratio modulation is an important tool used to adjust primary and secondary metabolites content and improve berry quality (Kliewer and Dokoozlian, 2005). A severe reduction in leaf-to-fruit ratio resulted in a strong imbalance between sugars and anthocyanins content (Bobeica et al., 2015), which affected berry quality at harvest. To confirm the responses of primary and secondary metabolites to different degrees of leaf-to-fruit ratio and to determine whether plant hormone ABA can restore the coupling relationship between sugar and anthocyanins by inducing anthocyanin synthesis under low leaf-to-fruit ratios, a trial with different leaf-to-fruit ratios (including 2, 4, 6, 8, 10, 12 leaves per cluster) or exogenous ABA (400 $mg.L^{-1}$) on berries were conducted at pre-*véraison* during six growing seasons (2013-2018) with *Vitis vinifera L.* Cabernet Sauvignon fruiting-cuttings. Primary metabolites (sugars, organic acids, free amino acids), secondary metabolites (anthocyanins) and plant hormone (ABA and its catabolism products) were compared under different treatments. The results will be described and discussed in the form of a manuscript submitted for publication to the journal 'Australian journal of grape and wine research'.

Article: Differential response of the accumulation of primary and secondary metabolites to leaf-to-fruit ratio and exogenous abscisic acid

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Abstract

Background and Aims: Climate change is modifying grape berry composition and affecting wine quality and typicity. We evaluated the effects of leaf-to-fruit ratio manipulations and exogenous abscisic acid applications on the accumulation of primary and secondary metabolites in the berries, in order to optimize strategies for adaptation to climate change and maintain the sustainability of wine production.

Methods and Results: A trial including different leaf-to-fruit ratios (2, 4, 6, 8, 10, or 12 leaves per cluster) or exogenous ABA (400 mg/L) application on berries was conducted pre-*véraison* during six growing seasons (2013-2018) with *Vitis vinifera L.* Cabernet Sauvignon fruiting-cuttings in a greenhouse. Reducing the leaf-to-fruit ratio decreased both sugar and anthocyanin content and changed the balance between the two, slightly increased total organic acids, and modified the composition of free amino acids. Moreover, the response of berry composition to leaf-to-fruit ratio changes showed to be correlated with the degrees of leaf-to-fruit ratio. Exogenous ABA significantly enhanced sugar and anthocyanin concentrations and partially restored the balance of sugar and anthocyanins under low leaf-to-fruit ratios, without altering free amino acids or sugar to acids ratios.

Conclusions: Combining leaf-to-fruit ratio manipulation with exogenous ABA applications to fruiting-cuttings offers a potential method to reduce berry sugar concentration, whilst maintaining anthocyanin concentration.

Significance of the Study: This study paves the way for possible adaptation strategies for viticulture to global climate change.

Keywords

ABA, leaf-to-fruit ratio, free amino acids, organic acid, sugar to anthocyanins ratio.

2.1 Introduction

Grape is one of the most economically important fruit crops in the world, and its market value derives more from its quality (i.e. its fruit composition) than its absolute yield. Berry composition is environmentally sensitive and highly influenced by changes in climate, soil components and vineyard management. In the context of climate change, berry composition has been altered by elevated temperatures, light intensity changes, rise of atmospheric CO_2 levels or water deficit that occurred during the past few decades in many wine producing regions (Teixeira et al., 2013, Martínez-Lüscher, 2014, Kizildeniz et al., 2015, Martínez-Lüscher et al., 2016, Van Leeuwen and Darriet, 2016).

Among these changes, decoupling of sugar and anthocyanins accumulation by the elevated temperature is of particular concern as it may disrupt the balance of color and alcohol content in red wines (Sadras and Moran, 2012, Martínez de Toda et al., 2014, Martínez de Toda and Balda, 2015, Salazar-Parra et al., 2018). In France, Laget et al. (2008) observed that sugar concentrations increased, leading to up to 1.5% increase in potential alcohol at harvest in the Hérault region between 1997-2007. In the Languedoc region, the alcohol in wine has risen from 11% to 14% during the last 35 years (van Leeuwen et al., 2019). On the other hand, experiments in the field or greenhouse have shown that high temperature conditions reduce total anthocyanin content in grape berry and altered the anthocyanins profile (Kliewer, 1970, Buttrose et al., 1971, Kliewer and Torres, 1972, Coombe, 1987, Mori et al., 2007, Sadras and Moran, 2012, Fernandes de Oliveira et al., 2015, Martínez de Toda and Balda, 2015, Martínez-Lüscher et al., 2016, de Rosas et al., 2017). In addition, elevated temperature was also reported to affect other ingredients. Organic acids, in particular the malic acid concentration, were reduced during the last decades as the rising seasonal temperature (Jones and Davis, 2000, Vršič and Vodovnik, 2012, Van Leeuwen and Destrac-Irvine, 2017). The response of amino acids to long-term climate change was less studied, but elevated temperature was found to increase the accumulation of specific amino acids including arginine (Arg), proline (Pro), threonine (Thr) and glutamine (Glu) (Kliewer and Lider, 1970,

Torres et al., 2017). The aforementioned changes in berry metabolites may influence wine typicality and even wine industry sustainability in some areas.

To reduce sugar concentrations, one potential strategy is to control carbon source by reducing leaf-to-fruit ratio in order to delay berry ripening and slow the rate of sugar accumulation (Kliewer and Dokoozlian, 2005, Martínez de Toda et al., 2014). Berry sugar content at harvest largely depends on the carbon supply level as a result of photosynthesis in mature leaves. Leaf removal, which is a commonly used canopy management practice to improve berry quality, can reduce assimilates by reducing photosynthetic leaf area, thus reducing sugar input to the berry. By removing 35% of the leaf area apical to the bunch zone when berry sugar content was approximately 16–17°Brix, the time needed to reach the optimal total soluble solids (TSS) of Sangiovese berries was delayed by 2 weeks. Yet, this leaf removal treatment did not significantly change the total phenolic concentration in the berries, the chemical and chromatic characteristics of the wines and the replenishment of soluble sugars, starch and total nitrogen in the canes and roots (Palliotti et al., 2013). Similarly, Poni et al. (2013) found that leaf removal above the bunch zone at pre- and post-*véraison* delayed technological ripeness without affecting colour and phenolics.

However, many factors influence the effect of leaf removal on berry composition, and different treatment methods and processing times would lead to different results. After *véraison*, basal leaves are no longer the main source of assimilates (Poni et al., 1994), so the removal of the medial and apical part of the canopy around *véraison* has attracted attention in recent years as a field practice (Zhang et al., 2017). In general, a leaf-to-fruit ratio around 0.8 to 1.2 m^2/kg produces the maximum level of TSS, berry weight and berry coloration at harvest in vineyard (Kliewer and Dokoozlian, 2005). Although the modification of leaf-to-fruit (L/F) ratio did not affect total organic acids (Parker et al., 2015), it decreased the concentrations of total free amino acids; in particular, a linear decrease in the concentration of proline when the leaf-to-fruit ratio ranged between 1.2 to 0.4 m^2/kg (Kliewer and Ough, 1970). The effect of leaf-to-fruit ratio manipulations at *véraison* on berry composition also depended on the cultivar (Gutiérrez-Gamboa et al., 2019). In a previous study, Bobeica et al. (2015) reported that severe leaf removal (3 leaves per cluster versus 12 leaves per cluster) at

one week before *véraison* in Cabernet Sauvignon and Sangiovese significantly reduced the accumulation of sugar and anthocyanins in the berries. Interestingly, the decrease in anthocyanin level was greater, suggesting that other factors than carbohydrate limitation may also participate in the control of anthocyanins accumulation.

Conversely to carbohydrate limitation, abscisic acid (ABA) is thought to promote berry ripening (Wheeler et al., 2009, Koyama et al., 2010). At the onset of berry ripening, the initial increases in berry ABA may come from phloem import from the leaves (Antolín et al., 2003, Castellarin et al., 2016). Hence, leaf removal might not only reduce carbon source, but also ABA translocation from leaves to berries. The reduction in carbon source and ABA supply may both delay berry ripening, but their effects on berry metabolic content might be distinct. Exogenous ABA application on berries has been shown to stimulate anthocyanin accumulation, and the effects of sprayed ABA depend on the application stage, dose, frequency of application and cultivars (Peppi et al., 2006, Zhu et al., 2016, Koyama et al., 2018, Shahab et al., 2020). Although ABA may increase berry sugar concentration by promoting long-distance sugar transport via an increase in phloem area and by promoting the expression of some hexose transporter genes in leaves and/or berries (Murcia et al., 2016, Murcia et al., 2018), the extent to which ABA improves sugar accumulation remains elusive. Exogenous application of ABA on whole grapevines at pre-*véraison* stages enhanced the skin coloration and decreased sugar accumulation in the berries (Murcia et al., 2017). Yet, application of ABA to berries increased anthocyanin content of grape skins, but did not affect pH, berry weight and TSS (Kataoka, 1982, Mori et al., 2005, Sandhu et al., 2011). Moreover, Wheeler et al. (2009) reported that the application of ABA to berries advanced ripening as measured by skin coloration, berry size and to a lesser extent sugar accumulation. Therefore, to adapt to climate change and help to mitigate the decoupling of anthocyanins and sugars accumulations, exogenous ABA application appears as a potential strategy to re-balance the ratio between sugars and anthocyanins.

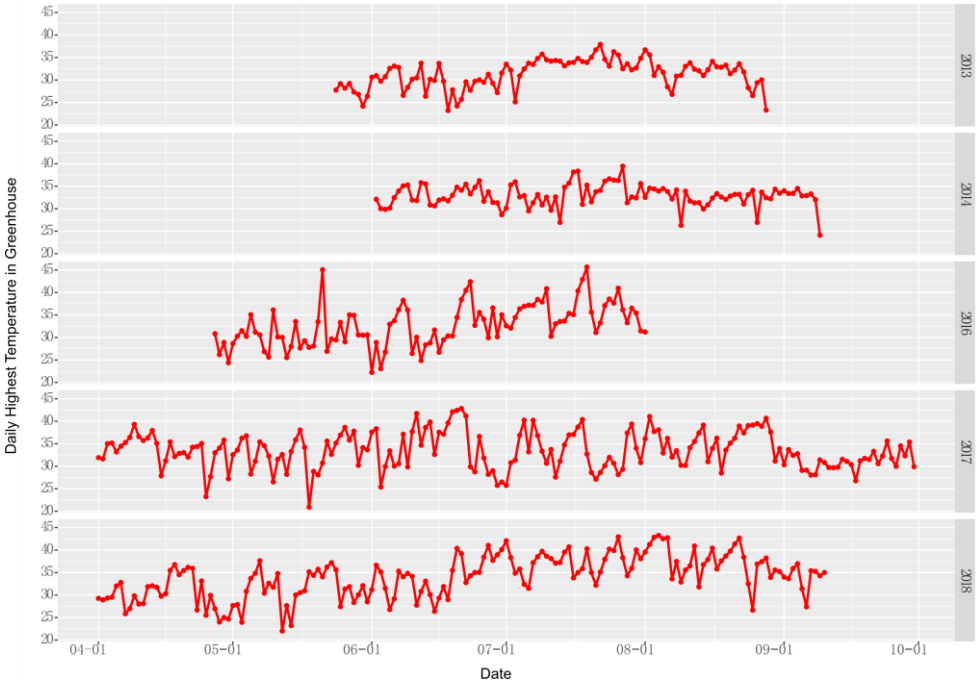
In the present work, a series of trials with different L/F were conducted during four growing seasons (2013-2016) with *Vitis vinifera L.* Cabernet Sauvignon fruiting-cuttings in greenhouse to comprehensively explore the effects of different intensities of leaf removal on

sugar and anthocyanins. On that basis, we combined exogenous ABA applications to gradients of L/F treatments in seasons 2017 and 2018, in order to explore the possibility of adjusting the ratio of sugar to anthocyanins, and further offer an adaptive strategy. Sugars are also used to provide the carbon skeletons for multiple primary and secondary metabolites produced during berry ripening and development changes in sugar concentration can easily affect other components. We thus also examined the contents of organic acids and amino acids in each treatment.

2.2 Materials and Methods

2.2.1 Plant material treatments and sampling

All experiments were conducted with *Vitis vinifera* L. cv. Cabernet Sauvignon in Bordeaux, France. Fruiting-cuttings made up of one vertical shoot with one grape cluster were prepared as described in (Mullins & Rajasekaran, 1981) and grown in a naturally lighted and semi-controlled greenhouse, with chemical disease control treatments applied every 2 weeks. In detail, after pre-rooting, fruiting-cuttings were transplanted into 0.5L volume pots containing a mixture of perlite, sand and vermiculite (1:1:1). After irrigation with water for the first 3 weeks, full strength Hoagland's solution was supplied to each pot with a drip irrigation system 3-5 times per day to avoid any water stress throughout the experimental period. The temperatures data inside the greenhouse during each growing season was shown in Sup Fig.1 and Sup Fig.2.



Sup Fig.1 The daily highest temperature in the greenhouse during the growing seasons from 2013-2018 (Data for 2015 was missing).

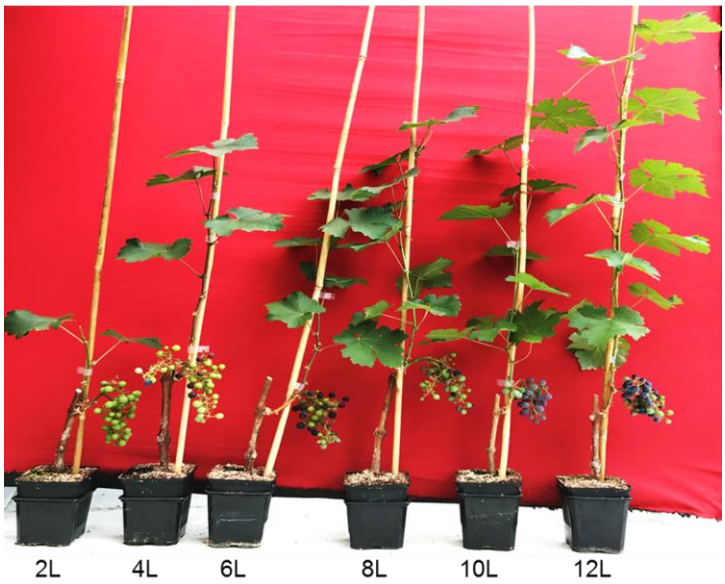
Sup Fig.2 The daily minimum temperature in the greenhouse during the growing seasons from 2013-2018 (Data for 2015 was missing).

The defoliation descending series (Sup. Fig.3), including 2 leaves (2L), 4 leaves (4L), 6 leaves (6L), 8 leaves (8L), 10 leaves (10L), 12 leaves (12L), and exogenous ABA treatments were performed one week before *véraison* stage. All treatments were randomly allocated into 3 blocks, with three homogeneous fruiting-cuttings for each treatment per block. All the leaves underneath and at the first node above the basal cluster were removed firstly in all the treatments when the leaf removal process was conducted to standardize the microclimate effects. Then the leaves were removed from top to bottom, and all secondary shoots were removed throughout the measurement period.

The ABA solution was prepared by dissolving 400 mg ABA in 10 mL 50% ethanol and bringing the solution to 1 L with distilled water added 0.5 mL Tween 20. The final concentration of ABA was 400 mg L^{-1}. The ABA solution was spray-applied directly to the clusters with a handheld sprayer until runoff (approximately 10 mL solution per cluster), ensuring that all berry surfaces were covered. 1L ethanol solution of the same concentration

added Tween 20 was applied similarly to the clusters as the controls. In this study, exogenous ABA was applied in a single dose, and all the treatments were sprayed at dusk (sunset) to minimize ABA photo-degradation.

The experiments were conducted in 6 consecutive growing seasons from 2013-2018, where 2013-2016 focused on the dose-response of berry metabolites to gradients of L/Fs and 2017-2018 focused on the combination effects of L/F with ABA treatments.



Sup Fig. 3 Different L/Fs (including 2, 4, 6, 8, 10, 12 leaves per cluster) treatments on fruiting-cuttings of cv. Cabernet Sauvignon. The photo was taken at 70 DAF in 2017.

2.2.2 L/F Measurement

One plant was randomly selected for each repetition of each treatment. The length and the width of all leaves in this plant were measured at harvest and were used for leaf surface area estimation according to the following equation Montero et al. (2000):

$$\text{Leaf surface area } (cm^2 \text{ /leaf}) = 0.587 \text{ (L x W)}$$

Where, L = Length of leaf blade, W = Width of leaf blade

All the berries were sampled at harvest, and weighed after pedicel removal to calculate the L/F.

2.2.3 Berry processing

All the berries were sampled at the same time defined by the berries of the 12L (reference) treatment reaching maturity, randomly from 3 clusters (vines) of a given treatment within a block and were pooled to form a biological replicate. The maturity of 12L berries were determined by three criteria: a) the seed color reached deep brawn as indicated by Ristic and Iland (2005); b) berry size (measured by an electronic calliper with ± 0.01 mm precision) reached the plateau; b) berry ºBrix reached the plateau before any clear dehydration. Three biological replicates were obtained for each treatment. Sampled berries were immediately put into a tube and dropped into liquid nitrogen. The tubes were reweighed after deep freeze to calculate berry fresh weight and then stored in -80°C for later biochemical analysis. Berries stored in -80°C were slightly thawed (without melting) and separated quickly into skin, pulp, and seed in the laboratory. The skin and pulp were immediately ground into fine powder in liquid nitrogen, using a ball grinder MM200 (Retsch, Haan, Germany).

2.2.4 Sugars, organic acids and free amino acids analysis

Aliquots of 500 mg fine powder of pulp or 250 mg of berry skin were used to perform the extraction of primary metabolites according to Torres et al. (2017) with minor modifications. Extraction of primary metabolites was done by adding 2 mL of 80% ethanol at 80°C for 15 min, centrifuged 10 min at 5000 x *g*, followed by another extraction step using 2 mL of 50% ethanol, and finally extracted using 2mL of de-ionized water. The extracts were dried in a Speed-Vac, re-dissolved in 2 mL ultrapure water and used for determinations of sugars, organic acids and amino acids. Glucose and fructose contents were measured enzymatically with an automated micro-plate reader (EPOCH2, Biotek Instruments Inc., Winooski, VT, USA) using the Glucose/Fructose kit from BioSenTec. Tartaric and malic acids were determined with an automated colorimetric method using the TRAACS 800 autoanalyzer (Bran-Luebbe) according to Bobeica et al. (2015). Amino acids were determined by UHPLC

(UltiMate 3000 UHPLC system with FLD-3000 Fluorescence Detector, Thermo Electron SAS, Waltham, MA USA) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate according to Torres et al (2017). All the amino acids were identified and quantified with external chemical standards purchased from Sigma (St Louis, MO, USA).

2.2.5 Anthocyanins analysis

A 250 mg berry skin powder aliquot was freeze-dried for 72 h and the dried powder (~20 mg) were extracted in 500 µL methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.2-µm polypropylene syringe filter for UHPLC analysis. Each individual anthocyanin was analyzed as described in De la Cruz et al. (2012) and modified by (Hilbert et al., 2015) by UHPLC coupled to a Diode Array Detector (UltiMate 3000, Therma Scientific, CA, USA). Quantification was carried out by peak area integration at 520 nm, and malvidin-3-glucoside (Extrasynthèse, Lyon, France) standard was used for quantifying anthocyanin concentrations.

2.2.6 ABA and ABA metabolites analysis

An aliquot of 250 mg of pulp or skin powder was freeze-dried for 72 h and the dried powder (20~50 mg) was extracted using a stable isotope dilution assay (Speirs et al., 2013). ABA, ABA-GE, PA, and DPA in samples were measured using liquid chromatography/mass spectrometry (Agilent 6410 Triple Quadrupole LC-MS/MS with Agilent 1200 series HPLC, Agilent Technologies Inc., Santa Clara, USA). The column used was a Phenomenex Luna 5 µm C18 (2) 75 mm x 4.6 mm and the column temperature was set at 40 °C. The solvents used were nanopure water and acetonitrile, both containing 0.05 % acetic acid (v/v). Samples were eluted with a linear 15 min gradient starting at 10 % acetonitrile (v/v) and ending with 90 % acetonitrile (v/v). Compounds were identified by retention times and multiple reactions monitoring of mass-to-charge ratio (m/z) for parent and product ions of native and deuterated internal standards (Rossdeutsch et al., 2016).

2.2.7 Data statistical analysis

All data analysis was conducted with R software (R Development Core Team, 2013). Differences between the treatments were tested for significance by applying the analysis of variance (ANOVA) followed by Tukey post-hoc test (p value < 0.05).

2.3 Results

2.3.1 Leaf-to-fruit ratio treatments validation

As shown in Fig.1, a gradient of L/F was achieved by removing leaves from fruiting Cabernet Sauvignon cuttings to leave 10, 8, 6, 4, and 2 leaves per cluster (10L, 8L, 6L, 4L and 2L, respectively) with 12 leaves per cluster (12L) considered the reference treatment. From 2014 to 2018 (not measured in 2013), except for a slightly lower ratio in 2014, the leaf area-to-fruit fresh mass ratio did not significantly differ among years for a given treatment, although the leaf area-to-fruit fresh mass ratio of 12L was slightly higher in 2015 and 2016 than in the other years. The data also showed that exogenous ABA treatments did not affect the L/F of vines significantly, which was expected because leaf growth had already stopped at the time of treatments. The average L/F were 5.36, 10.04, 13.00, 16.97, 21.67, 28.58 $cm^2\ g^{-1}$ as the number of leaves increased from 2 to 12. The average L/F of 2L was around 81.2% less than that of the control (12L).

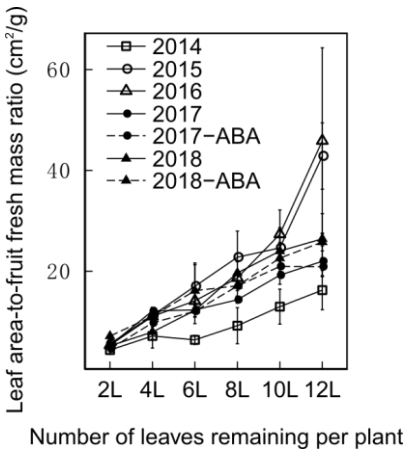


Fig.1 Effect of leaf removal on the L/F. Growing seasons: 2014 (□); 2015 (○); 2016 (△); 2017 (●); 2018 (▲). Treatments: L/F manipulation (─); L/F with exogenous ABA application (┄). Vertical bars indicate standard error (SE) (n=3).

2.3.2 Berry metabolite concentrations upon various leaf-to-fruit ratio treatments

2.3.2.1 Sugars

Although the reducing sugar (glucose and fructose) concentrations in control (12L) fluctuates with years, its concentrations in both of skin and pulp tended to gradually decrease with decreasing L/F (Fig. 2A, B). Moreover, the sugar concentration was likely to be a bit more sensitive to the change of L/F in the skin than in the pulp. The average reduction of sugar concentrations in the skin was 43.0% when comparing 2L with 12L, while that in the pulp was only 27.2%. The maximum reduction in skin reducing sugar reached as high as 63.1% when comparing 2L with 12L in 2016. The sugar concentration of 8L and 10L showed no significant difference in both of skin and pulp, compared with 12L.

2.3.2.2 Organic acids

The concentration of total organic acids slightly increased with low L/F in both pulp and skin (Fig. 2G, H). In the pulp, a marked increase was observed in 2015 and 2016, a moderate one in 2013 and no significant effect in 2014. In the skin, total organic acid concentration

increased with the low L/F in 2013, 2015 and 2016 and was not affected during the 2014 season. In addition, the growing season had a strong effect, with higher organic acid concentrations in 2015 and 2016 than in the other years. In the pulp, the average of total organic acid concentration in 2015 and 2016 was 3.2 times that in 2013 and 2014, while that was 3.8 times in the skin.

Malic (Fig. 2C, D) and tartaric (Fig. 2E, F) acid concentrations were differentially affected by L/F modulation at harvest. In the pulp, malic acid concentration increased at low L/F, while the concentration of tartaric acid was hardly affected. In the skin, malic acid concentrations also increased at low leaf-to-fruit ratio, while the tartaric acid concentrations were unchanged (2013, 2014 and 2016) or slightly decreased, with a maximal 35.8% reduction in the tartaric acid concentration in 2015 compared with 12L.

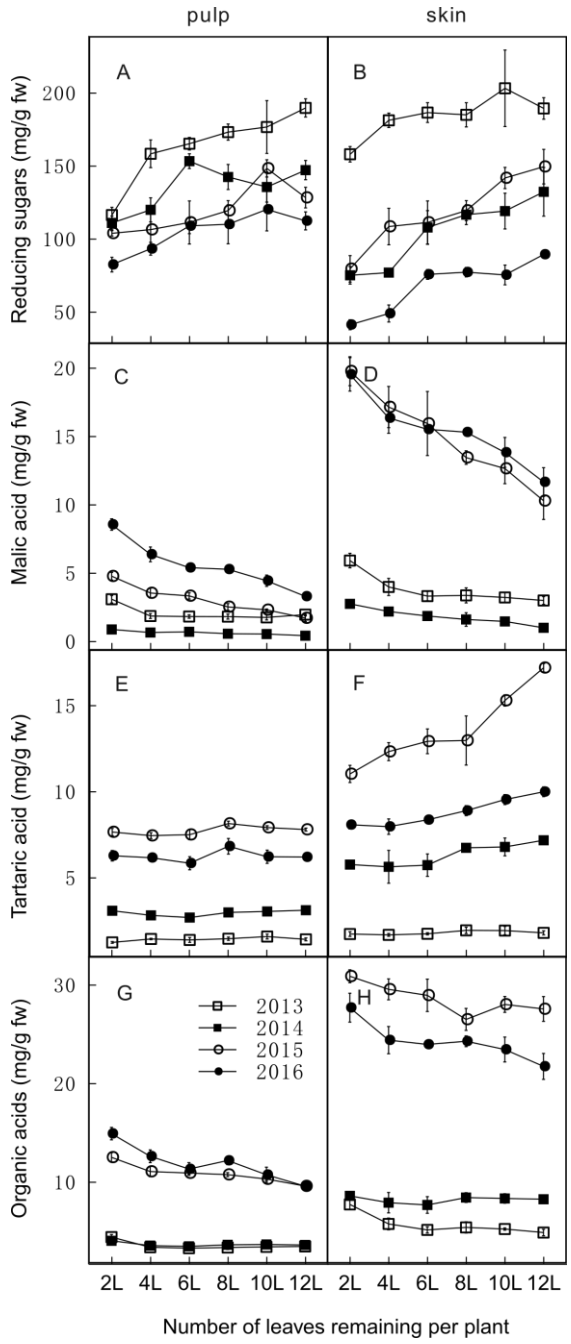


Fig.2 Effect of L/F modulation on the concentrations of reducing sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in skin (right panel) and pulp (left panel) at harvest from 2013 to 2016. Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).

2.3.2.3 Amino acids

Amino acids showed increase, decrease or bell-shaped responses in response to increasing L/F (Fig. 3). Moreover, these response patterns were also affected by growing season. A significant decrease in total amino acid concentrations was observed in pulps in 2015 as the decrease of L/F, while a large increase occurred in skins in 2013. In other growing seasons, the total free amino acid concentrations showed less difference between the L/F treatment in pulp and skins. Individual amino acids concentrations were also influenced by L/F and the growing seasons, with Pro, Arg and Thr being always the major amino acids in both skin and pulp. Amino acid profiles were found to vary between the berry components. Pulp of mature berries contained a very high concentration of Pro, but a much lower concentration of Arg and Thr in all treatments. Meanwhile, skin of berries contained moderate levels of Pro, Arg and Thr. In addition, in general, free amino acids associated with Glycolysis and TCA cycle process showed to vary with the gradient of L/F, including His, Ileu, Pro, Glu, GABA, Gln, Arg, Asp, Ala and Ser. Among them, Pro decreased with the decrease in the L/F both in pulp and skin, while Arg and Ser increased. Besides, Thr in pulp and skin increased with L/F decrease.

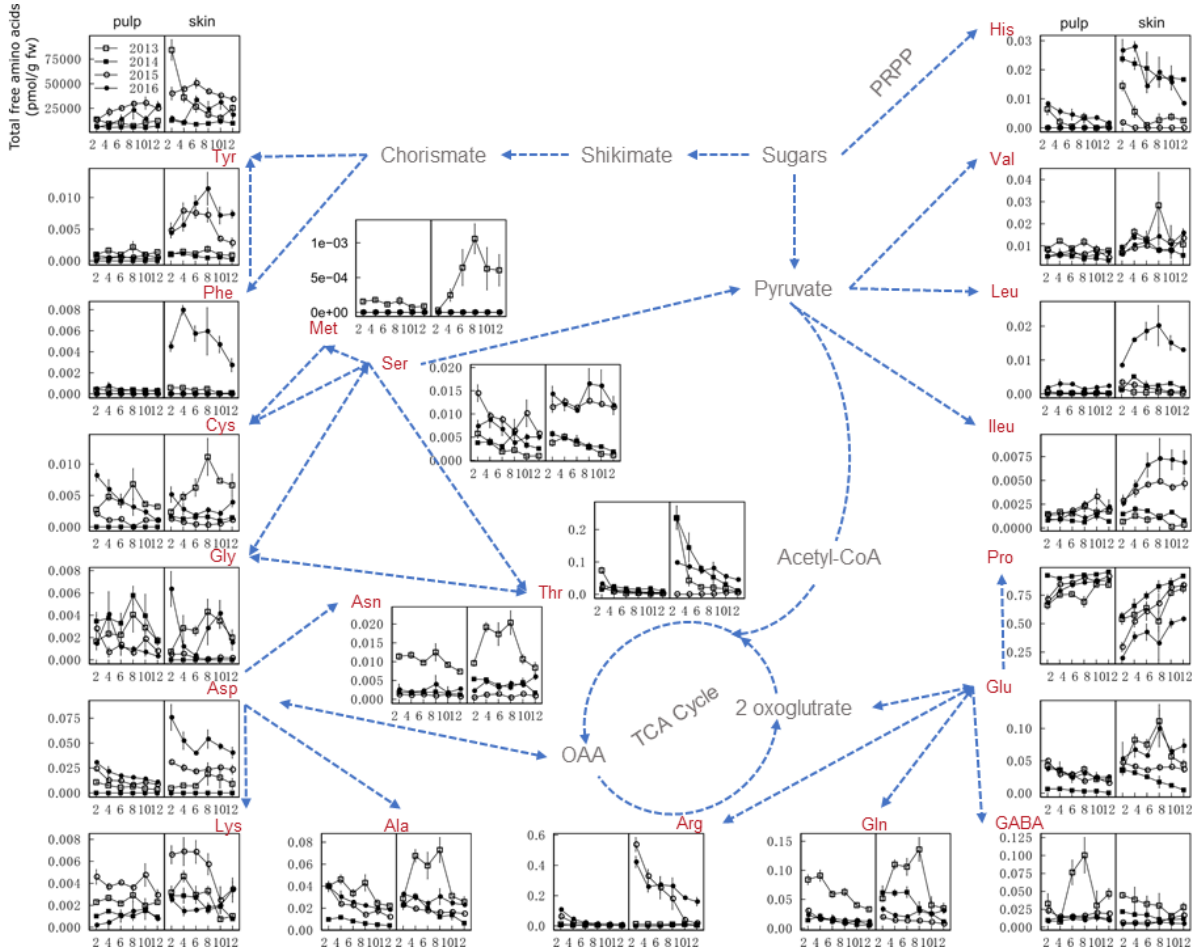


Fig.3 Effect of L/F modulation on free amino acid compositions of skin (right panel) and pulp (left panel) at harvest. The first figure at the top-left is the effect of L/F modulation on total free amino acids from 2013 to 2016. The remaining panels are the effect of L/F modulation on the proportion of individual amino acids in skin (right panel) and pulp (left panel) at harvest. X-axis and Y-axis represent the number of leaves and the proportion of individual amino acids to total amino acids (%), respectively. Amino acid pathways are inferred according to KEGG. Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).

2.3.2.4 Anthocyanin concentration and composition

Total anthocyanin concentrations in skins decreased significantly with the reduction of L/F (Fig. 4A). The average reduction of total anthocyanins across the 4 years was 77.8% when comparing 2L with 12L. The 2L treatment caused a maximum reduction of 82.6%, compared to 12L treatment, in 2015. The anthocyanin composition at harvest was also significantly affected by leaf removal (Fig. 4B, C and Sup. Fig. 4). The ratio of di-OH to tri-OH anthocyanins (Fig. 4B) tended to decrease with low L/F, indicating that the biosynthesis of di-OH anthocyanins was more sensitive than that of tri-OH ones to defoliation. In addition, reducing L/F decreased non-acylated anthocyanins more than acylated ones and accumulation of non-acylated anthocyanins was less than acylated anthocyanins in 2L and 4L in 2014 and 2016 (Fig. 4C).

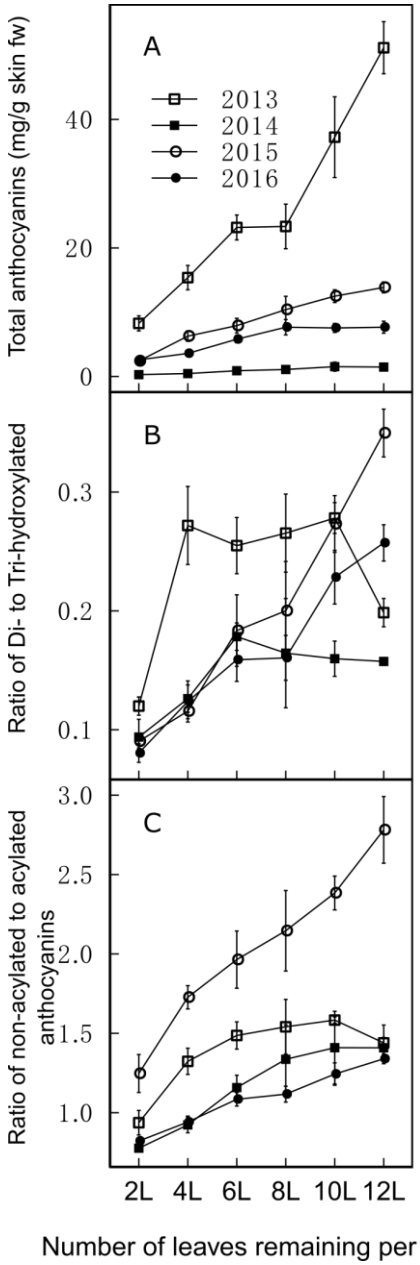
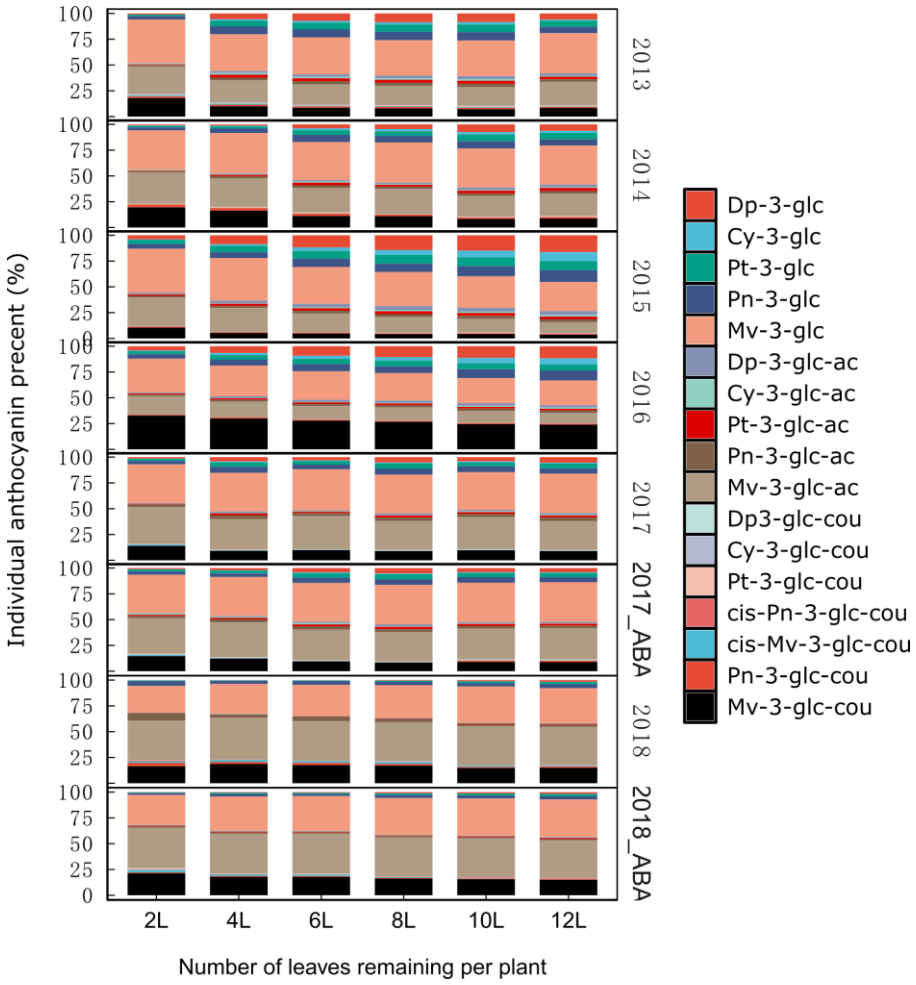


Fig.4 Effect of L/F modulation on anthocyanin concentrations (A), the ratio of di- to tri-hydroxylated anthocyanins (B), and the ratio non-acylated to acylated anthocyanins (C). Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).



Sup Fig.4 Effect of L/F modulation and exogenous ABA application on anthocyanins compositions of skin at harvest. Dp-3-glc: Delphinidin-3-glucoside; Cy-3-glc: Cyanidin-3-glucoside; Pt-3-glc: Petunidin3-glucoside; Pn-3-glc: Peonidin-3-glucoside; Mv-3-glc: Malvidin-3-glucoside; Dp-3-glc-ac: Delphinidin-3-acetyl-glucoside; Cy-3-glc-ac: Cyanidin-3-acetyl-glucosides; Pt-3-glc-ac: Petunidin-3-acetyl-glucosides; Pn-3-glc-ac: Peonidin-3-acetyl-glucosides; Dp-3-glc-cou: Delphinidin-3 *p*-coumaroyl-glucoside; Mv-3-glc-ac: Malvidin-3-acetylglucosides; Cy-3-glc-cou: Cyanidin-3 *p*-coumaroyl-glucoside; cis Pn-3-glc-cou: cis-Peonidin-3 p-coumaroyl-glucoside; Pt-3-glc-cou: Petunidin-3 *p*-coumaroyl-glucoside; cis-Mv-3-glc-cou: cis-Malvidin-3 *p*-coumaroyl-glucoside; Pn-3-glc-cou: Peonidin-3 *p*-coumaroyl-glucoside; Mv-3-glc-cou: Malvidin-3 *p*-coumaroyl-glucoside. Vertical bars indicate SE (n=3).

2.3.2.5 ABA and its catabolic products

To determine the effect of L/F treatment on the profile of ABA at harvest in berries, the concentration of free ABA, inactive ABA-glucose ester (ABA-GE), and ABA catabolism products, PA (phaseic acid) and DPA (dihydrophaseic acid), were determined in pulp and skins at harvest respectively for the 2015 to 2017 seasons (Fig.5). Meanwhile, to determine the effect of ABA application on ABA metabolism at harvest, ABA and ABA metabolites were also quantified in the pulp and skin at harvest in 2017. The results showed that, with or without exogenous ABA treatment, the concentration of free ABA was higher in the skin than in the pulp, as were ABA-GE and other degradation products (Fig 5). However, no significant differences were observed between different L/F for ABA and ABA metabolites. Conversely, after exogenous ABA treatment on pre-*véraison* berries in 2017, the concentrations of free ABA and ABA-GE were significantly higher compared to untreated berries in skin at harvest (Fig. 5B, D). However, no significant differences in the ABA catabolism products between ABA treated and untreated berries in 2017 were observed. In addition, ABA content varied significantly between years, the concentrations of ABA in 2015 in untreated berries were almost as much as after exogenous ABA treatment in 2017.

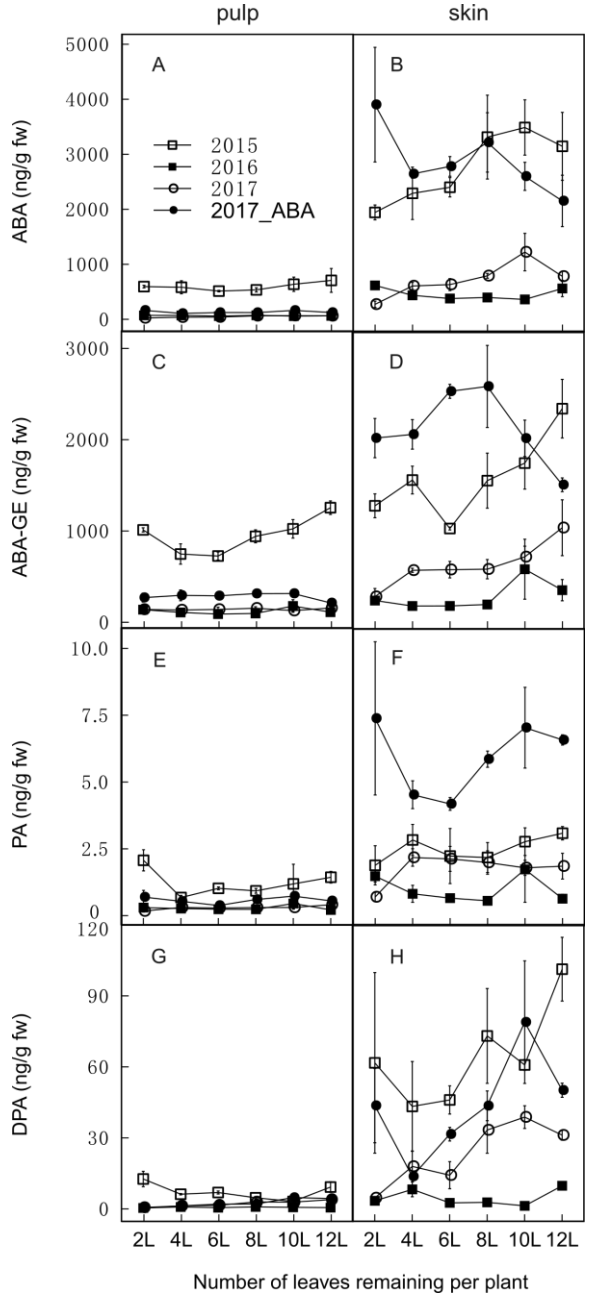


Fig.5 Concentrations of free ABA and ABA metabolites (ng/g fw) in skin (right panel) and pulp (left panel) of the berries at harvest with different L/Fs from 2015 (□), 2016 (■) and 2017 (○) or ABA treatment: 2017_ABA (●). ABA (A and B), abscisic acid; ABA-GE (C and D), ABA glucose ester; PA (E and F), phaseic acid; DPA (G and H), diphaseic acid. Vertical bars indicate SE (n=3).

2.3.2.6 Exogenous ABA treatment and berry metabolites under various L/Fs

As the results of L/F experiments in the previous four years (2013-2016) showed that the accumulation of anthocyanins was more sensitive to the influence of leaf removal than that of sugars and organic acids, experiments combining L/F manipulation with ABA application were conducted in 2017 and 2018. To determine the effect of such treatments on berry composition at harvest, reducing sugars, organic acids, free amino acids and anthocyanins were analyzed.

For a given L/F, exogenous ABA increased sugar concentrations in both pulp and skin, and this effect was amplified at lower L/F (Fig. 6A, B). Hence, compared with treatments without ABA treatment, the reduction trend of sugar concentration with the decrease of L/F was partly alleviated. In 2017 and 2018, the decrease of L/F led to a maximum reduction of 61.6% and 52.7% of sugar in the skin, while the L/F combined with ABA treatment led to a maximum reduction of 40.8% and 47.2%, respectively. Meanwhile, the maximum reductions of sugar concentrations in pulp, were 40.3% and 27.3% respectively in 2017 and 2018 in L/F treatment, while they were 26.3% and 27.9% respectively, in L/F combined with ABA treatment. Here, the reducing sugar concentrations in the skin of 2017 samples did not differ between various L/F after ABA treatment.

ABA reduced malic acid contents in pulp and skin, leading to no significant differences between the various L/Fs (Fig. 6C, D). ABA had little effect on tartaric acid concentration under high L/F, but under low L/F, ABA reduced the concentration of tartaric acid in the pulp and slightly increased the content in the skin (Fig. 6E, F). The total organic acid concentrations were unchanged after ABA treatment, except a slight decrease under low L/F in pulp in 2017 (Fig. 6G, H). ABA had little consistent effects over the two seasons (2017 and 2018) on the distributions and contents of free amino acids (Fig. 7).

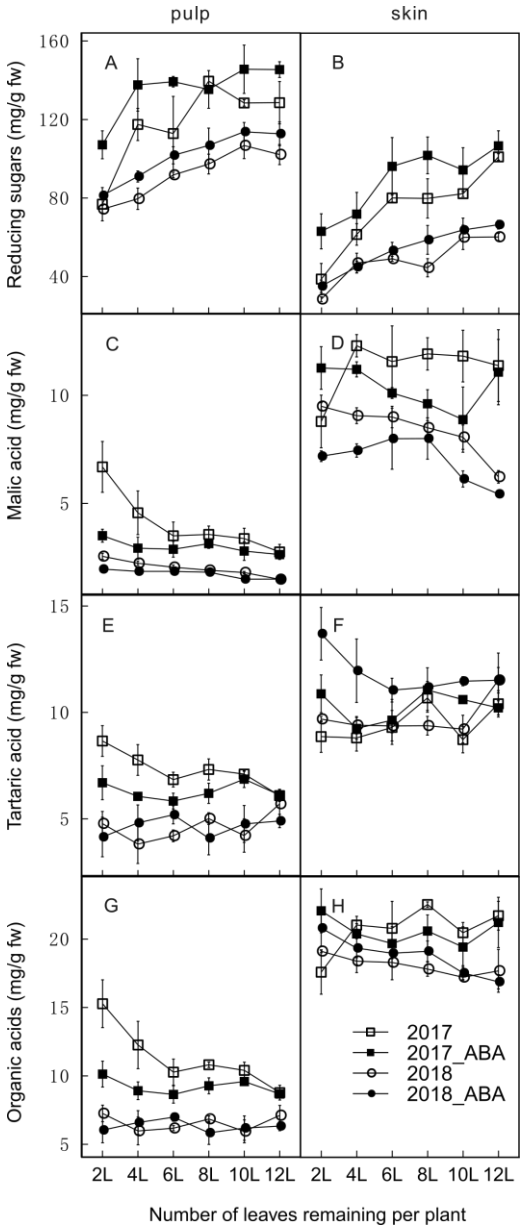


Fig.6 Effect of exogenous ABA application on the concentrations of reducing sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in skin (right panel) and pulp (left panel) with different L/F in 2017 and 2018 at harvest. Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

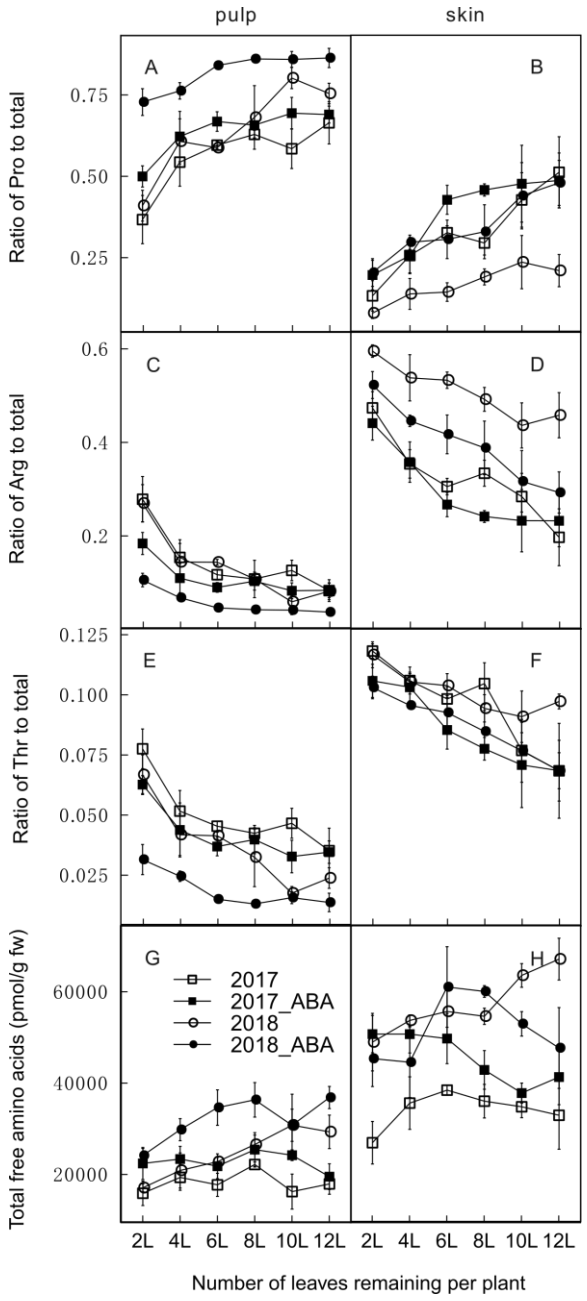


Fig.7 Effect of exogenous ABA application on free amino acid compositions of skin (right panel) and pulp (left panel) at harvest. The ratio of proline to total amino acids (A and B), the ratio of arginine to total amino acids (C and D), the ratio of threonine to total amino acids (E and F) and the total free amino acids (G and H). Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

ABA treatment markedly increased anthocyanins concentrations in skin under same L/Fs. The total anthocyanin concentrations decreased with the number of leaves, but the difference between different L/Fs in the same year was not significant (Fig. 8A). In 2017 and 2018, the maximum reductions of anthocyanins were 90.5% and 90.7% respectively in L/F treatment, while those were 52.4 % and 56.1%, respectively, in L/F combined with ABA treatment. ABA had a greater impact on tri-OH anthocyanins than on di-OH anthocyanins, resulting in a lower di- to tri-OH anthocyanins ratio in 2018, while the effect in 2017 was not significant (Fig. 8B). ABA had no significant effect on the ratio of non-acylated to acylated anthocyanins (Fig. 8C).

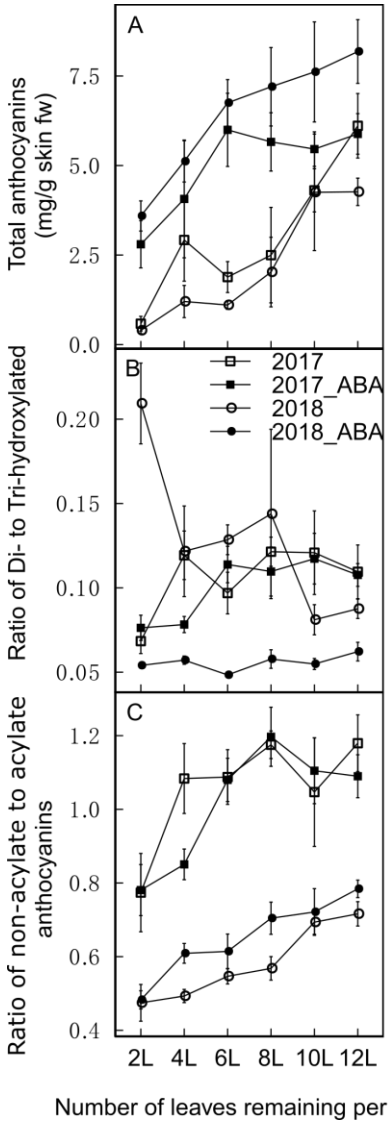


Fig.8 Effect of exogenous ABA application on total anthocyanin concentrations (A), the ratio of di- to tri-hydroxylated anthocyanins (B), and the ratio non-acylated to acylated anthocyanins (C) in the skin with different L/Fs at harvest in 2017 and 2018. Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

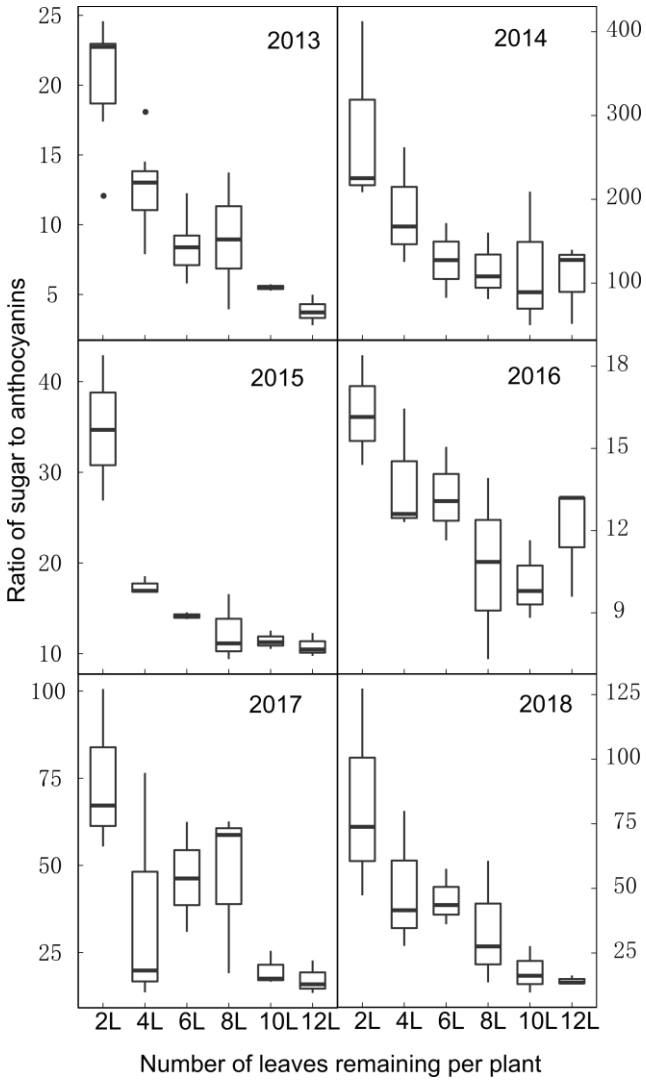
2.4 Discussion

2.4.1 Manipulation of L/F decoupled sugar and anthocyanins at harvest

Reducing L/F by leaf removal one week before *véraison* caused a significant reduction in the accumulation of sugars and anthocyanins in ripe berries. This result confirms previous studies reporting that the accumulation of both sugars and anthocyanins was affected by L/F manipulation (Weaver, 1963, Martínez de Toda and Balda, 2013, Pastore et al., 2013, Wu et al., 2013, Bobeica et al., 2015). However, other studies conversely showed that anthocyanin accumulation increased (Diago et al., 2012, Lee and Skinkis, 2013, Feng et al., 2017) or was unaffected (Palliotti et al., 2013, Poni et al., 2013, Pastore et al., 2017) after leaf removal. These contradictory observations are most likely due to the differences in several factors including the timing, position, degree of defoliation and harvest time (Lee and Skinkis, 2013, Martínez de Toda and Balda, 2013, Martínez de Toda et al., 2014). In fact, in most cases, early defoliation around the cluster zone increased anthocyanin accumulation in the berry due to better sunlight exposure and modification of the cluster microclimate (Diago et al., 2012, Gatti et al., 2012, Lee and Skinkis, 2013, Feng et al., 2017). In the present study, the leaves at the cluster zone were removed in all the vines one week before *véraison* to avoid any difference in microclimate around the cluster. Therefore, our results reflected mainly the effects of L/F linked to carbon supply to the berries.

Furthermore, the accumulation of total anthocyanins in the skin was more sensitive to leaf removal than skin sugar. The ratio of sugar to anthocyanins increased when L/F decreased, and its fluctuation range increased (Fig. 9A and Sup Fig. 5). More specifically, the decrease of leaf numbers from 12L to 2L resulted in an average 77.8% reduction in four growing seasons (2013-2016) in anthocyanin concentrations at harvest (Fig. 4A), paralleled by only 43.0% and 27.2% reductions in sugars in skin and pulp (Fig. 2A, B), respectively. The results clearly indicate that the accumulation of sugars and anthocyanins are uncoupled by reducing L/F. This might be due to preferential carbon allocation to sugar accumulation rather than anthocyanin biosynthesis under carbon limitation, as suggested by Bobeica et al. (2015).

However, Edwards et al. (2016) developed a system to directly manipulate whole vine carbon assimilation without affecting canopy size or cluster environment via reduced air CO_2 concentration, finding no impact in the ratio of anthocyanins to sugars during maturation, despite a 50% reduction in maturation rate. Therefore, the decoupling seen here may be due to more extreme differences in L/F or be influenced by additional factors, possibly interactively with L/F. As mentioned in the introduction, ABA in the berry is likely a combination of endogenous synthesis and import from the rest of the vine through the transpiration stream (Antolín et al., 2003, Castellarin et al., 2016). ABA is an important inductor of anthocyanin synthesis (Pilati et al., 2017). Moreover, Kataoka et al. (1982) reported that endogenous ABA levels in the skin and pulp did not increase throughout ripening period after leaf removal at *véraison*. Hence, the uncoupling of sugar and anthocyanins by reducing L/F might be caused by the reduction of ABA supply from leaves. No significant differences of ABA and its catabolites were observed at harvest between different L/Fs. Hence, it is necessary to compare the changes of ABA concentration with berry development under different L/Fs for verification. Moreover, further efforts are warranted to investigate why L/F manipulation uncoupled berry sugar and anthocyanin contents.



Sup Fig.5 Effect of L/F modulation on the ratio of sugar to anthocyanins from 2013-2018.

2.4.2 Effects of L/F manipulation on organic acids and free amino acids at harvest

The concentration of malic acid increased while tartaric acid was unaffected or even decreased, which together led to a slight increase of the total organic acids when the L/F was reduced. Combined with the effects on sugar and anthocyanins, the reduction of L/F resulted in a decrease in the ratio of sugar to acids and an increase in the ratio of acids to anthocyanins (Fig. 9B and C). Organic acids, in particular malic acid content, decrease at high temperature

as mentioned in introduction, but the efficiency of reducing L/F on the increase of the total organic acids was often below expectations (Parker et al., 2015, Gatti et al., 2019). Parker et al. (2015) thought that limited photosynthesis was not generally limiting organic acids, so reducing the L/F did not affect them. Our results showed that the effect of L/F on organic acid was correlated with the degrees. In 2015 and 2016 the L/F treatment 4L, 6L, 8L and 10L increased total organic acid in both skin and pulp, compared with 12L, whereas in 2013 only the 2L treatment did show an effect. No effect was noticed in 2014, maybe due to the leaf-to-fruit ratios achieved with 6L, 8L, 10L and 12L in this season were significantly lower than in the other years. Moreover, the effects of L/Fs on organic acids were different among growing seasons and tissues (skin and pulp). For example, in 2013, 2015 and 2016 total organic acid were influenced by L/Fs in both pulp and skins, while tartaric acid was hardly affected in pulp, in all the growing seasons. These findings suggest that the effects of L/F manipulation on organic acids are complex. On one hand, by reducing the leaf-fruit ratio, the onset of *véraison* was delayed, that is, the green period was prolonged, which meant that the duration of organic acids synthesis extended (Dharmadhikari, 1994). On the other hand, reducing carbon source by reducing L/F might result in an increase in the sugar converted from malic acid by gluconeogenesis (Hawker, 1969, Famiani et al., 2018), which may accelerate the degradation of malic acid after *véraison*. Hence, the concentrations of organic acids at harvest are the result of addition and subtraction. This may explain the variation in organic acids with the L/F manipulation.

Amino acids profiles were influenced by the modulation of L/F, especially for some free amino acids associated with Glycolysis and TCA cycle process. GABA is a four-carbon amino acid that is reported to be involved in maintaining C/N balance by regulating carbon flux into the TCA cycle (reviewed by Shelp et al., 1999, Hildebrandt et al., 2015), and it also can be catabolised to Glu and subsequently Asp to maintain osmotic homeostasis during fruit ripening (Michaeli and Fromm, 2015). The changes in these intermediates linking primary and secondary metabolites are likely to be in response to changes in sugar concentration in different L/Fs. Pro decreased with decreased L/F, while arginine and threonine increased. Several reports have shown that the concentrations of Pro, Arg and Thr in grape skins increased with the elevated temperature (Kliewer and Lider, 1970, Torres et al., 2017). Pro,

Arg, Thr and volatile compounds account for more than 82 % of the sensory characteristics of wines (Temerdashev et al., 2019). Pro may contribute to berry taste, but it is relatively non-assimilable as a nitrogen source under anaerobic fermentation conditions, and musts with high Pro content may lead to low yeast biomass (Ingledew et al., 1987, Stines et al., 2000). Arg is one of main yeast nitrogen sources (Garde-Cerdán et al., 2009). Hence, L/F manipulation could reduce the ratio of Pro to Arg in berries and improve some sensory characteristics. In addition, Phe, the substrate of phenylalanine ammonia-lyase (PAL), which is one of the key enzymes in phenolic biosynthesis pathway (Kataoka et al., 1983), showed an increase trend with the decrease of L/F in 2013 and 2016. Dai et al. (2014) reported that Phe was increased under low sugar supply, while anthocyanin accumulation decreased in an innovative in-vitro cultured berry model. Hence, this result suggests that the decrease of anthocyanins under low carbon supply might not be due to lower amounts of precursor molecules.

2.4.3 Effect of exogenous ABA application

Exogenous ABA application increased both the accumulation of sugar and anthocyanins in berries, causing 20.9% and 11% increases in sugar concentrations, paralleled by 131.3% and 345.2% in anthocyanins, as compared to untreated berries. Moreover, exogenous ABA led to a more marked anthocyanin increase in the berries under low L/F, suggesting that the ABA-induced increase in anthocyanins is L/F dependent. This is not the case for the ABA-induced increase in berry sugar concentration. As shown in Fig. 9A, compared with the increased ratio of sugar and anthocyanins caused by low L/F, the ratio of sugar to anthocyanins kept stable among different L/F treatments after exogenous ABA application. Hence, exogenous ABA application restored the uncoupling of sugar and anthocyanins resulting from L/F manipulation.

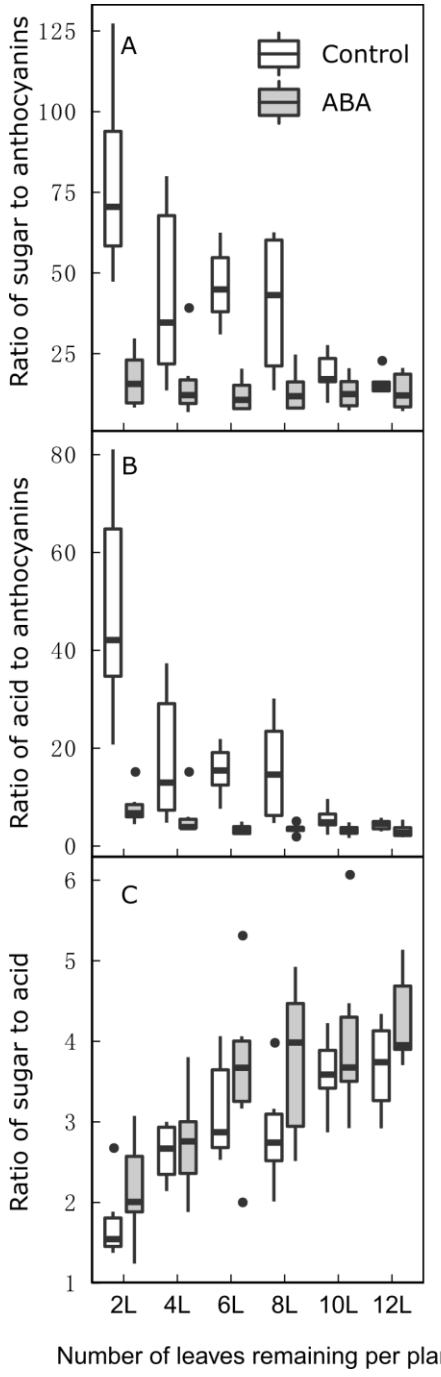


Fig.9 Effect of L/F modulation and exogenous ABA application on the ratio of sugar to anthocyanins (A), the ratio of acid to anthocyanins (B), and the ratio of sugar to acid (C). Data reported for 2017 and 2018.

Several studies investigated the biological mechanisms underlying ABA-induced increase of anthocyanins and sugar content in grape berries. The transcriptomic comparison between pre-

véraison and *véraison* revealed that exogenous ABA promoted anthocyanins accumulation by affecting the expression of genes related to anthocyanins biosynthesis (He et al., 2020). The structural genes *CHI*, *F3H*, *DFR*, *ANS* and *UFGT,* and the regulatory factors *MybA1,* and *MYBA2* in berries were found to be upregulated by exogenous ABA (Yamane et al., 2006, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018). About sugar, Moreno et al. (2011) found that exogenous ABA application on whole plant resulted in an increase in berry sugar by promoting carbon allocation to berries. Furthermore, Murcia et al. (2018) found that, ABA application on the whole plant upregulated the expression of *VvHT2*, *VvHT3* and *VvHT6* in berries. This would favor sucrose transport through a decrease of osmotic and turgor pressures in berry phloem and improvement of apoplastic unloading. Hence, ABA treatment on berry might also increase sugar accumulation in berry by regulating sugar transport. ASR proteins were induced by ABA, stress, and ripening in berry (Çakir et al., 2003), and over-expressing an *ASR* gene, *FaASR*, could increase anthocyanins, endogenous ABA and sucrose accumulation, and induced the transcription levels of *CHS* and *UFGT* in strawberry (Zhongjie et al., 2015). Moreover, an ASR protein, VvMSA showed to positively regulate the expression of *VvHT*1 by binding to its promoter (Çakir et al., 2003). Hence, ABA might induce anthocyanins and sugar accumulation by regulating ASR proteins expression.

There is no consensus on the effect of exogenous ABA on anthocyanins composition. Mori et al. (2005) found that the composition of anthocyanins in Pinot Noir was not significantly affected by ABA. He et al. (2020) found that exogenous ABA upregulated the expression of *F3'H* and increased the proportion of di-hydroxylated anthocyanins in Cabernet Sauvignon. A similar conclusion was reached by Villalobos-Gonzalez et al. (2016), and they found that di-hydroxylated anthocyanin concentrations after spaying ABA at pre-veraison increased from 65 days after veraison while the total anthocyanins began to decrease. In our study, the ratio of di- to tri-OH anthocyanins decreased after ABA application in berries in 2018 which were harvest after 56 days after veraison, but not in 2017 which were harvest after 33 days after veraison. This discrepancy may be the result of multiple factors including temperature, cultivars and sugar content. In fact, a study about the effect of temperature and ABA on anthocyanins profiles showed that in Cabernet Sauvignon, exogenous ABA decreased the

ratio of di- to tri-OH anthocyanins at 33 °C, and increased the ratio of di- to tri-OH at 40 °C, with no change at 25 °C (Deis et al., 2012). Moreover, the effect of ABA treatment under different temperatures on anthocyanins profiles in Malbec also depended on sugar content: at 22°Brix, ABA significantly decreased the ratio of di- to tri-OH anthocyanins at 33 °C, but at 24°Brix, ABA had no significant effect at the same temperature (Deis et al., 2012).

The ratio of acid to anthocyanins decreased after ABA application in response to decreased L/Fs (Fig 9B). As the reducing sugar concentration increased with minor change in acid concentration after ABA treatment, the ratio of sugar to acid increased slightly, especially in 2L (Fig 9C). Our results showed that ABA promoted the decrease of organic acids, especially for malic acid. This result was consistent with cytosolic NADP-dependent malic enzyme (ME) increasing in pulp after exogenous ABA treatment at *véraison* (Giribaldi et al., 2010), while NADP-malic enzyme is considered to play a key role to regulate the malate breakdown (Yinshan et al., 2017). In addition, part of malic acid might be another source of sugar increased after ABA treatment through gluconeogenesis (Hawker, 1969, Famiani et al., 2018).

2.5 Conclusions

In this study, reducing of L/F was shown to decrease sugar content at harvest significantly, but led to the uncoupling of sugar and anthocyanin accumulations during ripening. It also increased the sugar to anthocyanins ratio and decreased the sugar to acids ratio. Low L/F also affected berry free amino acid content, decreasing the proportion of proline and increasing that of arginine and threonine. The effects of L/F on compositions in berry depended on the extent of L/F modification. Exogenous ABA application alleviated the uncoupling of sugar and anthocyanins accumulation in berries under reduced L/F, but had a weak effect on organic acids and did not affect free amnio acids. In summary, the combination of different L/Fs manipulation with ABA application looks promising to adjust the concentrations of sugar and anthocyanins in response to climate change in grape berry. Further efforts need to be made to uncover the mechanisms underlying the differential responses of berry compositions during berry ripening development in response to L/F and ABA treatment.

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Figures

Fig.1 Effect of leaf removal on the L/F. Growing seasons: 2014 (□); 2015 (○); 2016 (△); 2017 (●); 2018 (▲). Treatments: L/F manipulation (─); L/F with exogenous ABA application (---). Vertical bars indicate standard error (SE) (n=3).

Fig.2 Effect of L/F modulation on the concentrations of reducing sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in skin (right panel) and pulp (left panel) at harvest from 2013 to 2016. Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).

Fig.3 Effect of L/F modulation on free amino acid compositions of skin (right panel) and pulp (left panel) at harvest. The first figure at the top-left is the effect of L/F modulation on total free amino acids from 2013 to 2016. The remaining panels are the effect of L/F modulation on the proportion of individual amino acids in skin (right panel) and pulp (left panel) at harvest. X-axis and Y-axis represent the number of leaves and the proportion of individual amino acids to total amino acids (%), respectively. Amino acid pathways are inferred according to KEGG. Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).

Fig.4 Effect of L/F modulation on anthocyanin concentrations (A), the ratio of di- to tri-hydroxylated anthocyanins (B), and the ratio non-acylated to acylated anthocyanins (C). Growing seasons: 2013 (□); 2014 (■); 2015 (○); 2016 (●). Vertical bars indicate SE (n=3).

Fig.5 Concentrations of free ABA and ABA metabolites (ng/g fw) in skin (right panel) and pulp (left panel) of the berries at harvest with different L/Fs from 2015 (□), 2016 (■) and 2017 (○) or ABA treatment: 2017_ABA (●). ABA (A and B), abscisic acid; ABA-GE (C and D), ABA glucose ester; PA (E and F), phaseic acid; DPA (G and H), diphaseic acid. Vertical bars indicate SE (n=3).

Fig.6 Effect of exogenous ABA application on the concentrations of reducing sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in skin (right panel) and pulp (left panel) with different L/F in 2017 and 2018 at harvest. Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

Fig.7 Effect of exogenous ABA application on free amino acid compositions of skin (right panel) and pulp (left panel) at harvest. The ratio of proline to total amino acids (A and B), the

ratio of arginine to total amino acids (C and D), the ratio of threonine to total amino acids (E and F) and the total free amino acids (G and H). Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

Fig.8 Effect of exogenous ABA application on total anthocyanin concentrations (A), the ratio of di- to tri-hydroxylated anthocyanins (B), and the ratio non-acylated to acylated anthocyanins (C) in the skin with different L/Fs at harvest in 2017 and 2018. Treatment: L/F manipulation in 2017 (□) and 2018 (○); L/F with exogenous ABA application in 2017 (■) and 2018 (●). Vertical bars indicate SE (n=3).

Fig.9 Effect of L/F modulation and exogenous ABA application on the ratio of sugar to anthocyanins (A), the ratio of acid to anthocyanins (B), and the ratio of sugar to acid (C). The data were derived from the concentrations of metabolites in the skin in 2017 and 2018.

Supplementary data

Sup Fig. 1 The daily highest temperature in the greenhouse during the growing seasons from 2013-2018 (Data for 2015 was missing)

Sup Fig. 2 The daily minimum temperature in the greenhouse during the growing seasons from 2013-2018 (Data for 2015 was missing)

Sup Fig. 3 Different L/Fs (including 2, 4, 6, 8, 10, 12 leaves per cluster) treatments on fruiting-cuttings of cv. Cabernet Sauvignon. The photo was taken at 70 *véraison* in 2017.

Sup Fig. 4 Effect of L/F modulation and exogenous ABA application on anthocyanins compositions of skin at harvest. Dp-3-glc: Delphinidin-3-glucoside; Cy-3-glc: Cyanidin-3-glucoside; Pt-3-glc: Petunidin3-glucoside; Pn-3-glc: Peonidin-3-glucoside; Mv-3-glc: Malvidin-3-glucoside; Dp-3-glc-ac: Delphinidin-3-acetyl-glucoside; Cy-3-glc-ac: Cyanidin-3-acetyl-glucosides; Pt-3-glc-ac: Petunidin-3-acetyl-glucosides; Pn-3-glc-ac: Peonidin-3-acetyl-glucosides; Dp-3-glc-cou: Delphinidin-3 *p*-coumaroyl-glucoside; Mv-3-glc-ac:

Malvidin-3-acetylglucosides; Cy-3-glc-cou: Cyanidin-3 *p*-coumaroyl-glucoside; cis Pn-3-glc-cou: cis-Peonidin-3 p-coumaroyl-glucoside; Pt-3-glc-cou: Petunidin-3 *p*-coumaroyl-glucoside; cis-Mv-3-glc-cou: cis-Malvidin-3 *p*-coumaroyl-glucoside; Pn-3-glc-cou: Peonidin-3 *p*-coumaroyl-glucoside; Mv-3-glc-cou: Malvidin-3 *p*-coumaroyl-glucoside. Vertical bars indicate SE (n=3).

Sup Fig. 5 Effect of L/F modulation on the ratio of sugar to anthocyanins from 2013-2018.

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Chapter 3 Mechanistic exploration on the regulation of anthocyanins and sugar accumulation by carbon source limitation and exogenous ABA application

Foreword

Our results in Chapter 2 and those from Bobeica et al. (2015) both confirmed an uncoupling of sugar and anthocyanins by carbon source limitation, and we also found that under carbon source limitation, exogenous ABA application partially restored the ratio of anthocyanin to sugar in berry.

To further explore the mechanisms of the regulation of carbon source limitation and exogenous ABA on berry composition, we compared the effect of three different ways to limit carbon source, including early leave removal at pea size, late leave removal at pre-*véraison*, and phloem girdling at pre- *véraison*, on berry composition, in parallel withthe changes on transcript levels associated with sugar, anthocyanin and ABA metabolism. The results will be described and discussed in the form of a manuscript submitted for publication.

Abstract

Previous studies have reported the uncoupling of sugar and anthocyanins by carbon source limitation, and we found that exogenous ABA application on berry partially restore the ratio of anthocyanin to sugar under carbon source limitation. To optimize these viticultural practices to adapt to climate change, we need more knowledge about the regulation mechanisms of metabolite accumulation under carbon source limitation and exogenous ABA application in grape. In this study, we first compared the effects of different ways of carbon source limitation, including limiting leaf area at pea sized (2L-P), and limiting leaf area (2L-V) or phloem girdling (12L-girdling) at pre-*véraison*, on berry composition. All three ways of carbon source limitation significantly reduced sugar and anthocyanin concentrations. Of them, 2L-P treatment delayed the onset of ripening, but 2L-V treatment resulted in the most severe decoupling of sugar and anthocyanin during berry ripening. The 12L-girdling treatment had less effect on anthocyanin compared to 2L-V, as a result of the late increase in endogenous ABA that up-regulates the expression level of *MybA1*. By analyzing the expression level of genes involved in anthocyanin biosynthesis, we found the genes related to anthocyanins synthesis, including *CHS2*, *CHS3*, *CHI*, *F3H*, *DFR*, *LODX*, *UFGT*, *MybA1* and *MybA2* were down-regulated under low leaf-to-fruit ratio, while seven genes, including *CHI*, *F3H*, *F3'5'H*, *LODX*, *UFGT*, *MybA1* and *MybA2* were up-regulated after exogenous ABA treatment. In addition, exogenous ABA caused an increase in the expression of *CHS2* and *CHS3* only under low leaf-to-fruit ratio. Meanwhile, exogenous ABA was showed to up-regulate the expression of *NCED1* and *NCED2* but had little effect on the genes related to sugar, and leaf-to-fruit ratio also had little effect on the genes related to ABA. This result suggested that both source limitation and exogenous ABA regulated the concentration of sugar and anthocyanins through differential gene expression, and exogenous ABA can partially offset the effect of carbon source limitation on the ratio of sugar to anthocyanins by up-regulating anthocyanins synthesis genes.

Keywords

Vitis vinifera cv. Cabernet-Sauvignon, source limitation, ABA, decoupling of anthocyanins and sugar, Real-time qPCR

3.1 Introduction

Ongoing climate changes is impacting grape and wine quality by affecting berry ripening dynamics and composition at harvest. Elevated temperature affects both primary (sugars, organic and amino acids) and secondary (polyphenols, aroma and aroma precursors) berry metabolite content. Indeed, in the past decades, it has been noted that berry sugar content increased, whereas malic acid and anthocyanin content decreased (Pillet et al., 2015, Pons et al., 2017, Torregrosa et al., 2017) leading to unbalanced berry composition. The increase in sugars and the decrease in anthocyanin accumulation led to uncoupled accumulation kinetics of anthocyanins and sugars in berry under high temperature, which affects the quality of red wines (Sadras and Moran, 2012, Arrizabalaga et al., 2018).

To try to cope with the uncoupling of anthocyanins and sugars under elevated temperature, adaptation strategies should be taken to increase anthocyanin contents, and/or reduce sugar contents relatively. Modifying the relationship between carbon sources and sinks is a common strategy widely used to control sugar contents in grape berry. Martínez de Toda et al. (2014) and Poni et al. (2013) reported that delaying the berry ripening process by source limitation could partially restore the anthocyanins to sugars ratio disrupted by elevated temperatures. However, the effects of carbon source limitation on adjusting sugar and anthocyanins concentrations in berries varied with the timing (Kliewer, 1970, Tardáguila et al., 2008), position (Zhang et al., 2017), degree of leave removal (Kliewer, 1970, Kliewer and Dokoozlian, 2005), cultivars (Lanari et al., 2013) and harvesting time (Martínez de Toda and Balda, 2013, Martínez de Toda et al., 2014). Generally, early leaf removal, such as at pre-bloom or fruit-set stages, improved both sugar and anthocyanin content (Diago et al., 2012, Gatti et al., 2012, Lee and Skinkis, 2013, Pastore et al., 2013, Risco et al., 2014, Feng et al.,

2017). On the contrary, late leaf removal (i.e. around *véraison*) significantly reduced the accumulation of sugars and total anthocyanins during grape ripening (Weaver, 1963, Martínez de Toda and Balda, 2013, Pastore et al., 2013, Wu et al., 2013, Bobeica et al., 2015). Moreover, berry anthocyanins content was more affected by source limitation than sugar content, which led to lower anthocyanin to sugar ratio at harvest (Bobeica et al., 2015), hampering the modification of source-sink ratios as a direct adaptation strategy under climate change.

ABA has been proposed as a key hormone in triggering the onset of the ripening process in grapes (Fortes et al., 2015, Villalobos-Gonzalez et al., 2016, Pilati et al., 2017). The application of exogenous ABA on berry at pre-*véraison* or post-*véraison* stages effectively increased anthocyanin content at harvest, and may also cause a lesser extent increase in sugar concentration (Wheeler et al., 2009, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018), thus improving the ratio of anthocyanins to sugar. Our results in Chapter 2 showed that exogenous abscisic acid (ABA) application at pre-*véraison* stages can partially restore the decoupling of anthocyanins and sugars at harvest under low source limitation (Wang et al. unpublished). Thus, exogenous ABA application combined with appropriate carbon source limitation may regulate the concentration of sugar and anthocyanins in berry to adapt to climate change. However, the underlying mechanisms of the source limitation and exogenous ABA application affecting sugar and anthocyanins accumulation in berry during development and ripening are poorly understood, which may affect the actual outcome and the application of this strategy at the vineyard.

Sugar accumulation in ripening berries is mainly affected by the carbon input from the sources (photosynthetic leaves). Sugar is mainly produced through photosynthesis in mature leaves and transported to the berries via phloem during berry development and ripening in the form of sucrose. In berries, upon phloem unloading, sucrose is hydrolysed into glucose and fructose that are stored in vacuoles of pericarp cells (Swanson and El-Shishiny, 1958, Conde et al., 2007). Multiple transporters and enzymes are involved in sugar transport and accumulation during berry repining. Two sucrose transporters VvSUC11 and VvSUC12 were reported to affect sugar accumulation in berry by facilitating the loading of sucrose from the

apoplast into the parenchyma cells (Manning et al., 2001). Of 20 putative hexose transporters, 7 *VvHT*s including *VvHT*1, *VvHT*2, *VvHT*3 ((also named *VvHT7*), *VvHT*4, *VvHT*5, *VvHT*6 (also identified as *VvTMT1*) and *VvHT*11 have been identified to be most probably involved into berry hexose accumulation (reviewed by Afoufa-Bastien et al., 2010). Several Sugars Will Eventually be Exported Transporter (SWEET) efflux proteins were shown to be highly expressed in berries, and *VvSWEET10* was reported to contribute to berry glucose and fructose contents and thus influence the soluble sugar composition in grapes (Zhang et al., 2019). Hence, a combination of ABA applications and carbon source limitation manipulations might help to reduce sugar content in berry by affecting the activity and expression of these transporters and enzymes. In fact, in tomato, it was confirmed that the increase of leaf-to-fruit ratio could modulate the activity of the key sugar metabolism enzymes and increase the expression of hexose transporter genes (*LeHTs*) (Aslani et al., 2020). In grape, Murcia et al. (2016) reported that ABA improved long-distance sugar translocation by enhancing phloem area and promoting the expression of *VvHT2* and *VvHT6* in leaves at *véraison*. Furthermore, they showed that ABA may improve apoplastic unloading by increasing the expression of *VvHT2*, *VvHT3* and *VvHT6*, favouring sucrose transport and hexoses accumulation in berries at the onset of ripening (Murcia et al., 2018).

Anthocyanins are synthesized via the phenylpropanoid pathway, starting with the amino acid phenylalanine (Boss et al., 1996). The genes encoding anthocyanin biosynthetic enzymes and the transcription factors that activate or repress them have been identified in grape (Kobayashi et al., 2002, Ageorges et al., 2006, Deluc et al., 2006, Bogs et al., 2007, Deluc et al., 2008, Matus et al., 2009, Koyama et al., 2014, Pérez-Díaz et al., 2016). All the genes of this pathway except *UFGT* are expressed in the early stages of berry development, and the expressions of *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, and *UFGT* peak around *véraison* (Boss et al., 1996, Bogs et al., 2006). ABA and carbon source limitation manipulation may affect anthocyanins concentration by regulating the expression of these genes. However, only few studies focused on the regulation of carbon source limitation on anthocyanins synthesis in grapes.

Sugars may affect anthocyanins biosynthesis in two ways. On one hand, sugars are important metabolites for the biosynthesis of anthocyanins, since they provide carbon skeletons for phenylalanine biosynthesis. Indeed, the anthocyanin concentrations in grape berry are often positively correlated with the sugar level, especially in the skin (González-SanJosé and Diez, 1992, Liang et al., 2011). When reducing source carbon supply levels by reducing atmospheric CO_2 concentration, anthocyanins decreased at the same rate as the concentration of sugar during the ripening of grape berries (Smith et al., 2019). However, severe source limitation by reducing leaf-to-fruit ratio might cause less carbon to be allocated for anthocyanins synthesis, and lead to the decoupling of the accumulation of sugars and anthocyanins in ripening berry (Bobeica et al., 2015). On the other hand, sugars are also important signalling molecules involved in anthocyanins synthesis regulation; and the phosphorylation of hexose by hexokinase (HXK) has been reported to account for the sugar signal transduction leading to anthocyanin accumulation (Vitrac et al., 2000, Zheng et al., 2009). The structural genes, *F3H*, *DFR* and *LDOX*/*ANS*, encoding the enzymes that directly participate in the formation of anthocyanins, were demonstrated to be induced by sugars in *vitis vinifera* cells (Gollop et al., 2001, Gollop et al., 2002, Zheng et al., 2009).

Finally, ABA was reported to accelerate anthocyanins accumulation by upregulating the expression of anthocyanins biosynthesis related genes, including structural genes *CHI*, *F3H*, *DFR*, *ANS* and *UFGT,* and the regulatory transcription factors *MybA1* and *MYBA2* (Yamane et al., 2006, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018). Furthermore, exogenous ABA application and endogenous ABA levels may affect anthocyanin biosynthetic genes in different ways (Saito et al., 2018). Although both application of exogenous ABA or Abz (Abscinazole, a specific inhibitor of abscisic acid (ABA) 8'-hydroxylase CYP707A that induces endogenous ABA levels to increase) promoted the expression of *VvUFGT* and *VvMYBA2* genes and the accumulation of anthocyanin in 'Kyoho' grape berries (Jia et al., 2018), different genes expression profiles were found by RNA-seq analysis by Saito et al. (2018).

In this work, we firstly compared the impact of different source limitation conditions, including leaf removal at two developmental stages (pea sized and one week before *véraison*),

as well as phloem girdling, on berry quality traits, including soluble sugars, organic acids, free amino acids and anthocyanins. The effects of exogenous ABA application and carbon source limitation on berry composition also were analysed, and the expression of genes involved in sugar and anthocyanins accumulation and endogenous ABA synthesis and response were detected. By integrating the metabolites data and related gene expression levels, we were able to explore the mechanism of source limitation and exogenous ABA application on anthocyanins and sugar accumulation during berry ripening, which is important in choosing efficient adaptation strategies in viticultural management practices to mitigate the effect of climate change.

3.2 Materials and methods

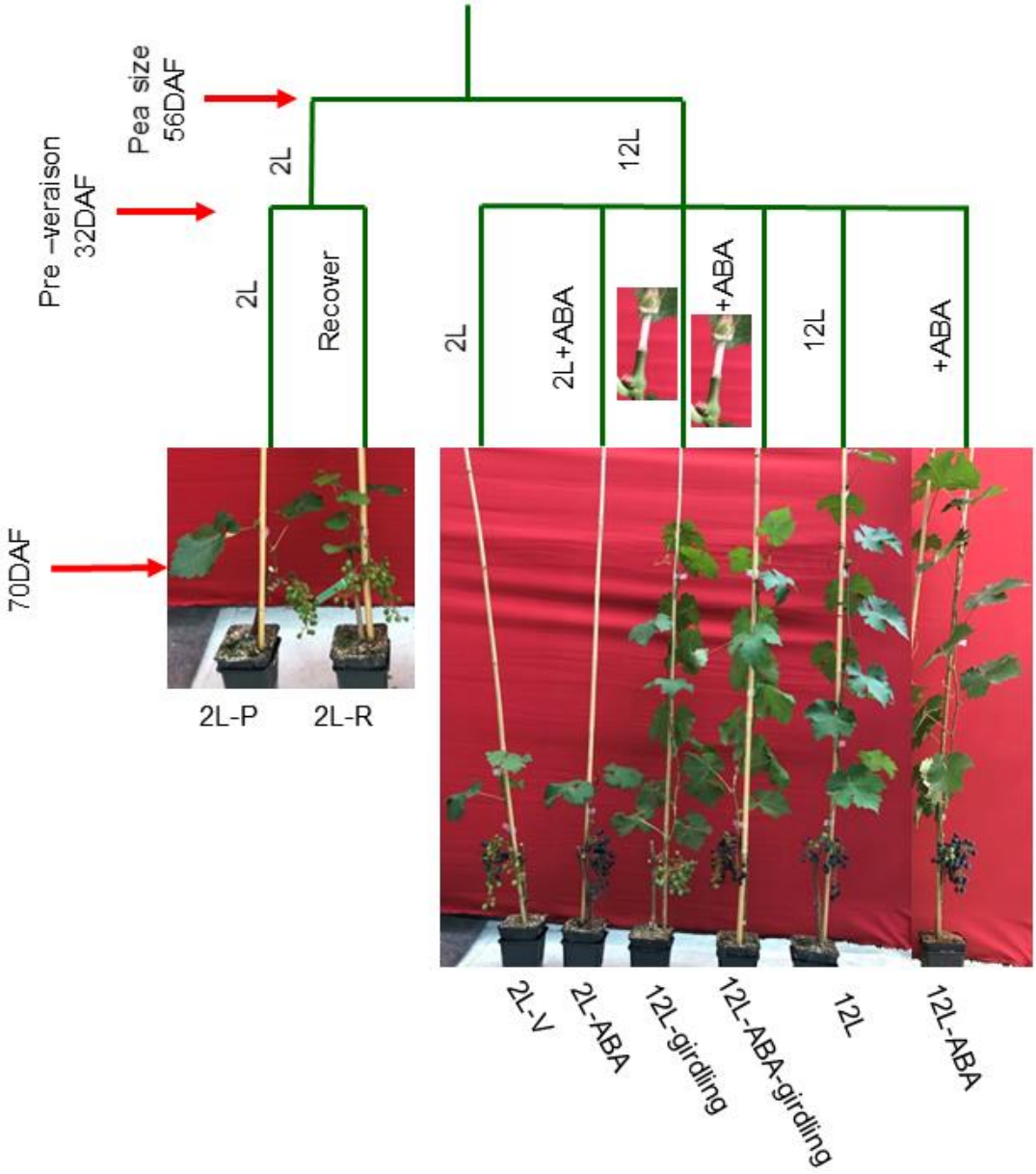
3.2.1 Plant material and sampling

This study was conducted in 2017 and 2018 on fruiting-cuttings of *Vitis vinifera* cv. Cabernet Sauvignon in Bordeaux, France. Fruiting-cuttings consisting in one vertical shoot bearing a single grape cluster were prepared as described in (Mullins and Rajasekaran, 1981) and grown in a naturally lighted and semi-controlled greenhouse, with chemical disease control applied every 2 weeks.

In 2017, three kinds of different source limitation treatments were conducted to compare their effects on berry compositions with a no source limitation control (12 leaves per vine, 12L). The carbon source limitation treatments were early leaf removal (2L-P) at 32 days after flowering (DAF), late leaf removal (2L-V) at 56 DAF and a single stem-girdling around 1 cm long between the second and the third leaf above the cluster on the plants with 12 leaves (12L-girdling) at 56 DAF, respectively (Sup Fig. 1). In addition, a treatment with a recovery of leaf area by allowing new leaf development at 56 DAF based on 2L-P (2L-R) was performed in order to explore if the recovery of leaf area is able to restore the effect of carbon source limitation. Meanwhile, exogenous ABA application was conducted on the treatment 2L-V, 12L-girdling and 12L, named 2L-ABA, 12L-ABA-girdling and 12L-ABA, respectively. The ABA solution was prepared by dissolving 400 mg ABA in 10 mL 50% ethanol and bringing the solution to 1 L with distilled water supplemented with 0.5 mL Tween 20. The final concentration of ABA was 400 mg. L^{-1}. The ABA solution was spray-applied directly to the clusters with a handheld sprayer until runoff (approximately 10 mL solution per cluster), ensuring that all berry surfaces were covered. A water/ethanol/Tween 20 spray was applied similarly to the clusters as the controls. In 2017, we repeated 2L-V, 12L-girdling and 12L treatments to confirm the effect of carbon source limitation. All the treatments were sprayed at sunset to minimize ABA photo-degradation. All treatments were randomly located into three blocks within the greenhouse, with six homogeneous fruiting-cuttings for each treatment in each block. Leaves underneath the basal cluster were removed

firstly in all the treatments to standardize the microclimate effects. Then the leaves were removed from top to bottom, and the remaining leaves and all secondary shoots were removed throughout the measurement period.

In 2017, berries of treatments 2L-P, 2L-R, and 12L were sampled 8 times at 32, 40, 49, 56, 63, 70, 83 and 96 DAF, and 2L-V, 2L-ABA,12L-girdling, 12L-ABA-girdling and 12L-ABA were sampled at 56, 63, 70, 83 and 96 DAF. In 2018, berries for all treatments were sampled at 47, 54, 68, 82 and 96 DAF. *Véraison* was determined at the time when 50 % of the berries in 12L had changed colour and corresponded to 63 and 54 DAF in 2017 and 2018 respectively. At each sampling date, between 15 and 30 berries were sampled in randomized way from the top, middle and bottom of the four clusters which represented one biological replicate. Three biological replicates were obtained for each treatment at each sampling date. At harvest (96 DAF), all remaining berries were sampled. Sampled berries were immediately put into a tube and dropped into liquid nitrogen. The tubes were reweighed after deep freeze to calculate berry fresh weight and then stored in -80°C for further analysis.



Sup. Fig.1 Schematic representation of different treatments. The legend is the same with Fig.2. Photograph was taken at 70 day after flowering.

3.2.2 Leaf area measurement

One plant was randomly selected for each repetition of each treatment. The length and the width of all leaves in this plant were measured at harvest and were used for leaf surface area estimation according to the following equation of Montero et al. (2000):

$$\text{Leaf surface area } (cm^2/\text{leaf}) = 0.587\ (L \times W)$$

Where, L = Length of leaf blade, W = Width of leaf blade

3.2.3 Berry biochemical composition

3.2.3.1 Sugars, organic acids and amino acids

Aliquots of 500 mg fine powder of berry were used to perform the extraction of primary metabolites according to Torres et al. (2017) with minor modifications. Extractions of primary metabolites was done by adding 2 mL of 80% ethanol at 80° C for 15 min, centrifuged 10 min at 5000 *g*, followed by another extraction step using 2 mL of 50% ethanol, and finally extracted once more using 2 mL of de-ionized water. The extracts were pooled, dried in a Speed-Vac and re-dissolved in 2 mL ultrapure water for determinations of sugars, organic acids and amino acids. Glucose and fructose content were measured enzymatically with an automated micro-plate reader (EPOCH2, Biotek Instruments Inc., Winooski, VT, USA) using the Glucose/Fructose kit from BioSenTec. Tartaric and malic acids were determined with an automated colorimetric method using the TRAACS 800 autoanalyzer (Bran-Luebbe) according to the method of (Bobeica et al., 2015). Amino acids were determined by UHPLC (UltiMate 3000 UHPLC system with FLD-3000 Fluorescence Detector, Thermo Electron SAS, Waltham, MA USA) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate according to Torres et al (2017). All the amino acids were identified and quantified with external chemical standards purchased from Sigma (St Louis, MO, USA).

3.2.3.2 Anthocyanin profiling

Five hundred mg FW of frozen powdered berry material were lyophilized during 72h. The extraction was done with 500 µL methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.2-µm polypropylene syringe filter for UHPLC analysis. Each individual anthocyanin was analyzed by UHPLC coupled to a Diode Array Detector (UltiMate 3000, Therma Scientific, CA, USA) as described in (Acevedo De la Cruz et al., 2012) and modified by (Hilbert et al., 2015). Anthocyanin quantification was carried out by peak area integration at 520 nm, using Malvidin-3-glucoside (Extrasynthèse, Lyon, France) as external standard.

3.2.3.3 Analysis of ABA and its derivatives

Two hundred mg of berry powder were freeze-dried for 72 h and the dried powder (20~50mg) was extracted using a stable isotope dilution assay (Speirs et al., 2013). ABA, ABA-glucose ester (ABA-GE), phaseic acid (PA) and dihydrophaseic acid (DPA) in samples with a deuterated internal standard were measured using liquid chromatography/mass spectrometry (Agilent 6410 Triple Quadrupole LC-MS/MS with Agilent 1200 series HPLC, Agilent Technologies Inc., Santa Clara, USA). The column used was a Phenomenex C18(2) 75 mm x 4.6 mm x 5 µm and column temperature was set at 40 ° C. The solvents used were nanopure water and acetonitrile, both supplemented with 0.05 % acetic acid (v/v). Samples were eluted with a linear 15 min gradient starting at 10 % acetonitrile (v/v) and ending with 90 % acetonitrile (v/v). Eluted compounds were identified by retention times and multiple reactions monitoring of mass-to-charge ratio (m/z) for parent and product ions of native and deuterated internal standards (Rossdeutsch et al., 2016).

3.2.3.4 RNA extraction and RT-qPCR analysis

Total RNA was isolated from 1 g of berry frozen according to (Reid et al., 2006) RNase-free DNase (Turbo DNA-free^{TM} kit, Ambion, Austin, TX, USA) was used to degrade DNA from total RNA. First-strand cDNA was synthesized from 1.5 ug purified RNA using iScript^{TM} advanced cDNA Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's

instructions. Obtained cDNAs were diluted twentyfold in ultrapure 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 regular PCR. Quantitative real-time PCR reactions were performed SYBR Green on an iCycler iQH (Bio-Rad, Hercules, CA, USA) according to the procedure described by the supplier, with 0.2 µM of primers for each gene. Each sample had three biological and two technical replicates to ensure the accuracy of results. Gene expression was calculated as normalized relative quantities (Hellemans et al., 2007) with *GAPDH*, *ACTIN* and *EF1γ* as house-keeping genes. Primer sequences are listed in Table S1.

3.2.3.5 Data Analysis

All data analysis was conducted with R software (R Development Core Team, 2010). Differences between the treatments were tested for significance by applying the analysis of variance (ANOVA) followed by Tukey post-hoc test (p value < 0.05). The hierarchical clustering analysis used the R package pheatmap (distance: euclidean; cluster method: complete) (Kolde and Kolde, 2015)

3.3 Results

3.3.1 Leaf area evolution

In this study, we designed three gradient leaf area treatments. The first one is two leaves, including 2L-P (early leaf removal at pea size (32DAF) and 2L-V (late leaf removal at pre-*véraison*), with an average leaf area keep around 120 cm^2 per vine. The second one is 2L-R, with early leaf removal at pea sized (32 DAF) and then retention of new leaves one week before *véraison* (56 DAF), and the leaf area showed a gradual increase and eventually reached to about 500 cm^2 per vine at harvest. The third one is twelve leaves, including 12L and 12L-girdling, with the leaf area increased slightly over time and reached aver 800 cm^2 per vine at harvest (Fig.1). The 12L-girdling treatment changed the available leaf number per cluster to two via phloem-girdling that isolated the above 10 leaves from the cluster (Sup Fig. 1), but its whole vine leaf area maintained the same as those of 12L treatment. Exogenous ABA treatment did not change leaf area in comparison with their non-treated counterparts, because leaf growth stopped at the moment of ABA spraying.

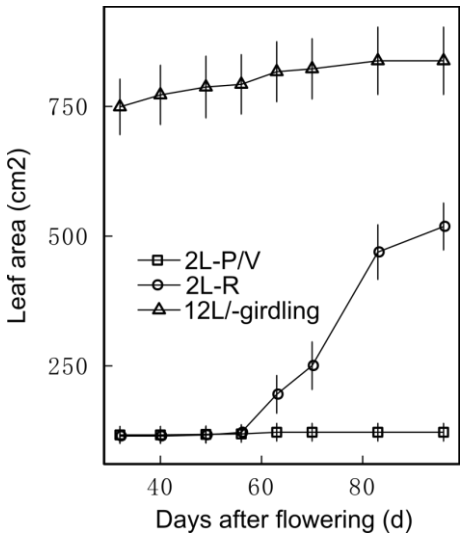


Fig.1 Effect of leaf manipulation on the leaf area. 2L-P/V: reserving 2 leaves from pea size (early leaf removal at pea size (32DAF)) and reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-R: early leaf removal and reserving 2 leaves at pea sized (32 DAF) and then

retention of new leaves one week before *véraison* (56 DAF); 12L/-girdling: 12 leaves and 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster. Vertical bars indicate SE (n=3).

3.3.2 Metabolite accumulations in berries

To explore the effect of reducing carbon source, the content of berries in primary metabolites (hexoses, organic acids and free amino acids), and in secondary metabolites (anthocyanins) were determined during berry development and ripening.

3.3.2.1 Sugars

Soluble sugar (glucose and fructose) concentrations were significantly reduced by source limitation (Fig. 2A, B). In 2017, early leaf removal induced a greater delay in sugar accumulation compared to late leaf removal. A twenty-day delay in sugar accumulation was observed in 2L-P, compared to 12L (reference treatment), while less than one-week delay was noticed in 2L-V treatment (Fig. 2A, B). The soluble sugar concentrations in 12L treatment rose rapidly from 63 DAF, while sugars in 2L-V and 12L-girdling treatments began to accumulate rapidly between 63 and 70 DAF, and the soluble sugar concentrations in 2L-P treatment started to increase only after 83 DAF. However, at harvest, the concentration of soluble sugar in 2L-P, 2L-V and 12-girdling treatments were reduced by 46.2%, 57.2% and 44.2% compared to 12L treatment, respectively. After recovery of leaf growth from 56 DAF (2L-R treatment), the soluble sugar concentrations increased by 43.3% compared to 2L-P treatment and were significantly higher than in 2L-V treatment but still less than in 12L treatment at harvest. In 2018, the concentration of soluble sugar in 2L-V and 12-girdling treatments were reduced by 36.6% and 37.2% compared to 12L treatment, respectively. The soluble sugar concentrations in 12L treatment rose rapidly after 54 DAF, while they accumulated rapidly in 2L-V and 12-girdling treatments after 68 DAF.

Exogenous ABA application promoted the accumulation of soluble sugars in ripening berries. Under no-source limitation (12L treatment), exogenous ABA led to earlier sugar accumulation, while under the condition of source limitation exogenous ABA improved the

rate of sugar accumulation (Fig. 2A, 2L-ABA, 12L-ABA-girdling and 12L-ABA). At harvest, there was no significant differences in sugar concentration between 12L-ABA and 12L treatment, but a 30.3% increase in 2L-ABA treatment compared to 2L-V was observed, as well as a 47.1% increase in 12L-ABA-girdling compared to 12L-girdling treatment.

3.3.2.2 Organic Acids

Contrary to the trend in soluble sugars, organic acids (malic acid and tartaric acid) concentrations decreased after *véraison* stage. In 2017, malic acid in 12L treatment increased before 63 DAF (*véraison*), then decreased sharply (Fig. 2C). Early carbon limitation treatments (2L-P) led to a slower accumulation of malic acid compared to 12L treatment. Both 2L-P and 2L-V treatment resulted in a significantly higher content of malic acid than that in 12L treatment at harvest (Fig. 2C). Meanwhile, the 2L-P treatment resulted in a 10-day delay in the decrease of malic acid. The recovery of leaf growth in 2L-R treatment significantly decreased its concentration at harvest, compared with 2L-P (Fig. 2C). Furthermore, the results in 2018 were similar to those in 2017 (Fig. 2D).

Exogenous ABA application accelerated the decrease of malic acid after *véraison* but had no significant effect on its accumulation earlier during berry development. (Fig. 2C, 2L-ABA, 12L-ABA-girdling and 12L-ABA). More specifically, the effects of ABA on source limitation treatments were more significant (Fig. 2C 2L-ABA and 12L-ABA-girdling). In comparison, the concentrations of malic acid in 2L-ABA and 12L-ABA-girdling at harvest were significantly lower than that without ABA treatments, but there was no significant difference in that between 12L and 12L-ABA treatments.

Source limitation and exogenous ABA application had less effect on tartaric acid berry content. Source limitation did not affect its concentration at harvest in 2017 and 2018 (Fig. 2E, F). Both the recovery of leaf growth and ABA application decreased slightly the concentration of tartaric acid at harvest (Fig. 2E, 2L-R, 2L-ABA, 12L-ABA-girdling and 12L-ABA).

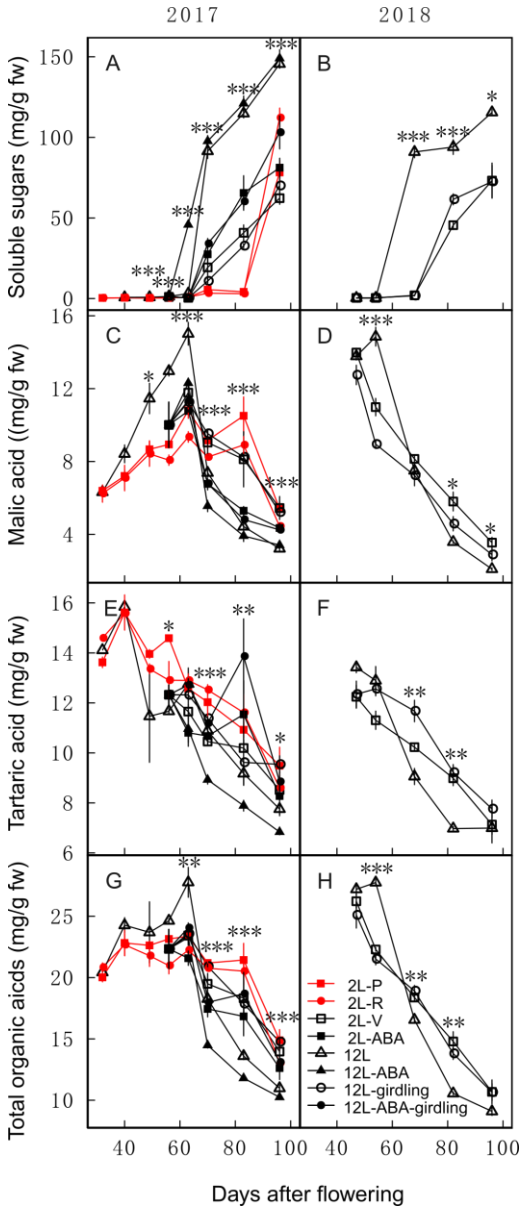


Fig.2 Effect of source limitation and exogenous ABA application on the concentrations of soluble sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in 2017 (right panel) and 2018 (left panel). 2L-P: reserving 2 leaves from pea size (early leaf removal at pea size (32DAF)); 2L-R: early leaf removal and reserving 2 leaves at pea sized (32 DAF) and then retention of new leaves one week before *véraison* (56 DAF); 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenous ABA sprayed on berries at pre-*véraison*; 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from

the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

3.3.2.3 Amino acids

The concentrations of 19 free amino acids in berry were determined at various stages during berry development and ripening. Total free amino acid concentrations remained rather stable before *véraison* but increased sharply after *véraison* (Fig. 3G, H). The results showed that source limitation and exogenous ABA application had significant effects on total free amino acids accumulation during berry ripening in 2017, but source limitation had no significant effect in 2018 (Fig. 3G, H). There was no significant difference in the accumulation of total free amino acids between early and late leaf removal treatments (Fig. 3G 2L-P and 2L-V, respectively), but the recovery of leaf growth at pre-*véraison* restored the concentration of total amino acid at harvest under early leaf removal treatment (Fig. 3G, 2L-R). ABA promotes the accumulation of total free amino acids in berry, especially in source limitation treatments (Fig. 3G).

Proline, arginine and threonine were affected by source limitation and exogenous ABA application (Fig. 3A to F). Proline, the most abundant amino acid in berry at harvest, was extremely low before *véraison*, but its concentration increased sharply after *véraison* (Fig. 3E, F). Carbon source limitation decreased the accumulation of proline, but the reduction caused by early leaf removal was slighter (Fig. 3E, F, 2L-P and 2L-V, respectively). Moreover, the recovery of leaf growth at pre-*véraison* had no significant effect on the accumulation of proline compared to 2L-P, whereas ABA treatment promoted it, especially under source limitation situations (Fig. 3E, 2L-P, 2L-R, 2L-ABA and 12L-ABA-girdling). Arginine was the second major amino acid in berry at harvest, and its concentration decreased with berry development in 12L treatment (Fig. 3C, D). Arginine concentration was found to increase after *véraison* under the condition of source limitation (Fig. 3C, D 2L-P,2L-V and 12L-girdling). The recovery of leaf growth at pre-*véraison* resulted in a decrease of arginine

concentration with the berry development after *véraison* (Fig. 3C, 2L-R). ABA increased the concentration of arginine in source limitation treatments (Fig. 3C, 2L-ABA and 12L-ABA-girdling). The concentration of threonine decreased at pre-*véraison* stages and remained stable after *véraison* in 12L treatment (Fig. 3A, B). Source limitation (2L-V, 2L-P,12L-girdling) sharply increased the accumulation of threonine after *véraison*, but 2L-P increased faster, followed by 2L-V, and 12L-girdling ends. Similar to arginine, the recovery of leaf growth at pre-*véraison* (2L-R) resulted in a decrease of threonine concentration during berry post-*véraison* development. Finally, ABA increased the concentration of threonine at same stage when applied under source limitation treatments (Fig. 3A, 2L-ABA).

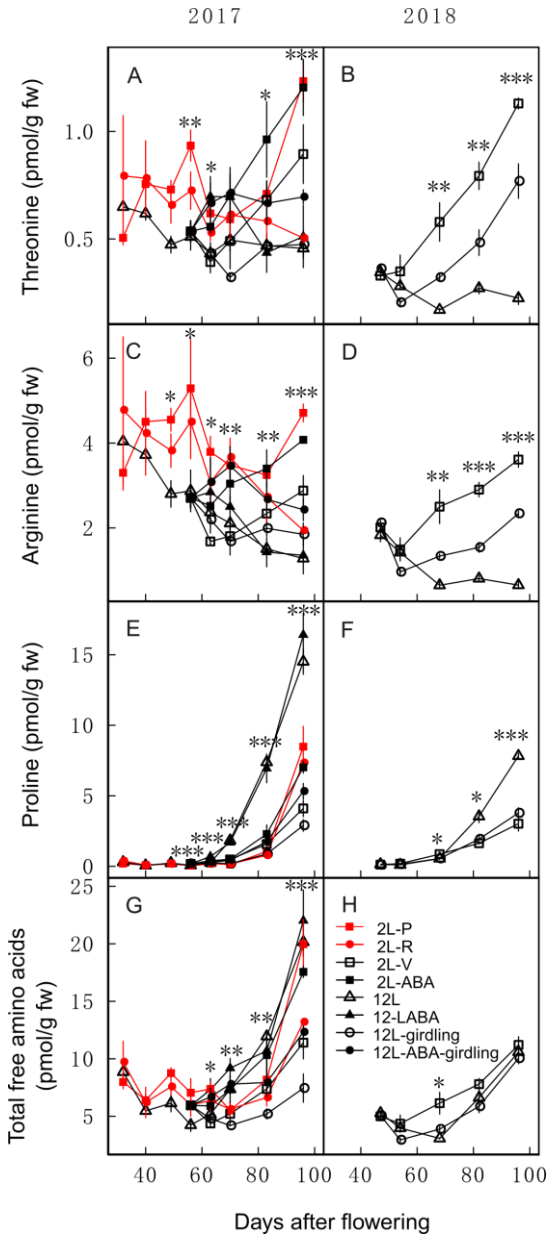


Fig.3 Effect of source limitation and exogenous ABA application on threonine (A and B), arginine (C and D), proline (E and F) and total free amino acids (G and H) in 2017 (right panel) and 2018 (left panel). The legend is the same with Fig.2. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

3.3.2.4 Anthocyanins

Carbon source limitation and exogenous ABA application affected the concentration of total anthocyanins in berry during berry development. The 12L treatment sharply increased berry anthocyanin content from 63 DAF in 2017 and 54 DAF in 2018, and total anthocyanin concentrations were systematically higher in the 12L berries than in berries submitted to lower carbon source treatments, throughout berry development (Fig. 4A, B). The concentrations of anthocyanins in 2L-P, 2L-V and 12L-girdling treatments were reduced by 74.7%, 87.4% and 70.8% at harvest compared to 12L, respectively. Total anthocyanin concentrations in 2L-P and 12L-girdling treatments exhibited a similar developmental trend with a slower accumulation compared to 12L, but higher than that in 2L-V. Total anthocyanin accumulation in 2L-R (recovery) treatment increased during late developmental stages and partially alleviated the decrease in anthocyanins observed in earlier developmental stages under carbon limitation treatments (2L-V, 2L-P). Exogenous ABA application improved the concentration of the total anthocyanins at each stage during berry development in both 12L and carbon limitation treatments (Fig. 4A, 12L-ABA).

Besides these quantitative effects, anthocyanin composition profile was also significantly affected by source limitation and exogenous ABA application. Berries had a higher proportion of tri-hydroxylated anthocyanins than di-hydroxylated ones in all the treatments; and the ratio of di- to tri-hydroxylated anthocyanins decreased alongside berry developmental curve. Source limitation treatments resulted in less ratio of di- to tri-hydroxylated anthocyanins compared to the reference 12L treatment (Fig. 4C, D). Early leaf removal (2L-P) delayed the accumulation of anthocyanins compared with 2L-V treatment, but there was no significant difference in the ratio of di- to tri-hydroxylated anthocyanins at harvest between both treatments. There was no significant difference in the ratio of di- to tri-hydroxylated anthocyanins between 2L-R and 12L treatments. The ratio of di- to tri-hydroxylated anthocyanins in 12L-girdling increased slightly after 83 DAF and showed no significant difference with 12L at harvest (Fig. 4C, D). Exogenous ABA decreased the ratio of di- to tri-hydroxylated anthocyanins during berry development in both 12L and source limitation treatments (Fig. 4C).

The ratio of non-acylated to acylated anthocyanins was also affected by source limitation and ABA treatments. Berries in 12L and 2L-R treatments had higher proportions of non-acylated anthocyanins than acylated ones at harvest, while berries under low carbon limitation treatments exhibited the opposite trend (Fig. 4E, F). Exogenous ABA decreased the ratio of non-acylated to acylated anthocyanins during berry development in low carbon limitation treatments but had not significant effect on the reference 12L treatment at harvest.

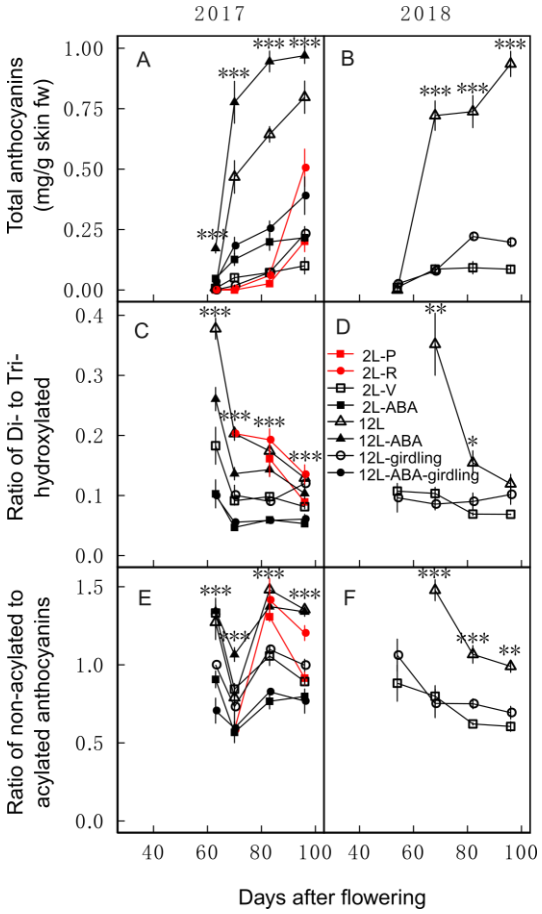


Fig.4 Effect of source limitation and exogenous ABA application on anthocyanin concentrations (A and B), the ratio of Di- to Tri-hydroxylated anthocyanins (C and D), and the ratio Glc to Acetyl and Glc anthocyanins (E and F) in 2017 (right panel) and 2018 (left panel). The legend is the same with Fig.2. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

3.3.3 ABA and its catabolism products in berries

To determine the effect of source limitation and exogenous ABA treatment on endogenous ABA, we analysed free ABA and its catabolism products, including ABA-GE (abscisic acid D-glucopyranosyl ester), PA (phaseic acid) and DPA (dihydrophaseic acid) contents in berry for growing season 2017 (Fig. 5). In 12L berries, the concentrations of free ABA and ABA-GE peaked at 68 DAF and subsequently decreased quickly. Carbon limitation significantly decreased the concentrations of free ABA during berry development, especially during the *véraison* stage (Fig. 5A). The recovery of leaf growth in 2L-R increased the concentration of free ABA at harvest, and 2L-P and 12L-girdling treatments had higher concentrations of free ABA at harvest compared to 2L-V. Additionally, early source limitation (2L-P and 2L-R) decreased the concentration of ABA-GE during *véraison* stage, but late source limitation (2L-V) had no effect on it (Fig. 5B).

Exogenous ABA application greatly increased the concentrations of free ABA and its catabolism products during *véraison* stage (Fig. 5). The concentrations of free ABA and its catabolism products in berries with exogenous ABA application were significantly higher than that without, at harvest. Furthermore, the concentration of free ABA in treatment 12L-ABA-girdling steadily increased until harvest, while free ABA in other treatments with exogenous ABA (2L-ABA, 12L-ABA) decreased after *véraison* (Fig. 5A).

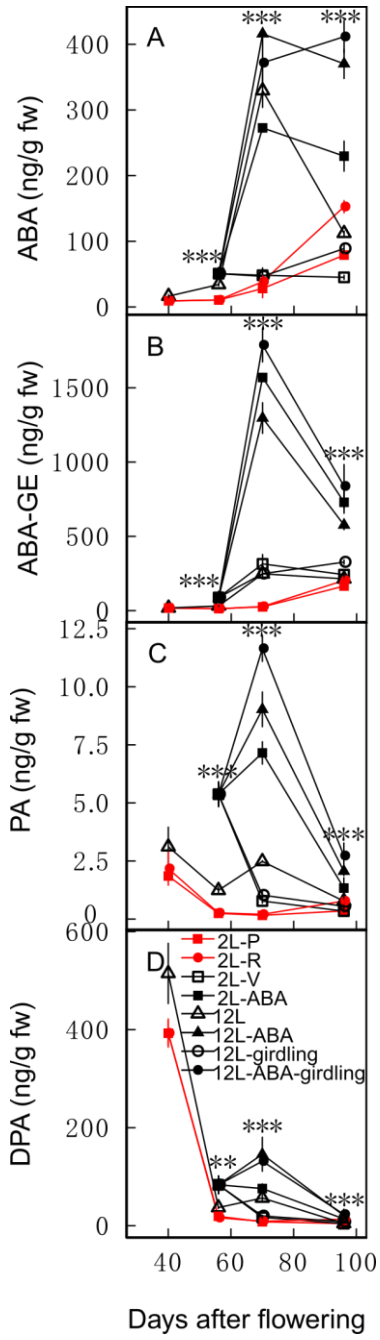


Fig.5 Concentrations of free ABA and ABA metabolites (ng/g) in berries in 2017 under source limitation and exogenous ABA application. ABA (A and B), abscisic acid; ABA-GE (C and D), ABA glucose ester; PA (E and F), phaseic acid; DPA (G and H). The legend is the same with Fig.2. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

3.3.4 Gene expression patterns in berries

To further explore the molecular mechanism of carbon source limitation and exogenous ABA application on berry metabolism, a total of 21 transcripts were quantitatively detected at 5 developmental stages for 6 treatments on samples from growing season 2017 (Fig. 6, 7 and Sup. Fig. 2). These included 11 genes involved into anthocyanins synthesis pathway, 5 related to sugar accumulation in berry and 5 related to ABA signalling pathway.

Regarding anthocyanin biosynthetic pathway, the transcript abundance of *CHS2*, *CHS3*, *ANS*, *UFGT*, *MYBA1* and *MYBA2* peaked around 70 DAF in 12L treatment, while the expression of *CHI*, *F3H*, *F3'5'H* and *DFR* genes peaked around 63 DAF (Fig. 6). Additionally, *F3'H* expression was down-regulated expression alongside berry developmental curve. With the noticeable exception of *F3H* and *F3'5'H*, the transcript abundances of all other anthocyanin biosynthetic genes were affected by carbon source limitation. Transcript abundances for *CHS2*, *CHS3*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT* in 2L-V treatment were lower than those in 12L at the peaks, without affecting the timing of the peak (around 70 DAF), while the peaks for *MYBA1* and *MYBA2* transcripts were delayed to 83 DAF in 2L-V treatment. Exogenous ABA application had a great influence on anthocyanins biosynthesis, by up-regulating the expression of both structural and regulatory genes. Seven genes, including *CHI*, *F3H*, *F3'5'H*, *ANS*, *UFGT*, *MYBA1* and *MYBA2*, had their transcript abundance increased after exogenous ABA treatment, and ABA led to the expression peaks of *ANS*, *UFGT*, *MYBA1* and *MYBA2* genes to occur a week earlier. Additionally, ABA caused the expression of *CHS2* and *CHS3* in 2L-V treatment to increase.

Among the genes related to ABA biosynthesis or response, the transcript abundance of *NCED1* was regulated by both source limitation and exogenous ABA (Fig. 7A). Source limitation downregulated the expression of *NCED1*, while exogenous ABA application increased its transcript abundance at *véraison*. ABA up-regulated the expression of *NCED2* under source limitation (Fig. 7B), while ABA up regulated the expression of *ABF2* under no-source limitation (Fig. 7C). Source limitation and exogenous ABA had little influence on

SnRK2.1 and *SnRK2.6* transcript abundance, and the maximum fold changes were lower than threefold (Fig. 7E, G).

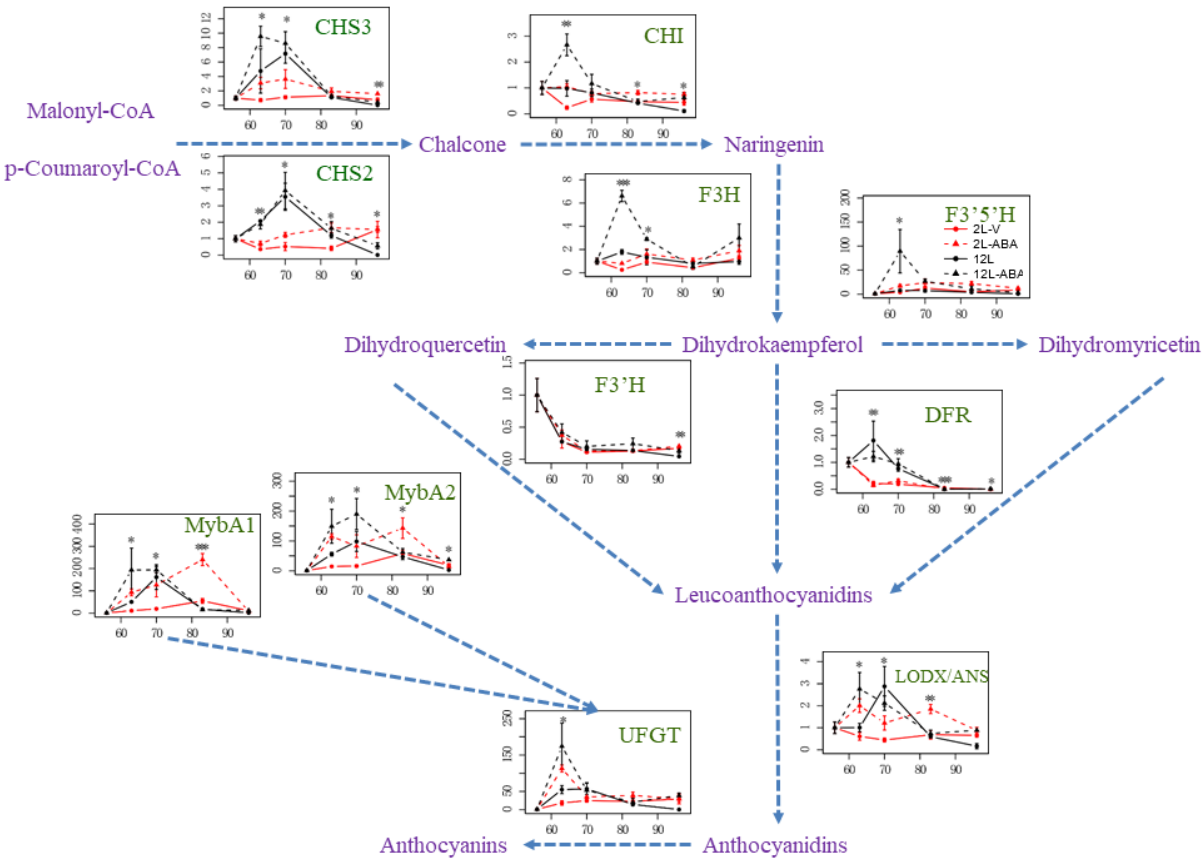


Fig.6 Effect of source limitation and exogenous ABA application on the expression of genes related to anthocyanin synthesis in 2017. X-axis and Y-axis represent the days after flowering and the expression fold change relative to 56 DAF, respectively. 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

Among the genes related to sugar accumulation, the transcript levels of *SUC11*, *SUC12*, *HT1* and *SnRK1* in 12L treatment were downregulated during berry development and maturation (Fig. 7). Source limitation caused *SUC11* and *SnRK1* transcript abundance to decrease more

slowly compared with 12L treatment. The transcript abundance of *SWEET10* was up-regulated in 12L treatment at 63 DAF and 70 DAF in 2L-V, with a delay of about a week under source limitation treatment. Meanwhile, exogenous ABA had smaller effect on the expression of these genes. ABA increased the transcript abundance of *HT1* at 70 DAF by 8.42 times under source limitation but had little effect on other genes.

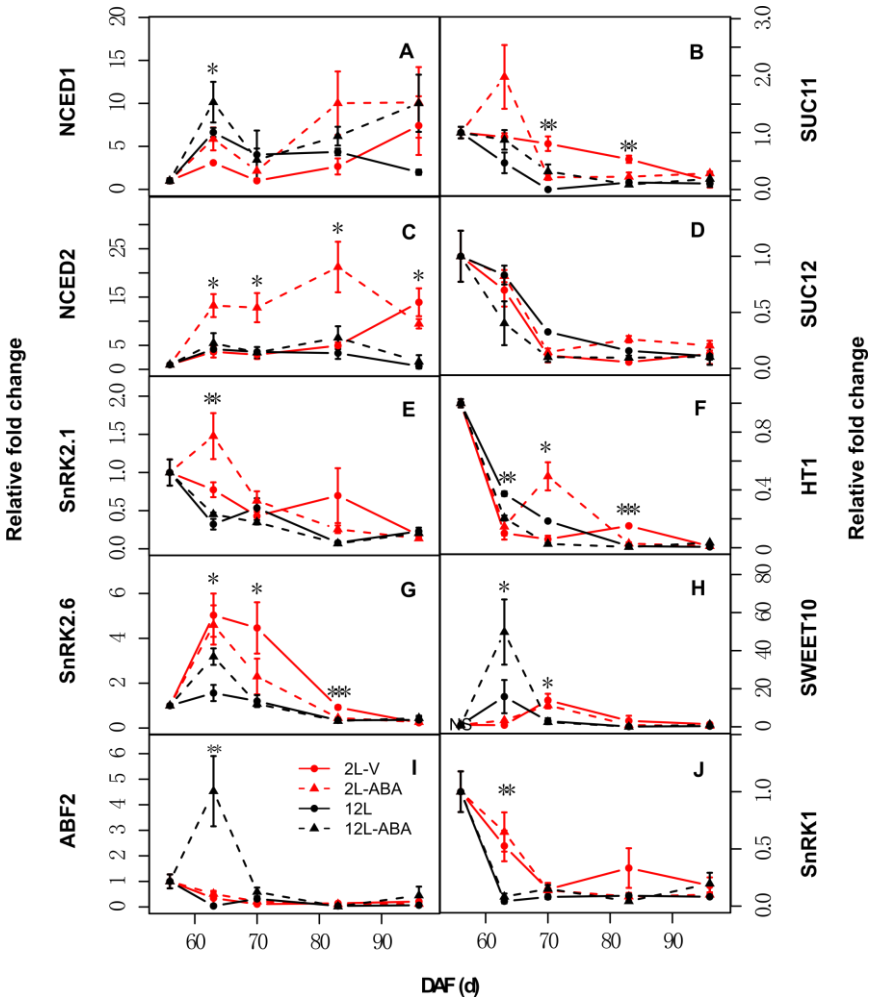


Fig.7 Effect of source limitation and exogenous ABA application on the expression of genes related to ABA and sugar in 2017. The legend is the same with Fig.6. Vertical bars indicate SE (n=3). * represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$.

To seek for correlations between gene expression for ABA, sugar and anthocyanins metabolism, a hierarchical clustering analysis was performed. As shown in Fig. 8, genes in response to carbon source limitation and exogenous ABA application fall in two clusters, A and B. Ten out of the 21 genes were in cluster A, including all the genes related to sugar metabolism (*SWEET10*, *HT1*, *SUC11*, *SUC12*, *SnRK1*) and three genes related to ABA responses (*ABF2*, *SnRK2.1*, *SnRK2.6*) and two structural genes of the anthocyanin biosynthetic pathway (*DFR* and *F3'H*); while the remaining nine genes related to anthocyanins synthesis (*F3H*, *CHS2*, *CHS3*, *CHI2*, *LDOX/ANS*, *UFGT*, *MYBA1*, *MYBA2*) and two ABA biosynthetic enzymes genes (*NCED1* and *NCED2*) were in cluster B. On the other hand, samples formed three clusters, with only one sample (12L at 96 DAF) in the first cluster, six samples in cluster 2, and the rest of the samples were in cluster 3. The samples in cluster 2 were from all source limitation treatment without exogenous ABA. Cluster 3 can be further divided into two sub-cluster, sub-cluster 1 and sub-cluster 2. Samples in sub-cluster 1 all came from post- *véraison* sampling stages (83 and 96 DAF), while sub-cluster 2 samples were all from *véraison* sampling (63 and 70 DAF).

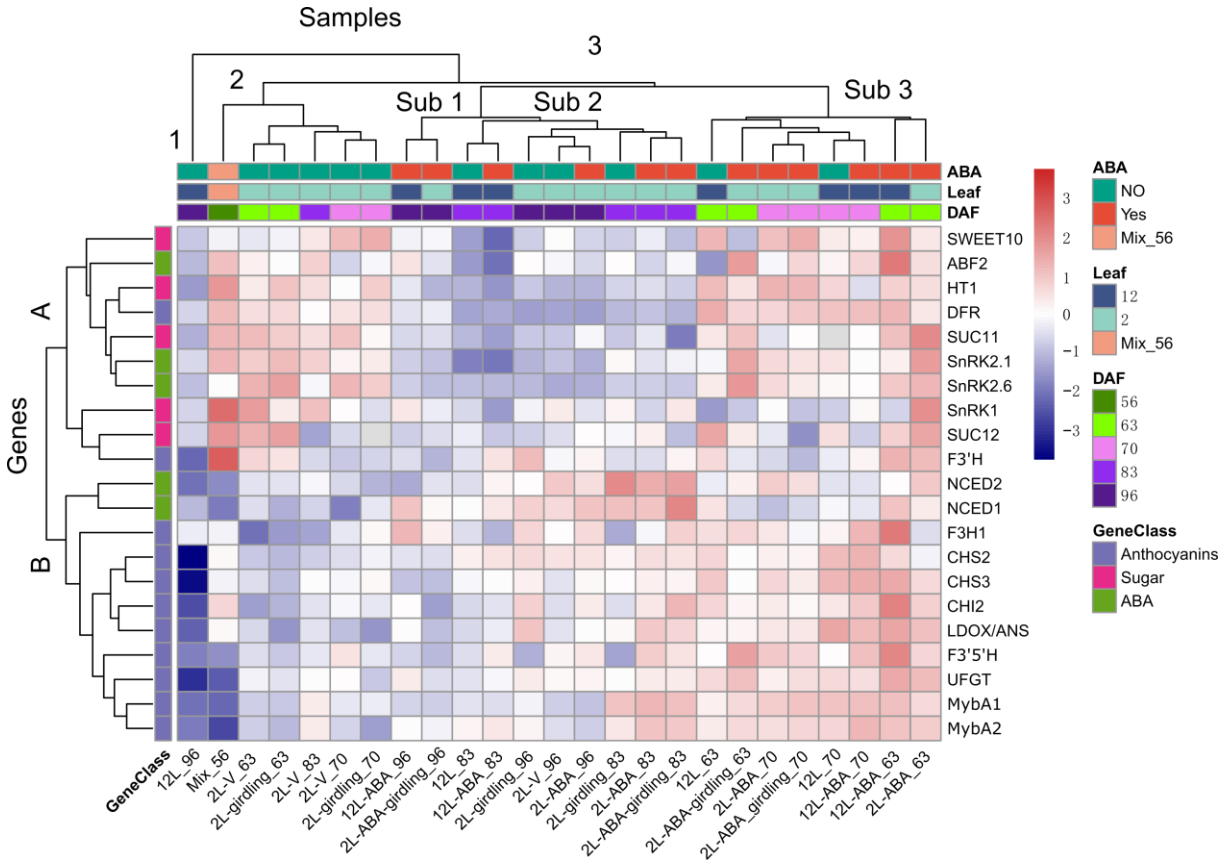


Fig.8 Cluster analyses of the genes related to anthocyanins, sugar and ABA based on the relative gene expression under source limitation and exogenous ABA application constructed using the module of pheamap of ggplot software. The red, black and blue elements in the matrix indicate up-regulated, no change and down-regulated genes, respectively. The rows and columns are the 21 genes and 25 treatments, respectively. 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenous ABA sprayed on berries at pre-*véraison*; 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. The number after treatment represents the sampling rime. ABA: with or without exogenous application. Leaf: the available leaf number per cluster. DAF: days after flowering. Mix_56: the mix samplings at 56 days after flowering. Geneclass: gene related to different pathway. The data used was RT- qPCR analysis data.

3.4 Discussion

3.4.1 Source limitation resulted in the reduction of sugar and anthocyanins concentrations through differential gene expression

Modifying the source-to-sink ratio is potentially one of the simplest and efficient ways to mitigate the climate change effect by trying to re-balance primary to secondary metabolite ratio at harvest. In reviewing the literature, we found that majority early leaf removal (before pea sized) resulted in the increase in both sugar and anthocyanin content (Diago et al., 2012, Gatti et al., 2012, Lee and Skinkis, 2013, Pastore et al., 2013, Risco et al., 2014, Feng et al., 2017), while late leaf removal (i.e. around *véraison*) significantly reduced the accumulation of sugars and total anthocyanins during grape ripening (Weaver, 1963, Martínez de Toda and Balda, 2013, Pastore et al., 2013, Wu et al., 2013, Bobeica et al., 2015). However, Ollat and Gaudillere (1998) found that early carbon limitation via leaf removal after fruit set largely reduced berry size and sugar concentration, and leaf area restoration caused increase in berry size and sugar concentration. Unfortunately, these authors did not quantify the effect of early leaf removal and leaf restoration on berry anthocyanins. Moreover, how the altered relationship of source and sink regulates sugar and anthocyanins concentrations is still poorly understood. In this study, we confirmed previous results that both early (2L-P) and late (2L-V) severe leaf removal reduced sugar and anthocyanins concentrations at harvest. The concentrations of sugar and anthocyanins in 2L-P were reduced by 46.2% and 74.7%, while those in 2L-V being reduced by 57.2% and 87.4, respectively. Moreover, our gene expression analysis provides further hints about the underlying mechanisms for the reduced sugar and anthocyanins under carbon limitation.

Sugar is produced through photosynthesis in the mature leaves and transported via phloem to berries in the form of sucrose (Swanson and El-Shishiny, 1958, Agasse et al., 2009), multiple transporters are involved in this translocation process (Zhang et al., 2019). SWEET10 is one of transporter proteins related to long-distance sucrose transport from leaves to other sink organs, which was shown to be strongly expressed at the onset of berry ripening and

contributed to glucose and fructose contents and influenced the soluble sugar composition in grapes (Zhang et al., 2019). In our study, the expression of *SWEET10* was higher at its expression peak for 12L berries compared with that of 2L berries, in line with the lower sugar concentration in 2L berries. The other sugar transporters, such as *SUC11*, *SUC12*, and *HT1* did not show clear differences between the two treatments, suggesting *SWEET10* may play a major role in carbon limitation responses. In addition to sugar transporters, sugar sensing players were also affected by carbon limitation. *SnRK1* was reported as an energy availability sensor that inhibited plant growth and development during metabolic stress to maintain energy homeostasis (Gazzarrini and Tsai, 2014). Overexpression of *AtSnRK1.1* (*KIN10*) gene blocked sucrose-induce accumulation of anthocyanin under a sucrose concentration of 3%, and repressed the expression of *MYB75/PAP1* which is a key transcription factor for anthocyanin biosynthesis in Arabidopsis (Baena-González et al., 2007). Interestingly, *MYB75/PAP1* is essential for the sucrose-mediated expression of the *DFR* gene in Arabidopsis (Li et al., 2014). The expression level of *SnRK1* in 2L-V at 63 DAF was 12.35 times that in 12L, while the expression level *DFR* in 2L-V DAF was also down-regulated by 61.8% at 63 compared to 12L, which may lead to carbon preferentially allocated for sugar accumulation rather than anthocyanins, because anthocyanin biosynthesis is a very high energy consumption process (Soubeyrand et al., 2018).

Gene expression pattern revealed that the decreased anthocyanins coincided with the down-regulated expression of *CHS2*, *CHS3*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT*, and the peak delay of *MYBA1* and *MYBA2* in 2L-V. Furthermore, the developmental profiles of anthocyanin composition were also significantly affected by source limitation. Both the ratio of di- to tri-hydroxylated anthocyanins and the ratio of non-acylated to acylated anthocyanins were reduced during the berry development by source limitation. The expression of structural genes *F3'H* and *F3'5'H* decides the ratio of di- to tri- hydroxylated anthocyanins that are eventually synthesized (Castellarin et al., 2006, He et al., 2010). However, our results showed that source limitation had little effect on the expression of *F3'H* and *F3'5'H*. It suggested that other copies of *F3'H* and *F3'5'H* may response to source limitation, or post-transcriptional regulation might affect the enzymatic activity. In fact, there are two copies of *F3'H* and

sixteen copies of *F3'5'H* in grape (Castellarin et al., 2006), hence, more effort is needed to detect if these copies response to source limitation specially.

3.4.2 Exogenous ABA application partially restore the decoupling sugar and anthocyanins under source limitation via differential expression of the associated genes and altered endogenous ABA

Our results showed that exogenous ABA application increased the concentrations of both sugar and anthocyanins in berry at harvest, in line with previous reports (Villalobos-Gonzalez et al., 2016). However, the increase of anthocyanins was higher than that of sugar after exogenous ABA application under source limitation. Sugar concentration in 2L-ABA increased by 30.4% and anthocyanin concentration by 115.95% compared with 2L-V, while sugar concentration in 12L-ABA-girdling increased by 40.17% and anthocyanin concentration by 67.58% compared with 12L-girdling. Therefore, exogenous ABA application can partially restore the decoupling sugar and anthocyanins under source limitation.

Previous studies observed the concentration of ABA increased in grape berries at the onset of ripening, and concluded that the increase of ABA concentration was a key factor in triggering *véraison* (Gagné et al., 2006, Deluc et al., 2009, Owen et al., 2009, Castellarin et al., 2016). By the application of exogenous ABA at fruit set stage, Gagné et al. (2006) changed the concentration of ABA in berry which caused an earlier accumulation of anthocyanins. By analysis in individual berries, Castellarin et al. (2016) confirmed that the increase in ABA during the onset of ripening occurred earlier than sugar and anthocyanins accumulation, and the source of initial increases in ABA was the decreases in catabolism and/or exogenous import. In our study, by quantitative RT-PCR analysis, the expression of anthocyanins synthesis genes, *CHI*, *F3H*, *F3'5'H*, *ANS*, *UFGT*, *MYBA1* and *MYBA2* were up-regulated with exogenous ABA, which was well matched with the increase of anthocyanins concentration. These results together with those previous studies (Yamane et al., 2006, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018) confirmed ABA accelerated

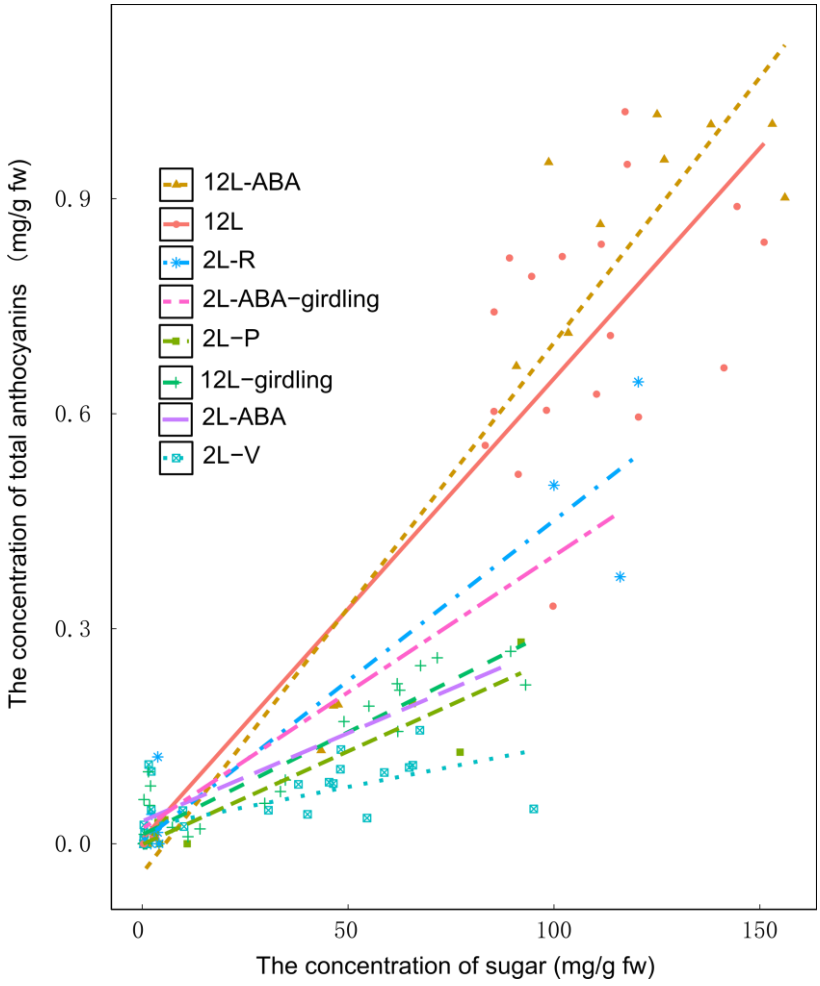
anthocyanin accumulation by up-regulating the expression of structural genes and regulators in anthocyanin synthesis pathway. In particular, the expression of *ANS*, *UFGT*, *MYBA1* and *MYBA2* genes reached peaks one week earlier, which was coincident with the advance of anthocyanin accumulation with exogenous ABA.

Exogenous ABA application on the whole vine was reported to increase the concentration of sugar in berry by enhancing sugar transport to berry rather than increasing carbon fixation (Moreno et al., 2011). ABA in berry promoted the accumulation of sugar by improving monosaccharide transport from apoplast to berry cells. ABA increased the expression level of *cwINV* to promote the hydrolysis of sucrose into hexose (Wang et al., 2017). In our study, we tested the expression of three sucrose transporters, including SUC11, SUC12 and SWEET10, and one hexose transporter HT1 during berry development. The expression of *HT1* was induced by both sucrose and ABA by inducing the expression of its binding protein VvMSA (Çakir et al., 2003). Since HT1 was localized in the plasma membrane of the sieve element/companion cell interface and of the flesh cells in berry, and mainly expressed before *véraison*, Vignault et al. (2005) speculated that HT1 retrieves the monosaccharides needed to provide the energy necessary for cell division and cell growth at an early stage of berry development Interestingly, in our result, the expression of *HT1* under low source limitation showed to be affected by exogenous ABA, while ABA did not change *HT1* under 12L condition. In contrast, the expression patterns of *SWEET10*, *SUC11* and *SUC12* were not significantly affected by ABA, nor the SnRK1. Therefore, it seems that the effect of exogenous ABA on sugar accumulation is mediated by the *HT1* gene, particularly under carbon limitation conditions.

To further explore the mechanisms by which ABA promoted sugar and anthocyanins accumulation, the genes involved in ABA synthesis (*NCED1* and *NCED2*) and response (*SnRK2.1*, *SnRK2.6* and *ABF2*) were detected. *SnRK2.1* and *SnRK2.6* were considered as the major *SnRK2* candidates for the ABA-regulated stomata response to abiotic stress (Boneh et al., 2012). The bZIP transcription factor *ABF2* is an important transcriptional regulator of ABA-dependent grape berry ripening processes (Nicolas et al., 2014). Our results showed *ABF2* expression was induced by ABA only under no-source limitation, and *SnRK2.1* and

SnRK2.6 expressions were higher under source limitation but had less response to ABA. These results suggest that ABA promotes sugar and anthocyanins accumulation by a pathway independent of *ABF2* and *SnRK2s* pathway. The upregulation of *NCED1* and *NCED2* after ABA application supported the conclusion of Pilati et al. (2017) that exogenous ABA application induced endogenous ABA synthesis.

In summary, exogenous ABA increased the concentration of anthocyanins by increasing the expression levels of anthocyanins synthesis genes, especially *MybA1*, *MybA2* and *UFGT*. Meanwhile exogenous ABA also increased the expression of hexose transporter rather than sucrose transporter to promote the accumulation of sugar. However, ABA had a greater influence on genes involved in anthocyanins synthesis than sugar transporter or *SnRK1*, which explains why exogenous ABA application partial restore the decoupling sugar and anthocyanins under source limitation.

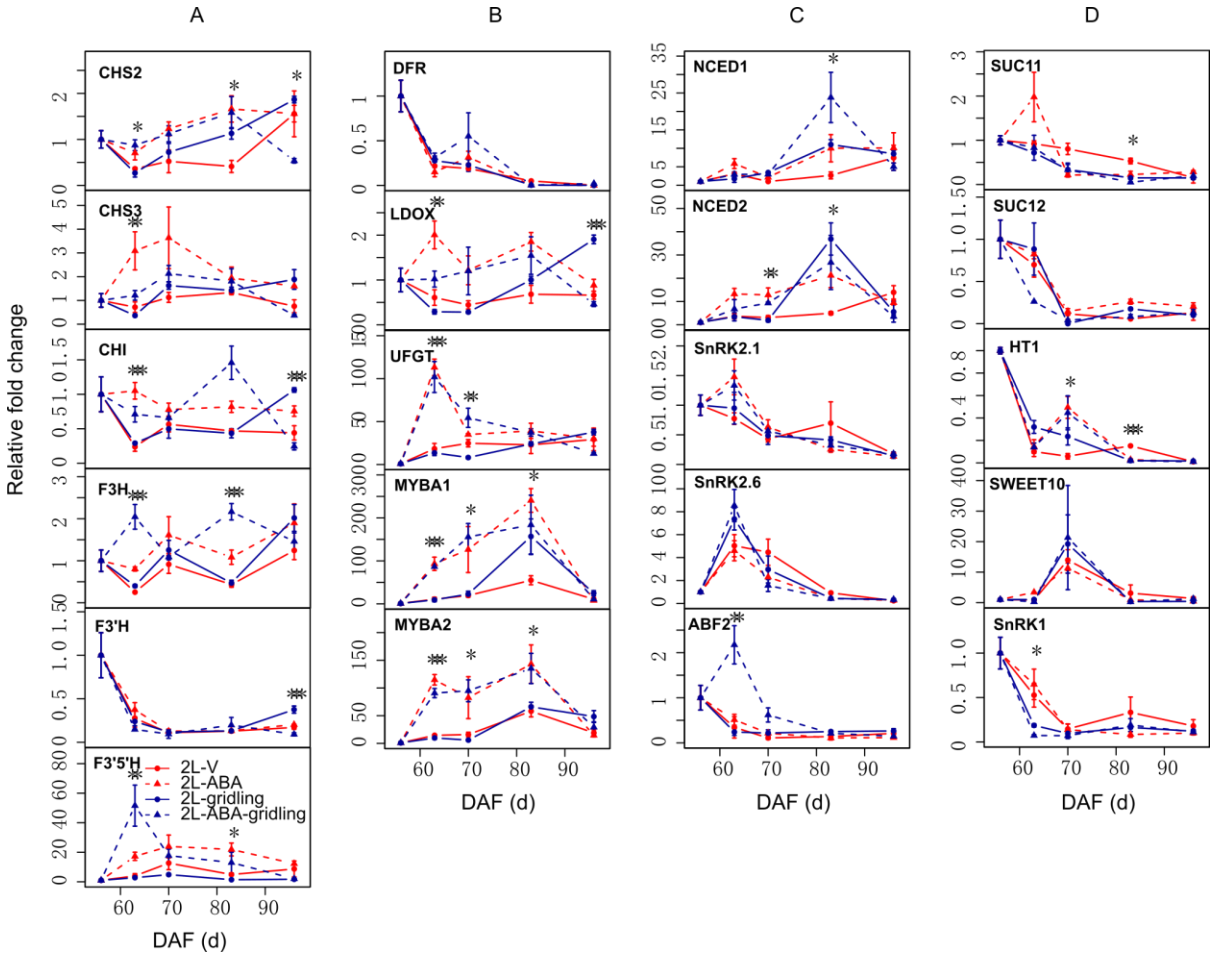


Sup. Fig.2 The relationship of sugar and total anthocyanins concentrations. The legend is the same with Fig.2.

3.4.3 The relative effects of carbon limitation and whole-vine transpiration on berry sugar and anthocyanins

The 2L treated vines had much lower leaf area and consequently had lower whole-vine transpiration water fluxes through the xylem, in comparison with 12L vines. Since the whole-plant water fluxes may have some influences on fruit growth (Bustan et al., 2016), we designed the 12L-girdling treatment to verify the relative effects of carbon limitation and whole-vine transpiration on berry quality. In fact, the berries of 2L-V and 12L-girdling had the same supply level of carbons, both with 2 leaves per cluster. The only difference between

them was the whole-vine xylem water fluxes from roots to leaves, with a higher water fluxes for 12L-girdling berries than that of 2L-V berries. The effect of 12L-girdling on sugar was similar to that of 2L-V, but the concentration of anthocyanins in 12L-girdling increased significantly compared with 2L-V at harvest. By analysing the genes involved, we found the expression of *NCED1*, *NCED2* and *MybA1* in 12L-girdling was up-regulated at 83 DAF, and *CHI* and *ANS* in12L-girdling was up-regulated at 96 DAF. Moreover, the free ABA concentration in 12L-girdling increased from 83 DAF. These results suggested that the increase of anthocyanin in 12L-girdling might due to the synthesis/import of ABA at 83DAF which consequently induced anthocyanin synthesis. In fact, prior studies have shown that phloem girdling not only reduced the assimilate supply, but also reduced the accumulation of berry ABA (Düring et al., 1978, Zhang et al., 1997). Comparing 12L with 12L-girdling, our results also confirmed those previous studies, showing reduced ABA concentration in 12L-girdling compared with 12L. However, these authors did not compare 2L-V with 12L-girdling, where the berries have the same level assimilate supply but different leaves for whole-vine transpirations. Interestingly, recent work showed that leaves are the main site of ABA biosynthesis in plant (McAdam et al., 2016, Mitchell et al., 2017, Zhang et al., 2018), and ABA can be transported both via xylem and phloem (Slovik et al., 1995, Hartung et al., 2002). Therefore, the higher ABA concentration in 12L-girdling than 2L-V might be a result of ABA importation from the additional leaves in 12L-girdling that are connected with the berries via xylem. Carbon limitation in combination with shoot girdling as we did for the 12L-girdling treatment might be an innovative way to increase anthocyanins while reduce or maintain sugars.



Sup. Fig.3 Effect of source limitation and exogenous ABA application on the expression of genes related to ABA and sugar in 2017. 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). *represent p<0.05; **represent p<0.01; ***represent p<0.001.

Figures

Fig.1 Effect of leaf manipulation on the leaf area. 2L-P/V: reserving 2 leaves from pea size (early leaf removal at pea size (32DAF)) and reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-R: early leaf removal and reserving 2 leaves at pea sized (32 DAF) and then retention of new leaves one week before *véraison* (56 DAF); 12L/-

girdling: 12 leaves and 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster. Vertical bars indicate SE (n=3).

Fig.2 Effect of source limitation and exogenous ABA application on the concentrations of soluble sugars (glucose + fructose) (A and B), malic acid (C and D), tartaric acid (E and F) and total organic acids (malic acid + tartaric acid) (G and H) in 2017 (right panel) and 2018 (left panel). 2L-P: reserving 2 leaves from pea size (early leaf removal at pea size (32DAF)); 2L-R: early leaf removal and reserving 2 leaves at pea sized (32 DAF) and then retention of new leaves one week before *véraison* (56 DAF); 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenous ABA sprayed on berries at pre-*véraison*; 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). *represent $p<0.05$; **represent $p<0.01$; ***represent $p<0.001$.

Fig.3 Effect of source limitation and exogenous ABA application on threonine (A and B), arginine (C and D), proline (E and F) and total free amino acids (G and H) in 2017 (right panel) and 2018 (left panel). The legend is the same with Fig.2. Vertical bars indicate SE (n=3). *represent $p<0.05$; **represent $p<0.01$; ***represent $p<0.001$.

Fig.4 Effect of source limitation and exogenous ABA application on anthocyanin concentrations (A and B), the ratio of Di- to Tri-hydroxylated anthocyanins (C and D), and the ratio Glc to Acetyl and Glc anthocyanins (E and F) in 2017 (right panel) and 2018 (left panel). The legend is the same with Fig.2. Vertical bars indicate SE (n=3). *represent $p<0.05$; **represent $p<0.01$; ***represent $p<0.001$.

Fig.5 Concentrations of free ABA and ABA metabolites (ng/g) in berries in 2017 under source limitation and exogenous ABA application. ABA (A and B), abscisic acid; ABA-GE (C and D), ABA glucose ester; PA (E and F), phaseic acid; DPA (G and H). The legend is the

same with Fig.2. Vertical bars indicate SE (n=3). *represent p<0.05; **represent p<0.01; ***represent p<0.001.

Fig.6 Effect of source limitation and exogenous ABA application on the expression of genes related to anthocyanin synthesis in 2017. X-axis and Y-axis represent the days after flowering and the expression fold change relative to 56 DAF, respectively. 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenouse ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). *represent p<0.05; **represent p<0.01; ***represent p<0.001.

Fig.7 Effect of source limitation and exogenous ABA application on the expression of genes related to ABA and sugar in 2017. The legend is the same with Fig.6. Vertical bars indicate SE (n=3). *represent p<0.05; **represent p<0.01; ***represent p<0.001.

Fig.8 Cluster analyses of the genes related to anthocyanins, sugar and ABA based on the relative gene expression under source limitation and exogenous ABA application constructed using the module of pheamap of ggplot software. The red, black and blue elements in the matrix indicate up-regulated, no change and down-regulated genes, respectively. The rows and columns are the 21 genes and 25 treatments, respectively. 2L-V: reserving 2 leaves from pre-*véraison* (late leaf removal at pre-*véraison* (56DAF)); 2L-ABA: reserving 2 leaves from pre-*véraison* and exogenous ABA sprayed on berries at pre-*véraison*; 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. The number after treatment represents the sampling rime. ABA: with or without exogenous application. Leaf: the available leaf number per cluster. DAF: days after flowering. Mix_56: the mix samplings at 56 days after flowering. Geneclass: gene related to different pathway. The data used was RT-qPCR analysis data.

Supplementary data

Table S1: Primers used for real-time PCR analysis.

Sup. Fig.1 Schematic representation of different treatments. The legend is the same with Fig.2. Photograph was taken at 70 day after flowering.

Sup. Fig.2 Effect of source limitation and exogenous ABA application on the expression of genes related to ABA and sugar in 2017. 12L-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster; 12L-ABA-girdling: 12 leaves with a phloem-girdling that isolated the above 10 leaves from the cluster and exogenous ABA sprayed on berries at pre-*véraison*; 12L: 12 leaves as control; 12L-ABA: 12 leaves and exogenous ABA sprayed on berries at pre-*véraison*. Vertical bars indicate SE (n=3). *represent $p<0.05$; **represent $p<0.01$; ***represent $p<0.001$.

Sup. Fig.3 The relationship of sugar and total anthocyanins concentrations. The legend is the same with Fig.2.

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Table S1 Primers used for real-time PCR analysis

Gene name	Accession number	Forward primer 5'-3'	Reverse primer 5'-3'	References related
VvSUC11	VIT_18s0001g08210	CTCACGCCTGGTCCAGTATC	CCGATGTCAGCCGAGAATCC	
VvSUC12	VIT_01s0026g01960	CAAGAATCTGAAGCAGGCTGAG	GGCGGCAATCATACAACTGAG	
LDOX/ANS	VIT_02s0025g04720	AGGGAAGGGAAAACAAGTAG	ACTCTTTGGGGATTGACTGG	Jeong et al., (2004)
F3'5'H	VIT_06s0009g02840	AAACCGCTCAGACCAAAACC	ACTAAGCCACAGGAAACTAA	Jeong et al., (2006)
UFGT	VIT_16s0039g02230	GGTGGTACAAGCAACAGTTC	AATCTGAGAGCCCTAAGAGA	
F3'H	VIT_17s0000g07200	GCCTCCGTTGCTGCTCAGTT	GAGAAGAGGTGGACGGAGCAAATC	Jeong et al.,(2006)
NCED2	VIT_10s0003g03750	AGTTCCATACGGGTTTCATGGG	CCATTTTCCAAATCCAGGGTGT	Wheeler et al.,(2009)
NCED1	VIT_19s0093g00550	GAGACCCCAACTCTGGCAGG	AAGGTGCCGTGGAATCCATAG	Wheeler et al.,(2009)
VvHT1	VIT_00s0181g00010	CGTTGTTCACATCGTCGCTTTATC	GAGCAGTCCTCCGAATAGCATTG	Lecourieux et al.,(2010)
VvABF2	VIT_18s0001g10450	GGAGTTGGAAGCAGAGGTTG	AGTGTGCGTCTCAAGCAACG	Nicolas et al.,(2013)
SnRK1	VIT_14s0108g01230	GTTGAGAATGATGGTGTTTCCAGG	TGAGCAAGGAAAGCTGCACAA	Gambetta et al.,(2010)
CHS2	VIT_14s0068g00920	GTTCTGTTTGGATTTGGACCAG	GTGAGTCGATTGTGTAGCAAGG	Belhadj et al., (2008)
CHS3	VIT_05s0136g00260	GGTGCTCCACAGTGTGTCTACT	TACCAACAAGAGAAGGGGAAAA	Belhadj et al.,(2008)
CHI2	VIT_13s0067g03820	AGGAGTTAGCGGATTCGGTTGAC	AAGGCAACACAATTCTCTGACACC	Guan et al., (2014)
F3H1	VIT_04s0023g03370	GCAGACTGTCCATAGCAACATTCC	CACTGCCTTCTCTCCCTCTCTTATC	Soubeyrand et al.,(2013)
DRF	VIT_18s0001g12800	GAAACCTGTAGATGGCAGGA	GGCCAAATCAAACTACCAGA	Jeong et al.,(2006)
MybA1	VIT_02s0033g00410	AAGCCATCATCCACTTCACC	TCTCTCCAGAAGCCGAAAAG	Xie et al.,(2019)
MybA2	VIT_02s0033g00390	GACTCGATGAAGAGCTTAGG	CTTTAGGCATCTATTCAACC	
SWEET10	VIT_17s0000g00830	CATCCTGGGCTTCGTCTTC	ATTGCTGTGCTGCTGGTTAGT	Zhang et al.,(2019)
SnRK2.1	VIT_18s0001g06310	TTTTTGTGGCAAACCCAGAT	CAGCTTCCTCCATCCATCAT	Boneh et al, (2012)
SnRK2.6	VIT_03s0063g01080	CACCAACCCACCTTGCTATT	AAACGTGCCTCATCCTCACT	Boneh et al, (2012)
GAPDH	VIT_17s0000g10430	CCACAGACTTCATCGGTGACA	TTCTCGTTGAGGGCTATTCCA	Reid et al., (2006)
Actin	VIT_04s0044g00580	CTTGCATCCCTCAGCACCTT	TCCTGTGGACAATGGATGGA	Reid et al., (2006)
EF1ɣ	VIT_12s0035g01130	CAAGAGAAACAATCCCTAGCTG	TCAATCTGTCTAGGAAAGGAAG	Reid et al., (2006)

Chapter 4 The roles of sugar and ABA in inducing anthocyanin synthesis at *véraison* in grape berries cultured *in vitro*

Foreword

Through the experiments in chapter 2 and 3, we confirmed that sugar and ABA may be the key factors determining anthocyanin accumulation in grape berry. Most anthocyanins syntheses genes respond to both sugar and ABA. However, only few studies have focused on the actual co-regulation mechanisms of ABA and sugar anthocyanins biosynthesis during berry ripening, which would help us gain novel insights into the relationship between sugar, anthocyanins and ABA, and find some clues about the regulation of the carbon allocation between primary and secondary metabolites.

In this study, we used an *in vitro* culture system of intact detached grape berries pre-*véraison* with different concentrations of glucose, HXK (hexokinase) inhibitors, exogenous ABA or ABA synthesis inhibitor NDGA (nordihydroguaiaretic acid) to investigated the role of sugar and ABA in inducing anthocyanin synthesis in berry. The results will be described and discussed in the form of a manuscript submitted for publication.

Abstract

Both sugar and ABA were reported to induce anthocyanins synthesis in grape berry, and they were considered to be the trigger for berry *véraison*. However, the biological mechanisms by which sugar and ABA induce anthocyanins synthesis at *véraison* in berry are not well established, especially the possible interactions between sugar and ABA. In this study, an *in vitro* culture system of intact detached grape berries pre-*véraison* with different concentrations of glucose, HXK (hexokinase) inhibitors, exogenous ABA or ABA synthesis inhibitor NDGA (nordihydroguaiaretic acid) was used to investigated the role of sugar and ABA in inducing anthocyanin synthesis in berry. The results confirmed that ABA and high concentrations of glucose induced anthocyanins biosynthesis, and a synergetic effect existed on anthocyanins accumulation when ABA and high concentration of glucose were applied simultaneously to berries. However, high glucose had no significant effect on ABA level, and NDGA had no significant effect on anthocyanin synthesis. This result suggested that sugar can induce anthocyanin without strictly requiring ABA synthesis. Besides, this induction was not due to an increase in phenylalanine (Phe), since the Phe content was decreased under high glucose, which means sugar can induce anthocyanins synthesis as a signal molecule. Exogenous ABA application decreased berry Phe content and induced anthocyanin synthesis without increasing berry sugar content, suggesting that the synthesis of anthocyanins induced by ABA was not through sugar-induced anthocyanin pathway. Moreover, we found that highly effective HXK activity inhibitor NAG (N-acetyl-glucosamine) did not inhibit the induction of anthocyanins by high glucose, suggesting sugar-induced anthocyanin synthesis can be independent of HXK. While a weak HXK inhibitor DGH (D-(+)-Glucosamine hydrochloride), which specifically affected protein kinase activity, effectively inhibited the induction of anthocyanins by high glucose and ABA, implying there are interactions between ABA and sugar induced anthocyanins biosynthesis pathway. The inhibition of Phe accumulation by DGH might confirm the role of sugar as carbon source to limit anthocyanins synthesis. In conclusion, sugar can be used as carbon source or signal molecule to influence

anthocyanin synthesis, and there are interactions between sugar and ABA-induced anthocyanins synthesis pathways.

Keyword

Sugar, ABA, Anthocyanins, interaction, *in vitro* culture berry

4.1 Introduction

The concentrations of sugar and anthocyanins, alongside aromas and aroma precursors at harvest, largely determine the quality of grape berries and wine. The increase in sugar concentration during the last several decades as an effect of the ongoing climate change has attracted much attention from the viticultural scientific community and the vine and wine industry. Viticultural practices based on carbon source reduction can reduce sugar concentration at harvest but also affects anthocyanin concentration (Bobeica et al., 2015, Smith et al., 2019). Severe source limitation achieved by reducing leaf-to-fruit ratio caused the uncoupling of sugar and anthocyanin accumulations during berry ripening (Bobeica et al., 2015), which affects berry quality at harvest. Hence, in order to harvest high quality berries, in the context of a globally changing environment and maintain the sustainability of wine production, we need explore in depth the links between sugars and anthocyanins metabolisms.

Previous studies have explored the relationship between sugars and anthocyanins accumulation in grapes. Sugars were reported to induce the biosynthesis of anthocyanins (Larronde et al., 1998, Vitrac et al., 2000, Zheng et al., 2009, Dai et al., 2014). In addition to providing carbon skeleton and glucosides moieties for anthocyanins biosynthesis, sugars can also act as signalling molecules to regulate anthocyanins biosynthetic genes (Hara et al., 2003, Hu et al., 2016). Using an innovative in-vitro cultured berry system, Dai et al. (2014) reported that high concentration of sugar can increase the concentration of anthocyanin and advance its synthesis, while the concentration of its precursor, phenylalanine, was decreased by the high sugar supply. Moreover, Vitrac et al. (2000) reported that sugar-induced anthocyanin

biosynthesis may be mediated by Ca^{2+}-calmodulin dependant kinases and hexokinases (HXK). Zheng et al. (2009) also found that sugar-induced anthocyanin synthesis was dependent on HXK phosphorylation activity in sliced grape berries. These authors found that, mannose, which can be phosphorylated by hexokinase but is poorly metabolized, was able to induce the anthocyanin accumulation and *F3H* expression in grape; whereas 3-O-methylglucose and 6-deoxyglucose, which are not hexokinase substrates, did not induce anthocyanin accumulation, indicating that sugar phosphorylation mediated by HXK is essential for sugar-induced anthocyanin accumulation. Furthermore, it was confirmed that in apple, the glucose sensor HXK1 promotes anthocyanins biosynthesis by phosphorylating and stabilizing transcription factor *MdbHLH3*, which bounds to the promoters of *DFR*, *UFGT* and *Myb*1 under low temperature to increase anthocyanin accumulation (Xie et al., 2012, Hu et al., 2016). In addition, sugars were also reported to regulate the expression levels of the anthocyanin biosynthetic structural genes, including *CHS*, *DFR*, *LDOX* and *UFGT* and regulatory genes *MYB* (Gollop et al., 2001, Payyavula et al., 2013, Hu et al., 2016). Thus, sugar may regulate anthocyanin synthesis more likely as a signalling molecule, and this signalling role is related to HXK phosphorylation activity.

In our above study (in Chapter 3), we found that in addition to sugar, the reduction of ABA in berries under low leaf-fruit ratio conditions may be the reason for the reduction of anthocyanin at harvest. ABA is considered as the trigger for the rapid accumulation of anthocyanins at the onset of ripening (Gagné et al., 2006, Gambetta et al., 2010, Castellarin et al., 2016). Moreover, exogenous ABA application up-regulated the expression of anthocyanins biosynthetic genes, including structural genes *CHI*, *F3H*, *DFR*, *ANS* and *UFGT,* and the regulatory transcriptional factors *MybA1* and *MYBA2* thereby increasing the concentration of anthocyanins in berry (Yamane et al., 2006, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018). Hence, both sugar and ABA were able to increase anthocyanins accumulation and regulate genes involved in anthocyanins biosynthesis. Finally, ABA may also affect the accumulation of sugar in ripening berries by regulating the expression level of some genes during berry development, such as *VvMSA* (Çakir et al., 2003), *VvSK1* (Lecourieux et al., 2010) and hexose transporters *VvHT*s (*VvHT1,VvHT2*, *VvHT3* and *VvHT6*)

(Çakir et al., 2003, Murcia et al., 2018). Hiratsuka et al. (2001) even found that ABA enhanced the promotive effect of sugar on anthocyanins accumulation in berry. Thus, ABA and sugar signalling pathways most probably achieved crosstalk in anthocyanins biosynthesis regulation. However, to the best of our knowledge, only few studies have focused on the actual co-regulation mechanisms of ABA and sugar anthocyanins biosynthesis during berry ripening.

NAG (N-acetyl-glucosamine) and DGH (D-(+)-Glucosamine hydrochloride) are hexokinase inhibitors which, to a different extent, reduce HXK activities (Hofmann and Roitsch, 2000). Hofmann and Roitsch (2000) confirmed that high concentration of NAG was able to abolish HXK activity; while the same concentration of DGH had a lesser inhibitory effective. However, NAG did not affect glucose-activated MAPK (Mitogen Activated Protein Kinase) activity, while DGH stimulated phosphate transfer to MBP (myelin basic protein) at low concentrations and partially inhibited it at higher concentrations. In 2017 and 2018, using these two HXK inhibitors, which have different inhibitory effects on HXK and glucose-activated MAPK (Hofmann and Roitsch, 2000), we attempted to analyse the role of HXK in sugar-induced anthocyanin synthesis. Furthermore, we combined these two hexokinase inhibitors with exogenous ABA and NDGA (nordihydroguaiaretic acid, an ABA biosynthesis inhibitor), on grape berries cultivated *in vitro* in various glucose concentrations to explore the respective effects of sugar and ABA on anthocyanins biosynthesis and clarify the mechanistic relationship between sugar and ABA signalling and anthocyanins accumulation.

4.2 Materials and methods

4.2.1 Berry *in vitro* culture, treatments and sampling

Explants of grape berries were obtained from greenhouse-grown fruiting-cuttings of *Vitis vinifera L*. cv. Cabernet Sauvignon at 52 days after flowering (DAF) in 2017 and 54 DAF in 2018 according to the method of Dai et al. (2015). Pre-*véraison* berries were screened out and sterilized with 70% ethanol and NaClO with available chlorine 2.5% for 2 minutes. Berries were rinsed 3 times in sterile de-ionized water, had their pedicel excised and were put onto MS solid culture medium (Murashige and Skoog, 1962) supplemented with various chemicals according to the various treatments applied in 6-wells plates. Twenty-one treatments were made (Table 1): 0% glucose, 0% glucose with 100 mM N-acetylglucosamine (NAG), 0% glucose with 100 mM D-(+)-Glucosamine hydrochloride (DGH), 0% glucose with 200 µM ABA, 0% glucose with 100 µM NDGA, 0% glucose with 100 µM NDGA and 100 mM DGH, 0% glucose with 200 µM ABA and 100 mM DGH, 2% glucose, 2% glucose with 100 mM NAG, 2% glucose with 100 mM DGH, 2% glucose with 200 µM ABA, 2% glucose with 100 µM NDGA, 2% glucose with 100 µM NDGA and 100 mM DGH, 2% glucose with 200 µM ABA and 100 mM DGH, 8% glucose, 8% glucose with 100 mM NAG, 8% glucose with 100 mM DGH, 8% glucose with 200 µM ABA, 8% glucose with 100 µM NDGA, 8% glucose with 100 µM NDGA and 100 mM DGH, 8% glucose with 200 µM ABA and 100 mM DGH. Each treatment included 18 berries in 3 separated MS plates (3 x 9 berries). All the plates were transferred into a culture chamber with constant temperature of 26±0.5 °C, a light period of 16h/8h day/night, and light intensity of ~50 µmol m^{-2} s^{-1}. In 2017, all the berries were sampled after 20 days culture, while in 2018, berries were sampled at 3 different dates: the first day of *in vitro* culture, one week after culture and two weeks after culture. Sampled berries were immediately put into a tube and dropped into liquid nitrogen for later analysis. The fresh weight of the berries was calculated with the tubes re-weighed after deep freeze, and berries were ground into fine powder in liquid nitrogen with a ball grinder MM200 (Retsch, Haan, Germany). The powder of berries stored in -80°C for later biochemical analysis.

Table 1 Twenty-one treatments in the in vitro culture system of intact detached grape berries pre-*véraison* with different concentrations of glucose, HXK inhibitors, exogenous ABA or ABA synthesis inhibitor NDGA.

Treatments		
Glucose (%,w/v)	ABA	HXK inhibitors
0	CT	100 mM NAG
2	CT	100 mM NAG
8	CT	100 mM NAG
0	CT	100 mM DGH
2	CT	100 mM DGH
8	CT	100 mM DGH
0	200 µM ABA	100 mM DGH
2	200 µM ABA	100 mM DGH
8	200 µM ABA	100 mM DGH
0	100 µM NDGA	100 mM DGH
2	100 µM NDGA	100 mM DGH
8	100 µM NDGA	100 mM DGH
0	CT	CT
2	CT	CT
8	CT	CT
0	200 µM ABA	CT
2	200 µM ABA	CT
8	200 µM ABA	CT
0	100 µM NDGA	CT
2	100 µM NDGA	CT
8	100 µM NDGA	CT

4.2.2 Sugars, organic acids and free amino acids analysis

Aliquots of 250 mg fine powder of berry were used to perform the extraction of primary metabolites according to (Torres et al., 2017) with minor modifications. Extractions of primary metabolites was done by adding 2 mL of 80% ethanol at 80°C for 15 min, centrifuged 10 min at 5000 *g*, followed by another extraction step using 2 mL of 50% ethanol, and finally extracted once more using 2mL of de-ionized water. The extracts were pooled, dried in a Speed-Vac and re-dissolved in 2 mL ultrapure water for determinations of sugars, organic acids and amino acids. Glucose and fructose content were measured enzymatically with an automated micro-plate reader (EPOCH2, Biotek Instruments Inc., Winooski, VT,

USA) using the Glucose/Fructose kit from BioSenTec. Tartaric and malic acids were determined with an automated colorimetric method using the TRAACS 800 autoanalyzer (Bran-Luebbe) according to the method of (Bobeica et al., 2015). Amino acids were determined by UHPLC (UltiMate 3000 UHPLC system with FLD-3000 Fluorescence Detector, Thermo Electron SAS, Waltham, MA USA) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate according to Torres et al (2017). All the amino acids were identified and quantified with external chemical standards purchased from Sigma (St Louis, MO, USA).

4.2.3 Anthocyanins analysis

A 250 mg berry powder aliquot was freeze-dried for 72 h and the dried powder (~20 mg) were extracted in 500 µL methanol containing 0.1% HCL (v/v). Extracts were filtered through a 0.2-µm polypropylene syringe filter for HPLC analysis. Each individual anthocyanin was analyzed as described in (Acevedo De la Cruz et al., 2012) and modified by (Hilbert et al., 2015) by UHPLC coupled to a Diode Array Detector (UltiMate 3000, Therma Scientific, CA, USA). Anthocyanin quantification was carried out by peak area integration at 520 nm, using Malvidin-3-glucoside (Extrasynthèse, Lyon, France) as external standard.

4.2.4 ABA and ABA metabolites analysis

An aliquot of 250 mg of berry powder was freeze-dried for 72 h and the dried powder were extracted overnight at 4 °C in 500 µL 20% aqueous methanol according to (Speirs et al., 2013). ABA, ABA-glucose ester (ABA-GE), phaseic acid (PA) and dihydrophaseic acid (DPA) in samples with a deuterated internal standard were measured using liquid chromatography/mass spectrometry (Agilent 6410 Triple Quadrupole LC-MS/MS with Agilent 1200 series HPLC, Agilent Technologies Inc., Santa Clara, USA). The column used was a Phenomenex C18(2) 75 mm x 4.6 mm x 5 µm and column temperature was set at 40 °C. The solvents used were nanopure water and acetonitrile, both supplemented with 0.05 % acetic acid (v/v). Samples were eluted with a linear 15 min gradient starting at 10 % acetonitrile (v/v) and ending with 90 % acetonitrile (v/v). Eluted compounds were identified

by retention times and multiple reactions monitoring of mass-to-charge ratio (m/z) for parent and product ions of native and deuterated internal standards (Rossdeutsch et al., 2016).

4.2.5 Data statistical analysis

All data analysis was conducted with R software (R Development Core Team, 2010). Two-way crossed ANOVA analysis were tested for significance and interaction by applying the analysis of variance (ANOVA) followed by interaction2wt from package HH (Heiberger and Heiberger, 2006).

4.3 Result and discussion

4.3.1 Effect of NAG and DGH hexokinase inhibitors combined with different concentrations of glucose on berry composition

In order to verify the effect of glucose concentration on the biosynthesis of anthocyanin accumulation berries cultivated *in vitro*, we selected three glucose concentrations (0, 2 and 8%, w/v) supplied in the culture medium according to the results of Dai et al. (2014). In Fig. 1A, in NAG, DGH and the control (CT), the reducing sugars concentrations in berry significantly increased over the time and with the concentrations of glucose in medium. In 2017, hexokinase inhibitor DGH significantly decreased the concentration of reducing sugars in 0% glucose medium at 3 WAT, but had no effect in 2% and 8% glucose medium. In 2018, the accumulation of reducing sugars was accelerated in 8% medium with DGH at 1 WAT and in all mediums with DGH at 2 WAT. Taken together, these results suggested that both hexokinase inhibitors did not affect exogenous glucose uptake into the berry.

Meanwhile, the total anthocyanins concentrations in berry also increased over the time, and high glucose concentration in the culture medium resulted in higher level of anthocyanins in berries when harvested at 3 WAT (Fig. 1B). This result confirmed that the accumulation of anthocyanins was positively correlated with the berry sugar concentration, in agreement with previous studies (Zheng et al., 2009, Dai et al., 2014). Interestingly, NAG and DGH had different effects on the accumulation of anthocyanins. Compared with CT, DGH obviously limited the synthesis of anthocyanins in both years regardless of glucose concentration. In contrast, in medium with 2% and 8% glucose, the concentration of total anthocyanins was similar between NAG and CT, while anthocyanins were increased significantly in NAG-containing medium without glucose (0%), compared to control. This implies that glucose-induced synthesis of anthocyanins might not (or not only) depend on the hexokinase phosphorylation activity, but rather depends on glucose-activated MAP kinases, as suggested by the differences between NAG and DGH in their inhibition of glucose-phosphorylating HXK activity (Hofmann and Roitsch, 2000).

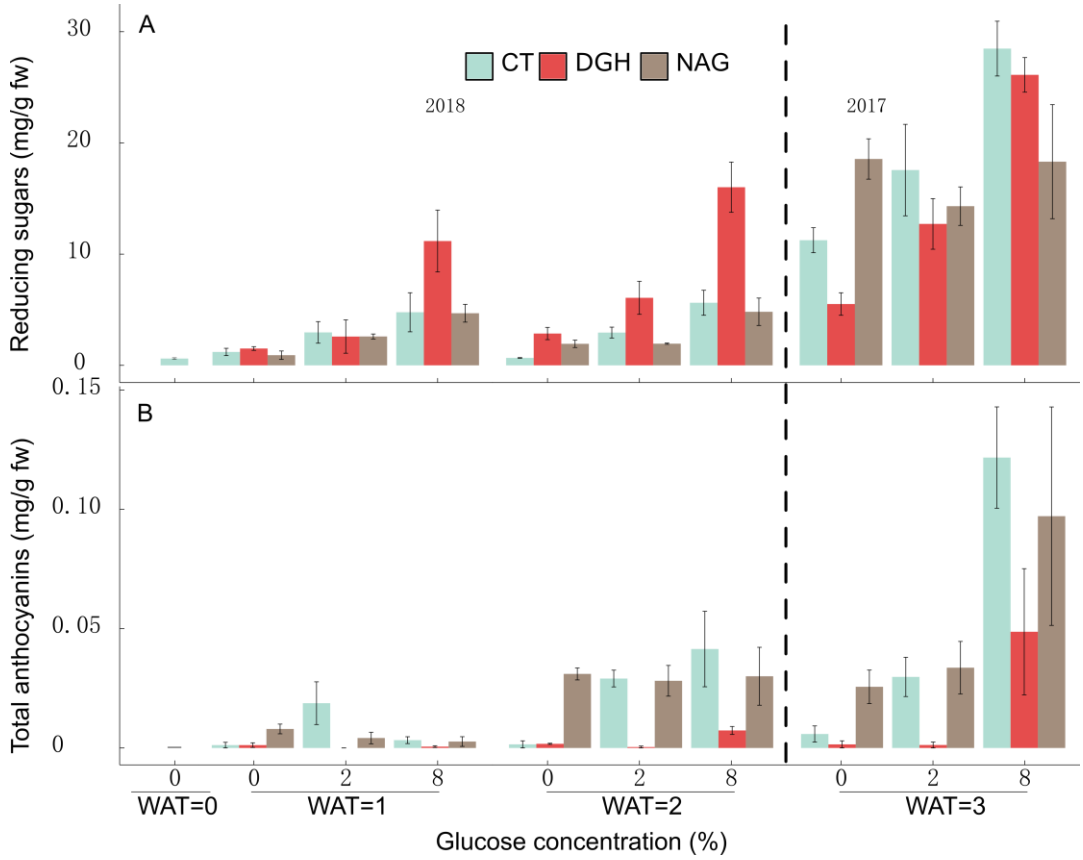

Fig.1 Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry reducing sugars (A) and anthocyanins (B). CT: control; WAT: weeks after treatment. WAT 0, 1 and 2 samplings were conducted in 2018 (the left part of the dashed line), and only WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

The concentration of total free amino acids increased slightly with the duration of *in vitro* culture (Fig. 2A). In 0% glucose in 2017, DGH resulted in a great increase in the total free amino acids due to a significant increase in the concentration of aspartate, but there was no same performance after two-week *in vitro* culture in 2018 (data not shown). Among the 21 free amino acids that we detected; GABA (γ-aminobutyric acid) increased significantly in DGH containing medium, under different glucose concentrations (Fig. 2B). GABA is a non-proteinaceous, four-carbon amino acid that is reported to be involved in multiple processes in plant, including the carbon flux into the TCA cycle, pH regulation, plant development and insect-defence, signalling (reviewed by Shelp et al., 1999, Hildebrandt et al., 2015). The increase in GABA may be due to the effect of DGH on the activity of glucose-related

enzymes, resulting in GABA shunting to provide intermediates for the tricarboxylic acid cycle of respiratory metabolism, thus maintaining metabolic homeostasis. In addition, exogenous GABA was able to significantly inhibit the expression of Chalcone synthase (CHS) related to anthocyanins synthesis under carbon limitation in *Arabidopsis* (Batushansky et al., 2014). Hence, GDH might inhibit anthocyanin synthesis via GABA shunting regulating the carbon flux. Since Phe is the entry point into the phenylpropanoid pathway which leads to anthocyanins biosynthesis (He et al., 2010), and since exogenous Phe was able to increase the production anthocyanins in grape cell suspensions (Krisa et al., 1999, Saw et al., 2010) or berries (Portu et al., 2015, Hattori et al., 2019), we tested the effects of various glucose concentrations and exogenous ABA application on Phe on *in vitro* cultured berries. Fig. 2C showed that the concentration of Phe was decreased by a high glucose supply to berries, which was consistent with the conclusion of Dai et al. (2014). As anthocyanins accumulation increased with the increase of glucose concentrations, a possible explanation for this negative correlation between Phe and anthocyanins concentrations might be that in high sugar concentration cultivated berries the metabolic flux in the L-phenylalanine ammonia lyase (PAL) is increased, thus depleting berry Phe concentration. Indeed, Soubeyrand et al. (2018) reported a 38% increase in PAL metabolic flux in grape cells cultivated under high C/N ratio (i.e. in high sugar, low nitrate medium), which support this hypothesis. However, the concentration of Phe in DGH treatment did not change with the different concentration of glucose, and remained relatively low compared with the control and NAG treatments. Take all together, this suggests that DGH might limit Phe synthesis and also affect the activity of PAL, leading to the decrease of anthocyanins in DGH.

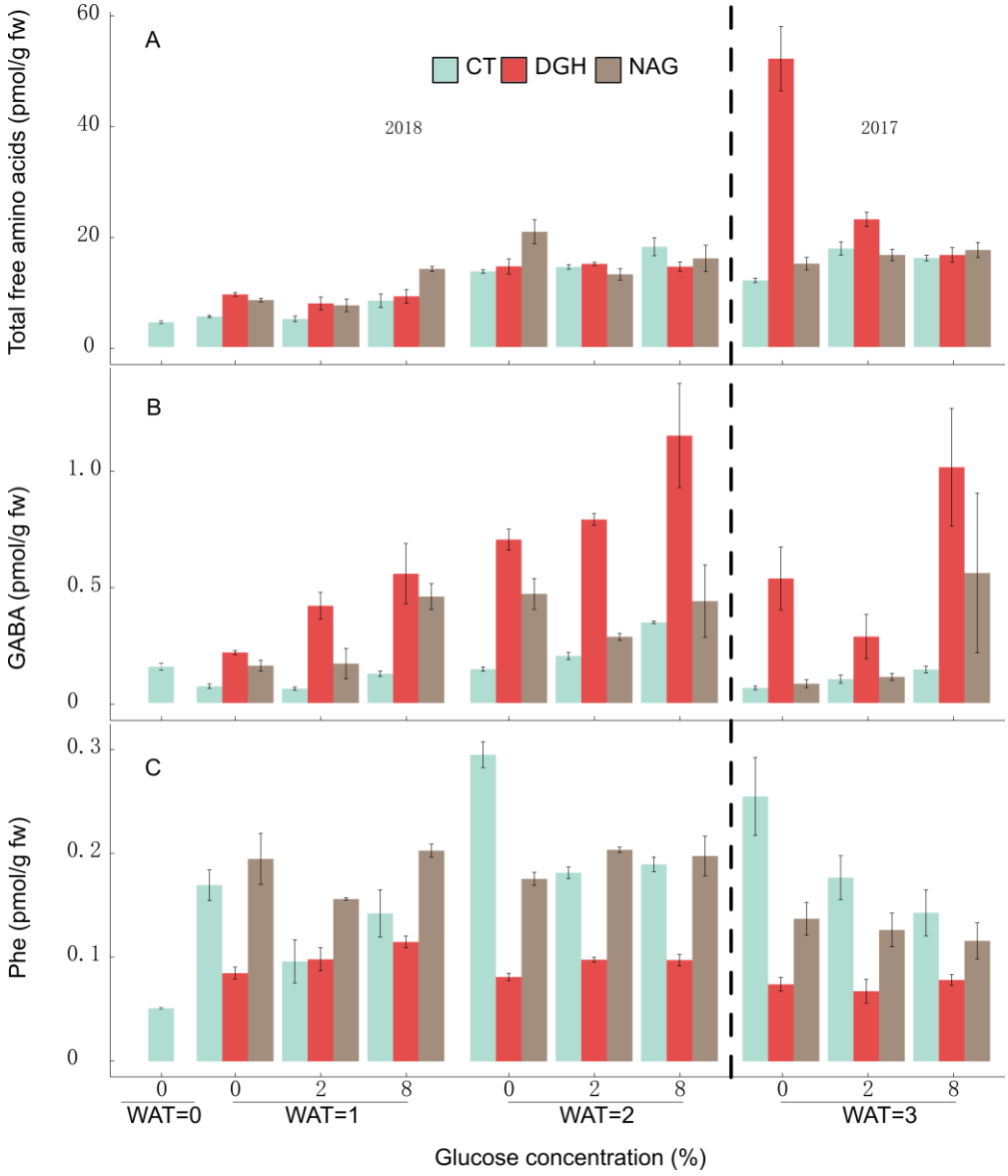

Fig.2 Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry total free amino acids (A), GABA (B) and Phe (C). CT: control; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Total organic acids in control and DGH appeared to decrease slightly with the duration of *in vitro* culture, while total organic acids in NAG increased at 2 WAT and decrease at 3 WAT (Fig. 3A). DGH led to a significant decrease in total organic acids at 2 WAT compared with NAG. The differences among the three treatments were largely due to the response of malic

acid to the HXK inhibitors. As shown in Fig. 3B, malic acid content in control treatment decreased with the duration of *in vitro* culture, more markedly in DGH-treated berries, and the decrease was delayed by a week in NAG-treated berries. Conversely, the different concentrations of glucose and the two HXK inhibitors had no significant effect on the accumulation of tartaric acid (Fig. 3C). In summary, both high glucose concentration (8%) and NAG treatment led to a delay in the degradation of malic acid in berries after *véraison*, but DGH had the opposite effect. As the effective inhibition of the phosphorylation activity of HXK (Hofmann and Roitsch, 2000), the result means that it is the glucose sensing function of HXK rather than phosphorylation affects the malic acid metabolism. As malic acid can be also used as carbon and energy source for respiration, which may occur more before *véraison* (Ruffner et al., 1984, Famiani et al., 2000), the reduction in malic acid exacerbated by DGH might be the result of GABA shunt regulation, resulting in the carbon flux change to TCA. Meanwhile, malic acid is able to be converted to sugars by gluconeogenesis *via* phosphoenolpyruvate carboxy kinase activity (Hawker, 1969, Famiani et al., 2018), and the concentrations of reducing sugars in berries added with DGH significantly increased compared with control at 2 WAT, hence, it is not ruled out a gluconeogenic carbon flux from organic acids into sugars.

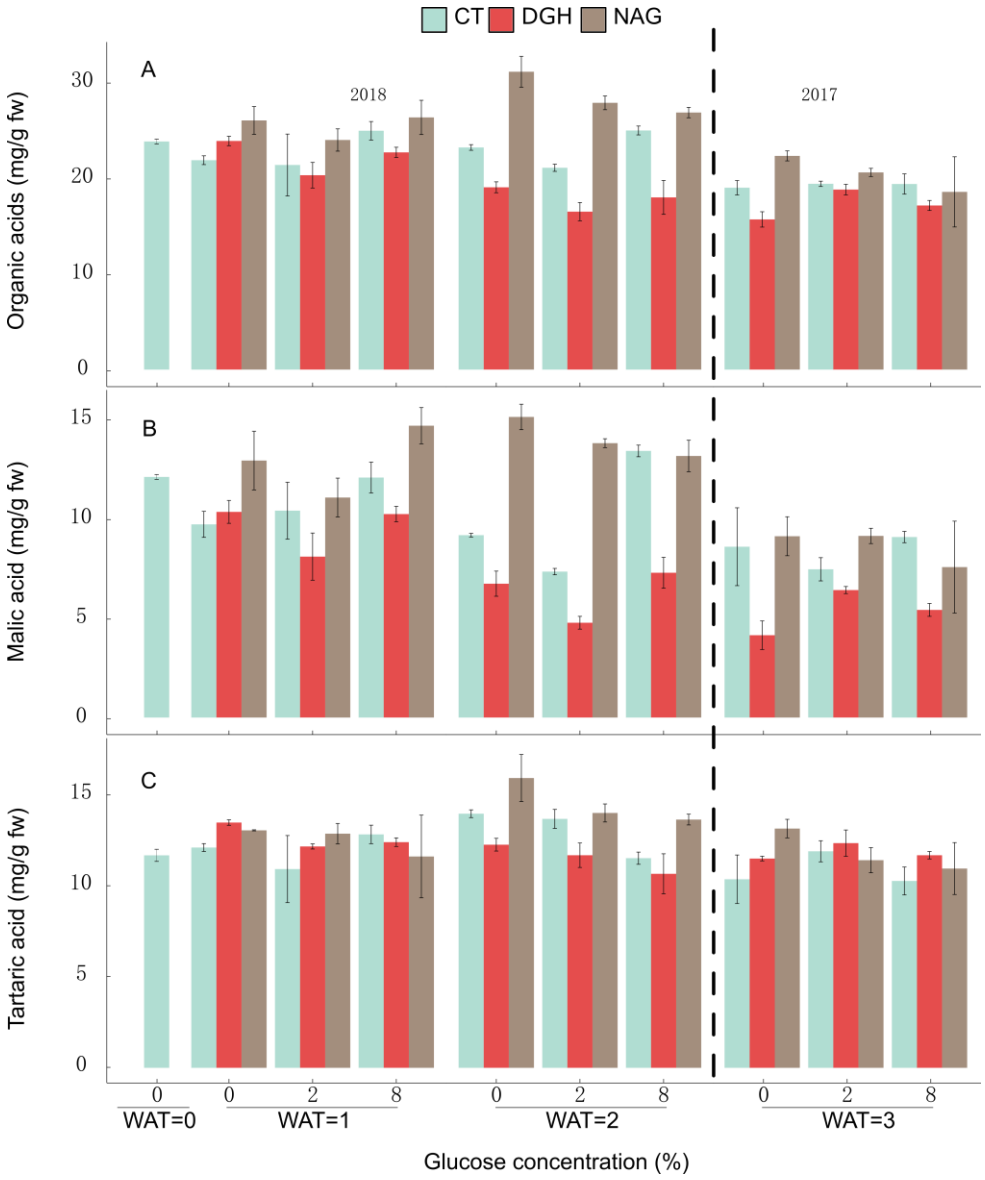

Fig.3 Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry total organic acids (A), malic acid (B) and Tartaric acid (C). CT: control; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

In conclusion, although both DGH and NAG are the inhibitors of HXK, but their effects on anthocyanins, malic acid, total free amino acid, GABA and Phe concentrations in berries were different. These differences might imply that DGH had an effect on sugar metabolism, leading to the reduction of carbon sources for secondary metabolites, which can provide some

support for the hypothesis mentioned by (Bobeica et al., 2015) that carbon allocation between primary and secondary metabolites has an effect on anthocyanins synthesis.

4.3.2 The relationship of sugar accumulation and ABA on the induction of anthocyanins accumulation

Considering the above-mentioned differential effects of DHG and NAG on primary metabolism, we chose DGH to further explore the relationship between sugar accumulation and ABA signalling on the induction of anthocyanins biosynthesis and accumulation.

Reports on the effect of exogenous ABA on berry or whole plant sugar content are contradictory. Several reports have shown that exogenous ABA application on berry or whole plant resulted in an increase in berry sugar content (Matsushima et al., 1989, Wheeler et al., 2009, Murcia et al., 2016, Murcia et al., 2018). Conversely other authors reported that ABA had no effect on or even led to a decrease in sugar content (Kataoka, 1982, Mori et al., 2005a, Sandhu et al., 2011, Murcia et al., 2017). Hence, we first examined whether exogenous ABA can promote sugar accumulation in berry, in our *in vitro* culture system. As shown in Fig. 4A, ABA had no significant effect on the concentration of sugar, nor did NDGA. Through the two-way crossed ANOVA analysis (Fig. 5), we found that glucose concentration in the medium was the main factor affecting the concentration of berry reducing sugar content, and that there was no significant interaction between ABA treatment and sugar content in the culture media on the accumulation of reducing sugars in berries.

Exogenous ABA can significantly increase anthocyanins concentrations in the mediums without inhibitors, but DGH inhibited this promoting effect (Fig. 4B). Moreover, the result of two-way crossed ANOVA analysis showed that both ABA and sugar concentrations had significant effects on anthocyanins accumulation, and there was an interaction between ABA and sugar in this process (Fig. 6A, D). These results corroborate the findings of a great deal of the previous work in ABA or sugar inducing the synthesis and accumulation of anthocyanins in berries (Larronde et al., 1998, Hiratsuka et al., 2001, Yamane et al., 2006, Zheng et al., 2009, Dai et al., 2014, Villalobos-Gonzalez et al., 2016, Koyama et al., 2018).

Although microarray analysis in *Arabidopsis* demonstrated that the expression of many genes is co-regulated by both sugar and ABA (Li et al., 2006), only a few studies had reported the interaction between ABA and sugar on promoting anthocyanins synthesis in grape berry (Das et al., 2012). Hiratsuka et al. (2001) found ABA enhanced the promotive effect of sugar on anthocyanins accumulation in grape berry cultured *in vitro*, but the mechanisms was not known at that time.

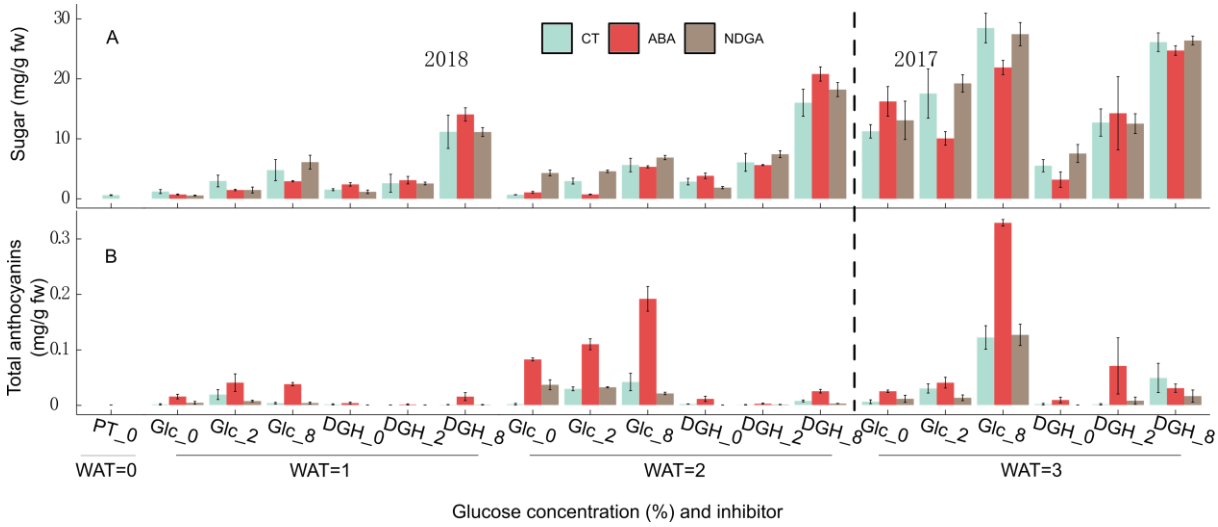

Fig.4 Effect of exogenous ABA and its biosynthesis inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry sugars (A) and anthocyanins (B). Glc_0, 2 and 8 represent 0%, 2% and 8% (w/v) glucose in medium respectively; DGH_0, 2 and 8 represent 100 mM DGH with 0%, 2% and 8% (w/v) glucose in medium respectively. CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0, 1, and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

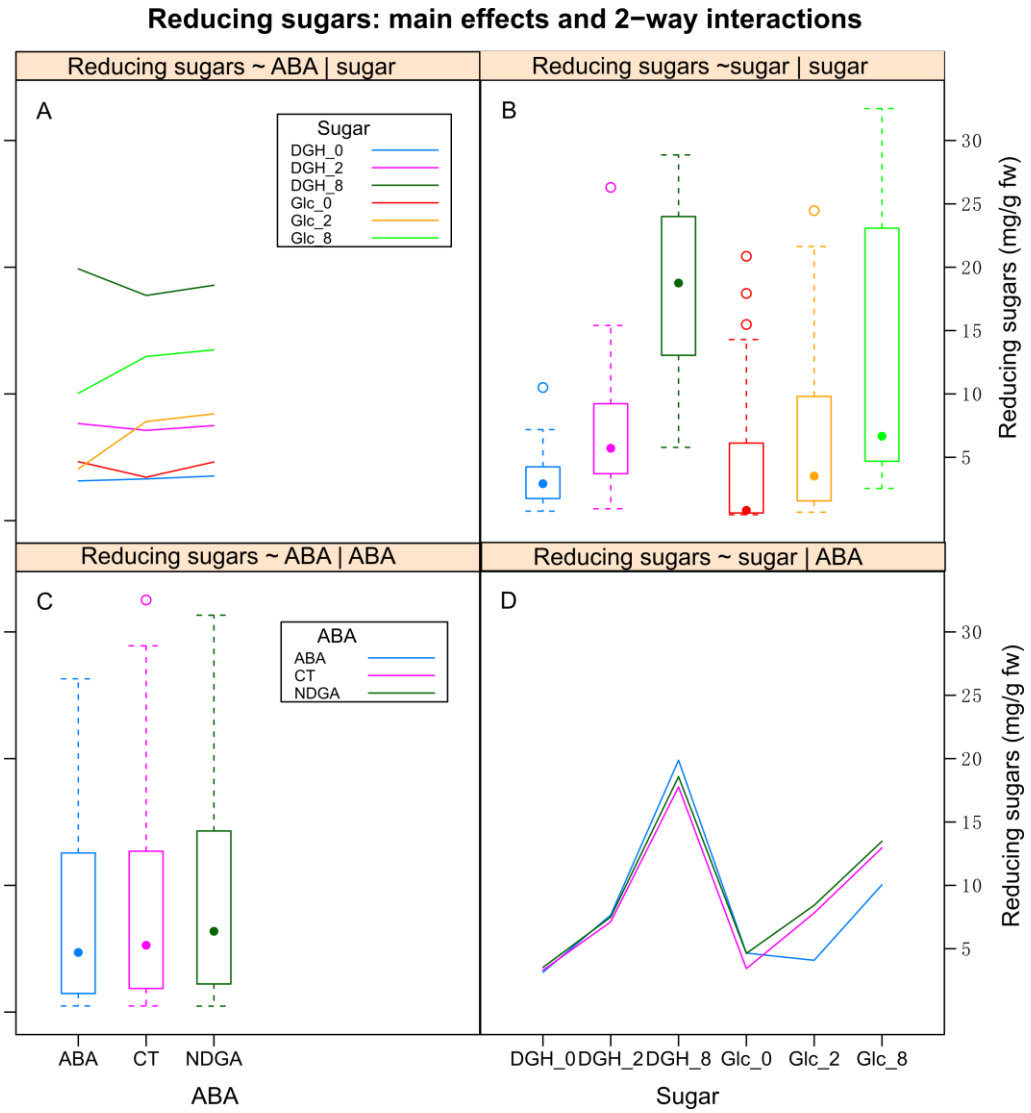

Fig.5 The main effects and 2-way interactions of sugar and ABA on berry reducing sugars. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 4A.

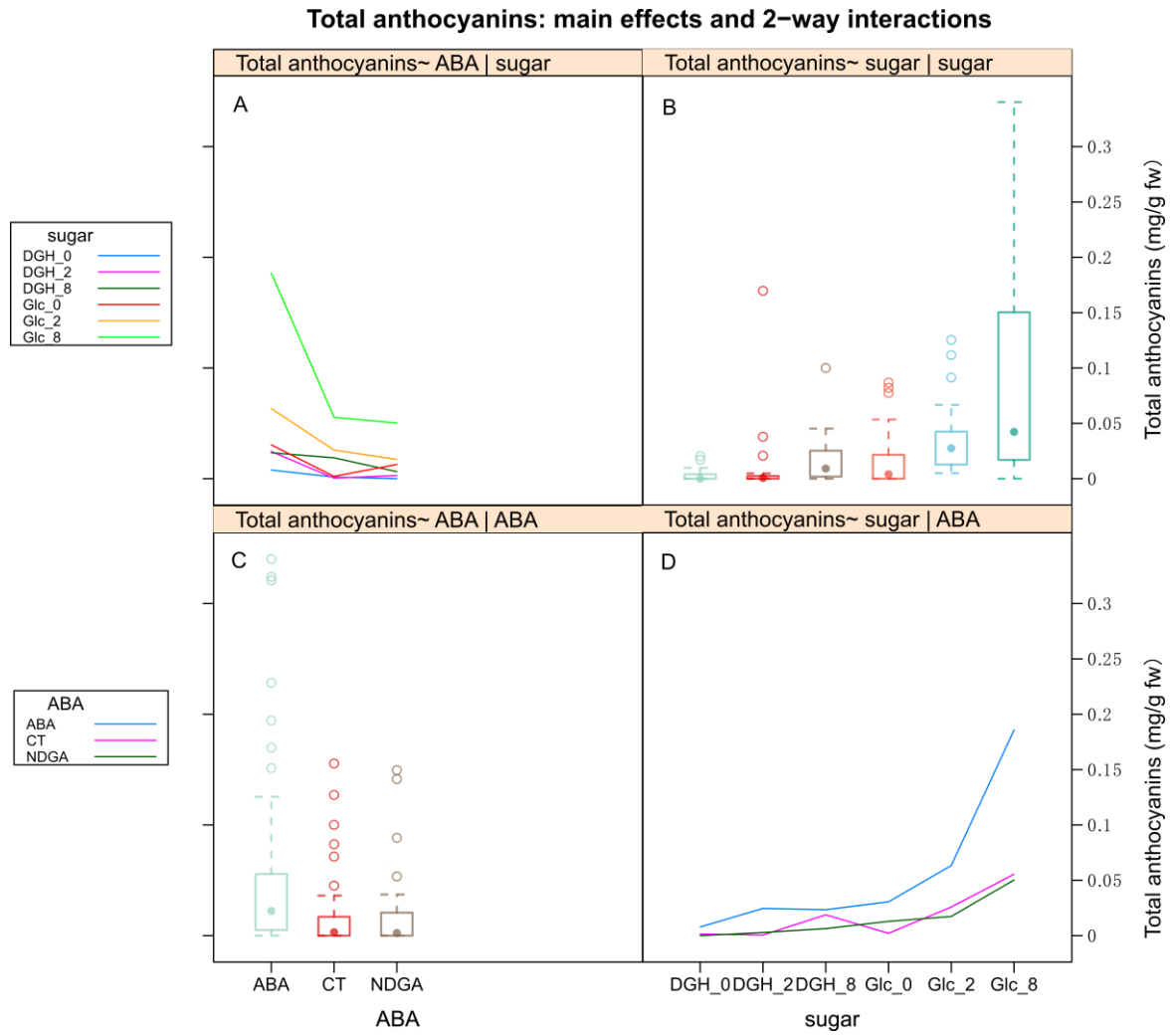

Fig.6 The main effects and 2-way interactions of sugar and ABA on berry anthocyanins. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The dataset used was the same as that in Fig. 4B.

As we found that DGH had significant negative effects on some free amino acid concentrations, especially on Phe, we expanded our analysis of the effects of ABA on free amino acids to further explore the possible interaction between ABA and sugar inducing anthocyanins biosynthesis. As shown in Fig. 7A, ABA had no significant effect on the concentration of total free amnio acids, nor did NDGA. However, the addition of exogenous ABA in medium led to the significant increase in GABA concentration in the medium with or

without DGH (Fig. 7B). It might be due to the effect of ABA on carbon allocation between sugar and anthocyanins. It is interesting to note that ABA led to a significant decrease in Phe concentrations in the berries cultivated in the mediums without DGH, but NDGA had no significant effect on it (Fig. 7C). Moreover, ABA did not affect the concentration of Phe significantly in the berries cultivated in the mediums supplemented with DGH. A possible explanation for this might be that, without DGH, ABA induces anthocyanins biosynthesis by increasing the expression of anthocyanins synthesis genes which increases the metabolism of Phe, while ABA had little effect the synthesis of Phe, which finally resulting in the decrease in the concentration of Phe in the berries; with DGH, DGH already lead to the low concentration of Phe, and may also inhibit the induction effect of ABA on anthocyanins, resulting in no difference of the Phe concentration between ABA and control. In addition, the result of two-way crossed ANOVA analysis showed that both ABA and sugar had significant effects on Phe concentration, and that there was an interaction between ABA and sugar in this process (Fig. 8), which is in agreement with the observed effects on anthocyanins accumulation in berries.

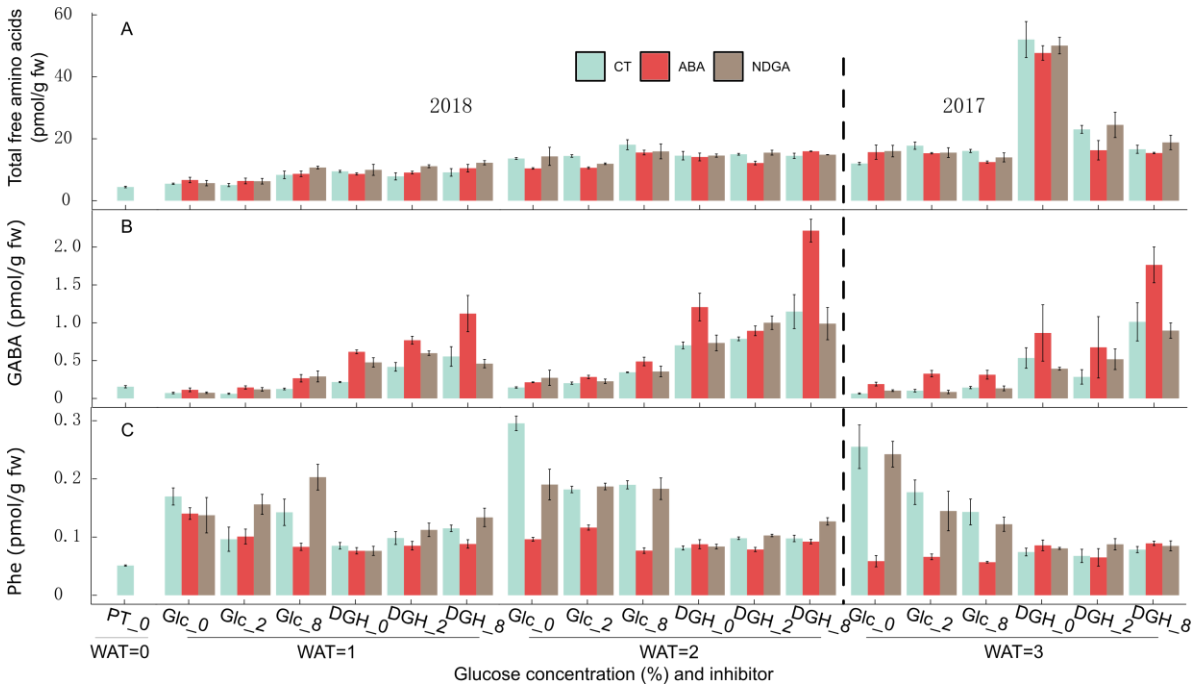

Fig.7 Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry total free amino acids (A), GABA (B) and Phe (C). CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

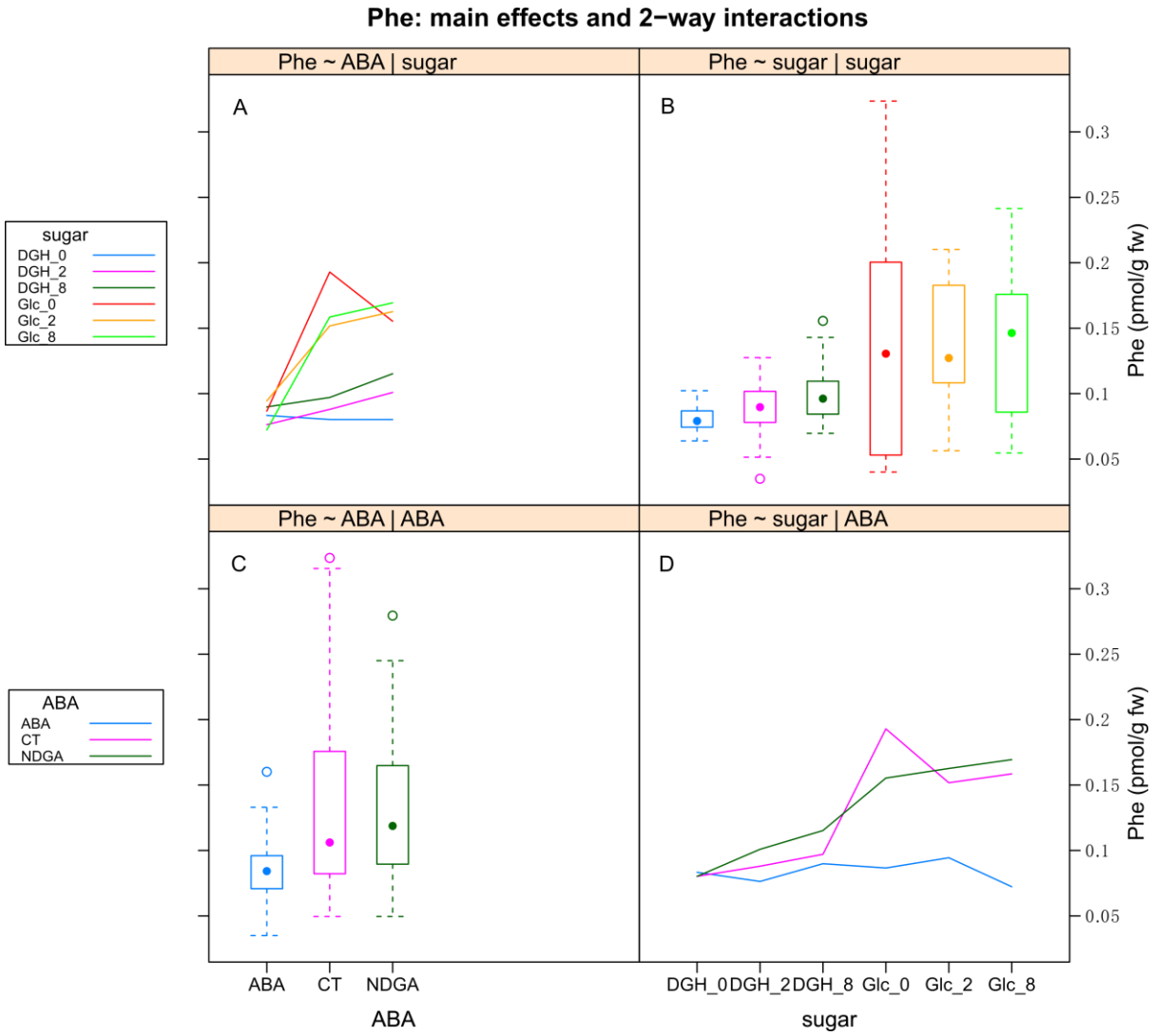

Fig.8 The main effects and 2-way interactions of sugar and ABA on berry phenylalanine. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 7C.

Finally, we found that total organic acid in berries without DGH had no significant change during 2 weeks after *in vitro* culture but decreased significantly in 3 WAT (Fig. 9A). This was consistent with changes in malic acid (Fig. 9B). In the berries cultivated in DGH conditions, the total organic acid decreased advanced a week. Interestingly, exogenous ABA addition led to a significant decrease in malic acid regardless of glucose concentration and of

the presence of DGH (Fig. 9B), while ABA reduced slightly the concentration of tartaric acid in the berries with DGH (Fig. 9C). This result is consistent with cytosolic NADP-dependent malic enzyme (ME) increasing in pulp after exogenous ABA treatment at *véraison* (Giribaldi et al., 2010), as NADP-malic enzyme is considered to play a key role to regulate the malate breakdown (Yinshan et al., 2017). Two-way crossed ANOVA analysis indicates that although DGH and ABA both resulted in significant reductions in malic acid during the late culture, but there was no interaction between them (Fig. 10).

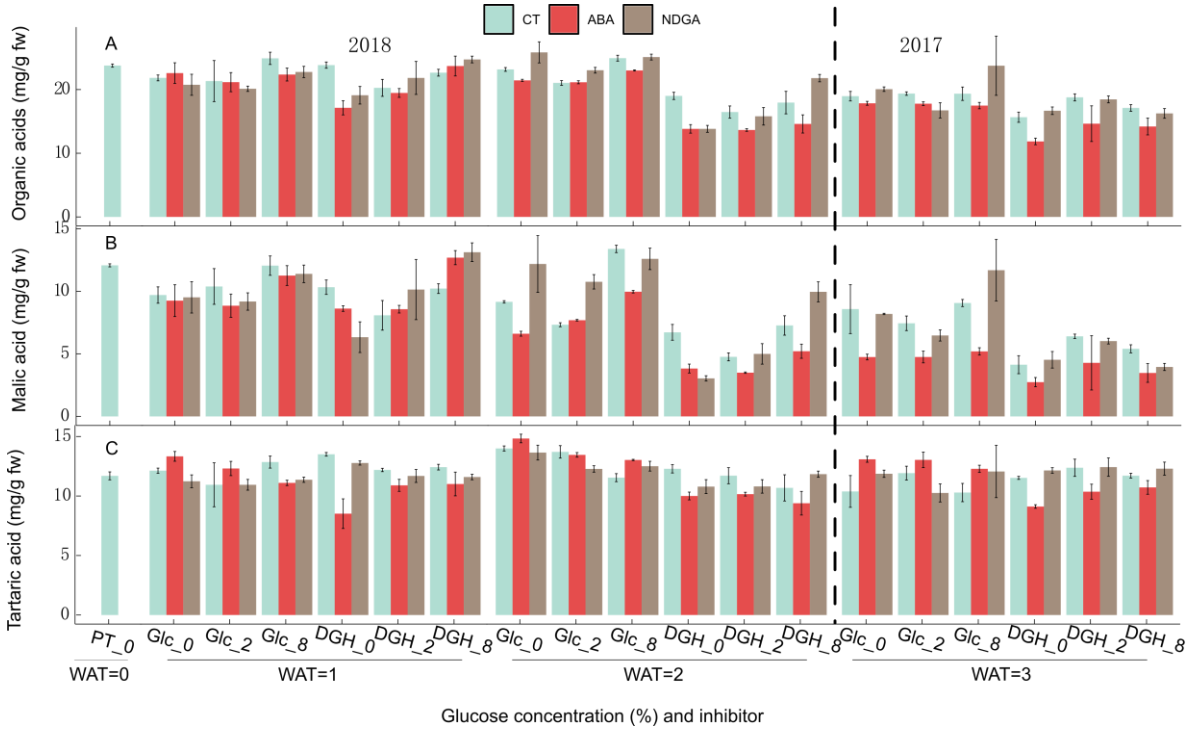

Fig.9 Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry total organic acids (A), malic acid (B) and Tartaric acid (C). CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

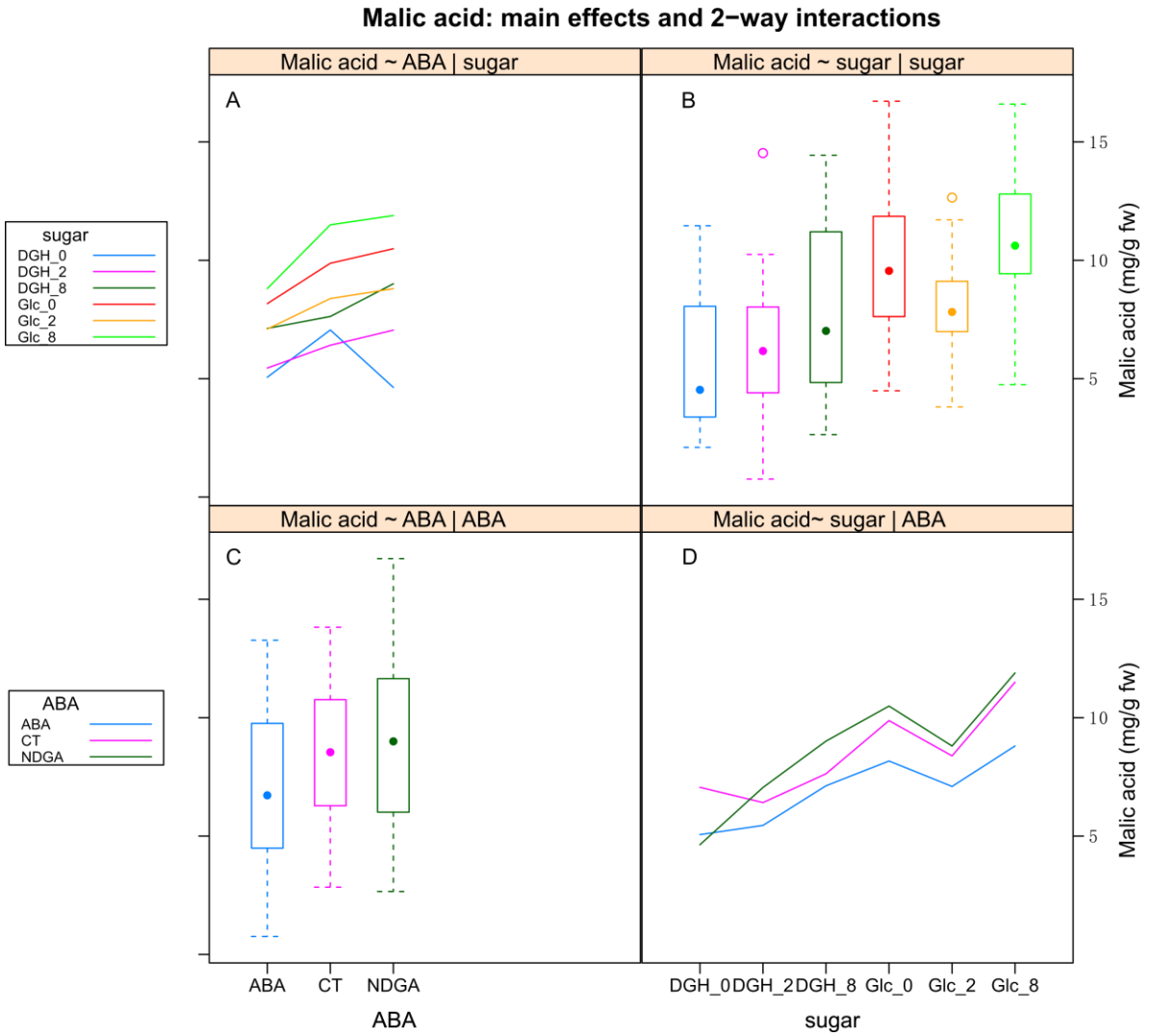

Fig.10 The main effects and 2-way interactions of sugar and ABA on berry malic acid. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 8B.

4.3.3 The effect of HXK inhibitors and exogenous ABA on berry ABA and its catabolism products

To explore the relationship between sugar, ABA and anthocyanins in ripening berries, we analysed ABA concentration in fruits after 3 weeks of *in vitro* culture in 2017. Both HXK inhibitors (DGH and NAG) did not significantly affected the concentration of endogenous ABA (Fig. 11A); but they did significantly increase the concentration of ABA-GE ($p = 0.00682$), and DPA ($p = 0.01661$) (Fig. 11B and D). Exogenous ABA can greatly increase ABA concentrations in berries as well as the concentrations of ABA catabolism products (Fig. 12A and B); thus, indicating that exogenous ABA can enter the berries cultivated *in vitro* and can be metabolized. Meanwhile, NDGA had no significant effect on berry ABA concentrations (Fig. 12A), but can decrease significantly ABA-GE in the berries cultured with DGH, and offset the effect of DGH on ABA-GE (Fig. 12B). This result support the findings by Castellarin et al. (2011, 2016) that the initial increase in berry ABA at *véraison* was not related to ABA biosynthesis in berries, but may result from decreases in catabolism (ABA-GE) and/or exogenous import. Prior studies have noted the possibility that ABA could be released by the hydrolysis of ABA-GE with β - glucosidase (Dietz et al., 2000, Lee et al., 2006, Xu et al., 2012, Hussain et al., 2020). However, the responsible enzymes did not change around *véraison* (Castellarin et al., 2016) when ABA concentrations increases, and therefore this pathway may not play an important role in modifying ABA pool in grape berry. Further work is warranted to examine the relevant genes or enzyme activities to obtain more detailed information.

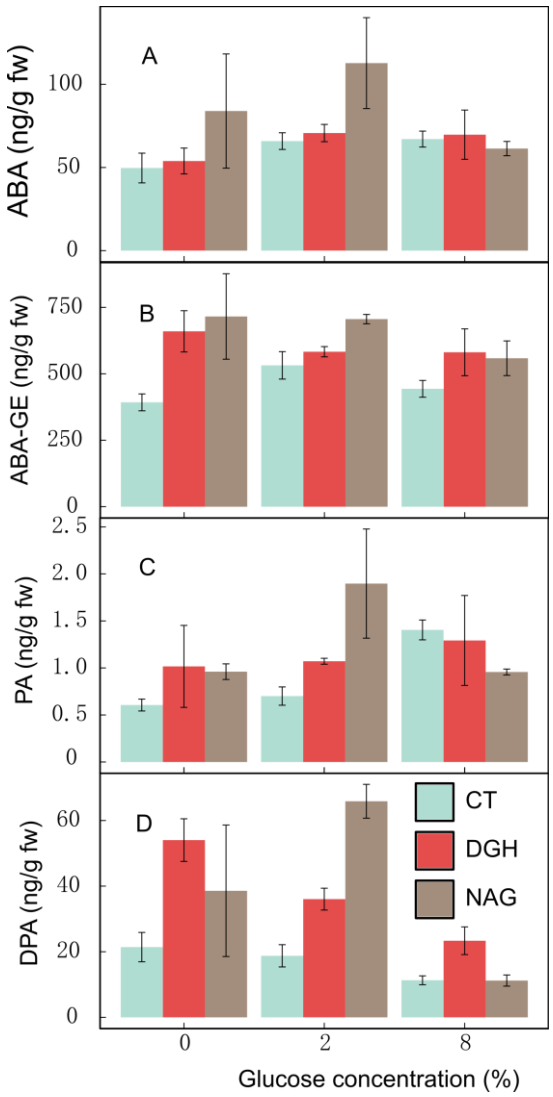

Fig.11 Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on ABA and its catabolism products. ABA (abscisic acid), A; ABA-GE (ABA glucose ester), B; PA (phaseic acid), C; neoPA (neophaseic acid), D; DPA (diphaseic acid), E. CT: control; The data used was from 3 week after treatment (WAT = 3, in 2017). Vertical bars indicate standard error (SE) (n=3).

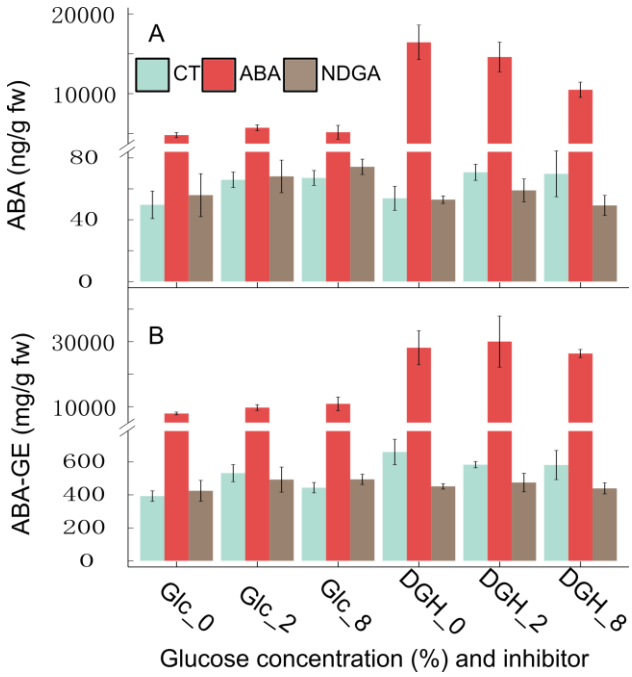

Fig.12 Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on ABA and its catabolism products. ABA (abscisic acid), A; ABA-GE (ABA glucose ester), B. CT: control; The data used was from 3 week after treatment (WAT = 3, in 2017). Vertical bars indicate standard error (SE) (n=3).

4.4 Conclusion

In this study, we designed a series of experiments to explore the mechanism by which sugar and ABA induce anthocyanins synthesis and accumulation in grape berry, and whether it involves interaction between sugar and ABA signalling, using an in berry *in vitro* culture system.

Firstly, we ruled out the possibility that ABA induced anthocyanin synthesis upstream or downstream of sugar signalling pathway. This study confirmed that sugar concentrations or HXK inhibitors had little effect on ABA synthesis in berry. Meanwhile, ABA did not significantly increase the concentration of reducing sugars in ripening berries. Therefore, ABA and sugar can independently induce anthocyanin synthesis at *véraison*, without the strict need for each other. Since sugar and ABA do have significant effects on both Phe and anthocyanins, the two signalling pathways of ABA- and sugar-induced anthocyanin biosynthesis may show some cross-talk as suggested by the interaction between sugar and ABA factors noticed in the two-way crossed ANOVA analysis. High concentration of glucose in the culture medium was able to increase anthocyanins accumulation and reduced the Phe concentration at the same time. Moreover, since NAG, which is able to abolish hexokinase activity at the concentration we used (100 mM, Hofmann and Roitsch (2000), had no significant effect on anthocyanins synthesis, the synthesis of anthocyanin induced by high glucose concentration does not depend on HXK phosphorylation activity. Hence, on the one hand, it could conceivably be hypothesised that sugar acted as a signalling molecule to induce the synthesis of anthocyanins, by promoting structural and regulatory genes expression and/or enzyme activities in the anthocyanin synthesis pathway, which led to the decrease of Phe observed with the increase in sugar concentration. On the other hand, DGH blocked the promoting effect of high sugar concentration on anthocyanins synthesis; and led to a decrease in Phe via inhibiting a glucose-activated MAP kinase. This finding confirmed the role of sugar as carbon source affecting the synthesis of anthocyanins. DGH can also block the promoting effect of ABA on anthocyanins, and a possible explanation for this result may be the lack of Phe caused by DGH. Finally, a last possible explanation is that the glucose-

activated MAP kinase inhibited by DGH may up-regulate anthocyanins synthesis pathway. This possibility is supported by the finding of Li et al. (2016) that a MAP kinase, MPK4, interacts with and phosphorylates MYB75, which regulates anthocyanin accumulation in *Arabidopsis*. Finally, since ABA alone had no effect on sugar concentration, we speculated that the target of ABA-induced anthocyanins increase was downstream of Phe (downstream the PAL enzymatic step). A recent study by Hattori et al. (2019) reported that both exogenous Phe and ABA applications resulted in an increase in anthocyanin accumulation, and there was an interaction between Phe and ABA, which is consistent with our results. This means that both sugar as signalling molecule and ABA can induce anthocyanins synthesis through promoting the expression of genes or enzyme activity in the anthocyanin synthesis pathway, and the specific signalling pathway remains to be further studied in depth.

To the best of our knowledge, this study demonstrates for the first time the dual role of sugar in inducing anthocyanins synthesis, and to find that ABA may only interact with sugar considered as a signalling molecule on the up-regulation of the expression of genes or enzyme activity involved in the anthocyanin synthesis pathway, without affecting the role of sugar as a carbon source.

Figures

Fig. 1. Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry reducing sugars (A) and anthocyanins (B). CT: control; WAT: weeks after treatment. WAT 0, 1 and 2 samplings were conducted in 2018 (the left part of the dashed line), and only WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 2. Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry total free amino acids (A), GABA (B) and Phe (C). CT: control; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 3. Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on berry total organic acids (A), malic acid (B) and Tartaric acid (C). CT: control; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 4. Effect of exogenous ABA and its biosynthesis inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry sugars (A) and anthocyanins (B). Glc_0, 2 and 8 represent 0%, 2% and 8% (w/v) glucose in medium respectively; DGH_0, 2 and 8 represent 100 mM DGH with 0%, 2% and 8% (w/v) glucose in medium respectively. CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0, 1, and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 5. The main effects and 2-way interactions of sugar and ABA on berry reducing sugars. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 4A.

Fig. 6. The main effects and 2-way interactions of sugar and ABA on berry anthocyanins. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The dataset used was the same as that in Fig. 4B.

Fig. 7. Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry total free amino acids (A), GABA (B) and Phe (C). CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0,1 and 2

was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 8. The main effects and 2-way interactions of sugar and ABA on berry phenylalanine. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 7C.

Fig. 9. Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on berry total organic acids (A), malic acid (B) and Tartaric acid (C). CT: control; PT: pre-treatment; WAT: weeks after treatment. WAT = 0,1 and 2 was conducted in 2018 (the left part of the dashed line), and WAT = 3 was conducted in 2017 (the right part of the dashed line). Vertical bars indicate standard error (SE) (n=3).

Fig. 10. The main effects and 2-way interactions of sugar and ABA on berry malic acid. The sugar factor was including DGH_0, DGH_2, DGH_8, Glc_0, Glc_2 and Glc_8 (B and D); the ABA factor was including ABA, CT and NDGA (A and C). The lower-left (C) and upper-right graphs (B) were the ABA and sugar main effects respectively, while the upper-left (A) and lower-right graphs (D) were interaction effects. CT: control. The data used was the same as that in Fig. 8B.

Fig. 11. Effect of hexokinase inhibitors DGH and NAG with different concentrations of glucose on ABA and its catabolism products. ABA (abscisic acid), A; ABA-GE (ABA glucose ester), B; PA (phaseic acid), C; neoPA (neophaseic acid), D; DPA (diphaseic acid), E. CT: control; The data used was from 3 week after treatment (WAT = 3, in 2017). Vertical bars indicate standard error (SE) (n=3).

Fig. 12. Effect of exogenous ABA and the inhibitor NDGA under different concentrations of glucose with or without the inhibitor DGH on ABA and its catabolism products. ABA

(abscisic acid), A; ABA-GE (ABA glucose ester), B. CT: control; The data used was from 3 week after treatment (WAT = 3, in 2017). Vertical bars indicate standard error (SE) (n=3).

Table 1. Twenty-one treatments in the in vitro culture system of intact detached grape berries pre-*véraison* with different concentrations of glucose, HXK inhibitors, exogenous ABA or ABA synthesis inhibitor NDGA.

Chapter 5 General discussion

Global climate change is modifying the composition of berries, causing higher sugar concentrations in berry and lowering secondary metabolites content, such as anthocyanins, consequently impairing wine quality and typicality (Van Leeuwen and Destrac-Irvine, 2017). Modifying vine canopy to regulate leaf-to-fruit ratio is one classical and efficient ways to control berry quality in viticulture. To maintain wine style and quality and reach the optimum berry composition, the effect of adaptation strategies on berry composition and its mechanism need to be carefully studied.

This study mainly investigated the phenomenon and biological mechanisms underlying the unbalance of sugar and anthocyanin accumulation during berry ripening caused by reducing leaf-to-fruit ratio, as well as the reasons why this unbalance can be partially restored by exogenous ABA application on berries. By combining metabolic analysis with the transcript level analysis of genes related to sugar, anthocyanins and ABA metabolisms, we sought to identify key factors regulating the carbon flux between sugar (the primary metabolites) and anthocyanins (secondary metabolites).

5.1 A decrease in ABA concentration in berry caused by reducing leaf-to-fruit ratio could be the reason for the unbalanced decrease in sugar and anthocyanins

Bobeica et al. (2015) observed that source limitation led to the decoupling of the accumulation of sugars and anthocyanins, and they speculated that this was caused by the adjustment of carbon allocation. That is, under the condition of source limitation, carbon was preferentially used for sugar accumulation rather than anthocyanins biosynthesis. In fact, an Arabidopsis transcription factor ectopically expressed in tomato, *AtMYB12*, was confirmed to bind directly to the promoter region of genes encoding enzymes of primary metabolism to promote carbon allocating to secondary metabolism, resulting in the decrease in sugar and

Phe concentrations and the increase of anthocyanin biosynthesis (Zhang et al., 2015). Hence, in grape, one can hypothesize that there might be similar transcription factors respond to the changes of carbon supply, regulating the flux of carbon between primary and secondary metabolism, and resulting in the unbalanced decrease in sugar and anthocyanin accumulations by reducing leaf-to-fruit ratio. A BLAST search using the Ensembl40 annotation and AtMYB12 protein sequence as a probe return 50 hits with highly significant E-values, the closest orthologous sequence being the protein corresponding to the VIT_05s0077g01360.t01 transcript, corresponding to gene model VIT_05s0077g01360.

In addition to being a carbon source, sugar has also been shown to regulate the expression level of some structural genes involved in anthocyanins synthesis pathway, such as *CHS*, *F3H*, *DFR* and *LDOX* or enzymes activity, such as PAL (Roubelakis-Angelakis and Kliewer, 1986, Gollop et al., 2001, Gollop et al., 2002, Zheng et al., 2009). Moreover, the anthocyanin contents in grape berry are often positively correlated with the sugar levels, especially in the skin (González-SanJosé and Diez, 1992, Liang et al., 2011). When reducing sugar accumulation in berry by reducing CO_2 concentration for the whole vine, anthocyanins decreased at the same rate as the concentration of sugar during the ripening of grape berries (Smith et al., 2019). The application of anti-transpiring Vapor Gard on vine only resulted in a decrease in berry sugar concentration, but did not affect anthocyanins accumulation (Di Vaio et al., 2019). These results suggested that if only the berry sugar concentration had been reduced, the ratio of sugar to anthocyanins did not increase. However, in our study, reducing leaf-to-fruit ratio resulted in a significant increase in the ratio of sugar and anthocyanins during berry ripening and at harvest, and the increase showed distinct dose-dependent response to the ratios. Hence, other anthocyanins regulators might be involved during berry ripening and affected by reducing leaf-to-fruit ratio.

ABA is an important inductor of anthocyanin synthesis in grape (Pilati et al., 2017). ABA signaling orthologs were found to be activated together with sugar signaling orthologs at the onset of ripening in grape (Gambetta et al., 2010), and the ABA initially increased at *véraison* in berry might come from leaves (Antolín et al., 2003, Castellarin et al., 2016). Moreover, Kataoka et al. (1982) reported that endogenous ABA levels in the skin and pulp did not increase throughout ripening period after leaves removal at *véraison*. Hence, the unbalanced

decreases in sugar and anthocyanins by reducing leaf-to-fruit ratio might be caused by the reduction of ABA supply from leaves.

By detecting the concentration of ABA and its catabolites in berry, similar to Kataoka et al. (1982), we found that decreasing the leaf-fruit ratio significantly reduced the concentration of free ABA during berry ripening, but had little effect on ABA-GE. Moreover, the concentration of free ABA could increase with the recovery of leaf area. By monitoring the relative expression of the key genes for ABA synthesis, *NCED1* and *NCED2* in berry, we found that decreasing the leaf-fruit ratio had less effect on them compared to the influence on berry ABA concentration. Moreover, by measuring the concentrations of ABA and its catabolites in berry cultured *in vitro*, we found out glucose concentration or HXK inhibitors had no effect on ABA concentration. All these results together indicate that the decrease in the concentration of free ABA in low leaf-to-fruit ratio berries was not due to the decrease of ABA biosynthesis in berry, nor the changes of ABA metabolism or catabolism. Instead, it is most likely due to the decrease of leaf area, since leaves are the main ABA biosynthesis location. In connection with the result that the concentration of anthocyanins under low leaf-to-fruit ratio was partially restored by exogenous ABA application on berry pre-*véraison*, we speculate that ABA from leaves plays a role in the regulation of anthocyanins synthesis under source limitation.

5.2 Sugar and ABA interaction in regulating anthocyanin synthesis in berries

Both sugar and ABA were reported to induce the synthesis of anthocyanins by regulating related genes of the flavonoid pathway. Previous studies have demonstrated that ABA and sugars have concerted actions on regulating diverse physiological processes (Gazzarrini and McCourt, 2001, Finkelstein and Gibson, 2002, Rook et al., 2006, Han et al., 2018a), including the induction effect of anthocyanin biosynthesis (Hiratsuka et al., 2001, Loreti et al., 2008, Ferrara et al., 2015). In present study, sugar and ABA also showed synergetic effects

on inducing anthocyanin accumulation in berries cultured *in intro*. However, the mechanism of ABA/sugar interaction is still unclear. Based the existing literature, we hypothesized three models for ABA and sugar inducing anthocyanins biosynthesis during berry development. The first one is ABA to promote sugar accumulation, which in turn would induce anthocyanins biosynthesis. The second possibility is sugar inducing ABA accumulation to finally induce anthocyanins biosynthesis, and the third possibility is ABA and sugar signalling pathways interacting to induce the biosynthesis of anthocyanins.

According to the results in this study, we first exclude the first possible way. Because ABA concentration was reduced in glucose insensitive mutants *gin5* in *Arabidopsis* and exogenous ABA can restore normal glucose responses in gin5, Arenas-Huertero et al. (2000) speculated that HXK (glucose signalling sensor) acts upstream of ABA signalling in glucose signalling. In addition, in strawberry, Jia et al. (2011, 2013) found exogenous sugars can significantly promote ABA accumulation and a sucrose transporter *FaSUT1* was able to affect ABA content. Hence, it is possible that sugar can promote ABA synthesis in fruit, and consequently promotes anthocyanin biosynthesis. However, as shown in Fig. 7 in Chapter 3, leaf-to-fruit ratio had little effect on the genes related to ABA biosynthesis, which suggested that sugar concentration had little effect on the *in situ* biosynthesis of ABA in berry. Moreover, *DFR* was a sucrose-specific inducible gene (Solfanelli et al., 2006), and in the present study, did not response to exogenous ABA, but its expression level decreased with the reducing of leaf-to-fruit ratio. These results suggested that the effect on anthocyanins synthesis by the decrease of sugar caused by reducing of leaf-to-fruit ratio might not be dependent on ABA. This conclusion was consistent with the study conducted in *Arabidopsis* ABA-deficient mutant (*aba1-3*) (Loreti et al., 2008), which showed that exogenous ABA could not induce the expression in both *aba1-3* and wild type, but *DFR* expression level in sucrose-treated *aba1-3* was only slightly lower than that of the wild type, suggesting that the sucrose-driven induction of the anthocyanin pathway is not strictly dependent on the synthesis of ABA. In agreement with these results, ABA concentration was not affected by different concentrations of glucose in berries cultured *in vitro*.

Secondly, this study was unable to demonstrate that ABA exerts its function in promoting anthocyanin accumulation by inducing sugar accumulations. Although exogenous ABA

caused an increase in berry sugar content to various extend, depending on the leaf-to-fruit ratio, ABA had little effect on the genes related to sugar metabolism and accumulation in this study, especially in berries with high leaf-to-fruit ratio. Moreover, in the experiment of *in vitro* culture, ABA had no significant effect on the berry sugar concentration compared to control. According to the literature, ABA might promote the accumulation of berry sugars by increasing the expression of CWIs and hexose transporters (Çakir et al., 2003, Murcia et al., 2016, Wang et al., 2017b, Murcia et al., 2018), but the extent of this promotion remains elusive, with some studies reporting an increase (Wheeler et al., 2009), no changes (Kataoka, 1982, Mori et al., 2005a, Sandhu et al., 2011) or even a decrease (Murcia et al., 2017). Because ABA was sprayed on the whole plants in the studies of Murcia et al. (2016; 2018), while we only sprayed it on berries, this difference may explain (at least in part) the different results about the ABA effect on berry sugar concentration. Another possible explanation might be related with the carbon supply situation. Rossouw et al. (2017) found that water deficit promoted starch remobilization from roots to berry, especially in grapevines with low leaf-to-fruit ratio. Water deficit often resulted in higher ABA levels (Mullet and Whitsitt, 1996), so we can assumed that under low carbon supply situation, ABA improved carbohydrate distribution to berry, possibly via altering the expression of CWIs and hexose transporters. Despite these results, there is still lack of direct evidence that the inductive effect of ABA on anthocyanins synthesis is sugar-mediated. Moreover, ABA showed a higher efficiency in promoting anthocyanin accumulation compared to sugars. Gambetta et al. (2010) found the immature berries at pre-*véraison* could produce anthocyanin accumulation when the *in vitro* cultured with a medium contained sucrose and ABA; while colour was not produced in a medium only containing sucrose. Hence, ABA is unlikely to induce anthocyanin synthesis via a sugar alone-mediated pathway during berry ripening.

The most likely possibility for sugar and ABA to interact to induce anthocyanins synthesis is that they do so independently but synergistically on the same set of genes regulating anthocyanins biosynthesis. In fact, during berry development, several genes were confirmed to respond to both sugar and ABA, including *VvMSA (Çakir et al., 2003)*, *VvSK1* (Lecourieux et al., 2010) and *VvHT*s (*VvHT1*, *VvHT2*, *VvHT3* and *VvHT6*) (Çakir et al., 2003,

Murcia et al., 2018). However, the effect of these genes on regulating anthocyanins biosynthesis were unknown. In this study, we propose a signalling pathway based on our results as shown in Fig.1. ABA might increase sugars concentration by increasing the transcript levels of some hexose transporters, like HT1, and ABA can also increase anthocyanin concentration by enhancing the transcript levels of genes related to anthocyanin synthesis independently of sugar concentration. While sugars might induce anthocyanin synthesis via SnRK1 pathway, but the effect of SnRK1 on anthocyanins stills need to be confirmed in berries. In the berries cultured *in vitro*, we found that HXK inhibitor DGH can not only block the sugar-induced anthocyanins synthesis pathway, but also block the one triggered by ABA. DGH was a weak HXK inhibitor but specifically affected the activity of a glucose activated MAP kinase (Hofmann and Roitsch, 2000). Although the function of this glucose-activated MAP kinase is still not clear, two possible explanations might be proposed for the inhibition effect of DGH on anthocyanins synthesis. The first one is that the MAP kinase plays a key role in the sugar/ABA interaction to induce anthocyanins biosynthesis, and its activity determines the induction of anthocyanin. As DGH also seemed to inhibit the accumulation of Phe which is the precursor for anthocyanins synthesis, resulting in the concentration of Phe remaining close to the amount before treatment. Hence, the second possible explanation is that the glucose-activated MAP kinase might also play a role on carbon allocation, and sugar or ABA regulation signalling functions downstream it.

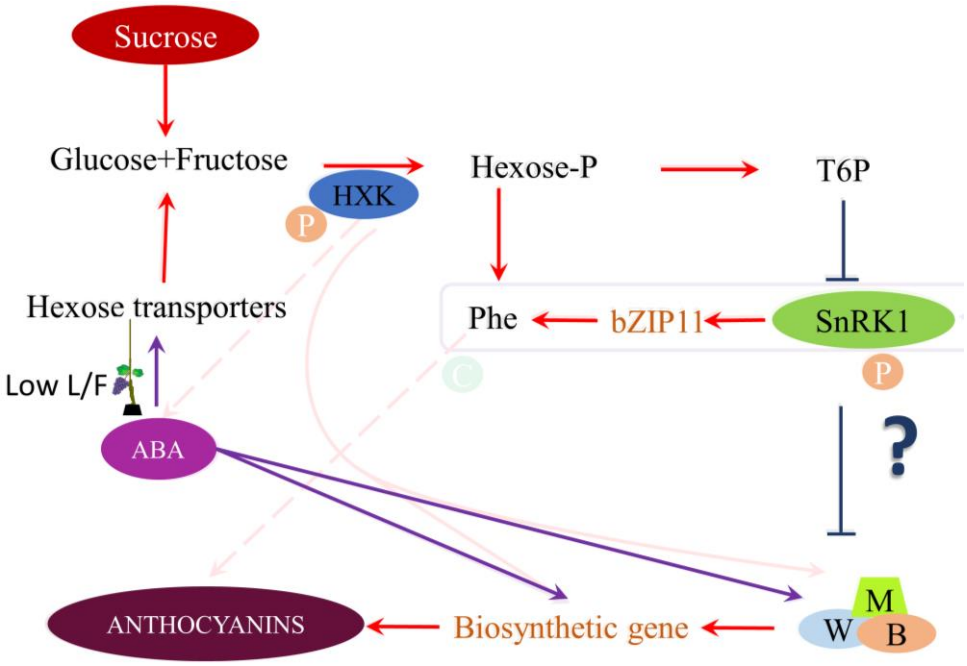

Fig.1 Possible model depicting the mechanism of ABA and sugars promoted anthocyanins biosynthesis in the berry based on present studies. ABA might increase sugars concentration by increasing the transcript levels of some hexose transporters, like HT1 under low L/F condition, and ABA can also increase anthocyanin concentration by enhancing the transcript levels of genes related to anthocyanin synthesis independently of sugar concentration, while sugars might induce anthocyanin synthesis via SnRK1 pathway, but the effect of SnRK1 on anthocyanins stills need to be confirmed in berries.

Chapter 6 Conclusion and prospective

Many viticultural practices involve changes in leaf-to-fruit ratio of vine. In the context of global climate change, we investigated the effect of leaf-to-fruit ratio manipulations on berry composition between 2013-2018. Our results confirmed that reducing leaf-to-fruit ratio (L/F) led to the decoupling of sugar and anthocyanin accumulations. Reducing L/F caused an increase in sugar-to-anthocyanins ratio but a decrease in sugar-to-acid ratio, affecting the concentration and composition of berry free amino acid. These alterations in berry composition might be detrimental to wine quality. In 2017 and 2018, with the application of exogenous ABA on berry at *véraison*, we partially restored the sugar-to-anthocyanins ratio, and reduced the side effects brought by L/F manipulation. In order to further analyse the regulatory mechanisms of L/F and ABA on sugar and anthocyanin, we analysed the physiological aspects and gene expression levels of sugar, anthocyanin and ABA-related changes with fruit development, under different treatments. The results indicated that both L/F and ABA indeed had significant effect on anthocyanins synthesis, by regulating anthocyanin related genes. The simultaneous reduction of sugar and ABA caused by removing leaves resulted in a reduction in anthocyanin accumulation and a decoupling of sugar and anthocyanin accumulation dynamics. In 2017 and 2018, berries at pre-*véraison* cultured *in vitro* with different concentrations of glucose, HXK inhibitors, exogenous ABA and ABA synthesis inhibitor NDGA were used to investigate the relationship between sugar, ABA and anthocyanins metabolism during berry ripening. The results showed that both sugar and ABA could induce anthocyanin synthesis in berry cultured *in intro*, while DGH, a weak inhibitor of HXK, could block the induction of them, whereas NAG, an effective inhibitor of HXK, could not.

These findings have deepened our understanding on the response of berry composition to L/F manipulation and ABA. According to these results, combining L/F manipulations with exogenous ABA applications might offer a fine-tuned way to reduce sugar concentration, while maintaining anthocyanin concentrations in grape berry, paving the way for possible

adaptation strategies for viticulture to global climate change. In future investigations, it might be possible to find the target proteins of DGH and study its function in regulating the flux of carbon between primary metabolism secondary metabolisms. It is anticipated that these proteins may play an important role in maintaining berry quality under predicted climate change future conditions.

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