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Effects of moderately elevated temperature on grape berry at metabolic and transcriptomic levels

Jing Wu

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POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTÉ
SPÉCIALITÉ BIOLOGIE VÉGÉTALE

Par Jing WU

**Impact d'une augmentation modérée de la température du raisin
sur le métabolome et le transcriptome**

Sous la direction de : Philippe PIERI

Soutenue le 13 Février 2018

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RESUME:

La viticulture dépend des conditions climatiques. Dans le contexte de réchauffement climatique, les changements de la vigne et du raisin sous l'effet des températures élevées vraisemblables pour les prochaines décennies pourraient modifier l'aire de répartition des cépages et même menacer la durabilité de la viticulture des régions chaudes. L'objectif de cette étude était donc d'analyser les effets de l'élévation de température sur la composition du raisin, du transcriptome au métabolome. L'utilisation d'un système « open top » passif au vignoble a permis d'augmenter la température autour des grappes de 0.5-1.6 °C en moyenne, une valeur compatible avec le réchauffement climatique prévisible. Les expérimentations ont été conduites sur Cabernet Sauvignon (CS) et Sauvignon Blanc (SB), de la nouaison à la surmaturité, à Bordeaux (France) et en Barossa Valley (Australie). Les analyses ont ciblé essentiellement les métabolites primaires, les composés phénoliques et aromatiques (l'IBMP, arôme de poivron vert; les précurseurs de 3SH, arôme de pamplemousse et la β -damascenone, arômes floraux). En complément, des analyses RNA-seq et q-PCR ont été réalisées pour explorer la réponse transcriptomique à cet échauffement modéré en conditions réalistes de vignoble.

L'échauffement modéré a peu affecté les concentrations en sucres, acides et total en acides aminés, mais a modifié la distribution des différents acides aminés. La composition en acides aminés s'est principalement différenciée suivant la variété, le stade de développement et le site expérimental.

Les concentrations finales en IBMP n'ont pas été affectées par l'échauffement. Cependant, à la fermeture de la grappe, les baies de CS échauffées avaient une moindre concentration en IBMP associée à une sous-expression de *VviOMT3*. La concentration en IBMP des baies de SB échauffées n'a pas montré de différenciation, bien que les niveaux d'expression de *VviOMT3* et *VviOMT4* soient diminués. Les effets limités et dépendant du génotype suggèrent qu'une augmentation modérée de la température ne serait pas suffisante pour modifier significativement l'IBMP.

Glut-3SH-Al était bien plus concentrée que Glut-3SH et Cys-3SH. le traitement échauffé a fait diminuer la présence de Glut-3SH-Al et Cys-3SH dans les baies de SB, en association avec une sous-expression de *VviGST4*. Par ailleurs, *VIT_08s0007g01420* (*GSTU8*) a été

réprimé par le traitement, et pourrait donc être un gène candidat potentiel impliqué dans la biosynthèse de précurseurs de 3SH.

Pour les baies de CS, les concentrations en caroténoïdes totaux et celles des deux caroténoïdes majeurs (la lutéine et le β -carotène) n'ont pas réagi à l'augmentation de température. La zéaxanthine a montré une tendance à la diminution sous l'effet de l'échauffement, jusqu'à une diminution significative. Cette concentration plus faible pourrait limiter la biosynthèse de β -damascenone et expliquer la plus faible teneur en β -damascenone observée dans les baies à sur-maturité en cas de température élevée.

Un total de 357 gènes (DEGs) ont été différenciellement exprimés en réponse à l'augmentation de température pour les échantillons de 2015 à Bordeaux. D'après l'analyse d'enrichissement « Gene Ontology », le traitement a principalement régulé quatre catégories en relation avec les microtubules, la paroi cellulaire, l'espace extracellulaire et l'activité des facteurs de transcription. 6 DEGs liés à la biosynthèse des anthocyanes ont été régulés négativement, ce qui pourrait expliquer, au moins en partie, la concentration réduite en anthocyanes totaux observée dans les baies de CS échauffées. En revanche, les tanins n'ont pas été affectés par l'augmentation de la température.

Les résultats permettent une meilleure compréhension des effets potentiels du réchauffement climatique sur la composition en métabolites des baies durant leur développement, et d'acquérir de nouvelles connaissances moléculaires concernant la réponse des baies de raisin à un échauffement modéré en conditions de vignoble.

MOTS-CLES: *Vitis vinifera*, maturation, réchauffement climatique, acide aminé, IBMP, précurseurs de 3SH, Caroténoïdes, norisoprénoides, q-PCR, polyphénol, RNA-seq.

Effects of moderately elevated temperature on grape berry at metabolic and transcriptomic levels

ABSTRACT:

Viticulture depends on climate conditions during the growing season. In the context of global warming, any changes in viticulture caused by the rising temperatures expected for the next decades may alter the geographical distribution of grape varieties and even threaten the sustainability of viticulture in hot areas. The objective of this research was to investigate the effects of moderately elevated temperature on grape composition, both at metabolic and transcriptomic levels. A passive open-top heating system was applied in Cabernet Sauvignon (CS) and Sauvignon Blanc (SB) vines grown with standard practice in Bordeaux, France and the Barossa Valley, Australia (CS only) to increase the bunch zone mean temperature by around 0.5-1.6 °C, which was commensurate with the projected global warming. This moderate heating was applied from fruit-set to two weeks after harvest. Metabolites related to technical, phenolic and aromatic maturities (IBMP, the green pepper aroma, precursors of 3SH, grapefruit aroma, and β -damascenone, floral aroma) were assessed, together with transcriptome analysis via RNA-seq and q-PCR, in order to obtain a comprehensive view of berry responses to this moderately elevated temperature in realistic vineyard conditions.

The moderately elevated temperature hardly affected the concentrations of sugars, organic acids and total amino acids, but it altered free amino acid composition depending on varieties, vintages and locations.

The final concentrations of IBMP were not affected by warming condition in mature berries. However, the elevated temperature significantly reduced IBMP content and expression level of *VviOMT3* (a known key gene of IBMP) in CS berries at bunch closure stage, while it reduced the expression levels of *VviOMT3* and *VviOMT4* at bunch closure stage without affecting IBMP concentration in SB berries. This limited and genotype-dependent effect of elevated temperature suggests that a moderate temperature elevation may not be sufficient to significantly modify IBMP.

Glut-3SH-Al was much more concentrated than Glut-3SH and Cys-3SH. Reduced Glut-3SH-Al and Cys-3SH concentrations were associated with a significantly lower expression level of

VviGST4 in heated SB berries. Meanwhile, *VIT_08s0007g01420* (*GSTU8*), was down-regulated by elevated temperature and might be a potential candidate gene involved in the biosynthesis of precursors of 3SH.

The concentrations of total carotenoids and two predominant carotenoids (lutein and β -carotene) were not altered by elevated temperature in CS berries, but zeaxanthin was reduced by elevated temperature and was significantly less concentrated at harvest. This lower concentration may limit the biosynthesis of β -damascenone and explain the observed lower β -damascenone concentration in post-ripening berries under elevated temperature.

A total of 357 genes were differentially expressed (DEGs) in response to the elevated temperature in Bordeaux samples in 2015. Enrichment analysis of Gene Ontology showed that temperature mainly regulated four GO categories, including microtubule, cell wall, extracellular region, and transcription factor activity. 6 DEGs related to anthocyanins synthesis were down-regulated and it could explain, at least in part, the observed lower total anthocyanins in warmed CS. Conversely, tannins were not affected by elevated temperature.

The results provide a better understanding of potential global warming effects on metabolite changes during berry development, along with novel molecular insights into the response of grape berry to moderate heating in vineyard conditions.

KEYWORDS: *Vitis vinifera*, temperature, maturation, global warming, amino acids, IBMP, 3SH precursors, carotenoids, norisoprenoids, q-PCR, polyphenol, RNA-seq.

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Chapter I: Literature Review

I.1 Global warming and its influences

I.1.1 Climate change and global warming

Global climate change is demonstrated by a very wide range of observations across the world. It is mainly caused by human activities and in particular the emission of greenhouse gases (Stocker, 2014). The Intergovernmental Panel on Climate Change (IPCC) is an organization made up of prominent climatologists around the world for assessment the scientific, environmental and socioeconomic impacts of climate change. Its latest synthesis report indicated that current levels of greenhouse gases in our atmosphere are higher than they have been in the last 800,000 years and recent technological and industrial advances have compounded this problem, with greenhouse gas emissions between the years 2000 and 2010 being the highest in recorded history (IPCC, 2014b). Based on several reports and studies, the most important but not the only effects of climate change can be summarized as follow:

- a) Increase in temperature
- b) Change in rainfall patterns
- c) Increase in incoming radiation, in particular UV-B
- d) Increase in carbon dioxide concentration

Temperature is a primary factor affecting the plant development (Hatfield and Prueger, 2015). A recent report shows that global annual average temperature has increased by more than 1.2°C since 1900 (through 2012) (Power et al., 2017). The IPCC expected the surface temperatures will rise throughout the 21st century between 0.3 and 0.7 °C by the year 2035 (IPCC, 2014b) (Figure 1). Besides the continuous increment of average temperature, both hot and cold temperature extremes will become more frequent (IPCC, 2014b). Until now, climate change already produced many effects on agriculture and the negative effects are expected to continue doing so unless adaptive measures are taken (Nelson et al., 2014; Rosenzweig et al., 2014)

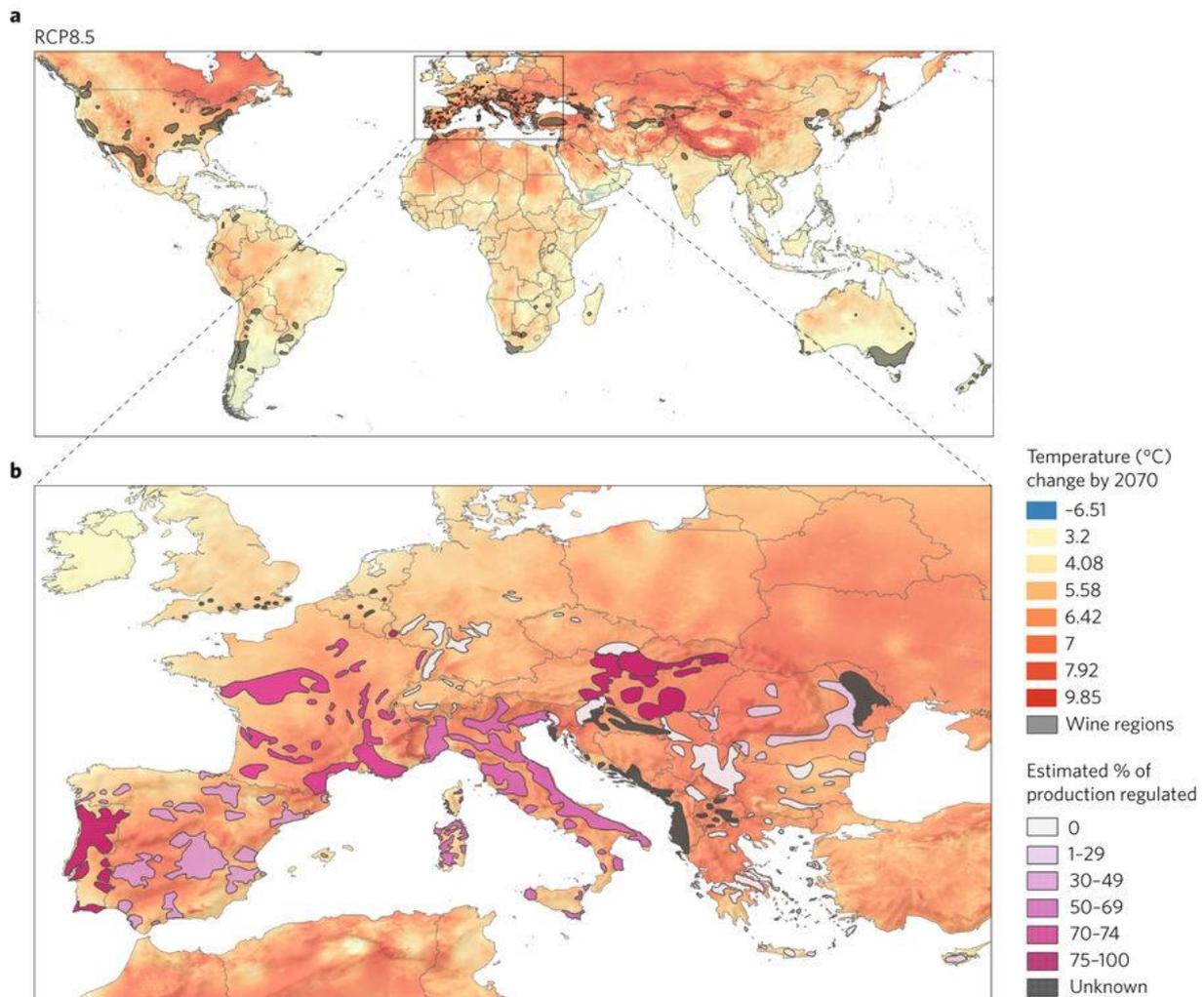


Figure 1: Projected changes in temperature (E. M. Wolkovich et al., 2018)

A global map (a) and an European map (b) are coloured based on the projected changes in temperature during the growing season by the year 2070 against 1970–2000 climate, as modelled by the general circulation model GFDL-CM3, and the high emissions scenario RCP8.5. Wine-growing regions are shaded in black within the global temperature change map (a). In the European map (b) they are shaded in a white to red colour depicting the estimated proportion of wine production under varietal restrictions, based on wines labelled by protected geographical indications (assessed at the country level).

I.1.2 Impacts of global warming on viticulture

Despite increasing knowledge in management practices to control and manipulate grapevine growth and development, viticulture is still a sector that highly depends on climate and is strongly affected by climate change, especially global warming (Jones, 2006). In summary, global warming effects on viticulture include, but not limited to, these listed below:

- a) Advance in phenology of vine
- b) Change on viticultural areas
- c) Modification in grape composition
- d) Increase in temperatures damage / Pests / Diseases
- e) Wine industry

Advance in phenology of grapevine

The phenology of grapevines is closely related to temperature. Previous research showed that the length of the growing season of grapevine depends on genotypes and the growing-season mean temperature (Jones, 2006). Earlier harvest dates associated with a significant increase in temperature were observed in the past several decades (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012; van Leeuwen and Darriet, 2016; García de Cortázar-Atauri et al., 2017; van Leeuwen and Destrac-Irvine, 2017). Moreover, grape ripen dates have been used as a climate indicator to extrapolate past climate conditions (Chuine et al., 2004). Not only the harvest dates, the dates of bud-break, flowering, and veraison (onset of ripening), are also driven by temperature (Parker et al., 2011; Martínez-Lüscher et al., 2016). Shortenings of the growing season coupled with earlier phenological events are reported in various grape production regions over the world (Jones et al., 2005; Lebon et al., 2006; Sadras and Petrie, 2011; Ferrise et al., 2016).

Change on viticultural areas

Temperature increase and precipitation pattern changes may significantly alter the present worldwide viticultural regions in the future scenarios (Meehl et al., 2007; Malheiro et al., 2010; Hannah et al., 2013). The hot regions will have more problems of suitability than relatively cooler region in the future (Hannah et al., 2013). An expected decrease viticultural suitability in southern Europe is reported (Jones et al., 2005; Stock et al., 2005; Fraga et al., 2012; Hannah et al., 2013); one third of the current Australian wine regions have been suggested that the growing season temperatures will be unsuitable for quality wine production by 2070 (Hall and Jones, 2009); a model constructed by temperature change and viticultural data showed that by the end of the 21st century suitable wine-grape production areas could decline by 81% in USA (White et al., 2006). Conversely, where viticulture is restricted by a shorter growing season and/or low summer temperatures, such as higher latitude or elevation regions, suitability is expected to improve under future climate conditions. In those cases,

higher temperature in future will permit the growing of a wider range of grape varieties, prolonged frost-free periods and growing seasons (Lough et al., 1983; Kenny and Harrison, 1992; Jones et al., 2005; Bertin, 2008). It may be beneficial for many regions in central and Western Europe, such as Alsace, Champagne, Bourgogne, Loire Valley, Mosel, and Rheingau (Stock et al., 2005; Malheiro et al., 2010; Neethling et al., 2012). Both positive and negative effects of global warming on viticulture are always associated with wine quality and typicity and suitable adaptation strategies are essential to maintain the suitability in the context of climate change (Van Leeuwen et al., 2013).

Modification in grape composition

Berry quality, the major determinant of wine quality and typicity, depends on the composition and concentration of primary and secondary metabolites, which are influenced by temperature (Seguin, 1986; Van Leeuwen et al., 2004; Deluc et al., 2007; De Orduna, 2010). Higher potential alcohol levels at harvest associated with a significant increase in temperature were observed in the past decades (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012). Accelerating the sugar accumulation is often associated with thermal increase, as indicated by the trend observed in the last decades (Coombe, 1986; Petrie and Sadras, 2008; Rienth et al., 2016). A reduction of total acidity (TA) coupled with increase in pH were also associated with high temperature (Keller, 2010). Loss of anthocyanins in red-wine grape under high temperature was reported (Mori et al., 2007b). Conversely, tannins was less affected by temperature (Cohen et al., 2008). The increase of temperatures also has an impact on aroma compounds and their precursors (van Leeuwen and Darriet, 2016; Pons et al., 2017). More details about temperature effects on berry composition will be discussed in sector: I.1.3 Grape berry compositions.

Increase in temperature-related damages / Pests / Diseases

Warming temperatures are also expected to increase disease and pest pressure on grapevines. Pierce's disease, a bacterium that inhibits vine's water circulation and ultimately kills the plant, is predicted to move toward the North and South Poles as winter temperatures become more hospitable for its main vector, the glassy-winged sharpshooter. For instance, in the United States, Pierce's disease is expected to move from California northward into Oregon and Washington (Hoddle, 2004; Daugherty et al., 2009). Likewise, warmer weather may promote fungal diseases such as black rot, downy mildew, powdery mildew, and botrytis. All

of these diseases are driven by climatic factors and particularly benefit from warmer night temperatures (Chakraborty et al., 1998; Francesca et al., 2006). However, dryer growing conditions may partially or fully curb the detrimental effects of increasing temperatures. On the other hand, more frequent hot and cold temperature extremes will couple with global warming. Spring frosts injure developing shoots and frosts after bud burst reduce the current season's crop yield. Exposure to late spring frosts has been projected to increase under some future climate-warming scenarios despite warmer temperatures due to an advance in bud-break (Molitor et al., 2014; Mosedale et al., 2015). Similarly, extreme maximum temperatures in summer also can cause substantial heat damage by inhibiting photosynthesis, growth, and ripening. Exposed grape bunches are particularly vulnerable to heat damage during veraison. As red wines more than whites depend on skin-derived components such as pigments and tannins, they could be more affected than whites because the skin is the part of the berry that is most sensitive to heat damage (Gladstones, 1992). In particular, with temperatures rise during the growing season, berries become more susceptible to sunburn and serious damage can kill the whole berry, and sometimes at least half the bunch can be destroyed (Jackson, 1997, Webb et al.2009).

Wine industry

In 2016, total world area under grapevines was 7.6 million hectares and global grape production was 7.8 million tons, among which around 50% were used for wine production (OIV, 2017). Moreover, this percentage reached 78% in Australia (OIV, 2017). All changes by global warming on viticulture will finally affect winemaking and wine quality and typicity. Higher potential alcohol levels can pose problems or bring advantages for winemaking depending on regions. Regarding acidity, the most relevant indicator is must and wine pH. When pH increases, wines are perceived as being rounder, sweeter, and less aggressive, which is considered as a positive change. However, wines can lack freshness when pH is too high, and wine stability is also negatively impacted by higher pH. The wild yeast *Brettanomyces bruxellensis* can spoil wine during aging in barrels or tanks, even after bottling, when pH is high (Ribéreau-Gayon et al., 2006). In addition, socio-economic impacts of climate change associated with wine industry also have to be considered. For instance, with the change of wine regions by global warming, retention of employment in some isolated wine regions could be affected (Ollat and Touzard, 2014)..

Overall, understanding global warming and the potential impacts on grape and wine has become increasingly important (Van Leeuwen et al., 2013).

I.2 Grape berry development and quality

I.2.1 Grape berry development

Grape, classified as a non-climacteric fruit, comprises skin, flesh and seed (Figure 2). They account for about 15%, 80%, and 5% of total berry fresh weight, respectively (Roby and Matthews, 2004). The growth and development of grape berry is characterized by a double sigmoid pattern that defines two main phases of formation and ripening, being separated by a lag phase (Coombe, 1992; Coombe and McCarthy, 2000; Ollat et al., 2002). The two main periods are sometimes called “herbaceous” phase and “ripening” phase (Figure 3).

The “herbaceous” phase is characterized by cell divisions and cell enlargement. When the fruit has been set, pericarp cells divide and expand rapidly (Coombe and McCarthy, 2000). Rapid cell division occurs through the first few weeks, and by the end of this period, the total number of cells within the berry has been established. During this period, the berry is formed and the seed embryos are produced. Increases in berry volume and weight are associated with intense water flows into the berry, with a progressive accumulation of organic acids (Coombe and McCarthy, 2000) and proanthocyanidins in berry skin and seeds (Downey et al., 2003); Two organic acids, tartaric acid and malic acid, accumulate at high concentration in skin and mesocarp cells. These acids can represent up to 92% of total organic acids of grape berries throughout different developmental stages (Kliwer and Nassar, 1966; Conde et al., 2007). At the end of the herbaceous phase, the berries are green, hard, acid, and seeds have reached their final size (Ristic and Iland, 2005).

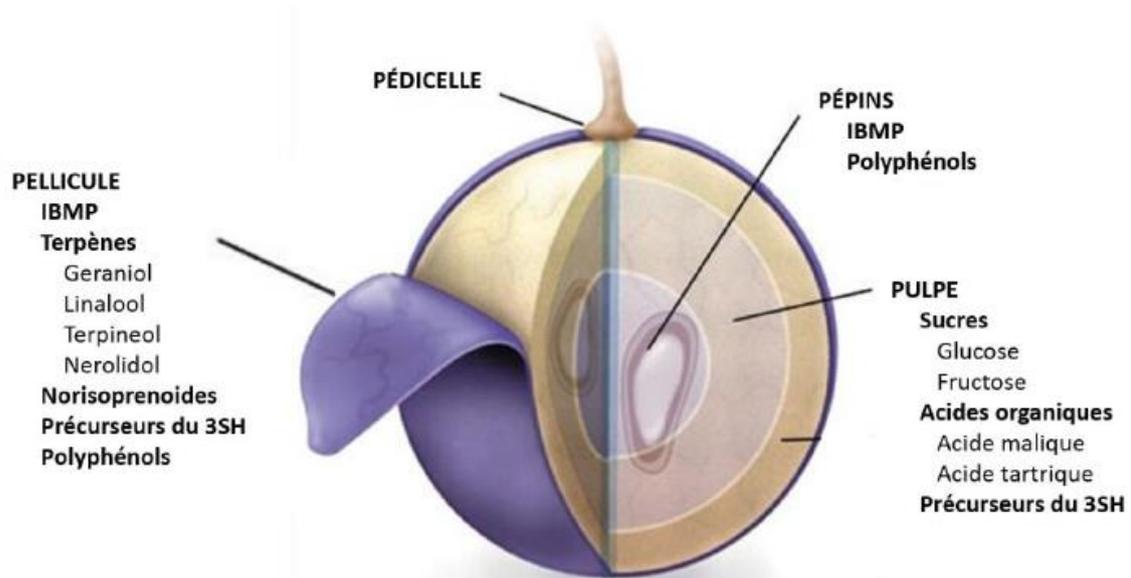


Figure 2: Berry structure and certain metabolite locations. Adapted from Lund and Bohlmann (2016)

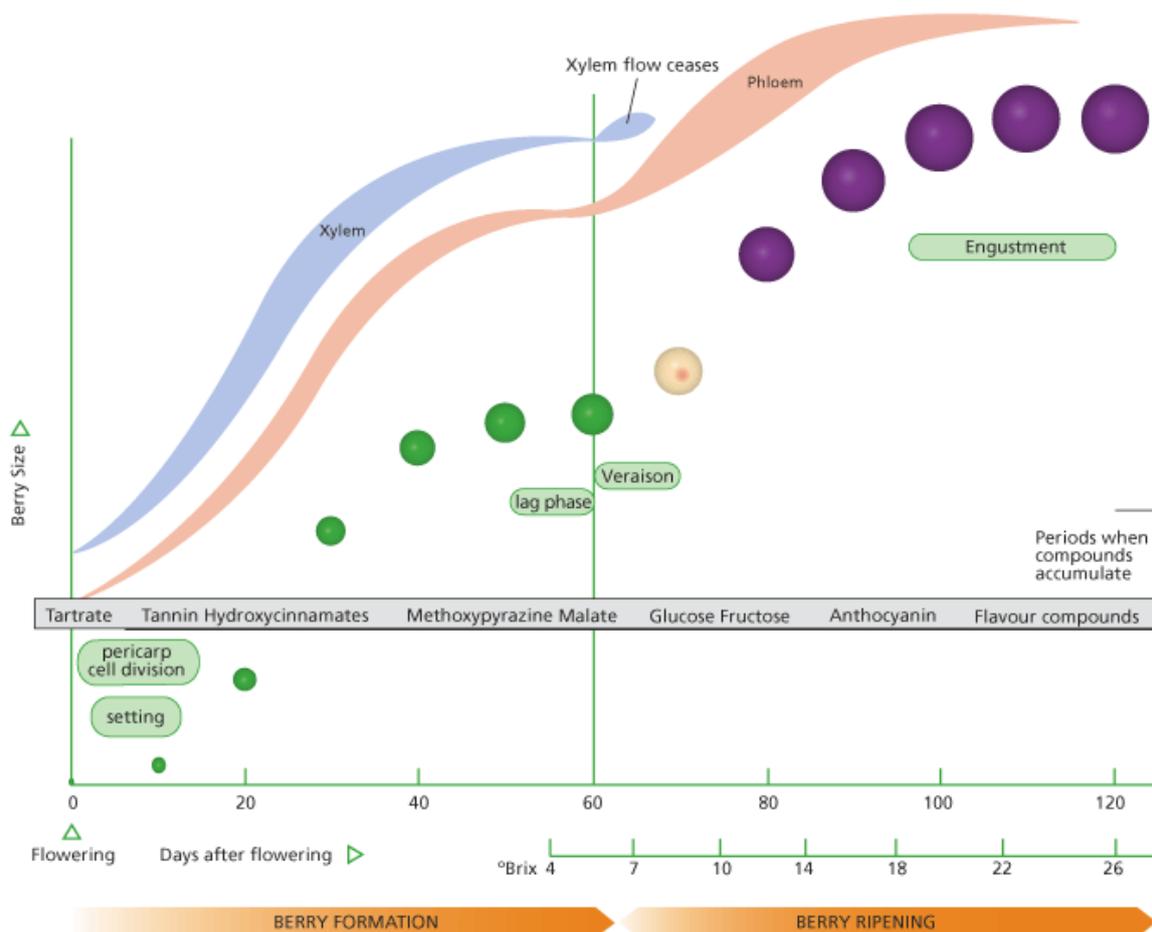


Figure 3: Dynamics of berry growth and solution transport (Kennedy, 2002)

After the cell division phase, there is a pause in berry development called the “lag phase”, during which seed formation progresses. Berry turgor pressure also declines during this period (Bondada et al., 2005). The duration of the lag phase (8 to 12 days) depends principally on the cultivar and is important in determining the time of fruit ripeness (early vs. late ripening varieties). After the lag phase, the number of cells in each berry is set. Berries are still small, green and hard and remain high in acid and low in sugar content.

At the end of lag phase, veraison indicates the beginning of the ripening phase. This phase is characterized by a decrease in acidity, a steep increase in sugar accumulation, color change (from green to red/black in red varieties, from green to semi-transparent in white varieties) and berry softening (Castellarin et al., 2011; Castellarin et al., 2015). Red pigment (anthocyanins) accumulates in the exocarp and sugars (glucose and fructose) accumulate in the pericarp (Figure 2), whereas organic acids (malate) and chlorophyll are degraded (Coombe and McCarthy, 2000; Ollat et al., 2002). The development of these characteristics is essential in the quality of the final product. Fruit ripening ends about 120 days after flowering (Carbonneau et al., 2015). Throughout this phase, the size of the mesocarp cells significantly expands, mainly due to the accumulation of water and sugars in the berries (Huglin and Schneider, 1998; Dai et al., 2010).

I.2.2 Factors influencing grape quality

The quality of grape berry, which largely shape the quality of wine, is affected by “terroirs”. According the definition of OIV, terroir includes soil type, topography, climate, landscape characteristics, biodiversity features and human factors. With the development of viti-vini technology and the deepening understanding about viticulture, the concept of grape quality has changed and “desired quality” might be more accurate to describe different compositional patterns (Poni et al., 2017). The factors which impacting on grape quality can be summarized the three as followed (Dai et al., 2011; Kuhn et al., 2013; Poni et al., 2017):

- a) Genotype
- b) Environmental factors
- c) Human factors

Genotype

High genetic diversity of grapevine not only promises the geographic distribution but also provides opportunities to adapt global warming (Nicotra et al., 2010; Duchêne, 2016). Meanwhile, it also plays a critical role to diversity of grape and wine quality (This et al., 2006; Dai et al., 2011). Sugar and acid composition and concentration vary with cultivars and clones in grape berries (Kliewer, 1967; Van Leeuwen et al., 2012; Duchêne et al., 2014). More importantly, secondary metabolites of grape berries which significantly affect wine typicity and quality, such as polyphenol and aroma potential are also variable between cultivars (Mattivi et al., 2006; Mattivi et al., 2009; Darriet et al., 2012). Pinot Noir berries have no acetate forms of the anthocyanins in skin (Dimitrovska et al., 2011) and have two times higher concentration of catechin than epicatechin in seeds (Mattivi et al., 2009). Differentially, the relative amount of acetate derivative anthocyanins was accounted 35% in Cabernet Sauvignon skin and similar concentrations of catechin and epicatechin were found in its seeds (Mattivi et al., 2009; Dimitrovska et al., 2011). Monoterpenes alcohols are more concentrated in Muscat family than many other varieties, such as Cabernet Sauvignon and Syrah (Darriet et al., 2012).

Environmental factors

Soil, nutrient supply, light, temperature and water availability are five environmental factors that are main determiners of berry quality (Poni et al., 2017). Although soil characteristics are believed to affect berry and wine quality in tradition, some studies showed that the effects of soil type and quality were quite limited (White, 2003; Van Leeuwen et al., 2004). Conversely, many studies demonstrated that physical properties, such as texture, porosity and infiltration rates, play an important role in grape quality (Seguin, 1986; Pomerol, 1995).

Nitrogen and 17 mineral nutrients are considered essential for grapevine growth because they are involved in the structure of compounds, activating enzymes, a charge carrier, and osmoregulatory (Roubelakis-Angelakis, 2009). Nitrogen showed direct effects on grape composition (Habran et al., 2016; Helwi et al., 2016; Poni et al., 2017) and also has a major impact on vine vigor and yield (Keller et al., 2001; Holzzapfel and Treeby, 2007). Vigorous vines, defined by a high leaf area index, result in shaded grapes and in turn influence berry metabolites such as methoxypyrazines (Ryona et al., 2008).

Incoming solar radiation provides the energy necessary for grape growth and maturation (Mullins et al., 1992). The amount and quality of radiation is important to maintain the photosynthesis during grapevine growing stage (Schwartz, 2003). Besides photosynthetic and phytochrome effects, light can affect berry composition. Un-uniform response of berry weight, TSS and TA to bunch shading (light reduction) were reported by previous studies (Morrison and Noble, 1990; Dokoozlian and Kliewer, 1996; Downey et al., 2004). These different responses may explain by vintage, variety or treated timing differences. Low and excessive light exposure were reported to reduce phenolic concentration in different cultivars (Dokoozlian and Kliewer, 1996; Chorti et al., 2010; Guan et al., 2016). Nowadays, genetic analysis are used to study the effect of cluster light regime on berry development and composition for a comprehensive understanding (Poni et al., 2017).

Grapevine productivity and berry quality is highly dependent on temperature, and the influence of temperature on viticulture is manifold. Based on the data of the past several decades, earlier harvest date and higher potential alcohol levels at harvest associated with a significant increase in temperature were observed (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012). Extreme temperatures could significantly impact berry quality and yield. In 2017, the exceptional weather events, including frosts and high summer temperature in summer with low precipitation, reduced of 23% of total wine production in Italy, 19% in France and 15% in Spain compared with 2016 (OIV 2017). More details about temperature effects on berry composition will be discussed in sector: 1.3 Grape berry compositions.

Water is a prerequisite for grapevine growth and survival. Water availability impacts vegetative growth, yield, and fruit composition (Medrano et al., 2003), but the effect of water supply on berry quality is complex because it lies with many factors, such as amount, timing and mode of irrigation (Poni et al., 2017). In general, excessive soil water availability can increase yield but water limitation reduces the yield (Matthews et al., 1990; Esteban et al., 1999; Merli et al., 2016). The yield decreases often couple with reduction of berry size and weight and also higher concentration of sugar and phenolic compounds (Ojeda et al., 2002; Castellarin et al., 2007). Moreover, water deficit has been reported to alter volatile compounds in berry (Ou et al., 2010; Song et al., 2012). Deluc et al. (2009) found that water deficit activated parts of the phenylpropanoid, energy, carotenoid and isoprenoid metabolic

pathways that contribute to increased concentrations of antheraxanthin, flavonols and aroma volatiles through both metabolite and transcriptomic analysis.

Human factors

Human or agronomic factors are important to grape quality. Mankind decides the varieties, locations and training systems based on their experiences. The purpose of all activities in vineyard is to improve the berry quality, ensure the reasonable production, keep the sustainability of vineyard, and reduce environmental impact. For example, a suitable treating system can help vines to better adapt to environments (Poni et al., 2017); early defoliation can improve berry quality (Poni et al., 2008; Diago et al., 2010; Sivilotti et al., 2017); shoot trimming can maintain canopy shape and contain vine vigor (Smart, 1985); water supply is necessary in many dry vine-grown regions such as Barossa Valley.

Overall, the environmental characteristics of a vineyard are major drivers of berry quality and cannot easily be changed by management techniques. In the context of global warming, a better understanding the effect of elevated temperature on berry quality in realistic vineyards is required.

I.3 Grape berry metabolites and elevated temperature effects

I.3.1 Primary metabolites

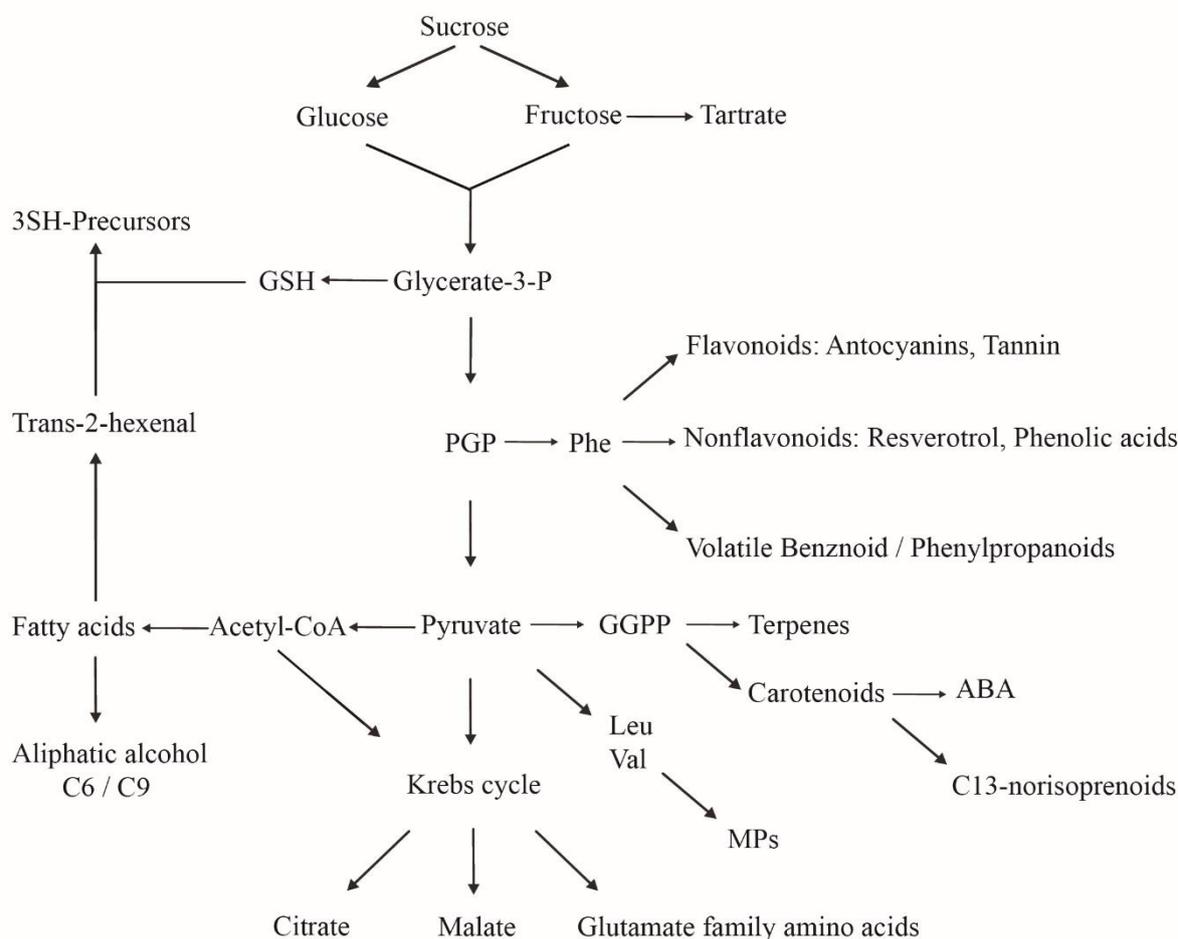


Figure 4: Simplified pathways of certain primary and secondary metabolites in grape.

Sugars

The main soluble sugars within grape berries are glucose, fructose, and sucrose, and sucrose is the main translocated sugar from phloem to fruit in grapevine (Hawker, 1969). Different sugar proportions and total sugar concentrations are varied between cultivars and clones (Kliwer, 1967; Van Leeuwen et al., 2012). They primarily accumulate in the pulp, but they are also present in grape skins (Possner and Kliwer, 1985; Coombe and McCarthy, 2000). Their concentrations shape berry sensory properties, final alcohol level in wine and provide precursors for biosynthesis of organic acids, amino acids, phenolic and aromatic compounds (Dai et al., 2011).

During the early stages of berry development, the total sugar concentration is very low (Possner and Kliewer, 1985). During berry ripening, sucrose is transported into the berries via the phloem from leaf and converted to glucose and fructose (Davis and Robinson, 1996). At harvest, glucose and fructose concentrations are similar, between 70 and 130 g/L (Ribereau-Gayon et al., 2000).

The sugar accumulation in grape berry is directly affected by temperature (Keller, 2010). Earlier harvest dates and higher potential alcohol levels at harvest associated with a significant increase in temperature were observed in the past several decades (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012). In general, accelerating the sugar accumulation is often associated with thermal increase, as indicated by the trend observed in the last decades (Coombe, 1986; Petrie and Sadras, 2008; Rienth et al., 2016). However, recent experiments with precise temperature regulations established that the effects of temperature on grape quality depend on the intensity of temperature elevation, stage of high temperature treatment, and grape genotypes. For example, a double of growing degree days did not change the timing of the onset of sugar accumulation of vines grown in growth chamber (Rienth et al., 2016). A 2-4°C increase in maximal temperature lasting 2 weeks at different phenological stages did not increase sugar concentration in Shiraz berries (Sadras and Moran, 2012; Sadras et al., 2013; Sweetman et al., 2014). Moreover, Lecourieux et al. (2017) showed that bunch zone high temperature imposed pre-veraison, during veraison or ripen stage did not change sugar concentrations.

Organic acids

Tartaric and malic acids are the principal organic acids of the grape berry, making up approximately 90% of total fruit acidity, along with small amounts of citric acid (Kliewer, 1967; Soyer et al., 2003). With sugar, they are principally employed to determine grape berry maturation (Philip and Nelson, 1973; Lamikanra et al., 1995; Dokoozlian, 2000). Organic acids present in grape pulp and skin, supporting many aspects of cellular metabolism (Possner and Kliewer, 1985; Coombe and McCarthy, 2000). Moreover, organic acids also affect wine stability, color and flavors (Sims and Morris, 1984; Ribereau-Gayon et al., 2000).

There is a high genetic diversity for grape berry organic acid concentrations (Dai et al., 2011; Duchêne et al., 2014). The accumulation of these acids occurs typically at the beginning of grape berry development, reaching their highest concentrations near the onset of ripening

(Dokoozlian, 2000; Conde et al., 2007; Sweetman et al., 2009). Tartaric acid remains fairly stable until berry ripen when concentrations decline. Malic acid continuously decline with berry ripening (Possner and Kliewer, 1985; Coombe and McCarthy, 2000). The decrease of tartaric acid post-veraison is mainly due to an increase in berry size. In contrast, degradations in malic acid concentration after veraison result from respiration, enzyme degradation, and dilution (Dokoozlian, 2000; Sweetman et al., 2009; Sweetman et al., 2014).

The observed warmer seasons result in greater ripening in grapes and the concentrations of organic acids decreases (Mullins et al., 1992). Malate concentrations decrease by elevated temperature while tartaric acid concentration does not appear to be significantly affected by temperature (Keller et al., 2005; Pereira et al., 2006; Parra et al., 2010; Rienth et al., 2016). Contrasted with the general consideration of negative relationship between elevated temperature and acid concentration, variety-dependent thermal responses of acidity were observed. Responses of juice TA and pH to elevated vineyard temperature over berry growing season were different between varieties (Sadras et al., 2013): heated Cabernet France et Chardonnay berries had an increase of pH and decrease of TA; both traits were not affected in Shiraz berries; pH was increased but total acidity was unresponsive in heated Semillon berries. Three weeks of elevated temperature treatments applied at veraison and ripening stages significantly decreased malate content in Shiraz berries, but no effect with pre-veraison treatment (Sweetman et al., 2014). Moreover, Lecourieux et al. (2017) also found that heat treatment applied during herbaceous stage did not alter malic acid. Rienth et al. (2016) focused on the change of organic acids accumulation profile by 10°C increase of day temperature. Higher day temperature brought forward malic and tartaric acid accumulation by 5.5 days without changing their maximal accumulation rates.

Amino acids

In grapes, free amino acids are the major nitrogen sources for yeast during winemaking (Marcy et al., 1981). Free amino acid concentrations and proportions in grape berries vary based on cultivars, rootstock/scion combinations, vine nutrient management, vineyard site, and growing season (Huang and Ough, 1989; Gump et al., 2002; Bell and Henscken, 2005). Generally, proline and arginine are two predominant amino acids in berries (Kliewer, 1968; Stines et al., 2000). During wine making, total nitrogen in the must can vary from 100 to 1200 mg/L, of which amino acids make up about 50% (Conde et al., 2007). Amino acids also serve

as critical subunits in the generation of enzymes and proteins and are present as precursors of some aroma compounds (Kliewer, 1970; Jackson, 2008) (Figure 4).

Temperature is known to affect the biosynthesis of amino acids in grapevine and different amino acids have different responses to elevated temperature. In greenhouse-grown grape, higher temperature (+8°C) applied to the bunch zone during veraison or ripening stages significantly increased 7 amino acids (Thr, Arg, Tyr, Phe, Cys, Lys and GABA) (Lecourieux et al., 2017); The treatment of elevated temperature (+5°C) lasting 11 days applied at veraison positively affected 11 free amino acids in Shiraz berries (Sweetman et al., 2014). In field-grown Riesling grape, leaf removal associated with higher temperature negatively affected the accumulation of amino acids (Friedel et al., 2015). However, Pieri et al (2016) found that south-exposed berries of Merlot in actual vineyard conditions with higher temperature (+5°C) and solar radiation had similar amino acid concentrations, compared with north-exposed berries.

I.3.2 Phenolic compounds: tannins and anthocyanins

Phenolic compounds are divided into flavonoid and nonflavonoid compounds. Flavonoids can be further divided into flavonols, flavones, flavan-3-ols, flavanones, and anthocyanins (Figure 5). Nonflavonoids include phenolic acids, hydroxycinnamic acids and their conjugated derivatives, and polyphenolic stilbenes (Monagas et al., 2005; Tsao, 2010; Perestrelo et al., 2012). The most important flavonoid compounds in grapes are anthocyanins and tannins. Tannins are mainly found in the skins and seeds, and anthocyanins are mainly accumulated in skins but also present in pulps in some teinturier cultivars (Montealegre et al., 2006; Guan et al., 2012). They can be partially extracted during winemaking and contribute to wine color, astringency, bitterness, stability, and structure (Revilla and Ryan, 2000). Moreover, tannins can combine with anthocyanins to form pigmented polymers that can improve pigment stabilisation (Waterhouse, 2002).

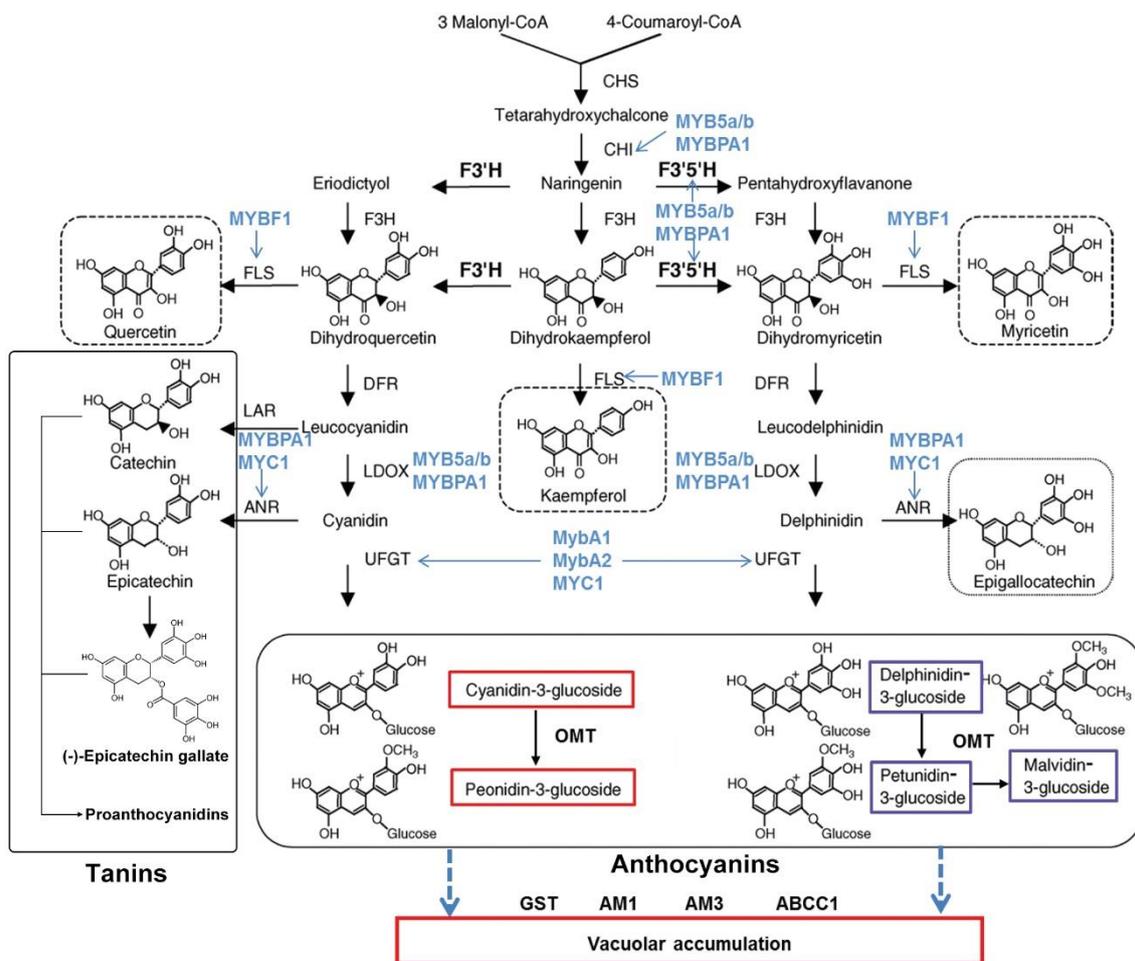


Figure 5: Simplified pathways of flavonoid biosynthesis and its regulation in grape. (Downey et al., 2003; Kuhn et al., 2013)

Structurally, anthocyanins are glycosides and acyl-glycosides of anthocyanidins. Glycoside of five anthocyanins are widely found in skin of red grape cultivars: cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside (Figure 5). They start to accumulate at the very beginning of the transition from unripe to ripening (veraison), and continue throughout ripening. Tannins are a group of flavan-3-ols which have monomers, oligomers and polymers, and are also called as condensed tannins or proanthocyanidins (Chira et al., 2011). Three free flavonols are widely detected in grape: C: (+)-Catechin and EC: (-)-epicatechin which are the majorities, and ECG: (-)-epicatechin-gallate with a low concentration (Downey et al., 2003). Tannins accumulate immediately after fruit set and reach the maximum levels around veraison. Thereafter, tannin concentrations decrease (Downey et al., 2003). Generally, it is considered that the decreases in tannin concentrations over berry development are caused by decreases in tannin

extractability rather than degradation or turnover (De Freitas and Glories, 1999; Kennedy et al., 2001; Downey et al., 2003).

The composition and concentration of tannins and anthocyanins mainly depends on genotype, climatic conditions and vineyard management practices. Elevated temperature can decrease the total anthocyanin concentrations and change their composition in berry skin (Spayd et al., 2002; Downey et al., 2006; Tarara et al., 2008; He et al., 2010; Azuma et al., 2012; Pastore et al., 2017). Several genes involved in anthocyanin biosynthesis, such as *CHS*, *F3H*, *DFR*, *ANS* and *UFGT*, were also down-regulated by high temperature (Mori et al., 2005; Yamane et al., 2006). Mori et al. (2007b) have used stable isotope-labelled tracer experiments and microarray analysis to establish that the negative effect of elevated temperature on anthocyanins is mediated by accelerating anthocyanins degradation and down-regulating the mRNA transcription of the anthocyanin biosynthetic genes. On the other hand, the timing of heating treatment also affects anthocyanin responses to temperature. Heating treatment applied at pre-veraison did not change total anthocyanins but those applied at veraison and ripen stages decreased total anthocyanins concentration by 50%. All three treatments decreased di-hydroxylated anthocyanin concentrations associated with repression of *F3'5'H*, without effect on tri-hydroxylated anthocyanin concentrations (Lecourieux et al., 2017). Kliewer and Torres (1972) found that high night temperature can also decrease berry coloration. In contrast, (Mori et al., 2007a) found that high night and morning temperature did not affect total anthocyanins concentrations but also decreased di-hydroxylated anthocyanin concentrations. The reasons behind those differences are still open questions. In contrast with anthocyanins, several studies found that tannin accumulation appears to be largely unaffected by high temperature in berry skin as well as in seeds (Cohen et al., 2008; Carbonell-Bejerano et al., 2013; Pastore et al., 2017).

I.3.3 Precursors of aroma and aroma compounds

Over 1000 volatile compounds have been detected in wine with concentrations ranging from a few ng/L to hundreds of mg/L level (Polášková et al., 2008; Ebeler and Thorngate, 2009). These volatile compounds contribute to olfactory and gustatory quality of wines. The different composition and concentration of volatile compounds helps to construct the characteristics of wine and their concentration differences distinguish one wine from another (Ribéreau-Gayon et al., 2006). Varietal aromas explain the unique flavours of wines made with different types of grapes. They are considered as key factors to recognition of a typicity

or sensory identity of wine, such as methoxypyrazines (vegetable like flavour), terpenes and norisoprenoids (floral aromas). Besides their commercial importance, plant volatiles are also associated with defensive and attractive roles (Pichersky et al., 2006)

Methoxypyrazines

Methoxypyrazines (MPs) are the main compounds responsible for the “green” flavours found in Cabernet Sauvignon and Sauvignon Blanc grape and wine (Lacey et al., 1991; Noble et al., 1995; Ryona et al., 2008). They have nitrogen-containing heterocyclic structures and probably result as a secondary product of amino acid catabolism in the grape (Figure 6). In grape berries, 7 MPs have been detected in grape and wine (Figure 7), and 2-methoxy-3-isobutylpyrazine (IBMP), 2-methoxy-3-isopropylpyrazine (IPMP) and 2-methoxy-3-sec-butylpyrazine (SBMP) are the predominant MPs (Darriet et al., 2012; Lei et al., 2017). They have been largely studied in grape and wine. The olfactory perception threshold of IBMP in water is very low, less than 1ng/L. The recognition threshold in wine, in range of 2-6 ng/L, is quite different from those determined in water and is variable due to the different composition from one wine to another (Roujou de Boubée et al., 2000; Pickering et al., 2007). An excessive level of IBMP concentration (≥ 15 ng/L) in wine has a negative effect as an obvious herbaceous off-flavours (Roujou de Boubée et al., 2000), but a concentration near its detection threshold can contribute to pleasant varietal aromas in Sauvignon Blanc wines (Allen et al., 1991). Therefore, controlling IBMP concentration at a suitable level is very important to wine quality. Based on previous studies, IBMP concentration in wine is highly correlated with its concentration in berry at harvest. It is easily extracted during maceration and difficult to reduce by oenological practice (de Boubée et al., 2002; Darriet et al., 2012). It has been found that IBMP accumulates in grape berries before veraison and degrades thereafter (Darriet et al., 2012). In general, high temperature has been reported to reduce its level (Lacey et al., 1991; Allen and Lacey, 1998; Marais et al., 1999; Falcão et al., 2007).

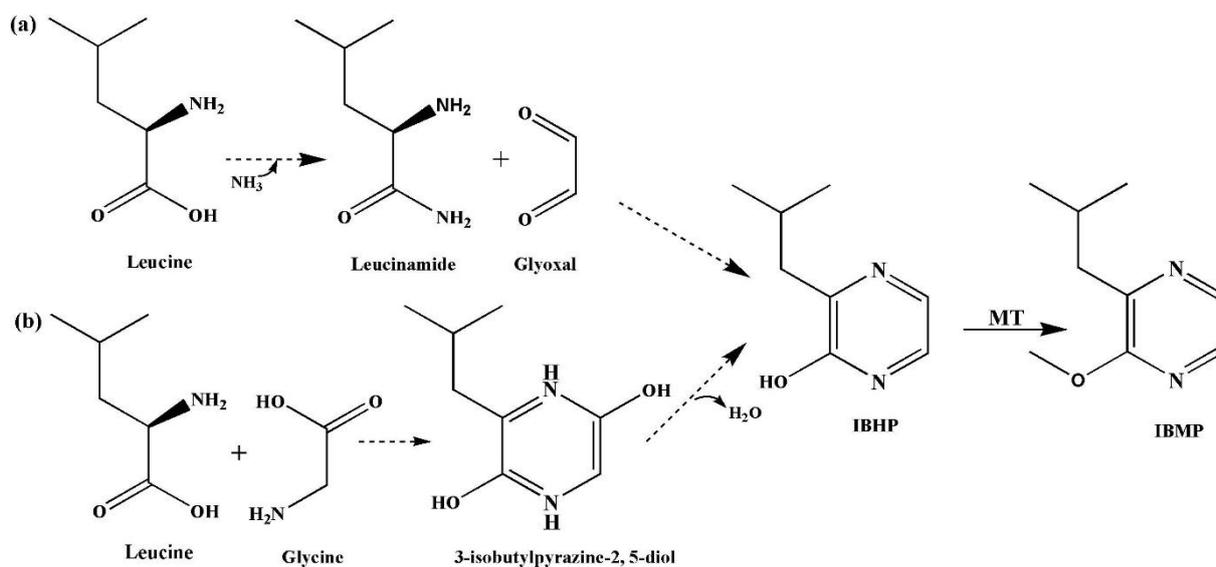


Figure 6: Two proposed pathways of 3-isobutyl-2-methoxypyrazine biosynthesis in living organisms (Dunlevy et al., 2013; Guillaumie et al., 2013).

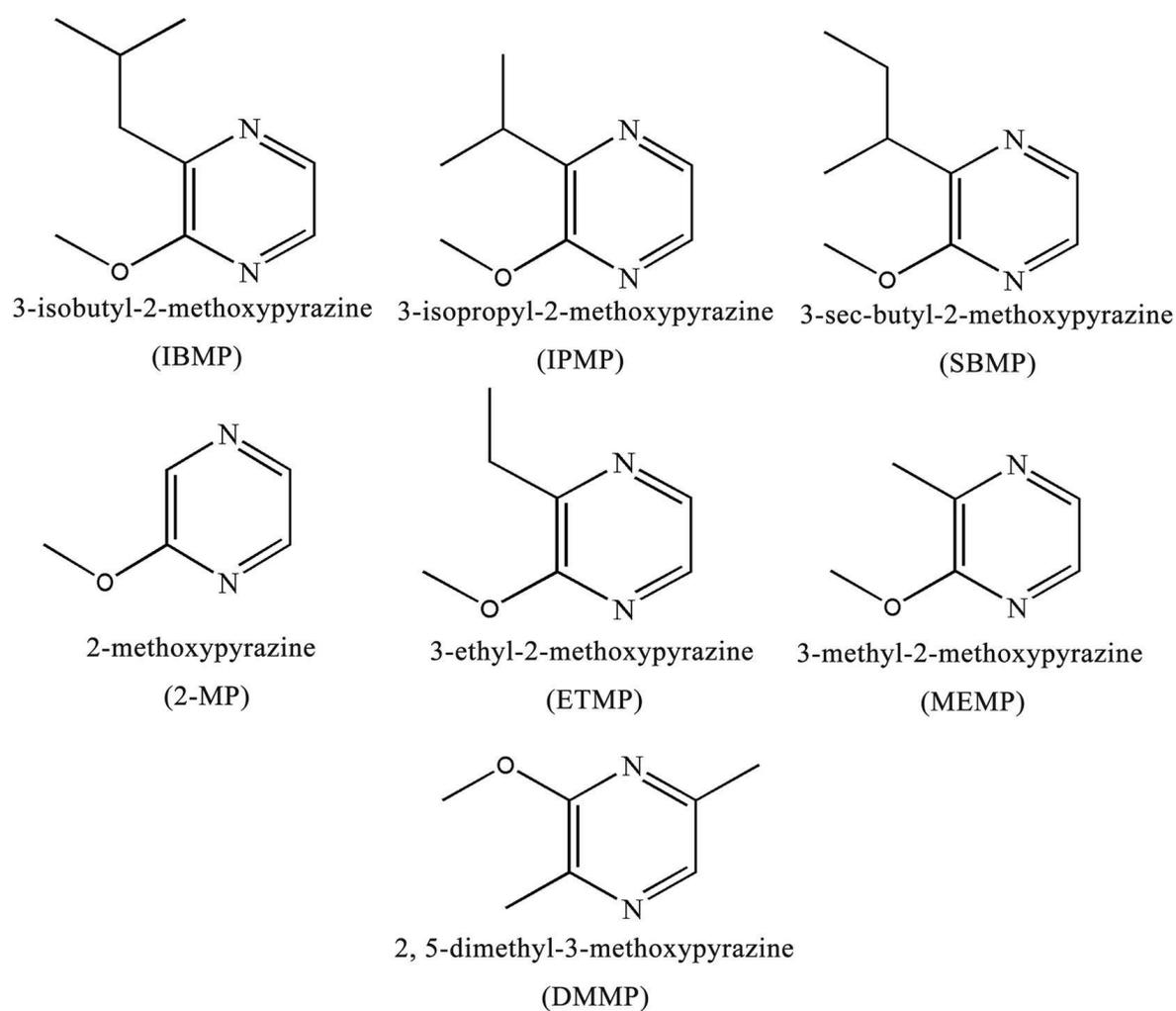


Figure 7: Chemical structures of MPs detected in grapes and wine (Lei et al., 2017).

Thiols and their precursors

Volatile thiols, a group of sulfur containing alcohols, can have a variety of aromas and smells. They have positive and negative contributions to wines depending on their chemical structure (Moreira et al., 2002). For example, hydrogen sulfide (H₂S), which is formed at the end of fermentation, smells like rotten eggs if it presents in wine (Winter et al., 2011). Among different thiols, 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexas-1-ol (3SH), and its acetate (A3SH) (Figure 8) have been identified as a character impacting compound in Sauvignon Blanc wines (Darriet et al., 1993; Tominaga and Dubourdieu, 1997; Tominaga et al., 1998a), and also detectable in wines made from other varieties such as Cabernet Sauvignon, Semillon and Merlot (Tominaga et al., 2000). 4MSP smells like box tree and blackcurrant buds when its concentrations are above 0.8 ng/L in model wine. 3SH and A3SH, which have reminiscent of grapefruit or passion fruit, have a smell-threshold of 60 ng/L and 4 ng/L in model wine (Darriet et al., 2012). They present in the odorless precursor forms in grape berries and musts, and are released from odorless precursors during alcoholic fermentation (Darriet et al., 1995) (Figure 7).

3SH is the most predominant thiol in wine with a concentration between 150-3500 ng/L (Darriet et al., 2012). Its precursors in grape berries and musts are S-3-(hexan-1-yl)-glutathione (Glut-3SH-Al), S-3-(hexan-1-yl)-glutathione (Glut-3SH) and S-3-(hexan-1-yl)-L-cysteine (Cys-3SH) (Tominaga et al., 1998b; Peyrot des Gachons et al., 2002; Thibon et al., 2016). Until now, the biosynthesis pathways of these precursors are still far away being well understood. Based on several studies, the hypothetic pathway is illustrated in Figure 8 (Peyrot des Gachons et al., 2002; Kobayashi et al., 2010; Thibon et al., 2011; Kobayashi et al., 2012; Thibon et al., 2016). The first step of biosynthesis of 3SH precursors is the formation of Glut-3SH-Al through the conjugation of glutathione on (*E*)-hex-2-enal by glutathione-*S*-transferase (GST). Next, Glut-3SH is synthesized by reduction of Glut-3SH-Al. Cys-3SH derives from Glut-3SH by removing of the glycine. This reaction needs two enzymes: a γ -glutamyltranspeptidase (*VviGGT*) and a carboxypeptidase (Peyrot des Gachons et al., 2002; Thibon et al., 2011; Thibon et al., 2016). Finally, 3SH is synthesized during the alcoholic fermentation by *Saccharomyces cerevisiae* (Pinu et al., 2012).

S-cysteinylated and/or S-glutathionylated thiol precursors increased during berry development (Cerreti et al., 2015; Helwi et al., 2016). Conversely, Capone et al. (2012) mentioned that the concentration of thiol precursors may have some fluctuation and even decreased during

ripening. To our best knowledge, nowadays no published work allows a direct assessment of elevated temperature effect on 3SH precursors in grapevine berries.

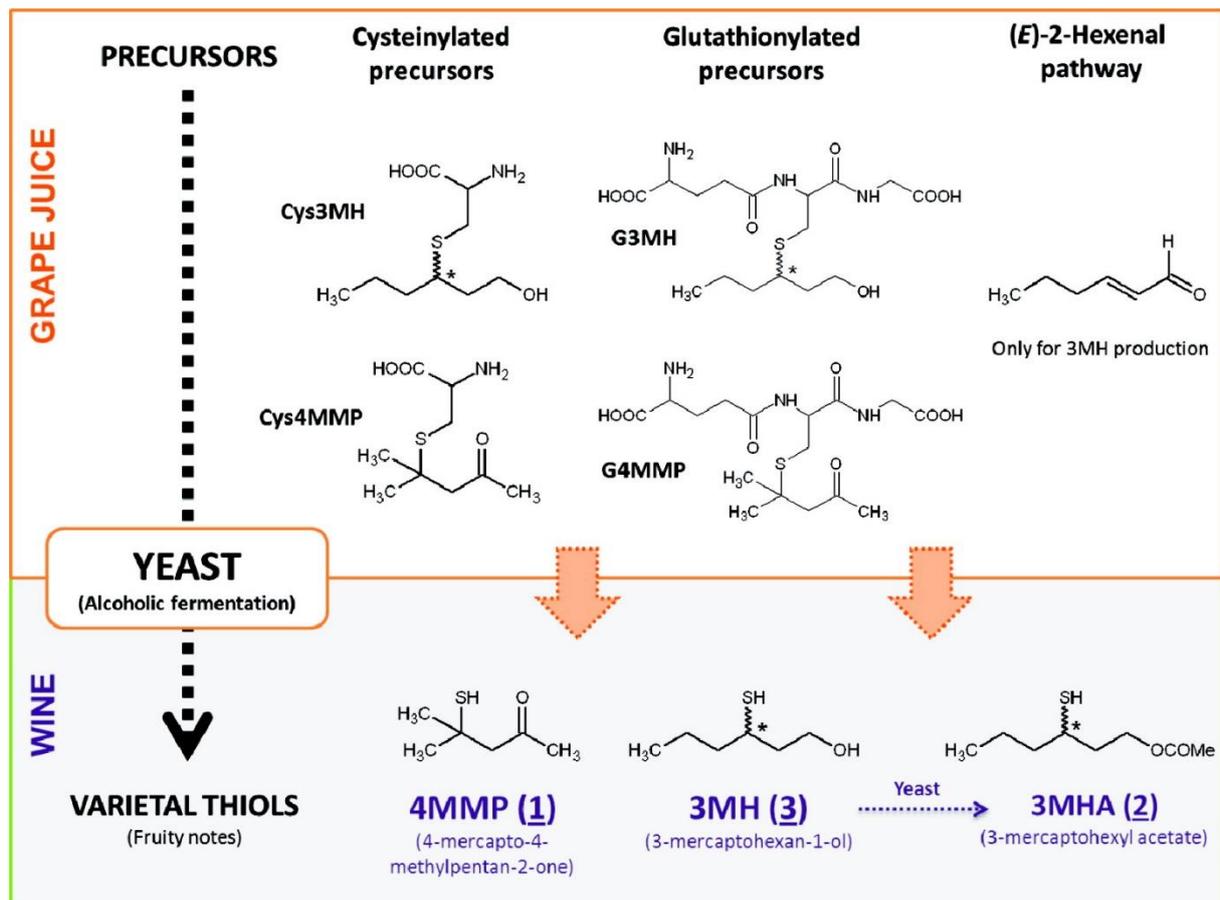


Figure 8: Three principle thiols in wine and their precursors in grape and musts.
(Roland et al., 2011)

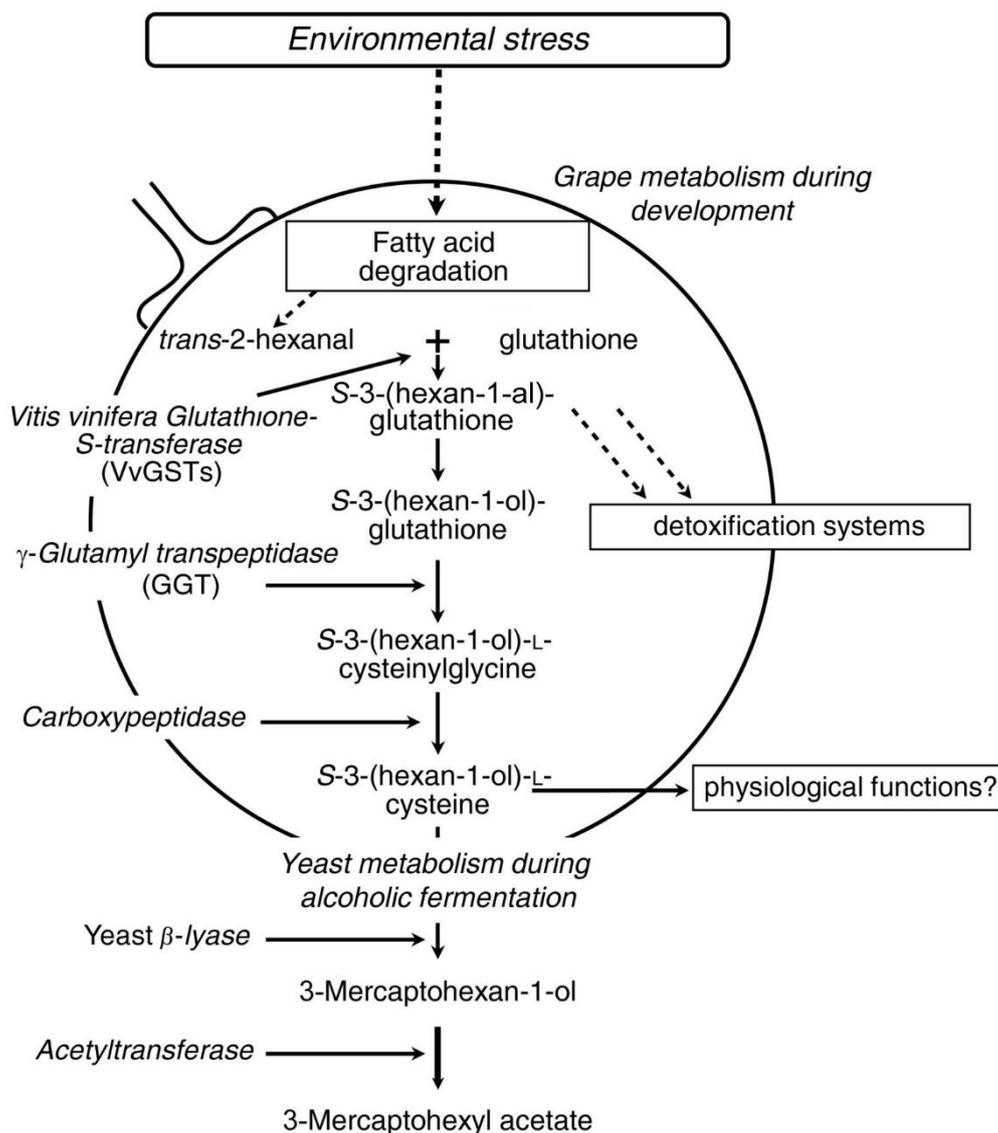


Figure 9: Hypothetic pathway of the glutathionylated precursors (Glut-3SH-Al and Glut-3SH) and cysteinylated precursor (Cys-3SH) in grape berries (Kobayashi et al., 2012).

C₁₃-norisoprenoids and carotenoids

Similar with thiols, C₁₃-norisoprenoids present in grape as non-volatile precursors and are released not only during alcohol fermentations and also during aging process (Sefton et al., 1994; Pinu et al., 2012). In wine, five kinds of C₁₃-norisoprenoids are identified: β-damascenone, β-ionone, TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), TPB (1-(2,3,6-trimethylphenyl)buta-1,3-diene) and vitispiranes (Mendes-Pinto, 2009; Darriet et al., 2012). β-damascenone and β-ionone are responsible for the floral and fruity pleasant notes; TDN is

considered to be responsible for petrol/kerosene-like aroma in Riesling wine (Black et al., 2012).

C₁₃-norisoprenoids are thought to be formed as biodegradation products of carotenoids. Their concentrations increase during berry development while carotenoids degrade (Razungles et al., 1988; Bindon et al., 2007). The biosynthetic pathway proposed for the formation of C₁₃-norisoprenoids from carotenoids combines three steps (Figure 10). Initially, the bio-oxidative cleavage of the carotenoids gives rise to primary degradation products, followed by enzymatic conversion to the aroma precursor involving glycosylation (catalysed by glycosyltransferases) of those norisoprenoids with a hydroxyl group. At harvest, C₁₃-norisoprenoids are present in grape as non-volatile glycosidically bounded precursors. The aglycones are released from the carbohydrate moiety (mono- and disaccharide) during maturation, storage, processing or aging through the action of enzymes, acids, or heat and undergo further transformation and rearrangement to result in the final aroma-active compound (Marais et al., 1992; Winterhalter and Rouseff, 2002; Mendes-Pinto, 2009; Winterhalter and Gök, 2013).

A linear relationship between the concentration of carotenoids and C₁₃-norisoprenoids was observed under various environmental conditions (Bindon et al., 2007; Kwasniewski et al., 2010). In grape berries, investigating the effect of temperature on carotenoids and C₁₃-norisoprenoids was always associated with alteration of light conditions. Higher concentration of C₁₃-norisoprenoidic glycosides was found under high light exposure conditions in Syrah and Pinot Noir (Bureau et al., 2000; Young et al., 2016). Conversely, Marais et al (1992a) found a higher TDN concentration but an unchangeable β -damascenone concentration in light exposed berries of Riesling and Chenin Blanc. Besides, higher altitude combined with lower temperature resulted in a higher carotenoids concentration in berries in Dauro Valley (Oliveira et al., 2004), but Falcão et al (2007) found that no significant relation was observed between α - and β -ionone and β -damascenone in wine with the vineyard's altitude in southern Brazil.

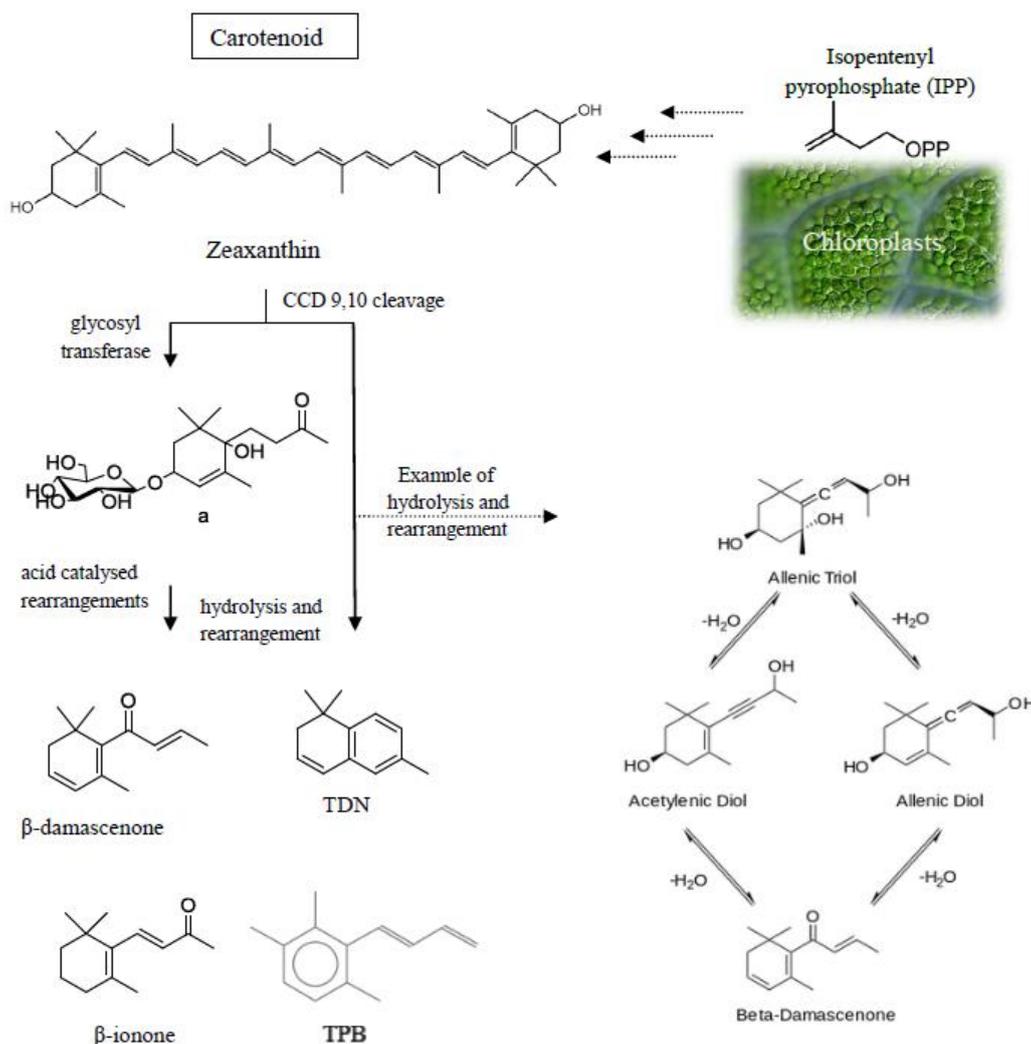


Figure 10: Putative pathway for the formation of important C₁₃-norisoprenoids, in detail for β-damascenone (Mendes-Pinto, 2009; Winterhalter and Gök, 2013).

I.4 Method for investigation temperature effects

Until recently, three kinds of methods have been used to investigate the influence of temperature on grape berries and vines (Sadras and Soar, 2009). The first one is indirect by comparing seasonal and/or regional differences with the aid of statistical analysis (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012). This method cannot avoid the bias caused by other environmental factors. Secondly, a direct comparison under artefactual conditions, like greenhouse (Mori et al., 2007b; Rienth et al., 2016; Lecourieux et al., 2017). These studies in controlled conditions were conducted mostly with an abrupt thermal-shock with a temperature increment by more than 5 °C, which is much higher than projected global warming. The last one consists in changing the temperature closely surrounding specific plant

parts. To overcome these limitations, Sadras and Soar (2009) created a new and inexpensive open system to increase temperature in realistic vineyard conditions, a passive open-top heating system based on a very local greenhouse effect. This system gently increased maximum bunch temperature with 2.3-3.2°C that was commensurate with the projected global warming. Meanwhile it maintained daily temperature cycle without or with little effect on other microclimate factors (Sadras and Soar, 2009).

Objectives of the thesis

Based on above bibliographic research, the general objective of this thesis is focused on the responses of berry quality (from metabolic and transcriptomic levels) to elevated temperature.

The general objective is divided into the following partial objectives:

- Identify how moderately elevated temperature influence the concentrations of soluble sugars, organic acids and free amino acids in fruit over development;
- Gain a better understanding of the transcriptional regulations underlying berry responses to elevated temperature;
- Verify the effects of temperature elevation on phenolic compounds;
- Investigate the accumulation of IBMP and 3SH precursors during berry development and their biosynthetic genes expression levels under two different thermal regimes;
- Explore the effect of elevated temperature on carotenoids and their hydrolytically derived C₁₃-norisoprenoids.

For achieve this study, an open-top passive heating system was installed in the realistic vineyard in Bordeaux, France and Barossa Valley, Australia. Two representative red and white varieties in Bordeaux region, i.e. Cabernet Sauvignon and Sauvignon Blanc were investigated in Bordeaux during two vintages (2015 and 2016). Only Cabernet Sauvignon was studied in Barossa Valley in one season (2015-2016). Comprehensive metabolite analysis was combined with qPCR and RNA-seq analysis

**Chapter II: Effect of moderately elevated
temperature on free amino acids content of
Cabernet Sauvignon and Sauvignon Blanc berries**

Effect of moderately elevated temperature on free amino acids content of Cabernet Sauvignon and Sauvignon Blanc berries

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ABSTRACT

In the context of global warming, elevated temperature may alter berry quality and development of grapevine. Free amino acids are the major nitrogenous compounds in grape berries and can serve as an important nitrogen source for yeast during winemaking. The present study investigated the effects of elevated temperature on berry amino acid concentrations under actual vineyard conditions. A passive open-top heating system was used to increase temperature from fruit setting to over-maturity by 0.5-1.6°C at bunch zone. Eighteen amino acids were assessed in the berries of two varieties (Cabernet Sauvignon and Sauvignon Blanc) in 2015 and 2016. The elevated temperature did not affect the developmental profiles of individual amino acids nor the total amino acid concentration, except a significantly lower total amino acid concentration in the heated mature berry of Sauvignon Blanc in 2015. However, the elevated temperature affected the amino acid composition depending on varieties and developmental stages. All free amino acids concentrations were lower in heated mature berry of Cabernet Sauvignon, except Pro in 2015, and Pro, Tyr and Cys in 2016, which were more concentrated. Although 17 out of 18 amino acids concentrations (except Cys) were lower in SB heated berries at harvest, most of them were higher at mid-veraison stage. Conversely, in 2016, positive effects at ripen stage was found by elevated temperature. These results suggest that the developmental profile of free amino acids in grape berries is mainly determined by genetic factors, and that the elevated temperature only has a role of fine tuning their composition.

KEYWORDS: Amino acids, fruit quality, ripening, temperature, *Vitis vinifera*.

II.1 Introduction

Global climate change is demonstrated by a very wide range of observations across the world (IPCC, 2014a). A recent report shows that global annual average temperature has increased by more than 1.2°C since 1900 (through 2012) (Power et al., 2017). The quality of grape berry and development of grapevine *Vitis vinifera* L. depend on genotype and environmental factors (Seguin, 1986; Van Leeuwen et al., 2004; Deluc et al., 2007). As one of the most important climatic factors, the influence of temperature on viticulture is manifold. In the context of global warming, many studies investigated the effects of higher temperature on grape berry quality, and wine quality and typicality (Duchêne and Schneider, 2005; Jones et al., 2005; Mori et al., 2007b; Azuma et al., 2012; Sweetman et al., 2014; Bonada et al., 2015). In general, long-term increase in temperature can accelerate phenological development (Jones and Davis, 2000), decrease acid concentration, but increase sugar concentration (Coombe, 1986). It was also reported that grape phenolic antioxidants exhibit significant degradation at high temperature (Hamama and Nawar, 1991; Sadras and Moran, 2012).

In grapes, free amino acids are the major nitrogenous compounds and can serve as an important nitrogen source for yeast during winemaking (Marcy et al., 1981). During wine making, total nitrogen in the must can vary from 100 to 1200 mg/L, of which amino acids make up about 50% (Conde et al., 2007). Phenylalanine and branched chain amino acids can be metabolized into the precursors of phenylpropanoids and volatile compounds and therefore affect grape flavours (Guan et al., 2017). They can also influence wine flavours, because of their role as precursors for the synthesis of aromatic compounds, such as isoamyl-acetate (Marcy et al., 1981; Conde et al., 2007). On the other hand, temperature is known to affect the biosynthesis of amino acids in many plants. In *Arabidopsis*, an increased concentration of many amino acids (Asn, Leu, Ile, Thr, Ala, Leu, and Val) was observed under heat shock (Kaplan et al., 2004). Similarly, higher temperatures (+8°C) increased most amino acids, such as 4-aminobutyrate (GABA) and Glu, with the exception of Gln, Pro, His and Tyr in rice (*Oryza sativa*) (Yamakawa and Hakata, 2010). In greenhouse-grown grapevines, a significant increase of 7 amino acids (Thr, Arg, The, Cys, Lys and GABA) was observed after a heat treatment (+8°C) was applied to the bunch zone during veraison or ripening stages (Lecourieux et al., 2017). In field-grown Riesling grapevine, strong negative correlations were observed between the accumulation of amino acids and leaf removal which was coupled with a higher temperature (Friedel et al., 2015). However, Pieri et al (2016) found that south-

exposed Merlot berries in actual vineyard conditions with higher temperature (+5°C) and solar radiation had similar amino acid concentrations, compared with north-exposed berries. The studies in controlled conditions were conducted mostly with an abrupt thermal-shock stress; therefore, the results obtained could be questionable regarding the effects of long-term and gradient increase of temperature in the context of climate change and acclimation. Whether a modest but long-term temperature elevation influences amino acid accumulation and composition remains an open question.

Until recently, three kinds of methods were used to investigate the influence of temperature on grape berries and vines (Sadras and Soar, 2009). The first one is indirect and compares seasonal and/or regional differences with the aid of statistical analysis (Duchêne and Schneider, 2005); the second is a direct method that manipulates temperature under an artificial environment such as a phytotron (Mori et al., 2007b); the last one consist in changing the temperature closely surrounding specific plant parts (Petrie and Clingeleffer, 2005). All these three experimental methods come with their own limitations (see details in Sadras and Soar 2009 and Soar et al. 2009). To overcome these limitations, Sadras and Soar (2009) created a new and inexpensive open system to increase temperature in realistic vineyard conditions, a passive open-top heating system. This system gently increased maximum bunch temperature by 2.3-3.2°C and maintained daily temperature cycle with little effect on other climate factors, therefore providing a very good way to investigate the effects of future temperature elevation.

We applied this passive open-top heating system in Bordeaux, France, in order to identify how moderately elevated temperature influence fruit soluble sugars, organic acids and free amino acids concentrations in two of the most cultivated varieties in the Bordeaux region, Cabernet Sauvignon and Sauvignon Blanc.

II.2 Material and methods

II.2.1 Site and vines

Experiments were conducted in 2015 and 2016 in Bordeaux vineyard (44° 46' 46'' N, 00°34' 01'' W) with the varieties *V. vinifera* cv. Cabernet Sauvignon (CS) and Sauvignon Blanc (SB). They were planted in an experimental vineyard “VitAdapt” growing on level ground with no slope or geospatial variations. The field-grown grapevines were 8 years old, spur pruned, with

a spacing of 1.6 m between rows and 1 m between plants. Vineyard management followed the local standards.

II.2.2 Heating system and measurements

The passive open-top heating system (Figure 1) was used to increase temperature from fruit setting to over-maturity (14 days after harvest). This system was inspired by the system of Sadras and Soar (2009) with a little modification to better adapt to the training system used in Bordeaux. The system consisted of modular rectangular polycarbonate panels (100 cm high × 300 cm width); two panels were installed obliquely on the two sides of the vines for each block of 5 vines, and fixed by thick ropes; the top and two sides were kept open. Most of leaves were left outside of this very local “greenhouse” effect heating system, therefore global plant function and source-sink ratios were reasonably assumed to remain unaffected.

The treatment was conducted with five replicates of five vines each. The same plots were used for the experiments of the two years to get a repetition and simulate accumulative effect of elevated temperature. To avoid boundary effects, measurements and sample collections were made only in the central three vines within each treatment. Bunch zone and berry temperature and bunch zone relative humidity were recorded at 20 min intervals using temperature and humidity probes (Vaisala HMP155 air temperature and humidity probes, copper-constantan thermocouples for berry and soil temperature) and stored by data loggers (Campbell Sci. CR1000 Datalogger).



Figure 1: Passive open-top system for increasing fruit zone temperature in vineyard.

One hundred berries were randomly sampled from each replicate at five different harvest stages: bunch closure (BC), mid-veraison (MV), mid-ripening (MR), ripe (R) and post-ripe (PR, 2016 only). All samples were ground into powder in liquid nitrogen using a ball grinder MM200 (Retsch, Haan, Germany), and stored at -80°C for later analysis.

II.2.3 Soil and vine water status

Predawn and midday water potentials were measured with a pressure chamber equipped with a digital LCD manometer (SAM Precis 2000, 33175 Gradignan, France), as described in Choné et al. (2000). Five primary leaves per block were measured, two times (MV and MR) in 2015 and five times (BC, MV, MR, R and PR) in 2016.

II.2.4 Extraction and analysis of free amino acid

Amino acids were extracted from 500 mg (fresh weight) finely ground powder of whole berries. The powder was extracted at 80°C successively with 2 mL water/ethanol (1:4, v/v), 2 mL water/ethanol (1:1, v/v) and 2 mL water. The supernatants were combined and then evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, NY). The dry residue was dissolved in 2 mL deionized water.

Amino acids were determined by using HPLC (Waters, Milford, MA, USA) after derivatation with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AccQ-Fluor Reagent Kit, Waters), as described in Martínez-Lüscher et al. (2014). All the amino acids were identified and quantified with external chemical standards purchased from Sigma (St Louis, MO, USA).

II.2.5 Analysis of soluble sugars and organic acids

Glucose and fructose were measured enzymatically as described in Guan et al. (2016). Tartaric and malic acids were determined using the autoanalyser TRAACS 800 (Bran & Luebbe, Plaisir, France) according to the method of Berdeja et al. (2014).

II.2.6 Statistical analysis

To get overviews of correlations among amino acids, principal component analysis (PCA) and heatmaps were produced using R software (R development Core Team, 2010). The Student's t-test was used to test the significance of differences between berries under control

and elevated temperature at $P < 0.05$ level. Dynamic profiles of total and individual amino acids were drawn using sigmaplot 11.0 (Systat Software Inc.).

II.3 Results

II.3.1 Heating system performance

II.3.1.1 Bunch zone and berry temperature of control and treatment in the field

The seasonal dynamics of maximal, average and minimal bunch zone temperatures in control (Figure 2a and 2b) and the elevation of temperature in treatment (Figure 2c and 2d) were assessed. In 2015, the average elevations of maximal, average and minimal temperatures were $\sim 2.76^{\circ}\text{C}$, 1.18°C and 0.97°C respectively and they were 1.13°C , 0.81°C and 0.8°C respectively in 2016 (Figure 3). According to the regression between temperatures of treatment and control (Figure 2c), the system effectively increased the maximal, average and minimal temperatures. The heating effect in 2015 was slightly higher than in 2016 (Figure 3). Because the heating system is passive, the heating efficiency depends on the weather, especially the global solar radiation. In hot clear days, increase of maximal temperature could rise to a maximum of 5.3°C (Figure 2a and 2b). Conversely, in cloudy and rainy days, there was almost no heating effect and even a cooling effect (only three times in 2015 and four times in 2016) (Figure 2c and 2d). The average berry temperature during the treatment period was 22.03°C in 2015 and 22.6°C in 2016, increasing by 1.09°C and 0.8°C respectively (Figure 4), which was similar with the increase in bunch zone air temperature (Figure 3).

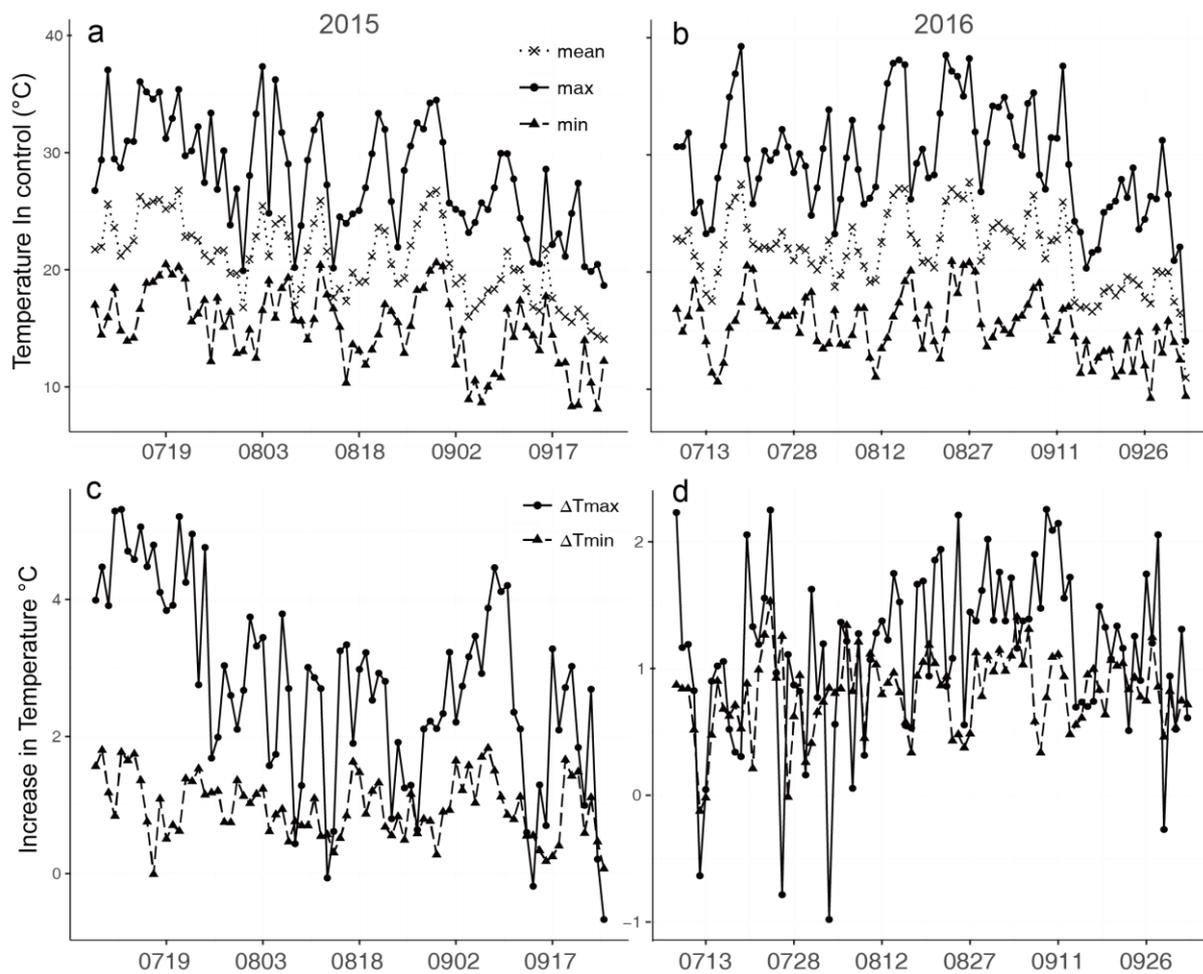


Figure 2: (a and b) seasonal dynamics of maximal, average and minimal bunch zone air temperatures in controls. (c and d) Difference of maximal and minimal temperature between heated (H) and control vines (C).

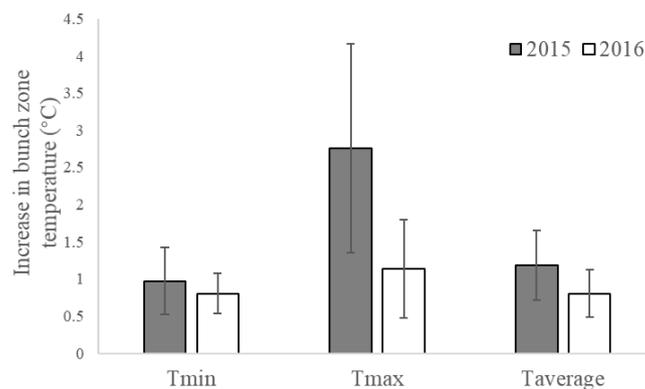


Figure 3: Increment of minimal, maximal and average air temperatures in bunch zone area during berry development in 2015 and 2016.

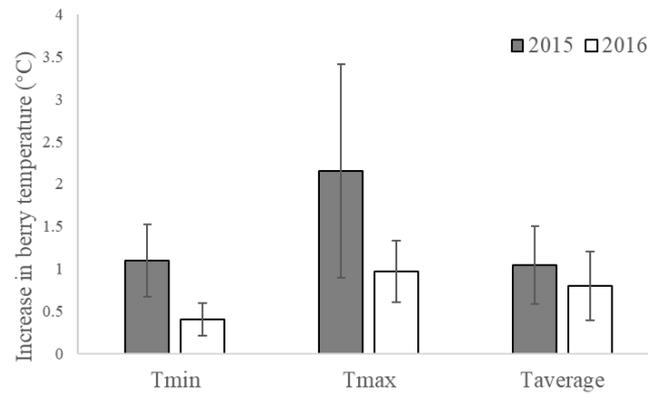


Figure 4: Increment of minimal, maximal and average berry temperature during berry development in 2015 and 2016.

Under vineyard conditions, the daily temperature cycle was maintained under elevated temperature condition (Figure 5a, 5b, 5c and 5d). As shown by the regressions between treated and control, the heating system slightly reduced the relative humidity (Figure 5e and 5g) and increased vapour pressure deficit (VPD) (Figure 5f and 5h), compared with control vines.

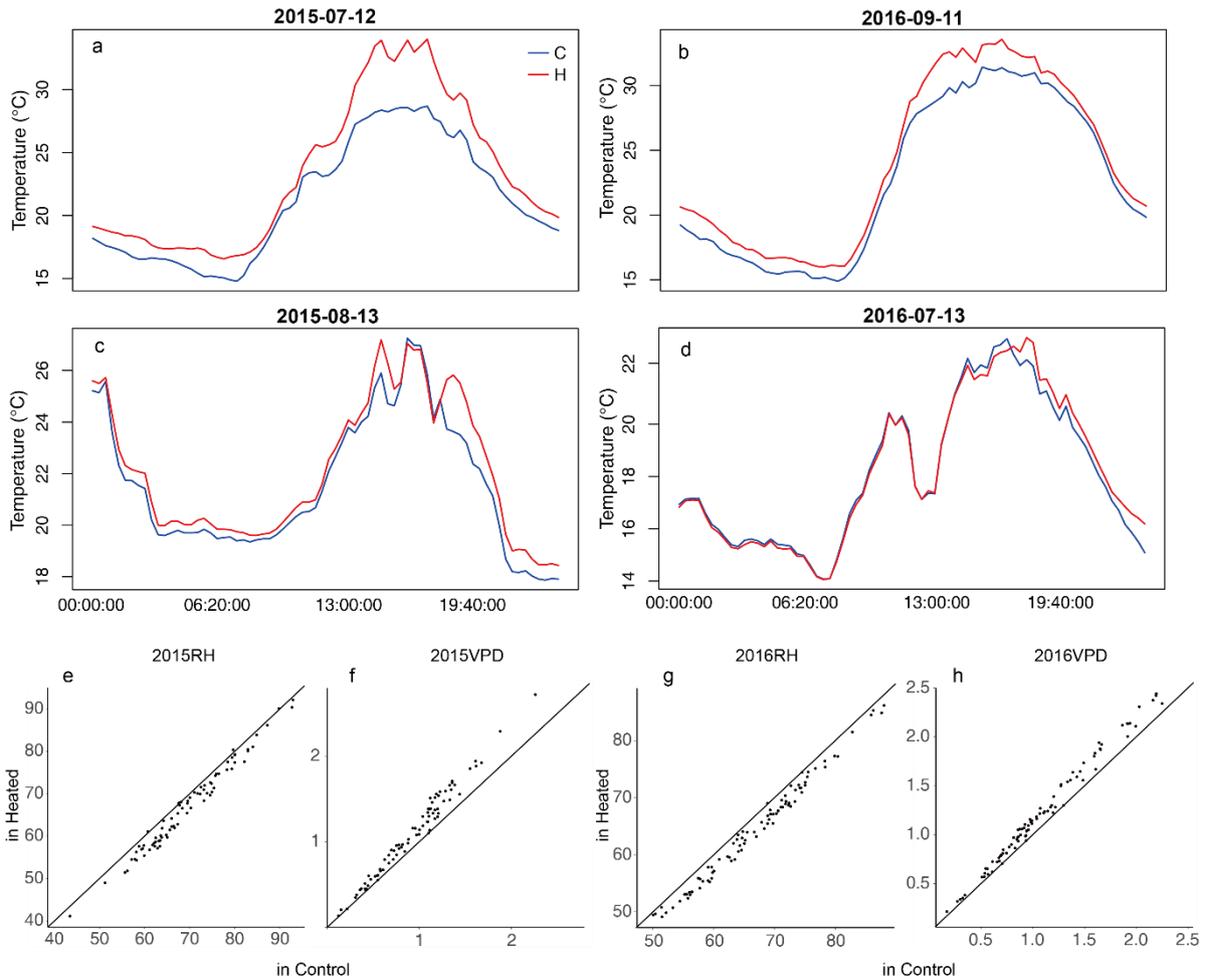


Figure 5: Daily temperature dynamics of representative sunny days (a and b) and cloudy days in each year (c and d). Regression of relative humidity (RH) (e and g) and vapour pressure deficit (VPD) (f and h) between control and heated.

The solid line is the 1:1 line

II.3.1.2 Plant and soil water status

Pre-dawn water potential is a good indicator of root zone soil water availability and stem water potential can be considered as a relevant discriminating indicator for plant water deficit (Choné et al., 2001; Williams and Araujo, 2002). The values of both predawn and stem water potentials did not show any significant difference between treatment and control (Figure 6). Using this open-top system to increase temperature affected neither soil water status nor plant water status; therefore, no bias from a water regime factor was expected to affect the results of berry biochemical compositions.

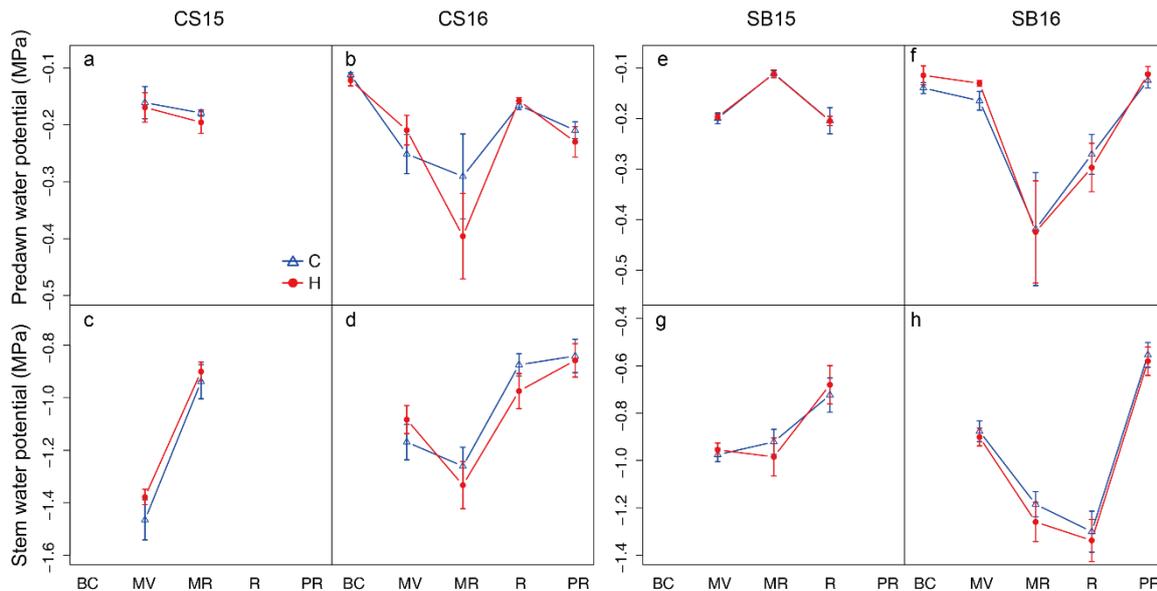


Figure 6: Soil water status as measured by pre-dawn water potential (MPa) and vine water status as measured by stem water potential (MPa) in the experimental plots at the same day of sample collection in 2015 and 2016.

Error bars indicate standard error. Each point is the mean of five replicates.

II.3.1.3 Berry weight, sugars and organic acids

For assessing whether higher temperature affected the parameters of berry ripeness, berry fresh weight, sugar concentration (glucose + fructose) and organic acids (malic and tartaric acids) were determined at each sampling time. Throughout the two years and two varieties, neither berry weight (Figure 7a) nor sugar concentration (Figure 7b) (147-166mg/g FW) at harvest was influenced by warming treatment. In contrast, the elevated temperature decreased malic acid concentration at harvest in comparison with the control, although its effect was significant only for CS in 2016 (Figure 7c). As expected, for CS in 2016, malic acid was lower in heated berries (2.34mg/g FW) than in control berries (2.96mg/g FW). Conversely, elevated temperature increased tartaric acid in the same sample from 6.5 mg/g FW to 7.7 mg/g FW (Figure 7d).

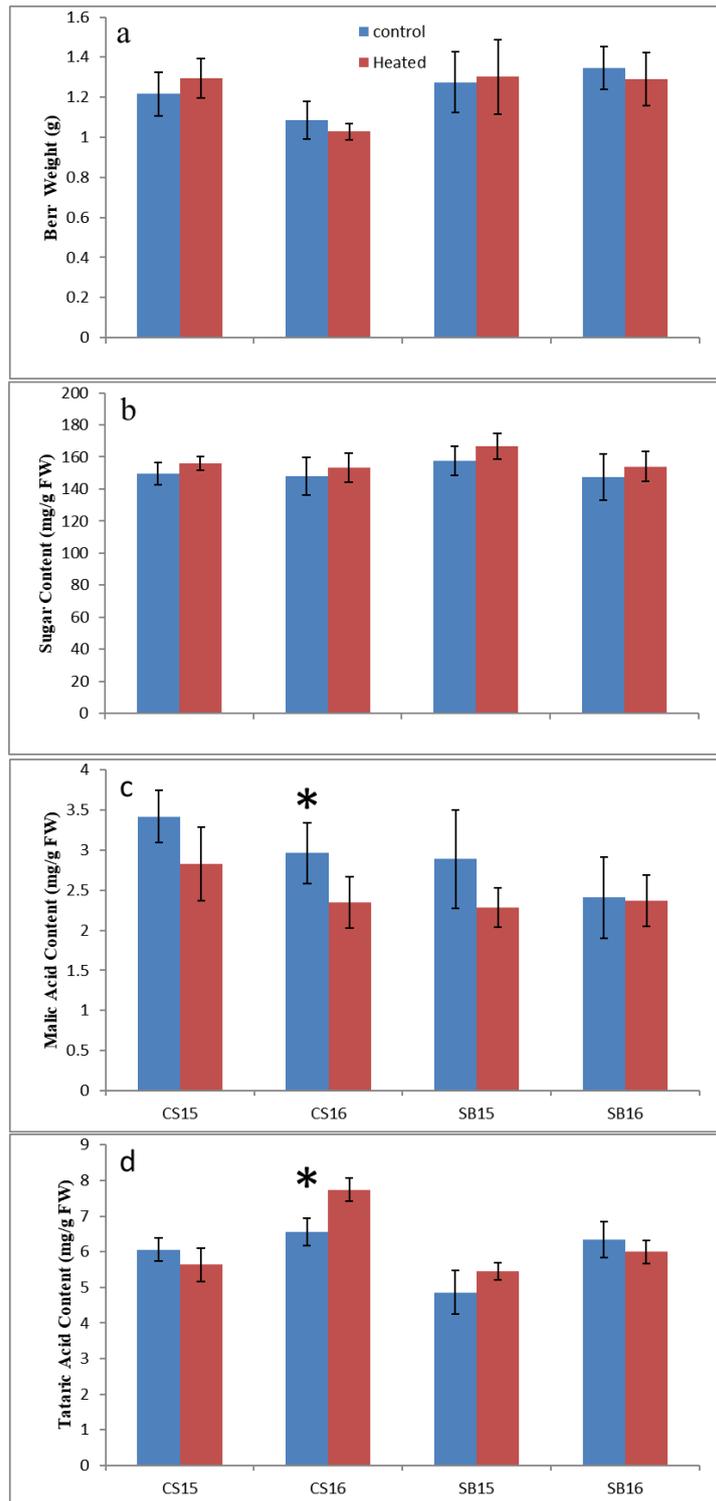


Figure 7: Fresh weight (a), sugar (b), malic acid (c) and tartaric acid (d) concentration at harvest.

Error bars indicate standard errors. * Significantly different from control (independent t-test, $P < 0.05$). Each point is the mean of five replicates.

II.3.2 Total amino acids and amino acid composition

The HPLC method used allowed us to identify and quantify 18 free amino acids in the berries. The total amino acid concentration was calculated by summing all 18 amino acids amounts and dividing by berry volume/weight. The developmental pattern of total amino acid concentration was similar (figure 8). It showed an increasing trend from BC to R/PR. During PR stage in 2016, the total amino acids concentration kept increasing under treatment while it slightly decreased in control. Generally, the total amino acid was present at a low concentration (around 5000 pmol/mg FW) at early stages (BC and MV), and thereafter increased dramatically. For CS, the concentration reached above 17000 pmol/mg FW at harvest and that of SB berries was between 10000 and 17000 pmol/mg FW. The total amino acid only showed a significantly higher concentration in SB control berries at R stage (16095 pmol/mg FW *versus* 12131pmol/mg FW). Comparing the two vintages, berries in 2015 contained more free amino acids than in 2016 in SB, while similar levels between the two vintages were observed for CS.

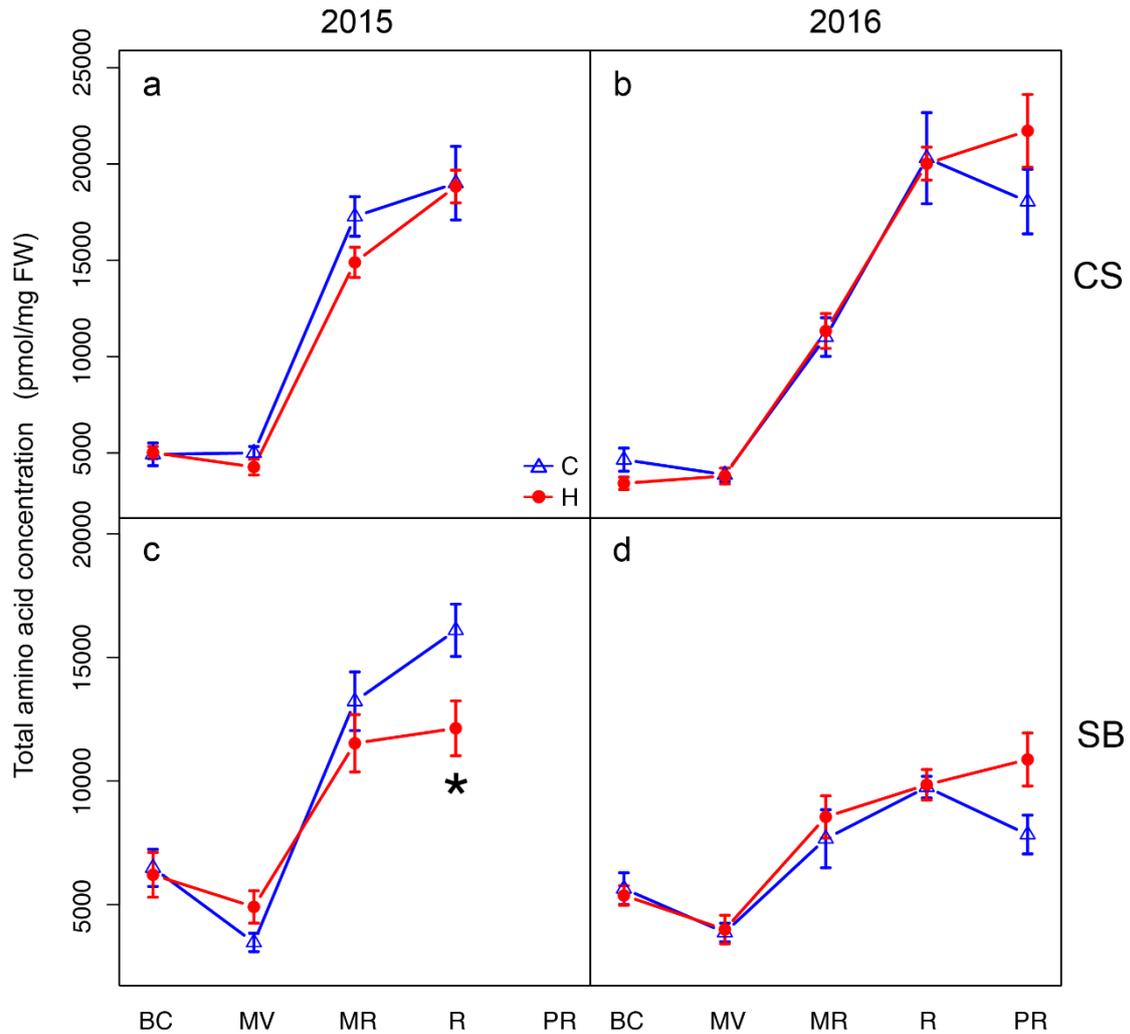


Figure 8: Seasonal variations of total amino acid concentrations in Cabernet Sauvignon and Sauvignon Blanc in 2015 and 2016.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$)

According to their biosynthetic pathway, the 18 amino acids are separated into six groups based on their metabolic precursor: glutamate family (Glu, Gln, Arg, Pro and GABA), aspartate family (Asp, Asn, Met, Thr and Ileu), serine family (Ser, Gly and Cys), Pyruvate family (Ala, Val), aromatic amino acid family (Tyr and Phe) and histidine family (only His) (Figure 10). The proportion of each amino acid family was relatively conserved during berry development and glutamate family was always present in the highest proportion (59.9-92.4%) (Figure 9).

In CS, only glutamate family increased during berry development and accounted for about 90% of total amino acid at harvest (figure 9). The five other families decreased during whole stage or increased till MV and decreased thereafter. At the early stage (BC to MV), Arg, Gln and Glu were dominant in CS, ranging from 10 to 46.6%. However, they decreased dramatically down to 1.2-5.7% at harvest. In contrast, Pro concentration increased sharply up to 84.3% of total amino acid at harvest.

In SB, glutamate family was also the most predominant group, accounting for 59.9-75.7% depending on stage. Histidine, serine and aromatic amino acid families were in small proportions, under 5.5% (figure 9). With a continuous increase, the pyruvate family accounted for 7.6-12.6% of total amino acid at harvest. Aspartate family proportion peaked at MV then decreased to the initial level (12.6-15.5%). At harvest, Pro was present at the highest proportion (26.4-34.1%) followed by Arg, Glu and Thr, ranging from 8-21.5%.

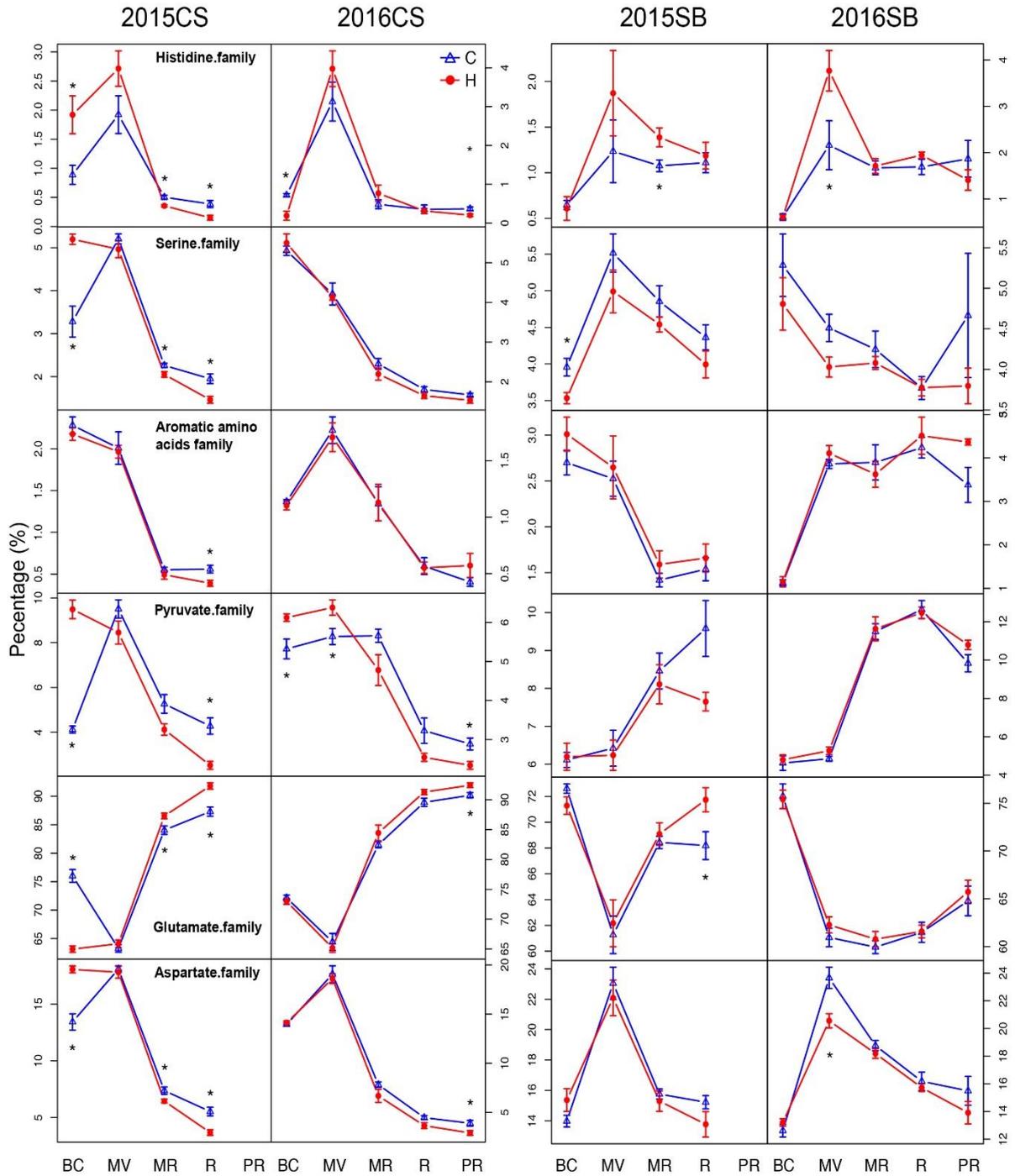


Figure 9: Seasonal variation of proportion of six amino acids families in Cabernet Sauvignon and Sauvignon Blanc berries in 2015 and 2016.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$)

II.3.3 Developmental changes of amino acid concentration

The dynamic changes of each amino acid are presented in detail according to their position in the biosynthesis pathway (Figure 10). To further clarify the difference between treatment and control of individual amino acid along berry development, heatmaps (Figure 11) were performed on the normalized ratio between treatment and control (H/C).

In CS, the elevated temperature mainly showed a negative effect during berry maturation (Figure 11), except BC stage in 2015 and PR stage in 2016 which showed a positive influence. Decreases in relative concentrations of most amino acids (except Pro, Tyr, Cys and His) were observed, but only Asp, Glu and Met concentrations exhibited significant differences in both years (Figure 10). Despite these differences, the concentration of total amino acid was not significantly affected by the temperature increase, because proline, the most predominant amino acid, did not significantly differ between heated and control.

In SB, vintage-dependent effects of elevated temperature on individual amino acids were observed between two years (Figure 11). In 2015, 17 out of 18 individual amino acids (except Cys) had a relative higher concentration and 7 of them showed a statistically higher level in control berries at harvest (figure 10). This resulted in a significantly higher concentration of total amino acid in control berries at harvest. In contrast, positive effects at R and PR stage were shown in 2016 (figure11), although only Gly and GABA significantly increased at R stage in heated berries.

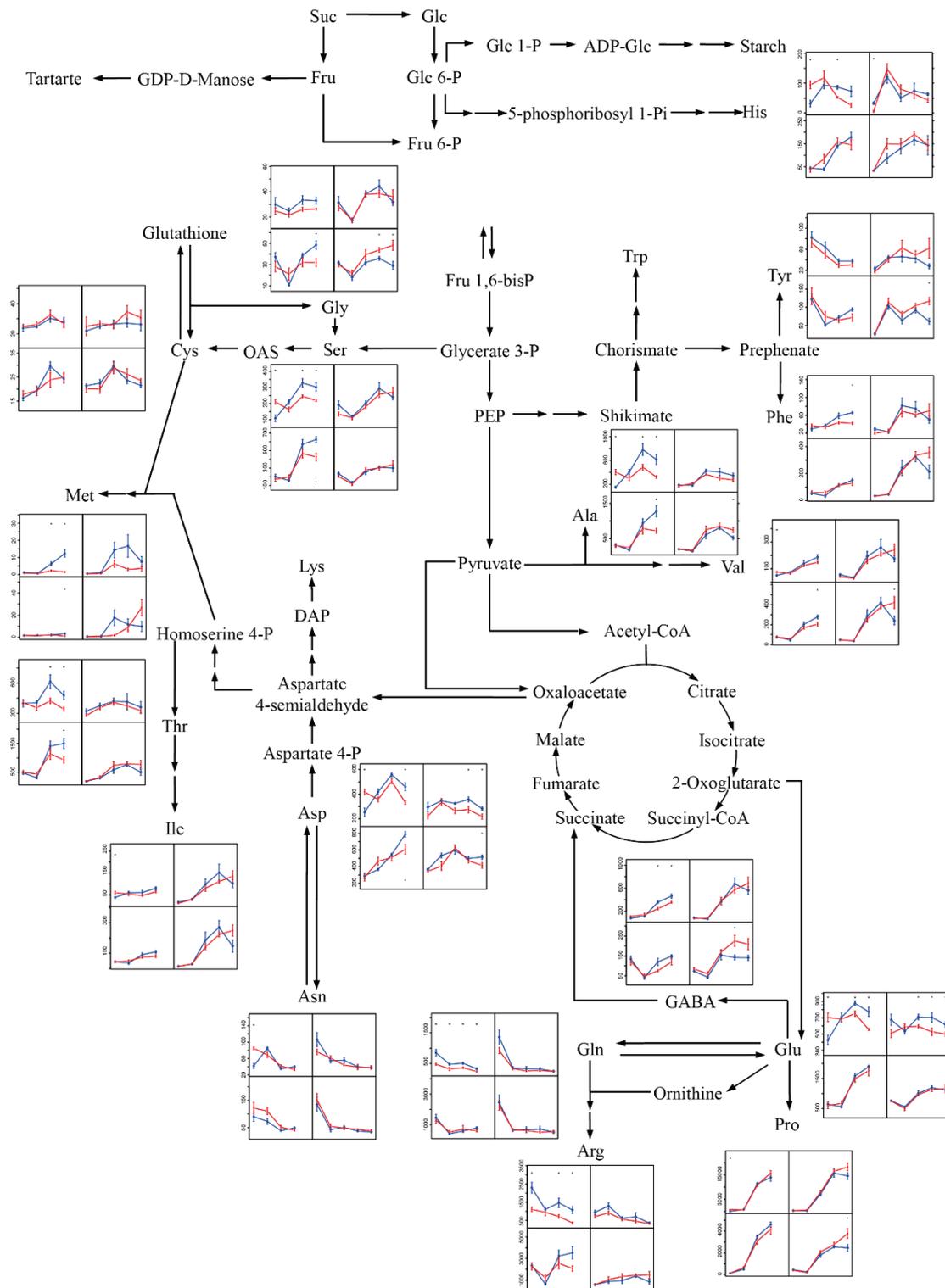


Figure 10: Sugar-amino Acid metabolism pathways. The graphs demonstrate the dynamic changes of each amino acid concentration (pmol/mg FW).

Each graph contains four small graphs, left panel shows data in 2015 and right in 2016. Top panel is for Cabernet Sauvignon and bottom panel for Sauvignon Blanc. Closed red circles represent heated berries and open blue circles represent control berries. Error bars indicate standard error. Each point is the mean of five replicates.

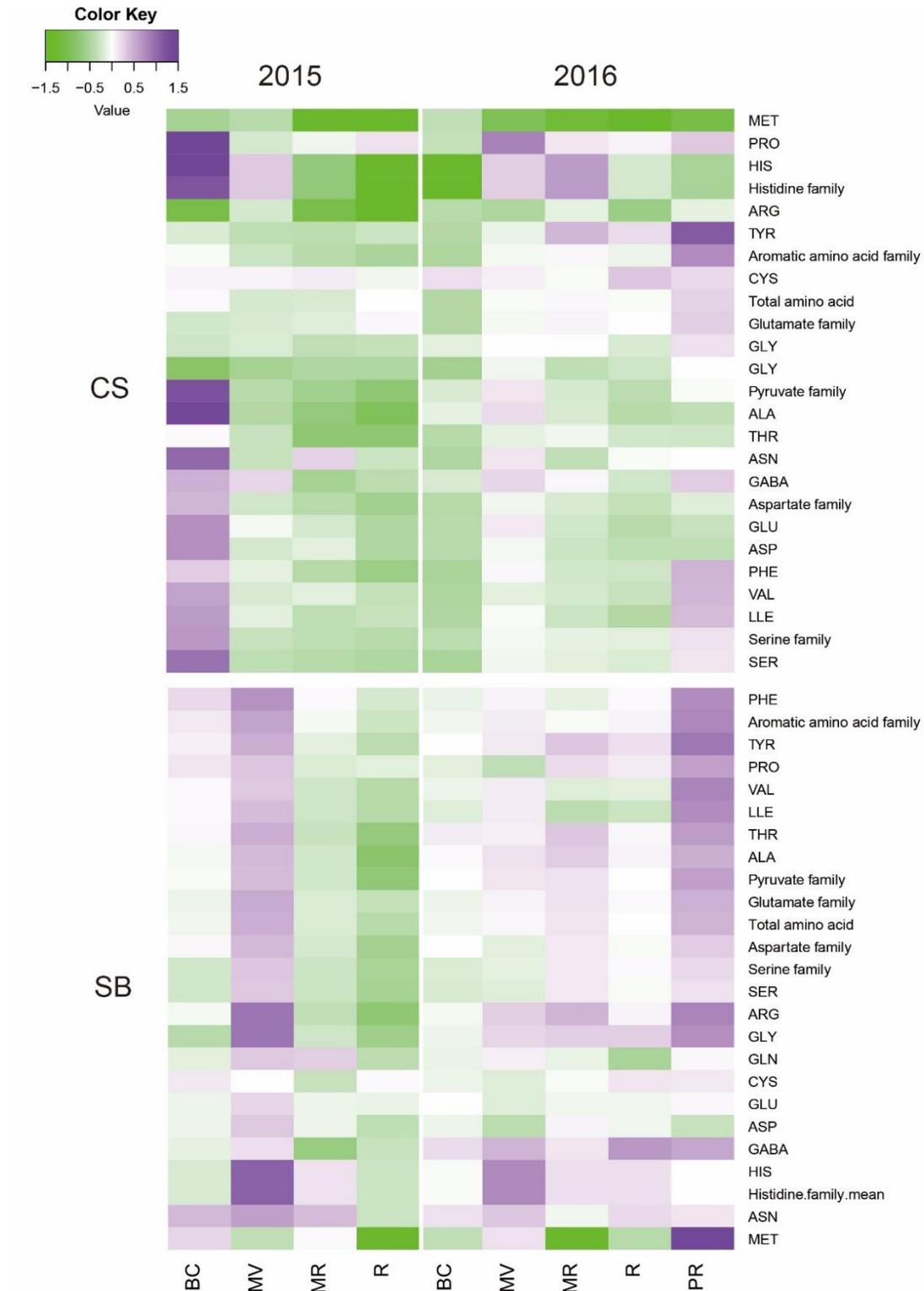


Figure 11: Heatmaps of temperature influence on each amino acid during berry development using the normalized ratio between treatment and control ($\log_2^{H/C}$) in Cabernet Sauvignon (upper) and in Sauvignon Blanc (lower).

Each column represents a developmental stage. The ratios of amino acid concentrations between treatment and control were represented as false colour with violet for higher concentration in heated berries and green for lower as illustrated in the colour key.

II.3.4 PCA analysis

To understand better the accumulation of amino acid under different conditions, a principal component analysis (PCA) was performed (Figure 12 and 13).

For CS, principal component 1 (PC1) explained 54.2% of the variance, PC2 16.8% and PC3 8.79% (Figure 12a and 12c). PC1 allowed the differentiation between developmental stages, and mainly correlated with the whole family of serine as well as GABA, Pro and Phe (Figure 12b). PC2 discriminated the temperature conditions and vintages, mainly determined by His, Asp and Glu. PC3 mainly correlated with Tyr and Gln (Figure 12d).

The PCA results for SB were similar. PC1, which explained the differences between developmental stages, accounted for 65.94% of the variance (figure 13a and 13c). Almost all individual amino acids contributed to PC1, especially Pro, Ala, Glu and His. PC2 (14.03%) separated vintage as well as treatment, strongly based on Met, Ileu and Arg (Figure 13b). PC3, which explained 7.94% of total variance, also discriminated vintage and was linked to Gly, GABA and Asp (figure 13d).

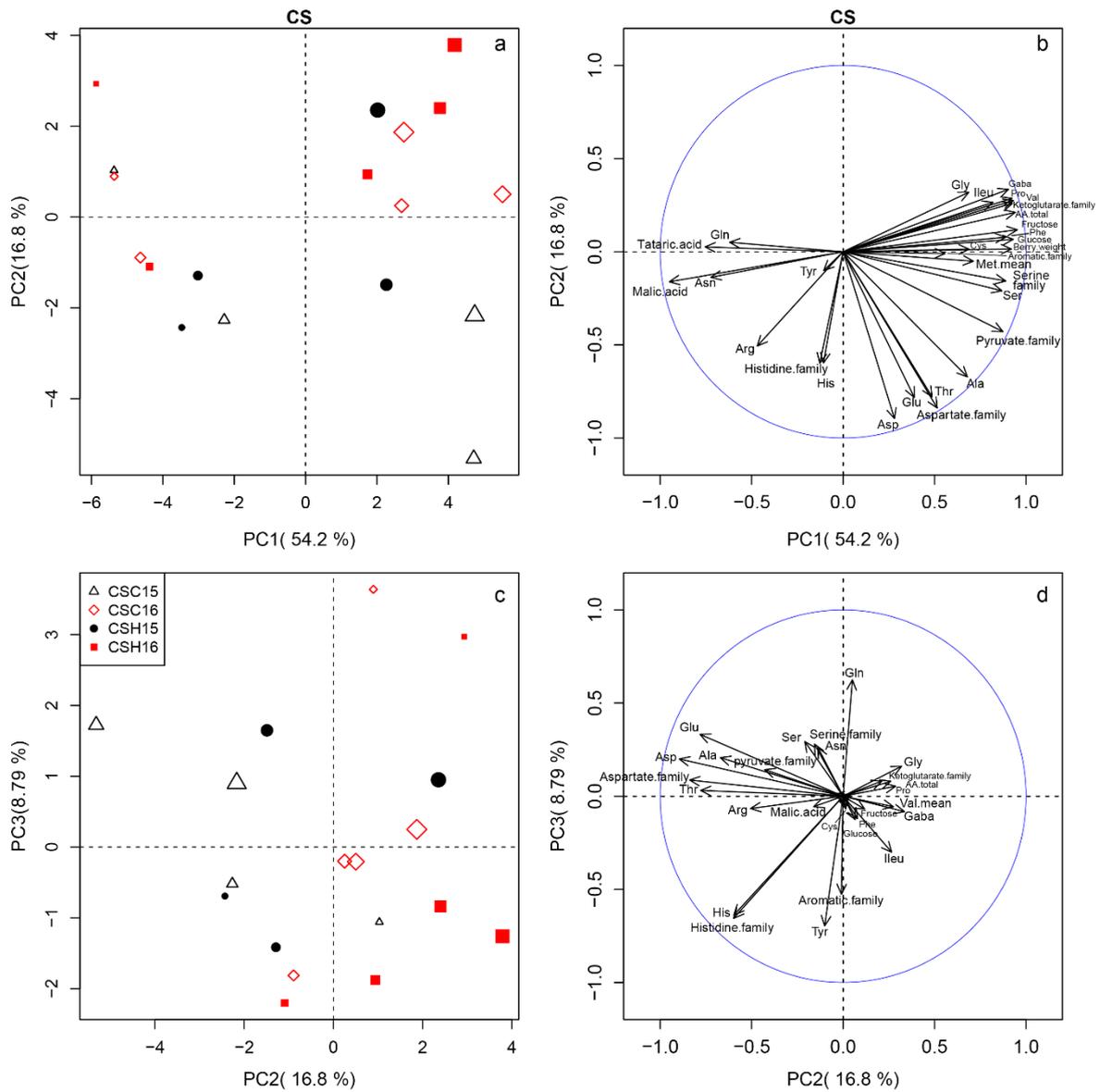


Figure 12: Principal component analysis of amino acid concentrations in Cabernet Sauvignon in two vintages. a and c discriminate years, treatment and developmental stages.

Berry size is represented by the size of the symbols. b and d load plots of primary metabolites and amino acid for the first three principal components (PC1 vs. PC2; PC2 vs. PC3)

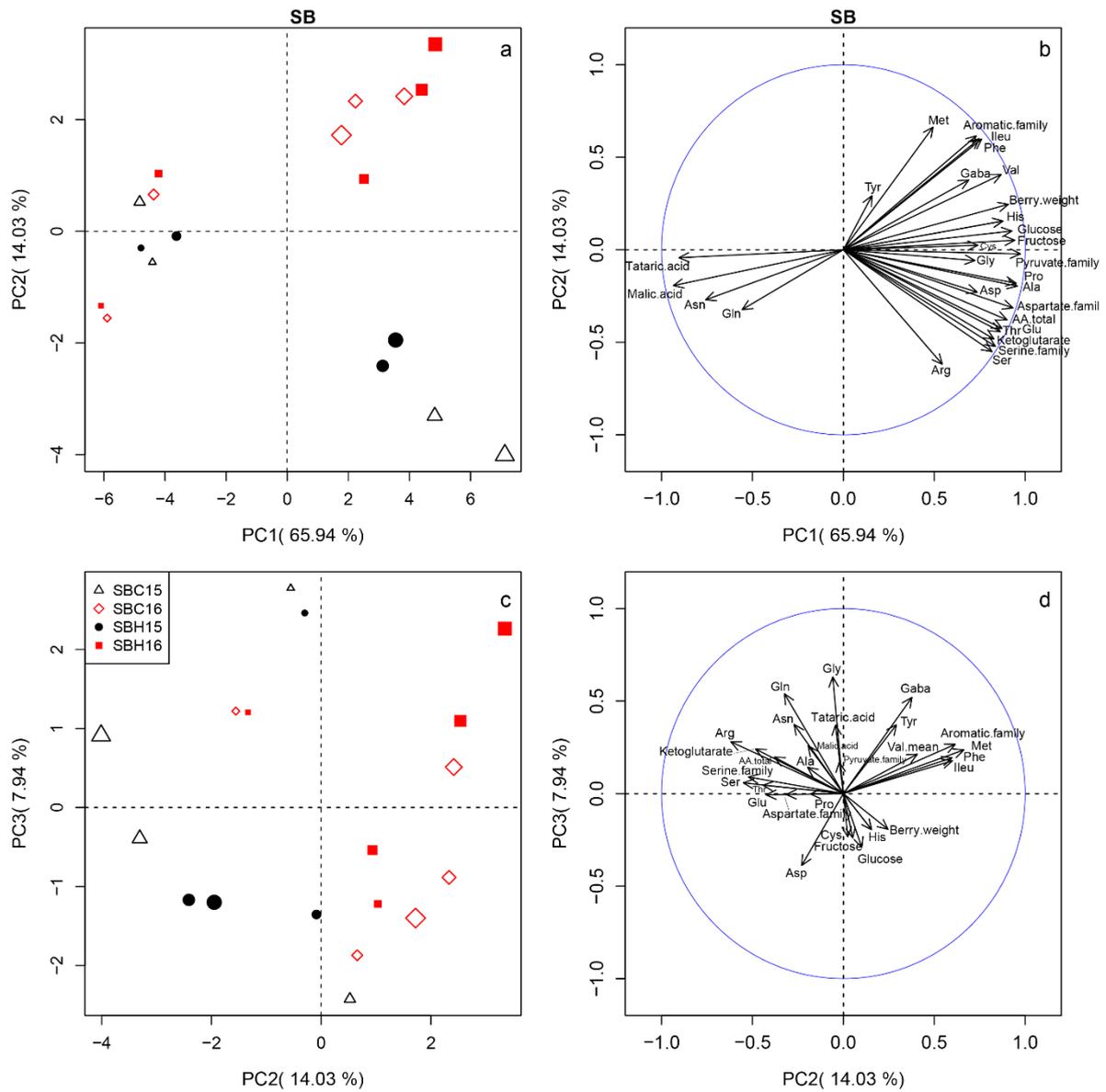


Figure 13: Principal component analysis of amino acid concentrations in Sauvignon Blanc two vintages. a and c discriminate years, treatment and developmental stages.

Berry size is represented by the size of the symbols. b and d load plots of primary metabolites and amino acid for the first three principal components (PC1 vs. PC2; PC2 vs. PC3)

II.4 Discussion

II.4.1 Global warming context and heating system performance

According to the report “Le climat de la France au XXI^e siècle” (Ouzeau et al., 2014), mean temperature is projected to rise by 0.6 to 1.3 °C in France by the mid-21st century (2021-2050) with respect to the 1976-2005 baseline and the summer mean temperature is projected to rise by 0.6-2°C by the late-21st century (2071-2100) with the same baseline. Simulating a similar increase temperature under realistic vineyard conditions can allow to better assess and help understand the potential impact of warming climate on grape berry development and quality. In the present study, the passive open-top heating system was installed in real vineyard during berry development in two consecutive years to manipulate *in-situ* temperature of grape. This system achieved to increase mean temperature by 0.5-1.6 °C, which was commensurate with the projected warming for the 21st century in France. The system maintained daily temperature dynamics with a slight decrease of the relative humidity and an increase of VPD. A similar performance was also obtained on cv. Shiraz in Adelaide under similar heating system (Sadras and Soar, 2009; Sadras et al., 2012; Sadras and Moran, 2012). However, the system also increased minimal temperature in Bordeaux vineyard, whereas it was not changed in Adelaide (Sadras and Soar, 2009). The open top and both ends insure air cycling and the clear transparent polycarbonate minimize radiation difference. With this magnitude of elevated temperature, soil and plant water status were not affected.

II.4.2 Response of technical maturity (sugars and organic acids)

In our study, season-long warming of approximately 1°C of mean temperature did not affect berry weight and sugar concentration in the two varieties. This finding matched previous studies which showed undetectable effect on Shiraz berry weight and sugar concentration at harvest with heating treatment at different key phenostages (Sadras and Soar, 2009; Sweetman et al., 2014). Malic acid concentration in SB was unresponsive to temperature, but it was 0.62 mg/g FW lower in warmed CS berries in 2016 than in control berries in our conditions. This strong seasonal/variety dependence to temperature is consistent with the results of Sadras et al. (2013), who observed that juice titratable acidity decreased in Cabernet Franc and Chardonnay, but was unresponsive in Shiraz and Semillon under elevated temperature conditions. According to Leeuwen et al. (2004), total variance of berry sugar concentration can be explained by varieties (41%), soil (32%) and climate (15%); conversely

malic acid concentration was more sensitive to climate, which explained 62% total variance than to variety (19%). This might explain in part why sugar concentration did not change under heating treatment, but malic acid showed a statistical difference in one year in CS berries. Moreover, responses of sugar and acid concentration to higher temperature in CS and SB did not confirm the results of many previous studies where an increase in sugar concentration and a reduction of acid concentration were found under warming conditions. The explanation might be linked to different temperature rise magnitude and/or temperature ranges, or otherwise the different experimental conditions that may confound the temperature effect with other environmental factors (Buttrose et al., 1971; Bowen et al., 2004; Duchêne and Schneider, 2005; Petrie and Sadras, 2008). These results emphasize the importance of considering the experimental set-up when comparing the effects of elevated temperatures on grape quality.

II.4.3 Amino acid response

To our best knowledge, nowadays no published work allows a direct assessment of season long warming effect on free amino acids in grapevine berries under realistic vineyard conditions. The present study showed that the response to temperature depends on individual amino acids, developmental stages, varieties and vintages; no significant effect of elevated temperature on total amino acid concentration was observed on CS, but a statistically lower concentration was found on SB, only in 2015.

Total amino acids concentration was much higher in CS than in SB for two years. Pro was predominant and its concentration in CS was more than three-fold higher as in SB. This result confirms earlier data obtained on grape juice of different varieties (Kliwer, 1968; Spayd and Andersen-Bagge, 1996; Stines et al., 1999; Hernández-Orte et al., 2002). Pro accumulation in many plant species differs from different environmental stresses including drought, high salinity and heavy metals (Hare and Cress, 1997; Szabados and Saviouré, 2010; Krasensky and Jonak, 2012), but high temperature (heat stress) did not lead to proline accumulation in tobacco and Arabidopsis plants (Rizhsky et al., 2004; Dobra et al., 2010). No response of Pro concentration to elevated temperature in our study matched this conclusion. However, a 0.5-1.6 °C increase of mean temperature could hardly cause an actual heat stress.

The next two abundant free amino acids in both varieties were Arg and Glu which belong to the glutamate family. In some studies, they were reported as major amino acids in grape

berries and must (Hernández-Orte et al., 1999; Stines et al., 2000; Guan et al., 2017). In CS berries, Arg decreased with time and Glu kept a similar concentration with some fluctuation regardless of temperature treatment. Both amino acids showed a negative response to elevated temperature. In SB, Arg accumulation had no uniform performance between vintages as well as treatments and Glu increased during berry development, but no significant difference was found between treated and un-treated berries. This finding contradicted previous studies where increases of glutamate family amino acids were observed with heating in Shiraz berries (Sweetman et al., 2014), in Arabidopsis (Kaplan et al., 2004) and in rice (Yamakawa and Hakata, 2010). However, two major differences distinguished the study of Sweetman (2014) from the present one. Firstly, the present study was conducted in a realistic vineyard, whereas the previous study was conducted in shade-house. Secondly, the magnitude and duration of warming was different, the present study manipulated temperature during whole berry ripening stage by 0.5-1.6 °C increase in mean temperature as opposed to heating at specific stages of berry development by increasing 10 °C and 5 °C of day and night temperature respectively.

Overall, free amino acids compositions were altered by elevated temperature. In CS, the other 15 free amino acids only represented 14.2% of total amino acids at maturity and ten of them represented even less than 1%. In SB, beside the three abundant amino acids, Thr, Ala, Asp and Gln were present in a relatively high proportion ($\geq 5\%$). This varietal difference of free amino acids composition was also confirmed by many previous studies (Spayd and Andersen-Bagge, 1996; Stines et al., 2000; Guan et al., 2017). Although those amino acids presented at such a low level, they are involved in the biosynthesis of many important secondary metabolites such as flavonoids and anthocyanins (Dai et al., 2014). In CS, Phe is a precursor for phenylpropanoids biosynthesis, and a lower concentration under elevated temperature may explain in part the anthocyanins loss under high temperature (Mori et al., 2004; Yamane et al., 2006). Met and Cys, precursors of sulfur volatile compounds in wine (Moreira et al., 2002), showed different responses to temperature. Higher Met concentration and relative lower Cys concentration were detected in heated berries. Further studies are needed to investigate whether those amino acids concentration could influence sulfur volatile compounds in wine. GABA, glycine, valine, and alanine have previously been found to accumulate in heat and cold stress-exposed Arabidopsis seedlings and are considered as part of a global response to temperature stress in different organs of different plant species (Kaplan et al., 2004; Mangelsen et al., 2011). In this experimental study, none of them over-accumulated under

warming conditions. The heterogeneous response to temperature of free amino acids in SB could explain why SB amino acid concentrations were strongly season- dependant.

II.5 Conclusion

The results obtained in actual vineyard conditions with standard practice in Bordeaux, increasing ambient mean temperature by a moderate 0.5-1.6 °C, similar with the projected global warming, unaffected individual amino acids developmental pattern and total amino acid concentration, except for SB berries at R stage in 2015. However, it affected differentially individual amino acid concentrations; amino acid composition of berries mainly varied with varieties and developmental stages. However, the elevated temperature affected the amino acid composition depending on varieties and developmental stages. All free amino acids concentrations were lower in heated mature berry of Cabernet Sauvignon, except Pro in 2015, and Pro, Tyr and Cys in 2016, which were more concentrated. Although 17 out of 18 amino acids concentrations (except Cys) were lower in SB heated berries at harvest, most of them were higher at mid-veraison stage. Conversely, in 2016, positive effects at ripen stage was found by elevated temperature. These results suggest that the developmental profile of free amino acids in grape berries is mainly determined by genetic factors, and that the elevated temperature only has a role of fine tuning their composition.

**Chapter III: Moderately elevated temperature
affects metabolism and transcriptome of grapevine
(*Vitis vinifera* L.) berries**

ABSTRACT

Temperature, one of the most important environmental factors, plays a key role in regulating grapevine phenology and berry composition. In the context of global warming, temperature becomes a threat to viticulture. To date, the mechanisms underlying the responses of grape berry to temperature elevation are still poorly understood. A passive open-top heating system was used to increase temperature from fruit setting to over-maturity by 0.5-1.6 °C at bunch zone under realistic vineyard conditions for Cabernet Sauvignon (CS) and Sauvignon Blanc (SB). Metabolite related to technical, phenolic and aromatic maturities were assessed in together with transcriptome via RNA-seq analysis in order to obtain a comprehensive view of berry responses to moderately elevated temperature. The elevated temperature hardly affected the concentrations of sugars, organic acids and tannins, but significantly reduced anthocyanins in CS and reduced two 3SH precursors (Glut-3SH-A1 and Cys-3SH) in SB. A total of 357 genes were differentially expressed in response to the moderate elevated temperature. Enrichment analysis of Gene Ontology showed that temperature mainly regulated four GO categories, including microtubule, cell wall, extracellular region, and transcription factor activity. Twenty-two transcripts encoding biosynthetic enzymes of phenylpropanoid and flavonoid pathways were down-regulated, and this down-regulation could explain the decreased concentration of anthocyanins in CS. A significantly lower expression level of *VviGST4* was associated with the lower concentration of 3-sulfanylhexas-1-ol precursors in SB. Meanwhile, *VIT_08s0007g01420*, a putative GST homologue, was down-regulated by elevated temperature and might be a potential candidate gene involved in the biosynthesis of 3SH precursors. By combing metabolite analysis and RNA-seq, our study provided novel molecular insights into the responses of grape berry to moderately elevated temperature in vineyard and could provide foundation for developing strategies for mitigating the effects of global warming.

KEYWORDS: RNA-seq, flavonoid, ripening, precursors, thiol, temperature, *Vitis vinifera*.

III.1 Introduction

Grape is one of the world's most economically important fruit crops, with a reported annual production higher than 74 million tons (FAOSTAT-FAO Statistical Database, 2017). Its high economic value comes from its multi-usage, such as fresh fruit, raisin, juice, wine and liquor. The quality and production of grapevine mainly depends on environmental conditions and viticultural practices. Among environmental factors, temperature plays a key role in regulating grapevine phenology and berry composition (Coombe, 1986; Bergqvist et al., 2001; Tomasi et al., 2011). A recent report showed that global annual average temperature has increased by more than 1.2°C since 1900 (through 2012) (Power et al., 2017). Global warming is becoming a real challenge to viticulture and grape industry, not only because it may influence grape quality and production, but also because it may threaten the sustainability of viticulture in hot regions (Keller, 2010).

In the context of global warming, it is essential to better understand of the effects of temperature on berry and wine quality (Duchêne and Schneider, 2005; Jones et al., 2005). Generally, higher temperature accelerates phenological development and decreases organic acid content while increasing sugar content (Coombe, 1986; Jones and Davis, 2000). However, recent studies with more precise temperature regulations showed that the temperature effects might be more complicated than previously reported. For example, Sweetman et al. (2014) showed that elevated night temperature imposed pre-veraison could increase the concentration of malic acid. In addition, Lecourieux et al. (2017) showed that bunch zone high temperature imposed pre-veraison could delay veraison date by two weeks. Moreover, the effects of high temperature on secondary metabolites, which largely influence berry quality and wine typicality, are less investigated and mainly focused on the phenylpropanoid biosynthetic pathway and aroma related components analysis (Jeandet et al., 2010; Peña-Gallego et al., 2012). Loss of anthocyanins in red-wine grape under high temperature was reported (Mori et al., 2007b). Conversely, tannins was less affected by temperature (Cohen et al., 2008). Lower 2-methoxy-3-isobutylpyrazine (IBMP, a green pepper aroma) content in berry, was always associated with a warmer vintage (Allen and Lacey, 1993; Falcão et al., 2007). Defoliation associated with a higher berry temperature only slightly but not significantly modified the precursors of thiols in Sauvignon Blanc berries (Sivilotti et al., 2017).

With the advance of plant genome projects, grapevine research is expanded from

metabolomic level to transcriptomic level. In 2007, the draft of the genome sequence of grapevine was finished (Jaillon et al., 2007). Grapevine transcriptomic analysis (via microarray or RNA sequencing) has provided different insights into mechanisms underlying the elevated temperature induced alteration in both primary and secondary metabolites. Several studies showed that the decreased anthocyanins content under high temperature was caused by the repression of related biosynthetic and regulatory genes such as flavonoids-3-O-glucosyltransferase and MYBA1 (Mori et al., 2007b; de Rosas et al., 2017). However, Carbonell-Bejerano et al. (2013) observed a reduction of anthocyanins without any different expressions of anthocyanin biosynthetic genes in Muscat Hamburg under elevated temperature. An alternative hypothesis for the negative effect of high temperature on anthocyanin content is the induction of anthocyanin degradation genes (Movahed et al., 2016). The effects of high temperature on aromatic potential have also been investigated at transcriptomic level. Lecourieux et al. (2017) found that heat treatment repressed three genes encoding for O-methyltransferase, which are responsible for synthesis of methoxypyrazine (Dunlevy et al., 2010; Guillaumie et al., 2013). The down-regulation of *VviOMT* coincides with lower concentration of IBMP found in warmer vintages (Ryona et al., 2008).

Three main kinds of methods have been used (Sadras and Soar, 2009). The first one consist in comparing, vintages and/or regions, but the temperature effect in this method is inherently confounded with other environmental factors, such as soil properties and composition, and rainfall (Duchêne and Schneider, 2005). The second one is the direct comparison under artefactual conditions, like greenhouse, but this impose limitations to apply the results to vineyard conditions (Mori et al., 2007b). The last method imposes temperature changes to specific plant parts or whole vine with plastic enclosures under realistic vineyard conditions. However, manipulation of specific plant parts only changes temperature in part and the enclosure around canopy changes other climatic factors as well as boundary layer of the canopy (Bowen et al., 2004; Petrie and Clingeleffer, 2005; Soar et al., 2009). To overcome these limitations, Sadras and Soar (2009) created a new and inexpensive open system leading to mild temperature increases under realistic vineyard conditions. This passive open-top heating system gently increased maximal bunch temperature by 2.3-3.2°C, that is commensurate with the projected global warming. Meanwhile it maintained daily temperature cycle without or little effect on other climate factors (Sadras and Soar, 2009).

In the present work, a passive open-top heating system was set up under realistic vineyard

conditions for the two representative red and white varieties in Bordeaux region, i.e. Cabernet Sauvignon (CS) and Sauvignon Blanc (SB), from fruit setting to two weeks after harvest. Berry primary and secondary metabolites were analysed and compared with transcriptome analysis via RNA sequencing. The objectives were 1) to verify the effects of temperature elevation at ~1.5-2°C in vineyard on berry metabolism in red and white cultivars; 2) to understand better the transcriptional regulations underlying the berry responses to elevated temperature. Our results show that grape berry metabolism and transcriptome changes induced by the elevated temperature, depended on the developmental stages and grape varieties. These results may provide a basis developing adaption strategies to mitigate the effects of global warming for wine industry.

III.2 Materials and methods

III.2.1 Location, vine material and experimental set up

The experimental study was conducted in 2015 in Bordeaux vineyard (44° 46' 46" N, 00°34' 01" W) with the varieties *V. vinifera* cv. Sauvignon Blanc and Cabernet Sauvignon. They were planted in an experimental vineyard "VitAdapt" growing in level ground with no slope or geospatial variations. The field-grown grapevines were 8 years old, spur pruned, with a density of 1.6 m between rows and 1m between plants. Vineyard management followed the local standards.

A passive open-top heating system was used to elevate temperature from fruit setting to post-maturity (14 days after harvest). Full details of the heating system, its performance and limitations are described in chapter II.

III.2.2 Metabolites quantification

One hundred fresh berries were randomly sampled from each replicate at four different berry developmental stages: bunch closure (BC), mid-veraison (MV), mid-ripening (MR) and ripening (R). All samples were ground into powder in liquid nitrogen using a ball grinder MM200 (Retsch, Haan, Germany), and stored at -80°C for later analysis.

Sugar and organic acid contents

An aliquot of 500 mg fine powder of whole berries was extracted sequentially with ethanol

(80% and 50%), dried in a Speed-Vac, and re-dissolved in 2.5 mL de-ionized water. Glucose and fructose content were measured enzymatically with an automated micro-plate reader (Elx800UV, Biotek Instruments Inc., Winooski, VT, USA) according to the method of Gomez et al. (2007). Malic acid was determined with an automated colorimetric method using the TRAACS 800 autoanalyzer (Bran-Luebbe) by using an enzymatic kit (R-Biopharm, Darmstadt, Germany).

Extraction and quantification of Cys-3SH, Glut-3SH and Glut-3SH-AI

An aliquot of 500 mg fine powder of whole berries was extracted in 1.5 mL methanol containing 0.1% HCl (v/v) and added 50 μ L 0.1 μ g/L the internal standard solution containing a deuterated form of the glutathionylated S-conjugate ((3-*S*-hexan-1-ol)-glutathione- d_3). Extracts were centrifuged and subsequently evaporated using a RapidVap Vertex Dry Evaporator (Labconco, Kansas City, MO, USA). The residues were filtered with a 0.45- μ m membrane before being analyzed by C18-RP-UHPLC-HRMS (Thermo Scientific, Illkirch, France).

The separation was performed on a Synchronis aQ column (100 \times 2.1 mm i.d., 1.7 μ m, Synchronis aQ, Thermo Scientific, Bremen, Germany) with a flow rate of 300 μ L min⁻¹ of solvent A (0.1% aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile). The gradient for solvent B was as follows: 0 min, 9%; 0.8 min, 9%; 5 min, 40%; 5.2 min, 90%. The column was equilibrated with 9% of solvent B for 1 min prior to an injection. The ion source was operated in the positive ion mode at 3.5 kV. The vaporizer temperature of the source was set at 300°C, the capillary temperature at 350°C, the nitrogen sheath gas at 80, and the auxiliary and sweep gas at 5 (arbitrary units). A mass range of 100-500 was acquired in full scan MS mode. The resolution setting was 25000 (m/ Δ m, fwhm at m/z 400). To quantify the metabolites in samples, standards were prepared at the same time as the berries samples, by adding 50 μ g L⁻¹ of the internal standard to solutions of the synthesized metabolites (mix of 1 mL water and 1 mL grape juice).

Anthocyanin quantification

An aliquot of 500 mg of berry skin powder was freeze-dried for 72 h and the dried powder (50 mg) was extracted in 1.0 mL methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.45 μ m polypropylene syringe filter (Pall Gelman Corp., Ann Harbor, MI, USA)

for HPLC analysis. Each individual anthocyanin was analyzed with HPLC as described in Soubeyrand et al. (2014). Quantification was carried out by peak area integration at 520 nm. The concentration of individual anthocyanins was calculated in milligrams per gram (mg. g^{-1}) of dry skin weight (DW) using malvidin 3-O-glucoside (Extrasynthese, Genay, France) as external standard.

Tannins quantification

An aliquot (1g) the obtained powder was extracted using 40mL methanol containing 0.1% HCl. This solution was stirred for 3 h at 20°C. The extract was filtered through 20 μm PTFE filters. Monomeric-dimeric tannins was analysed with HPLC as described in Cholet et al (2014). 600 μL of phenolic extract, desiccated under nitrogen flow, plus 200 μL of reagent (0.2 N methanol-HCl + ascorbic acid + phloroglucinol) were mixed and incubated at 50°C for 20 min. The reaction was stopped with 200 μL of sodium acetate. This extract was filtered and placed in a 2 mL HPLC sealed vial. Ten microliters was injected into the HPLC for analysis (column, 250 \times 4.6 μm , 5 μm , ODS (Beckman, RoissyCharles de Gaulle, France): precolumn, 10 \times 4.6 mm, 5 μm , BDS C18(Thermo Hypersil); flow rate, 1 mL/min; solvent A, water/acetic acid (19:1 v/v); solvent B, MeOH/acetic acid (19:1 v/v); gradient, 5% B from 0 to 30 min, 20% B from 30 to 55 min, 40% B from 55 to 60 min, 90% B from 60 to 75 min, 5% B from 75 to 80 min; injection volume, 10 μL ; detection wavelength, 280 nm.

III.2.3 RNA extraction and gene expression analysis

Total RNA from 1 g powder of whole berries was isolated according to the previous described method (Reid et al., 2006). RNA isolation was followed by DNase I treatment (Turbo DNA-free™ kit, Ambion, Austin, TX, USA). Afterward, total RNA was quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, DE), and the RNA quality was evaluated on 1% agarose gels. First-strand cDNA was synthesized from purified RNA using Superscript III enzyme (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA obtained was diluted (target concentration: 0.01 $\mu\text{g}/\mu\text{l}$) in distilled water for later analysis. Quantitative real-time PCR expression analysis was carried out using the CFX96 Real-Time PCR detection system (Bio-Rad). PCR reaction mix and specific oligonucleotide primer pairs were those from Helwi et al (2016). PCR conditions were 3 min at 95 °C, 40 cycles of 10 s at 95 °C, and 15 s at 60 °C. The amplification efficiencies were determined by serial dilution. Melting curves to control the annealing specificity of

oligonucleotides were recorded after each run. All experiments were performed with three biological replicates and two technical replicates. Normalized expression of each gene was calculated using Bio-Rad CFX Manager software. Gene transcripts were quantified upon normalization to *VviEF1* and *VviUBIQUITIN* as internal standards by comparing the cycle threshold of the target gene with those of standard genes.

III.2.4 RNA-Seq library construction and sequencing

RNA_Seq analysis was conducted through platform GenomEast (IGBMC, Strasbourg). RNA-Seq libraries were generated from 600 ng of total RNA using TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA), according to manufacturer's instructions. Briefly, following purification with poly-T oligo attached magnetic beads, the mRNA was fragmented using divalent cations at 94°C for 2 minutes. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. Strand specificity was achieved by replacing dTTP with dUTP during second strand cDNA synthesis using DNA Polymerase I and RNase H. Following addition of a single 'A' base and subsequent ligation of the adapter on double stranded cDNA fragments, the products were purified and enriched with PCR (30 sec at 98°C; [10 sec at 98°C, 30 sec at 60°C, 30 sec at 72°C] x 12 cycles; 5 min at 72°C) to create the cDNA library. Surplus PCR primers were further removed by purification using AMPure XP beads (Beckman-Coulter, Villepinte, France) and the final cDNA libraries were checked for quality and quantified using capillary electrophoresis. These libraries were then sequenced on the Illumina Hiseq 4000 as paired-end 50 base reads following Illumina's instructions.

III.2.5 Analysis of Illumina reads

Reads were mapped onto the 12X assembly1 of the *Vitis vinifera* genome using Tophat v2.0.14 (Kim et al., 2013) and the bowtie2 v2.1.0 aligner (Langmead and Salzberg, 2012). Only uniquely aligned reads have been retained for further analyses. Quantification of gene expression was performed using HTSeq v0.6.13 (Anders et al., 2015) and CRIBI V1 annotations. The gene expression level was calculated by using RPKM (reads per exon kilo base per million mapped sequence reads).

Read counts have been normalized across libraries with the method proposed by Anders and Huber (2010). Comparison of interest were performed using the method proposed by Love et

al., (2014) and implemented in the DESeq2 Bioconductor library (DESeq2v1.0.19). Resulting p values were adjusted for multiple testing using the Benjamini and Hochberg (1995) method. The log₂ Fold-Change was estimated using the method proposed by Love et al., (2014). Differentially expressed genes with an adjust p-value ≤ 0.05 and an absolute value of $\log_2(\text{ratio}) \geq 1$ were used as the thresholds to judge the significance of gene expression difference. RNA-seq data were averaged of three biological replicates.

III.3 Results and discussion

III.3.1 Heat treatment and berry characterization

Heat treatment was applied during berry development under realistic vineyard condition. The average elevations of maximal, average and minimal temperature was 2.76°C, 1.18°C and 0.97°C respectively (figure 1). The treatment slightly decreased relative humidity in bunch zone without changing plant and soil water status (chapterII). This system allowed to increase bunch zone mean air temperature 0.5-1.6 °C that was commensurate with the projected warming for the following 80 years in France (Ouzeau et al., 2014).

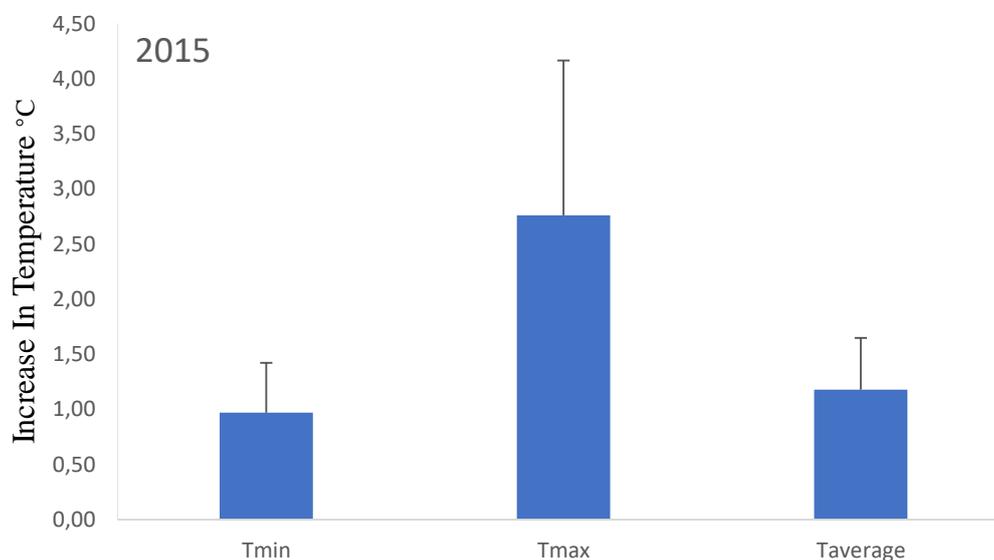


Figure 1: Increment of minimal, maximal and average temperature in bunch zone area during berry development

Berries of CS and SB from two different stages: bunch closure (BC) and mid-ripening (MR) were sampled for RNA sequencing analysis. The average berries weight was not affected by

the treatment at those two stages. At BC stage, there was no difference in the levels of primary metabolites between treatment and control, except that elevated temperature only significantly increased fructose and decreased malic acid content of CS berries at MR stage (Table 1).

Table 1: Effect of elevated temperature on berry weight, sugars and malic acid content measured at BC and MR stages.

	Bunch closure				Mid-ripening			
	Berry weight g	Glucose mg/g (FW)	Fructose mg/g (FW)	Malic acid	Berry weight g	Glucose mg/g (FW)	Fructose mg/g (FW)	Malic acid
Cabernet Sauvignon								
Control	0.6 ^a	3.17 ^a	0.7 ^a	17.12 ^a	1.17 ^a	71.16 ^a	67.19 ^a	4.28 ^a
Heated	0.52 ^a	2.63 ^a	0.78 ^a	15.89 ^a	1.06 ^a	74.39 ^a	70.7 ^b	3.42 ^b
Sauvignon Blanc								
Control	0.66 ^a	4.83 ^a	0.51 ^a	16.99 ^a	1.28 ^a	79.89 ^a	79.11 ^a	3.52 ^a
Heated	0.71 ^a	5.22 ^a	0.49 ^a	17.57 ^a	1.29 ^a	77.51 ^a	77.29 ^a	3.02 ^a

Values are mean of five replicates. Different letters within the same parameter indicate statistically significant differences between treatment and control as determined by Student's t test (p value < 0.05). FW, fresh weight

III.3.2 Global response of berry transcriptome to elevated temperature

Transcriptomic patterns in *Vitis vinifera* cv. CS and CS grape berries in response to elevated temperature were quantitatively assessed using the Illumina Hiseq 2500.

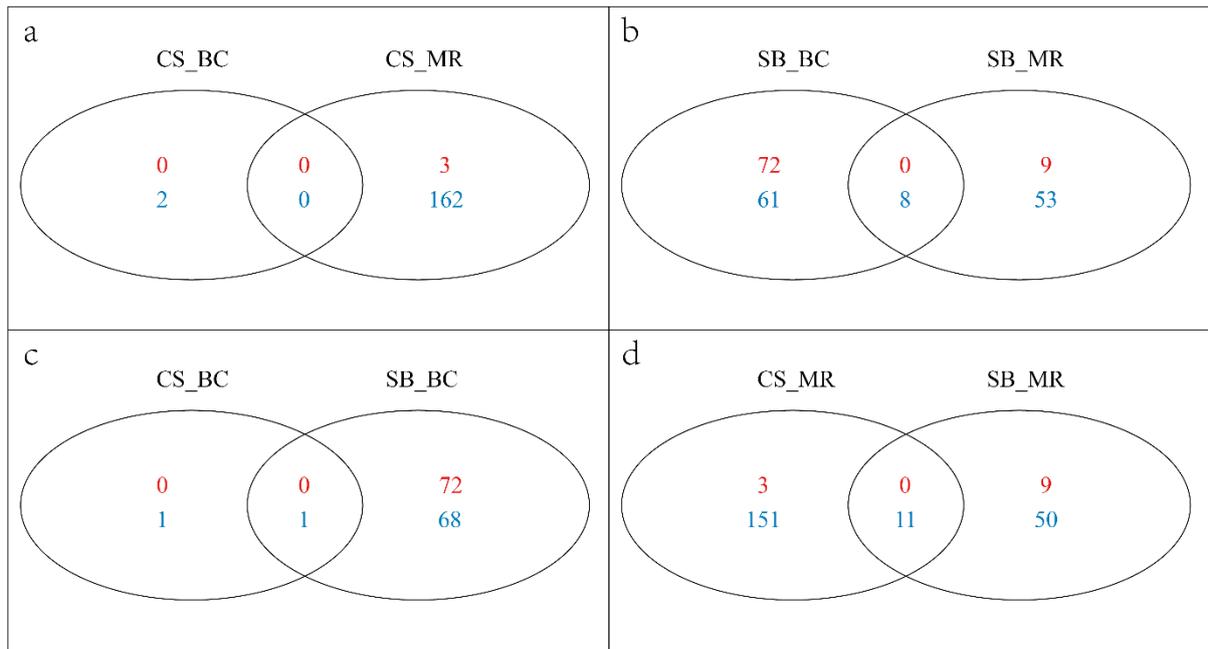


Figure 2: Venn diagrams representing the number of differentially expressed genes between the control and the treatment for (a) Cabernet Sauvignon (CS) and (b) Sauvignon Blanc (SB) at bunch closure (BC) and mid-ripeness (MR); and between varieties at same stages (b and c).

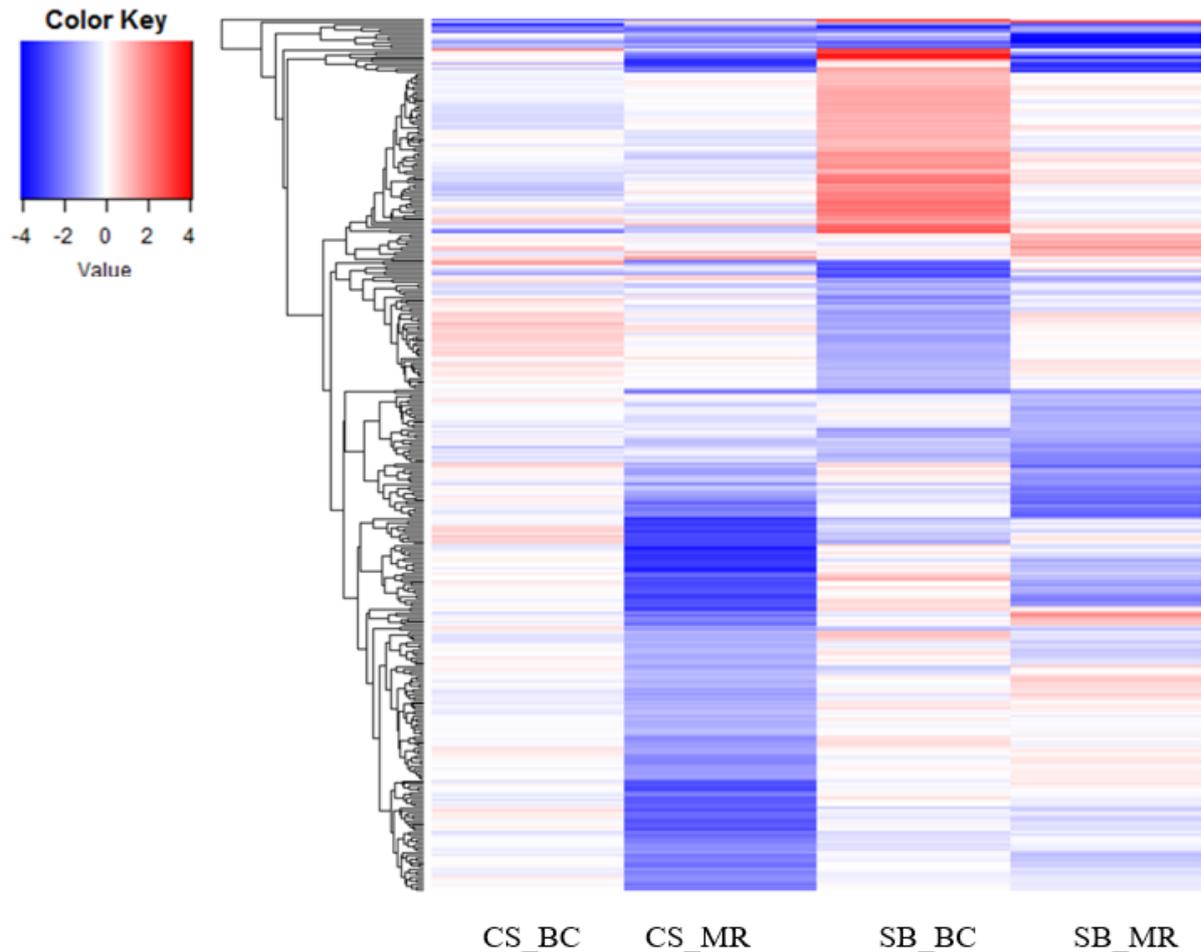


Figure 3: Heatmaps of temperature influence on DEGs at bunch closure (BC) and mid-ripening (MR) stage in Cabernet Sauvignon (CS) and in Sauvignon Blanc (SB).

The $\log_2(\text{Heated}/\text{Ccontrol})$ in Cabernet Sauvignon (two columns in left) and in Sauvignon Blanc (two columns in right) ratios were represented as false colour with red for higher expression level in heated berries and blue for lower as illustrated in the colour keys.

A total of 357 genes were differentially expressed in at least one of the four comparisons. The overlaps in differentially expressed genes (DEGs) under two conditions were shown with two-way Venn diagrams based on varieties and stages (figure 2). To get overviews of correlations among DEGs, heatmap was produced (figure 3).

For CS, the elevated temperature had less effect at BC stage than MR stage. At MR stage, most DEGs were down-regulated by elevated temperature. Only two genes were differentially expressed between control and treatment at BC stage, and both were down-regulated. A total number of 165 DEGs was found at MR stage and only 3 of them were up-regulated. Temperature have induced more changes at MR stage and show an inhibited effect.

For SB, different responses to elevated temperature between the two stages are illustrated in figure 3. The most remarkably down- and up-regulated genes found at BC stage were slightly induced or unaffected at MR stage. The down regulated genes at MR stage in SB berries were also repressed in CS berries at the same stage. Temperature effect was observed in SB berries with 141 DEGs at BC stage which was much more than the DEGs in CS berries at the same stage, and with 70 DEGs at MR stage which was less than half of that in CS berries. Among all DEGs, 69 and 61 genes were down-regulated at BC and MR stage, respectively. Meanwhile, there were 8 common down-regulated genes at two stages and one gene was up-regulated at first beginning then down-regulated at next stage. Comparing with CS berries, SB berries seemed to be influenced immediately by the treatment. Between two varieties, 11 down-regulated DEGs were shared between CS and SB at MR stage and three of them were also down-regulated at BC stage in SB berries. No up-regulated transcript was found neither between treatment within same varieties nor between varieties at a given stage (Figure 2).

Table 2: Common genes differentially expressed between varieties and stages.

Sequence ID Genoscope 12 X V1	Fold change								Best identity description
	CS stage	BC	CS stage	MR	SB stage	BC	SB stage	MR	
VIT_07s0031g01890	-2.5				-2				RING/U-box superfamily protein (<i>Arabidopsis thaliana</i>)
VIT_03s0038g04730			-1.83		-3.92		-1.81		polyphenol oxidase (<i>Vitis vinifera</i>)
VIT_10s0116g00410			-1.95		-2.52		-3.26		GA2OX8 (<i>Vitis vinifera</i>)
VIT_19s0177g00030			-2.17		-2.73		-4.12		GA2OX8 (<i>Vitis vinifera</i>)
VIT_04s0008g02070			-2.16				-2.53		protein kinase chloroplast (<i>Vitis vinifera</i>)
VIT_04s0008g04180			-1.54				-1.66		transporter arsb-like (<i>Vitis vinifera</i>)
VIT_05s0049g01650			-1.4				-1.98		cyclopropane-fatty-acyl-phospholipid synthase (<i>Vitis vinifera</i>)
VIT_05s0051g00680			-2.72				-1.26		SVR9 (<i>Arabidopsis thaliana</i>)
VIT_05s0094g00360			-2.25				-2.25		EP3 (<i>Vitis vinifera</i>)
VIT_07s0005g01210			-2.04				-2.6		MYB12 (<i>Vitis vinifera</i>)
VIT_07s0129g01010			-3.39				-3.32		CCT motif family protein (<i>Arabidopsis thaliana</i>)
VIT_08s0007g01420			-1.92				-1.81		GSTU8 (<i>Vitis vinifera</i>)
VIT_00s0317g00140					-1.36		-1.93		exocyst complex component 7 (<i>Arabidopsis thaliana</i>)
VIT_03s0038g03950					-1.11		-1.94		PHT3 (<i>Vitis vinifera</i>)
VIT_08s0007g03370					-1.33		-1.27		uncharacterized protein
VIT_12s0028g00320					-1.33		-1.25		LHB1B1 (<i>Vitis vinifera</i>)
VIT_16s0050g00020					-2.22		-2.86		transducin wd40 domain-containing protein (<i>Vitis vinifera</i>)
VIT_16s0100g00780					3.63		-3.67		TT4 (<i>Vitis vinifera</i>)

Adjust p value < 0.05 and $|\log_2(H/C)| \geq 1$. C, control. H, heated.

GA2OX8: gibberellin 2-beta-dioxygenase 8-like; SVR9: Suppressor of variegation 9; EP3: class iv chitinase; GSTU8: glutathione s-transferase protein; PHT3: mitochondrial phosphate carrier protein; LHB1B1: chlorophyll a b binding; TT4: stilbene synthase.

Enrichments of GO (Gene Ontology) categories

GO enrichment analysis was made to gain insight into the functional categories impacted by elevated temperature. Eighteen GO functional terms were significantly altered ($FDR \leq 0.05$) by heating treatment (table 3). Genes encoding microtubule (9 terms) and cell wall (6 terms) were the most enriched. One term (GO: 0030312) about extracellular region and two terms (GO: 0001071 and GO: 0003700) involved in transcription factor activity were also enriched. No terms related to biological process was significantly changed. But to a less extent, four GO terms about metabolic process and one term related to biotic stimulus (GO: 0009607) response were enriched with a p -value < 0.05 but $FDR > 0.05$. GO:009699, GO:009698, GO:0044550 and GO:0019748 were related to secondary metabolic process and two of them (GO:009699 and GO:009698) were specifically involved in phenylpropanoid pathway.

Table 3: GO functional analysis of elevated temperature related differentially expressed genes in grape berries

GO term	Description	Queryitem	Bgitem	Pvalue	FDR
GO:0048046	apoplast	14	186	4.50E-07	7.90E-05
GO:0005618	cell wall	14	249	1.10E-05	0.00074
GO:0030312	external encapsulating structure	14	252	1.30E-05	0.00074
GO:0005576	extracellular region	17	424	8.30E-05	0.0036
GO:0016762	xyloglucan: xyloglucosyl transferase activity	6	37	2.00E-05	0.0072
GO:0016798	hydrolase activity, acting on glycosyl bonds	19	486	4.40E-05	0.0079
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	18	466	8.10E-05	0.0097
GO:0005874	microtubule	8	127	0.00043	0.015
GO:0005875	microtubule associated complex	7	103	0.00064	0.017
GO:0099513	polymeric cytoskeletal fiber	8	140	0.00078	0.017
GO:0099512	supramolecular fiber	8	140	0.00078	0.017
GO:0005871	kinesin complex	6	79	0.0009	0.018
GO:0015630	microtubule cytoskeleton	9	185	0.0011	0.02
GO:0001071	nucleic acid binding transcription factor activity	24	821	0.00036	0.026
GO:0003700	transcription factor activity, sequence-specific DNA binding	24	821	0.00036	0.026
GO:0008017	microtubule binding	7	102	0.0006	0.036
GO:0003777	microtubule motor activity	6	79	0.0009	0.041
GO:0015631	tubulin binding	7	107	0.00079	0.041

FDR, False Discovery Rate.

III.3.3 Identification of significantly altered transcripts

III.3.3.1 Cell wall metabolism

Softening is an important process of the ripening in most fleshy fruits and it results from the hydrolytic breakdown of cell wall polymers (Cosgrove, 2000). In previous studies, cell wall metabolism and some genes involved in cell wall structural modifications were influenced by growth conditions including temperature (Nunan et al., 1998; Nunan et al., 2001). In the present study, thirty DEGs were related to the functional category “Cell wall” and mainly belonged to genes encoding cell wall degrading enzymes.

Two major kinds of cell wall degrading enzymes play an important role softening. They are pectin degrading enzymes (e.g., pectinesterase and polygalacturonase) and polysaccharides degrading enzymes (e.g., cellulase, xyloglucan endotransglucosylase/hydrolase and α -galactosidase) (Jenks and Bebeli, 2011). The mechanism underlying the actions of pectic

enzymes most likely involves the action of polygalacturonase (PGs), which acts on pectic substrates already suitably de-esterified by pectinesterase (PEs) (Deng et al., 2005). The expression level of two genes coding PEs (*VIT_15s0048g00480* and *VIT_02s0154g00600*) was promoted in SB green berries by elevated temperature. A decreased level of *VIT_05s0062g01160* (PEs) expression was observed in warmed mid-ripening CS berries. Meanwhile, *VIT_10s0071g01120*, a galactosidase, was down-regulated. Xyloglucans, a kind of polysaccharides, account for around 10% of cell wall material from grape berry mesocarp and reach 50% of the exocarp cell wall material (Doco et al., 2003). Xyloglucan endotransglycosylase/hydrolase (XTH), a xyloglucan modifying enzyme, has been proposed to have a role during fruit ripening by loosening the cell wall (Nunan et al., 1998; Munoz-Bertomeu et al., 2013). Six XTHs were altered by heat treatment in current study. *VIT_11s0052g01260* and *VIT_11s0052g01300* were up-regulated in warmed green SB berries and not during later stage. Consistently, an increased level of XET (Xyloglucan endotransglycosylase) transcripts was also measured in warmed green grape berries (Rienth et al., 2016; Lecourieux et al., 2017). Conversely, other two XTHs genes (*VIT_11s0052g01250* and *VIT_00s0386g00050*) were down-regulated at MR stage in CS berries. Besides that, down-regulated genes encoding cellulase (*VIT_07s0005g00740* and *VIT_00s2526g00010*) and glucanase (*VIT_05s0077g01150* and *VIT_18s0122g00980*) were found mostly in heated mid-ripening berries. In addition to its effects on the primary cell wall, elevated temperature also showed a negative effect on two genes involved in lignin degradation (*VIT_08s0007g01910* and *VIT_13s0067g01970*) in CS berries at MR stage. Overall, this suggested that the synthesis of cell wall components was affected by temperature and differed between genotypes. The changes of genes involved in cell wall metabolism support the idea that thermotolerance acquisition partially requires cell wall-remodelling enzymes, which could be involved in the biosynthesis or in subtle modification of major cell wall polymers (Yang et al., 2006; Le Gall et al., 2015). Moreover, the composition and structure of grape berry cell wall polysaccharides are of interest because of their effects on wine production. The cell wall of grape berries forms a barrier to the diffusion of components including aromas and phenols and acts as a protection against external factors (Doco et al., 2003; Vicens et al., 2009). Cell wall-remodelling by elevated temperature may alter extractabilities of the phenolic compounds and then affect juice and wine quality.

III.3.3.2 Microtubule

Microtubules (MTs) play important roles in regulation of cell division, cell expansion, and cell differentiation (Lloyd and Chan, 2004). 9 GO terms about microtubule including 11 DEGs were overrepresented. All the 11 DEGs were down regulated by elevated temperature, 9 of them in SB berries at bunch closure and only 2 in CS berries at mid-ripening stage. Cell division and expansion mainly occur during the green growth phase because of the increase in volume and weight (Ojeda et al., 1999). This could explain the enrichment of this category mainly in the BC stage.

III.3.3.3 Transcriptional factors

Presently, at least 64 families of transcriptional factors have been identified in the plant kingdom (Liu et al., 2014). In the present study, 24 DEGs were related to transcriptional factors and mainly belonged to “ethylene-responsive transcriptional factors” and “Basic leucine zipper/ HomeoDomain leucine zipper transcriptional factors”.

Ethylene-responsive (ERF) subfamily transcriptional factors belong to APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily which are not only involved in various biological functions and environmental stimuli but also participate in signal transduction pathways of jasmonic acid, ethylene and abscisic acid (Ohme-Takagi and Shinshi, 1995). Eight transcripts encoding ethylene-responsive transcriptional factors (*VIT_10s0003g00140*, *VIT_12s0028g03270*, *VIT_16s0013g01070*, *VIT_16s0013g01050*, *VIT_03s0063g00460*, *VIT_02s0025g04460*, *VIT_02s0234g00130* and *VIT_05s0049g00510*) were more abundant only in warmed SB berries at BC stage. Although ERF subfamily transcriptional factors are generally considered to be mediators of ethylene-related responses, they include members that respond to abiotic stresses, such as drought and high salinity, and can confer tolerance to these stresses by overexpression in transgenic plants (Fujimoto et al., 2000; Park et al., 2001). Genome-wide expression analyses of AP2/ERF family genes in poplar (*Populus trichocarpa*) (Zhuang et al., 2008), soybean (Zhang et al., 2008), tomato (Sharma et al., 2010) and rice (Sharoni et al., 2010) reveal that many ERF subfamily genes are also induced by low or high temperature.

VIT_16s0013g01070 and *VIT_16s0013g01050* were named ERF105 by Romero et al. (2015) and Nwafor et al. (2014) respectively. They are more orthologous with AtERF6 genes in

Arabidopsis and classed in *VviERF6* TF paralogs by Cramer et al. (2014). *VviERF6* expression pattern, especially *VviERF6L1*, showed a high correlation with terpenoid metabolism, pigment biosynthesis and fatty acid and alcohol metabolism during late stages of ripening of CS berries (Cramer et al., 2014). This suggested that ethylene may play a more important role in the late stages of ripening when flavours are produced than previously thought (Cramer et al., 2014). In the present study, these genes were only altered at the early stage and this alteration may have an indirect or less important influence on the synthesis of flavour compositions. *VIT_12s0028g03270*, named as ERF9, was involved in repressing the activation of pathogen related genes in Arabidopsis (Camehl and Oelmüller, 2010). Meanwhile, it was reported that the expression level was higher in the skin than in the flesh of a red cultivars from the cross ‘Syrah’× ‘Pinot Noir’ and was co-expressed with *VviMYBA1* (Costantini et al., 2015). *VIT_05s0049g00510*, named ERF1, was highly expressed after veraison in “Fujiminori” grape berry (Shangguan et al., 2017). The function of ERF1 is unknown, but it is considered to regulate ethylene during fruit development. The Up-regulations of *VIT_05s0049g00510* may accelerate the ripening process of SB berries. The transcripts of *VIT_02s0234g00130*, named as ERF2 which is under group IX (B-3) of ERF TFs subfamily, were reported to increase in CS leaves after 1h of dehydration (Hopper et al., 2016). *VIT_02s0025g04460* was the gene with the greatest increase in transcript abundance in the shoot tips after abscisic acid treatment (Rattanakon et al., 2016). The up-regulated genes coding ERF TFs, known regulators of abiotic stress in plant, play an important role in the response to temperature variation in SB grape berry at early stage.

The basic leucine zipper (bZIP) TFs family is one of the largest and most diverse families (Nijhawan et al., 2008; Liu et al., 2014). They play important roles in in all eukaryotes, not only in plant but also in animals (Gao et al., 2014). HomeoDomain leucine zipper (HD-Zip) TFs family also has a large number of TFs, but they seem be unique to the plant kingdom (Ariel et al., 2007). Both bZIP and HD-Zip TFs contain the Leu zipper (LZ) region. The bZIP TFs contains the basic region but HD-Zip TFs has Homeobox domain (HD). These two regions are highly conserved (Henriksson et al., 2005; Corrêa et al., 2008). Six transcripts encoding bZip/HD-Zip FTs and one CCAAT/enhancer-binding proteins (C/EBPs) TFs which contents a bZIP region were found differentially expressed in at least one of the four comparisons.

Six transcripts (*VIT_08s0007g06670*, *VIT_15s0021g01880*, *VIT_19s0015g01020*, *VIT_01s0026g01950*, *VIT_06s0080g00340* and *VIT_11s0016g01480*) were down-regulated in CS berries at mid-ripening stage. Only one transcript, *VIT_10s0003g00380*, was up-regulated in SB berries at BC stage. *VIT_19s0015g01020* and *VIT_06s0080g00340* are two Bzip TFs. They were named *VvibZIP47* and *VvibZIP19* by Liu et al. (2014), respectively. They were mainly expressed in seed at the end of fruit set (Liu et al., 2014). Note that these two genes were homologous with *AtbZIP67/DPBF2*, *AtbZIP39/ABI5* in Arabidopsis, which showed an important role in ABA-mediated seed development, germination, and embryo maturation (Bensmihen et al., 2005; Yamamoto et al., 2009). While studying with many plants, reduces seed weight and size were observed after heated treatment (Sinsawat et al., 2004; Yamamoto et al., 2008; Mohammed and Tarpley, 2010). *VIT_01s0026g01950*, *VIT_08s0007g06670*, *VIT_10s0003g00380* and *VIT_15s0021g01880* belong to HD-Zip TFs and were named *VviHDZ05*, *VviHDZ14*, *VviHDZ17* and *VviHDZ25* by Li et al (2017), respectively. HD-Zip genes are involved in embryo development (Prigge et al., 2005; Li et al., 2017). *VviHDZ05* and *VviHDZ17* were mainly expressed in Thompson Seedless while *VviHDZ25* had no differences in Pinot Noir and Thompson Seedless. But *VviHDZ11*, a homologous gene of *VviHDZ25*, had lower expression in Thompson Seedless (Li et al., 2017). It was postulated that *VviHDZ05*, *VviHDZ17* and *VviHDZ11* may be associated with embryo abortion and surviving seeds. In this study, these 5 down-regulated genes may alter seeds development and maturation.

Two transcripts (*VIT_07s0005g01210* and *VIT_05s0077g01360*) that belong to MYeloBlastosis (MYB) TFs family were down-regulated in berries at the MR stage. *VIT_07s0005g01210* (*VviMYBF1*), which was down-regulated by elevated temperature in CS and SB berries, is a regulator of the first step of flavonol biosynthesis (Czemmel et al., 2009). Meanwhile, *VIT_17s0000g06930* and *VIT_15s0021g02690*, members of The Basic Helix-Loop-Helix (bHLH) TFs, were also down-regulated in SB berries at BC and MR stage respectively. Previous results indicated that bHLH TFs participate in the control of flavonoids biosynthesis in many plants (Ludwig and Wessler, 1990; Quattrocchio et al., 2006; Bai et al., 2011). In grapes, MYB TFs regulate flavonoid pathway through an integration with basic bHLH proteins (Hichri et al., 2010). In addition, a bHLH TFs *VviCEBI* was shown be involved in cell expansion and auxin responses.

A WRKY transcriptional factor *VIT_08s0058g00690* was down regulated in SB berries at MR

stage. This gene is associated with antifungal defence and jasmonic acid signaling pathway (Domingos et al., 2016; Wong et al., 2016; Haile et al., 2017). Two zinc finger transcriptional factors (*VIT_07s0005g02580* and *VIT_04s0008g01290*) were down regulated only in SB berries. Heat treatment decreased the expression level of two B3 domain-containing transcriptional factor (*VIT_14s0068g01290* and *VIT_03s0063g00910*) in SB berries at BC stage and CS berries at MR stage, respectively.

III.3.3.4 Metabolism

Only a significant higher fructose and lower malic acid was found in treated CS berries at MR stage. No transcript related to sugar and organic acids synthesis was significantly altered. Opposite to the pattern of sugar concentration, three sugar transporters (*VIT_14s0030g00260*, *VIT_05s0077g02260* and *VIT_14s0030g00220*) were down-regulated in the same samples. This suggests that a mild elevated temperature may not alter sugar biosynthesis but changed sugar allocation between source and sink. Interestingly a sucrose synthase transcript (SuSy: *VIT_05s0077g01930*) was also down -regulated in warmed CS berries at MR stage. SuSy is thought to be cytoplasmic enzymes in plant cells and UDP-glucose for the synthesis of cell wall polysaccharides and starch (Kleczkowski et al., 2010; Rienth et al., 2014a). As fructose and UDP-glucose shared the same precursors: sucrose, the depressed pathway to UDP-glucose may explain in part the higher fructose concentration.

The ubiquitin-proteasome system (UPS) plays an integral and important role in plant response and adaptation to environmental stresses (Stone, 2014). In the present study, 9 DEGs potentially linked to ubiquitin machinery were putative ubiquitin ligases (E3). Only *VIT_16s0050g00020* was inhibited whatever the developmental stage in SB berries. It is also reported as a homologous gene to *AtRUP1* and *AtRUP2*, which in Arabidopsis are induced by UV-B radiation and increase flavonoid levels (Heijde and Ulm, 2013; Armijo et al., 2016). Only *VIT_01s0026g01030* which was increased in SB berries at BC stage. The rest were specifically deregulated at MR stage. Five of them (*VIT_18s0041g01090*, *VIT_11s0016g03590*, *VIT_01s0011g02360*, *VIT_09s0002g04720* and *VIT_11s0016g03580*) were in CS berries and only two transcripts (*VIT_01s0011g06140* and *VIT_06s0009g00370*) were in SB berries. *VIT_18s0041g01090* and other 110 genes potentially linked to UPS transcripts were conversely up-regulated in CS berries after a heat treatment (+8°C, 14 days) (Lecourieux et al., 2017). The UPS functions in grapevine are not clearly elucidated. The different responses to temperature may due to the magnitude and duration of warming.

The abundance of 22 transcripts encoding biosynthetic enzymes of phenylpropanoid and flavonoid pathways were changed by elevated temperature. 8 transcripts related to the flavonoid pathway were repressed in CS berries at MR stage by elevated temperature. Two flavonoid hydroxylases (*VIT_03s0063g01690* and *VIT_15s0048g01590*), which catalyze the addition of hydroxyl groups to B-ring of flavonoids (Bogs et al., 2006), were heat repressed. These two genes could contribute to variation in the composition of flavonoids. The repression of a flavonol synthase (*VIT_13s0106g00550*) may decrease the flavonol content in heated berries. Three anthocyanidin UDP-glycosyltransferases (UFGT: *VIT_08s0007g04570*, *VIT_03s0017g02000* and *VIT_08s0007g04570*), which catalyzes the transfer of glucose to the 3-position of anthocyanidins (Kovinich et al., 2010) and thereby increases their hydrophilicity and stability (Zheng et al., 2013), were also down-regulated. The transcript of two genes encoding O-methyltransferase (OMT) (*VIT_04s0008g02760* and *VIT_11s0016g02610*) were also repressed by elevated temperature. This result agrees with recent work reporting the repression of several genes related to the flavonoids pathway in grape berries subjected to elevated temperature (Carbonell-Bejerano, Santa María et al. 2013, Rienth, Torregrosa et al. 2016, Lecourieux, Kappel et al. 2017). Anthocyanins content can also be changed through catabolism depending on many other enzymes such as laccases, polyphenol oxidases, class III peroxidases, and β -glucosidases (Oren-Shamir, 2009). Four gene coding laccases (*VIT_13s0067g01970*, *VIT_18s0122g00420*, *VIT_13s0067g01970* and *VIT_08s0007g01910*) were deregulated by elevated temperature in CS mid-ripening berries. A polyphenol oxidase (*VIT_03s0038g04730*) was down-regulated in all warmed samples except CS berries at BC stage. That potentially impacted the polymerization rate of various phenolic compounds.

Compared with CS berries, SB berries responded to elevated temperature at early steps of phenylpropanoid pathway. Three phenylalanine ammonia lyases (PAL: *VIT_06s0004g02620*, *VIT_13s0019g04460* and *VIT_16s0039g01360*) and two Chalcone Synthases (CHS: *VIT_14s0068g00920* and *VIT_14s0068g00930*) were heat repressed in SB berries at MR stage. Two glycosyltransferases (GTs: *VIT_11s0052g01630* and *VIT_11s0052g01600*) were down regulated in SB berries. These genes contribute to the chemical diversity of flavonol (Kovinich et al., 2010).

Lignin and stilbene represent branching points in the phenylpropanoid pathway. A transcript (*VIT_09s0070g00240*) encoding cinnamoyl-CoA reductase (*VviCCR*) which is the first

committed enzyme of the lignin branch biosynthesis pathway (Lacombe et al., 1997) was down-regulated in CS berries at MR stage. Meanwhile two transcripts (*VIT_11s0016g02610* and *VIT_10s0042g00840*) linked to lignin and stilbene biosynthesis was repressed in CS and SB berries at MR stage, respectively. A gene (*VIT_03s0038g04730*) with an increased expression level at BC stage but decreased at MR-stage was found in SB berries. Interestingly, *VIT_16s0100g00780* which is also involved in lignin and stilbene synthesis was repressed in all heated samples except CS berries at BC stage. In plant, stilbenes and lignification respond to various biotic and abiotic stresses (Jeandet et al., 2010; Yun et al., 2013). Several genes related with stilbenes and lignin were down-regulated in grape berries (Rienth et al., 2014b; Lecourieux et al., 2017). The expression alterations of genes involved in stilbenes and lignins biosynthesis may change berry texture and tolerance to the environment.

Volatile thiols contributing to wine, are only present in grape and musts as their non-volatile S-Cysteinylylated and S-glutathionylated thiol precursors (Peña-Gallego et al., 2012). Two enzymes: γ -glutamyltranspeptidase (*VviGGT*) and glutathione-S-transferase (*VviGST*), were proposed to involve in the biosynthetic pathway from glutathione to precursors of thiols (Kobayashi et al., 2010). In present study, three GSTs transcripts were altered by heat treatment. *VIT_08s0007g01420* was repressed at MR stage both in CS and SB berries. *VIT_15s0024g01650* was only down-regulated in CS berries in the same stage. Conversely, *VIT_14s0060g02170* was induced in SB berries at BC stage. This indicates that precursors of thiols content probably decreased by treatment. This hypothesis is expounded in more detail in the part of content thiols non-volatile precursors and relative genes expression.

III.3.3.5 Hormone homeostasis and signaling

Abscisic acid (ABA) plays an important role in plant tolerance to extreme environmental conditions and regulate many important physiological and developmental processes (Salisbury and Marinos, 1985; Zeevaart and Creelman, 1988). Increase in ABA content induced by heat treatment in grapevine leaves resulted in improve thermotolerance (Abass and Rajashekar, 1993; Wang et al., 2005). *VIT_18s0041g01500* encoding ABA 8'-hydroxylase, a key step of ABA inactivation, was repressed in warmed CS berries at MR stage. The suppression of ABA inactivation enzyme can also increase or maintain ABA content and activity.

Auxin and cytokinin are the two most important hormones regulating cell division (Skoog and

Miller, 1957). Cytokinin can also influence many aspects of plant growth and development (Mok and Mok, 2001). In grape berries, it is considered to be related with fruit set and growth promotion (Zhang et al., 2003). Heat treatment may involve inhibition of cytokinin biosynthesis in roots of Creeping Bentgrass (Liu et al., 2002) and decreased cytokinin in roots of bean and maize seedlings (Hare et al., 1997). A cytokinin mediated signaling gene (*VIT_09s0002g03520*) and a histidine kinase (*VIT_19s0014g04420*), which belongs to the cytokinin receptor family, were down-regulated in CS berries at MR stage. In the present study, the biosynthesis of cytokinin was not influenced through alteration of transcript levels, but the activity of these hormones may be inhibited by down-regulation of these two genes involved in the signaling pathway. Beside cell division, auxin also plays a major role in controlling cell elongation and function at the intersection between environmental and developmental cues (Davies, 2010). In grape berries, auxin is related with fruit set and growth promotion (Zhang et al., 2003). A recent article indicated that auxin content always stayed at a stable level during berry development (Symons et al., 2006). A YUCCA gene (*VIT_07s0104g01260*), which is important for auxin biosynthesis (Böttcher et al., 2013), was down-regulated in SB berries at MR stage. Interestingly, a gene encoding auxin polar transport (*VIT_01s0127g00870*) was up-regulated in the same samples. This up-regulated auxin transport gene was perhaps to counterbalance the decrease in auxin biosynthesis.

Gibberellins (GAs) play an important role in induction of flowering, pollen development and fruit growth (Davies, 2010). They are widely used in the “seedless” table grape industry, because GAs can promote berry enlargement but inhibit seeds development (Acheampong et al., 2015). Little is known about the role of GAs in grape berry development. In present work, four GA-beta-dioxygenases were down-regulated by higher temperature. Lower expression level of *VIT_06s0004g06790* and *VIT_09s0002g05280* were only found in CS berries at MR stage, and *VIT_09s0002g05320* was observed just in SB berries at BC stage. *VIT_19s0177g00030* and *VIT_10s0116g00410* were commonly down-regulated gene in all warmed berries except CS berries at BC stage.

III.3.4 Anthocyanins and tannins content in CS berries

Flavonoids occur widely in plant and are a biologically major and chemically diverse group of plant secondary metabolites (Treutter, 2006). In grapes, anthocyanins and tannins are two well studied flavonoids because their importance for berry and wine quality as well as their properties for human health.

Anthocyanins are the major pigmented compounds in grapes which are in berry skin of most red cultivars. A total of 13 anthocyanins were identified in CS berry skin at harvest. All anthocyanins identified were monoglucosides, including malvidin, peonidin, petunidin, delphinidin and cyanidin derivatives, with -3-*O*-glucoside as the main derivative and 8 acylated derivatives. Only malvidin, peonidin and petunidin were detected coumaroyl derivatives. Total anthocyanins content in warmed berries skin (16.89mg/g DW) than in control berries (23.07mg/g DW) (table 4). This observation coincides with previous studies which showed a negative effect of high temperature on anthocyanins concentration (Mori et al., 2007b; Lecourieux et al., 2017; Pastore et al., 2017). According to the number of substituents on the B-ring of the anthocyanin, cyanidin and peonidin and their derivatives have two hydroxylated groups and are called 3'-substituted anthocyanins. Delphinidin, petunidin and malvidin and their derivatives have three hydroxylated groups and are called 3',5'-substituted anthocyanins. Elevated temperature not only decreased total anthocyanins content but also reduced the proportion of 3',5'-substituted anthocyanins (table 4). For both thermal conditions, malvidin-3-*O*-glucoside was the most abundant individual anthocyanins and showed a statistically lower content in treated berry skins. Meanwhile, delphinidin and cyanidin-3-*O*-glucoside and petunidin-3-(6'- β -coumaroyl) glucoside contents were also decreased by elevated temperature (figure 4). Under around 4°C higher temperature treatment, Sangiovese berry skin also showed a lower concentration of malvidin-3-*O*-glucoside (Pastore et al., 2017). Conversely, Lecourieux et al. (2017) and Mori et al. (2007) found that only malvidin derivatives were not significantly affected by temperature in CS berry skin. In these two studies, a 10°C higher temperature was applied to bunch zones for two weeks and whole plants from one week before veraison to harvest respectively. The different responses of individual anthocyanin may be caused by the different magnitude of increased temperature.

The significantly lower concentration of cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and malvidin-3-*O*-glucoside coincide with the repression of three anthocyanidin UDP-glycosyltransferase genes and two *O*-methyltransferase genes were also repressed expression at MR stage. This can explain in part that elevated temperature decreases anthocyanins content through down-regulation of related structural genes. Furthermore, the decrease in anthocyanins concentration at high temperature may be due to the down-regulation of genes encoding for transporters mediating the transport of anthocyanins into the vacuole (Azuma et al., 2012; Carbonell-Bejerano et al., 2013). Here, two MATE efflux protein-

(VIT_00s0225g00070 and VIT_10s0116g01880) were down-regulated by elevated temperature.

Table 4: Effect of elevated temperature on total anthocyanins and ratio of 3'-substituted anthocyanins to 3', 5'-substituted anthocyanins in Cabernet Sauvignon berry skin at harvest.

	Total anthocyanins	3'-substituted anthocyanins	3',5'-substituted anthocyanins	F3'H/F3',5'H
	mg/g DW			
Control	23.46±3.62 ^a	6.25±1.15 ^a	17.21±3.06 ^a	0.36±0.03 ^a
Heated	16.91±1.77 ^b	5.20±0.26 ^a	11.71±1.61 ^b	0.45±0.04 ^b

Different letters within the same parameter indicate statistically significant differences between treatment and control as determined by Student's t test (p value < 0.05). DW, dry weight

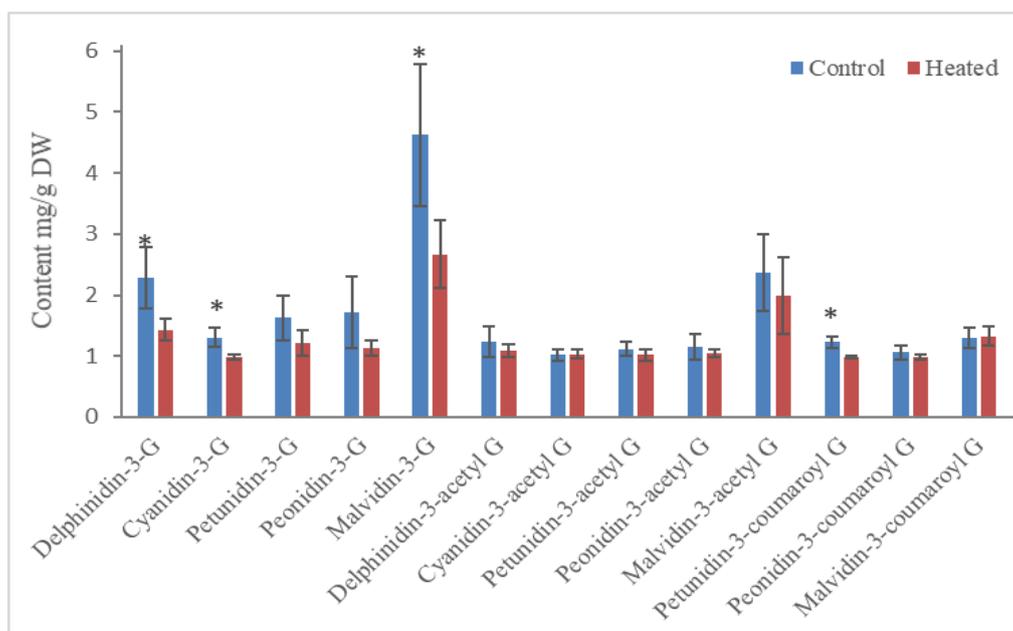


Figure 4: Effects of elevated temperature on the content of detected individual anthocyanins of Cabernet Sauvignon berry skin at harvest.

Error bars indicate STDEVA. * Significantly different from control (independent t-test, p<0.005)

Tannins are a group of flavan-3-ols including monomers, oligomers and polymers, and called also condensed tannins or proanthocyanidins (Chira et al., 2011). Because of their astringency and bitterness, they have a great importance for grape and red wine quality. Here, two procyanidins (C: (+)-Catechin and EC: (-)-epicatechin) and flavan-3-ol dimers (B1:(-)-

epicatechin-(4 β -8)-(+)-catechin; B2: (-)-epicatechin-(4 β -8)-(-)-epicatechin; B3: (+)-catechin-(4a-8)-(+)-catechin and B4: (+)-catechin-(4a-8)-(-)-epicatechin) were identified and quantified at harvest in both seeds and skin of CS berries. As seen in table 5, neither seeds nor skin tannins content were affected by heat treatment. The same result was also found in Sangiovese and Muscat Hamburg berry skin as well as in Merlot seeds (Cohen et al., 2008; Carbonell-Bejerano et al., 2013; Pastore et al., 2017). Seeds had a much higher tannins concentration than skin, which was agreement with previous studies (Cohen et al., 2008; Chira et al., 2011). Meanwhile, tannins concentration trend in whole CS berries were analysed (figure 5). B4 was only detected at BC and MV stage and showed a decreasing trend. The accumulation of other tannins peaked at MV stage and then decreased toward harvest, except B3 in control berries which decreased continuously until harvest. These observations are in line with previous studies suggesting that the maximal level of PAs appeared around veraison both in seeds and in skin (Kennedy et al., 2000; Downey et al., 2003). Although the final tannin concentration was not changed by elevated temperature, the concentrations at MV stage were significantly increased in heating berries. Therefore, elevated temperature resulted in a larger decrease (66%) in tannin concentration from MV to maturity than that under control condition (50%). Cohen et al. (2008) also observed that tannins level at veraison was positively related to elevated temperature, but was not altered at harvest.

In contrast to the reduction of final anthocyanins concentration, tannins contents were not influenced by elevated temperature. Consistently, there were no differentially expressed genes directly related to tannins biosynthesis. Moreover, the change of tannins concentration occurred around veraison, but no RNA-seq analysis was done for this stage. It should be an interesting point for further research. It generally considered that decreased tannins concentrations are caused by a decrease in tannin extractability rather than degradation or turnover (De Freitas and Glories, 1999; Kennedy et al., 2001; Downey et al., 2003). Tannin extraction is limited by tannin binding to cell walls. Renard et al. (2001) observed that tannin had greater affinity for pectic polysaccharides than xyloglucan and the least affinity for cellulose. Therefore, a cell wall with a higher proportion of pectic polysaccharides than neutral polysaccharides (xyloglucan and cellulose) may have a greater tannin-binding capacity and result in low tannin extractability. In warmed berries, a gene (*VIT_05s0062g01160*) encoding for pectinesterase was down-regulated at mi-maturation that may result in a higher proportion of pectic polysaccharides in cell wall and, in turn, lead to a lower tannin extractability. This lower tannin extractability at maturity stage in heated berry

may counteract the higher tannins observed at MV stage and result in a higher decrease from MV to maturity. An alternative possibility for the higher decrease in tannin monomer and dimer concentrations from MV to maturity might be that more tannin monomers and dimers have been transformed into high tannin polymers in heated berries. These hypotheses deserve further investigation by analysing tannin extractability, tannin polymers, tannin polymerization degree, in combine with analysing key genes involved in tannin biosynthesis.

Table 5: Effect of elevated temperature on monomers and oligomers tannins in Cabernet Sauvignon berry seeds and skin at harvest.

	B1	B3	C	B2	B4	EC
CS seeds	mg/g FW					
Control	0.14±0.04 ^a	0.56±0.11 ^a	2.10±0.40 ^a	0.28±0.02 ^a	0.14±0.05 ^a	1.43±0.14 ^a
Heated	0.11±0.01 ^a	0.56±0.03 ^a	2.00±0.26 ^a	0.28±0.03 ^a	0.13±0.02 ^a	1.35±0.08 ^a
CS skin						
Control	n.d.	0.03 ^a	0.09±0.01 ^a	0.02 ^a	0.02 ^a	0.04 ^a
Heated	n.d.	0.03 ^a	0.08±0.03 ^a	0.02 ^a	0.02 ^a	0.04 ^a

Different letters within the same parameter indicate statistically significant differences between treatment and control as determined by Student's t test (p value < 0.05). FW, fresh weight; n.d., not detected.

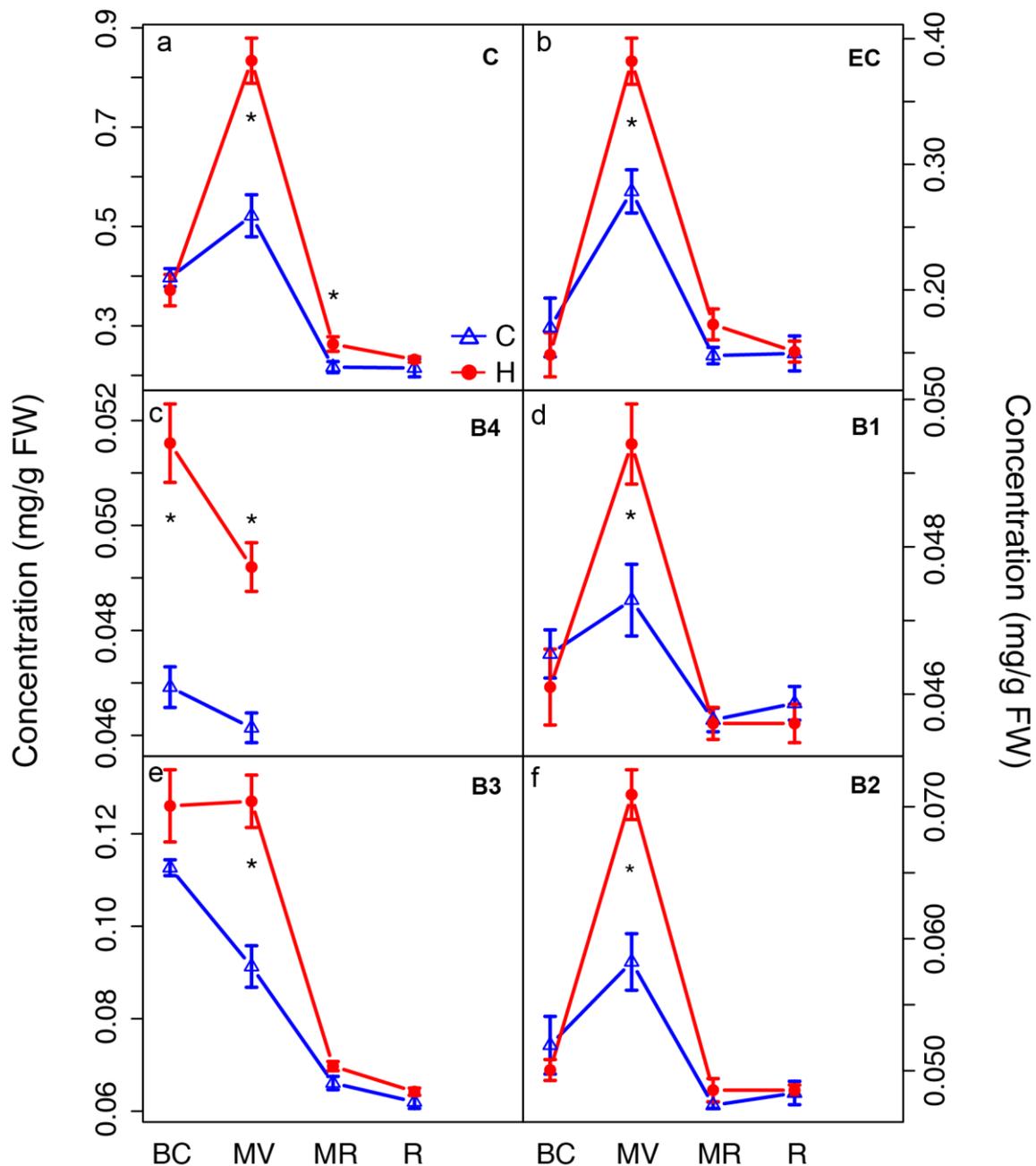


Figure 5: Concentration trend of monomers and oligomers tannins (mg/g FW) in control (blue) and heated (red) Cabernet Sauvignon berries.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.005$). BC, bunch closure stage; MV, mi-veraison stage; MR, mid-ripening stage; R, ripening stage; C, (+)-Catechin; EC, (-)-epicatechin; B1, (-)-epicatechin-(4 β -8)-(+)-catechin; B2, (-)-epicatechin-(4 β -8)-(-)-epicatechin; B3, (+)-catechin-(4a-8)-(+)-catechin; B4, (+)-catechin-(4a-8)-(-)-epicatechin

III.3.5 3SH precursors content and gene expressions in their pathway in berries

Volatile thiols, a group of sulfur containing alcohols, include a variety of aromas. Depending on their concentration and proportions, they contribute positively or negatively to wine flavor (Swiegers et al., 2007). Among them, 3-sulfanylhexan-1-ol (3SH) is a major aromatic compound in CS and SB wine which has a smell of grapefruit or passion fruit (Darriet et al., 2012). The 3SH does not exist in berries and musts, but is present as its odorless precursor form (Darriet et al., 1995). The precursors of 3SH in grape juice included S-3-(hexan-1-yl)-glutathione (Glut-3SH-Al), S-3-(hexan-1-ol)-glutathione (Glut-3SH) and S-3-(hexan-1-ol)-L-cysteine (Cys-3SH) (Tominaga et al., 1998b; Peyrot des Gachons et al., 2002; Thibon et al., 2016). Until now, the biosynthesis pathways of these precursors are still far away being well understood. Based on several studies, the hypothetic pathway is illustrated in Figure 6 (Peyrot des Gachons et al., 2002; Kobayashi et al., 2010; Thibon et al., 2011; Kobayashi et al., 2012; Thibon et al., 2016). The first step of biosynthesis of 3SH precursors is the formation of Glut-3SH-Al through the conjugation of glutathione on (E)-hex-2-enal by glutathione-S-transferase (GST). Next, Glut-3SH is synthesized by reduction of Glut-3SH-Al. Cys-3SH derives from Glut-3SH by removing of the glycine. This reaction needs two enzymes: a γ -glutamyltranspeptidase (*VviGGT*) and a carboxypeptidase (Peyrot des Gachons et al., 2002; Thibon et al., 2011; Thibon et al., 2016). Finally, 3SH is synthesized during the alcoholic fermentation by *Saccharomyces cerevisiae* (Pinu et al., 2012) (figure 6). In the present work, the accumulation of glutathione and three 3SH precursors in CS and SB grape berries during ripening was investigated. Moreover, relative genes expression profiling was evaluated to investigate their response to elevated temperature and their implication in 3SH synthesis.

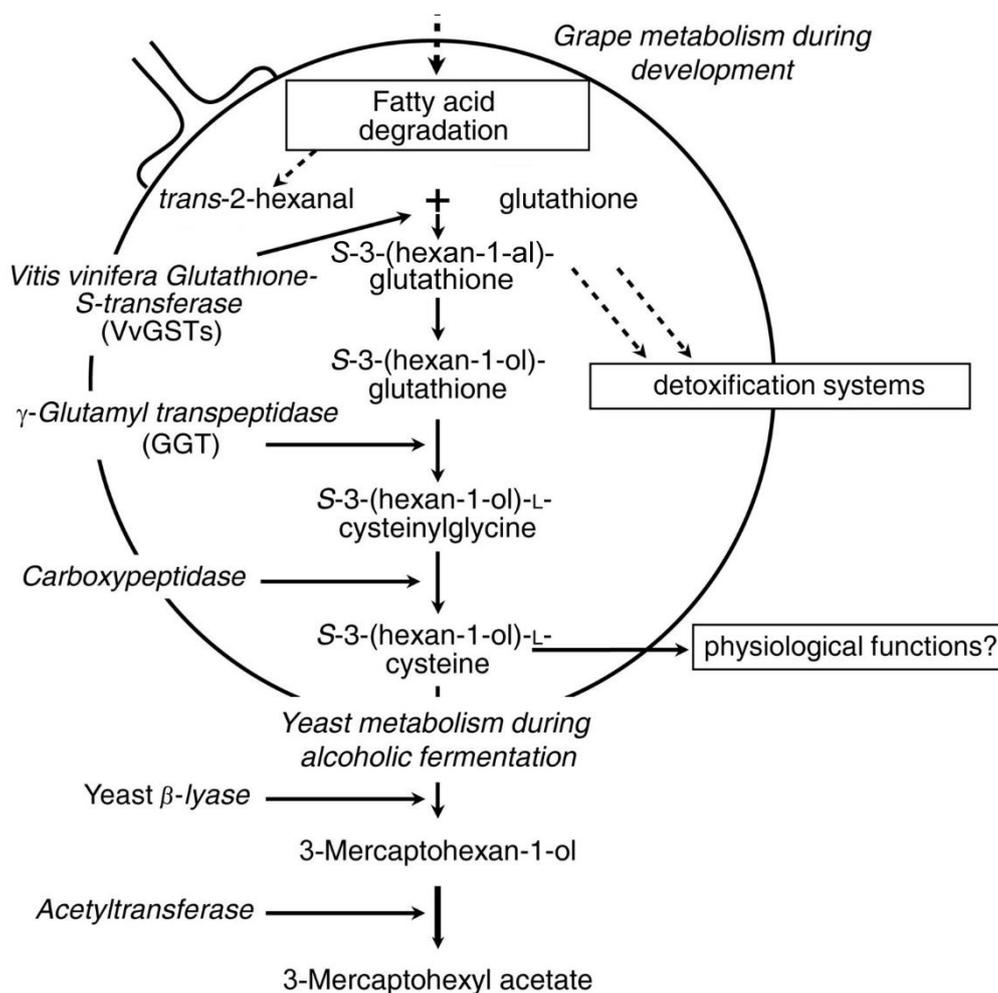


Figure 6: Hypothetic pathway of the glutathionylated precursor (Glut-3SH-Al and Glut-3SH) and cysteinylated precursor (Cys-3SH) in grape berries as described by Kobayashi *et al.*, 2010 and Thibon *et al.*, 2011 and 2016.

The production of 3-sulfanylhexan-1-ol (3SH) occurred during the alcoholic fermentation by the yeast.

To evaluate the effect of elevated temperature on Glut-3SH-Al, Glut-3SH and Cys-3SH in berries, their concentrations in control and warmed berries were monitored in four developmental stages (Figure 7). Glut-3SH and Cys-3SH concentrations were around limitation of quantification, and Glut-3SH was undetectable in CS berries. Glut-3SH-Al content was about 1000-fold higher than Cys-3SH content in all samples. Both compounds showed a similar accumulation profile with an important increase from MV to MR stage. Cys-3SH content was always higher in control berries even though it decreased during ripening stage in control berries, from 0.4 to 0.3 $\mu\text{g}/\text{kg}$ FW. In contrast, Glut-3SH-Al content only showed a significant higher level at harvest in control berries (847.2 *versus* 441.3 $\mu\text{g}/\text{kg}$ FW)

because of a dramatical decrease in heated berries from MR to R stage. In SB berries, all three precursors of 3SH were detected. Similar profiles were observed between control and treated berries for Glut-3SH-Al and Cys-3SH. Their concentrations remained low before veraison then increased rapidly from veraison onward. Significantly higher concentrations were always found in control berries than in heated berries. At harvest, Glut-3SH-Al content was 3.5-fold higher in control berries, with 3727.4 $\mu\text{g}/\text{kg}$ FW *versus* 1062.5 $\mu\text{g}/\text{kg}$ FW. Cys-3SH content was 2.5-fold higher in control berries, with 1.1 $\mu\text{g}/\text{kg}$ FW *versus* 0.4 $\mu\text{g}/\text{kg}$ FW. No significant difference of Glut-3SH between control and treated berries was found at any stage. The concentration increased from 0.4 $\mu\text{g}/\text{kg}$ FW to 0.9 $\mu\text{g}/\text{kg}$ FW and then decreased to around 0.7 $\mu\text{g}/\text{kg}$ FW at harvest.

The accumulation profiles of 3SH precursors depended on varieties but the general trends increased during development except Glut-3SH accumulation in SB berries. Several studies also found that S-cysteinylated and/or S-glutathionylated thiol precursors increased during berry development (Cerreti et al., 2015; Helwi et al., 2016). Conversely, Capone et al. (2012) mentioned that the concentration of thiol precursors may have some fluctuation and even decreased during ripening. 3SH precursors in CS berries and Glut-3SH in SB berries decreased during certain stages, in agreement with Capone's observation (2012). Capone et al. (2011) also detected but not quantified Glut-3-MH-Al in SB juices. It was considered as an intermediate in the formation of Glut-3SH and its concentration was estimated to be much higher than Glut-3SH based on an assumption of the same ionization efficiency. An extremely higher concentration of Glut-3SH-Al than other concentrations in this study agrees with the finding of Capone et al. (2011). In the present study, elevated temperature decreased 3SH precursors, and SB berries showed more significant differences between control and treated than CS berries. Kobayashi et al. (2010) observed that environmental stress, including cold shock, heat shock, UV-C irradiation, and biological stimulation, enhanced Glut-3SH and Cys-3SH in grape berries. This finding is in contradiction with the present study. The main differences between Kobayashi's study and the present one are the magnitude and duration of temperature treatment. Moreover, Glut-3SH-Al was identified as a new potential precursor of 3SH by Thibon and al. (2016), so that previous studies only quantified Glut-3SH and Cys-3SH contents in berry and must as precursors of 3SH (Cerreti et al., 2015; Helwi et al., 2016). Interestingly, Subileau et al. (2008) demonstrated that only 3-7% of total 3SH in wine was released by Cys-3SH lysis and considered that Cys-3SH is not the major 3SH precursor. Moreover, Winter et al. (2011) found that 3SH formation from Glut-3SH was significantly

less efficient than that of Cys-3MH. In view of an extremely higher concentration of Glut-3SH-AI than other precursors in berries, the hypothesis that Glut-3SH-AI, is a main precursor of 3SH can be made, but requires further investigations.

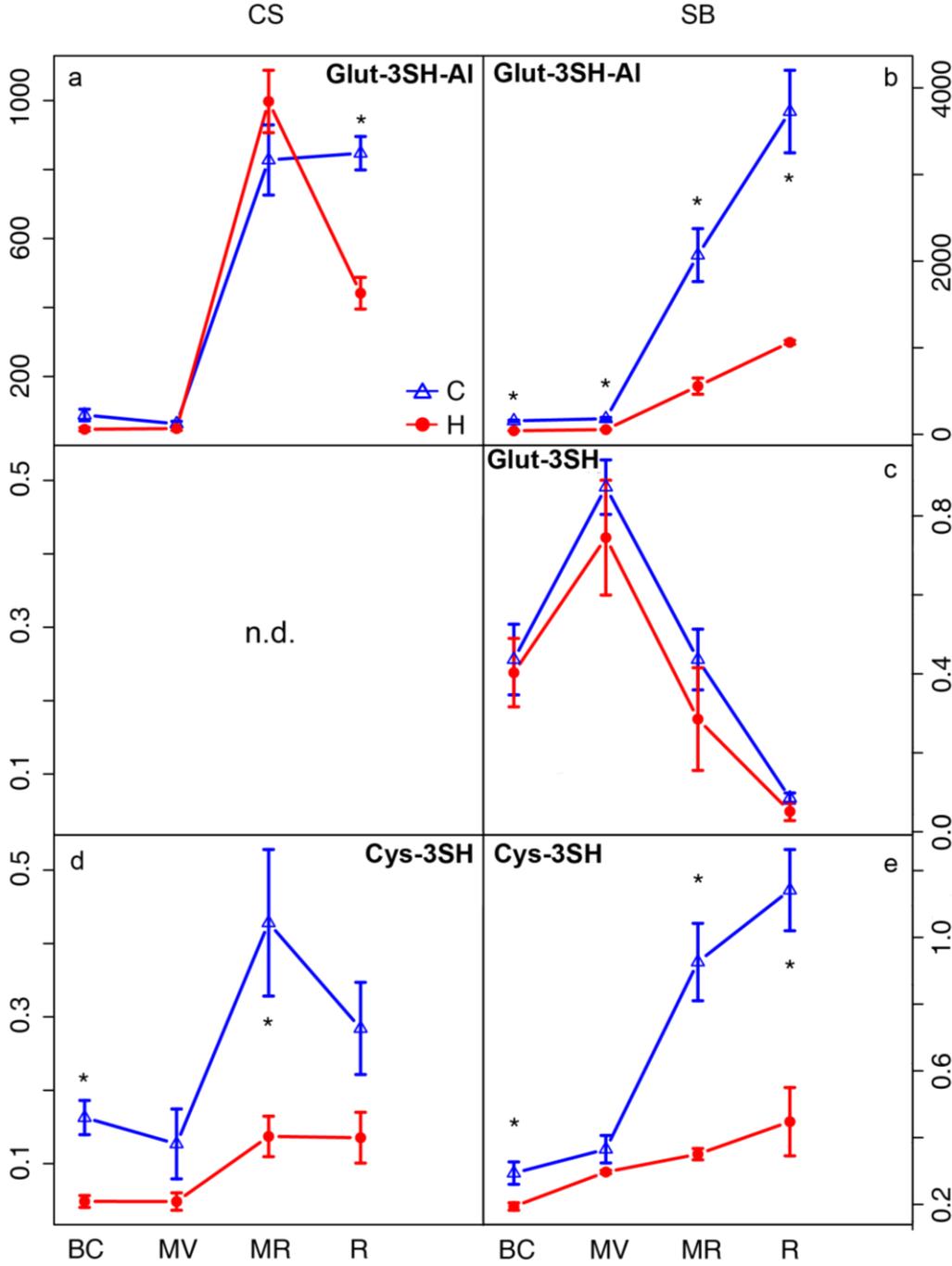


Figure 7: Concentration trend of Glut-3SH-AI, Cys-3SH and Glut-3SH ($\mu\text{g}/\text{kg}$ FW) in control (blue) and heated (red) Cabernet Sauvignon and Sauvignon Blanc berries in 2015.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$). n.d.: no detected.

Responses of the 3SH pathway to elevated temperature

OPT1, a gene encoding an oligopeptide transporter, was shown to be a high affinity glutathione transporter in *Saccharomyces cerevisiae* (Bourbouloux et al., 2000) and was identified in Helwi et al (2016) as potential candidate gene implicated in the transport of glutathione (GSH) or the GSH-S-conjugates, that are necessary for the genesis of 3SH precursors.. *VviGST3* and *VviGST4*, members of glutathione-s-transferase family, were described as two essential genes for the addition of glutathione to the trans-2-hexanal, producing Glut-3SH. Meanwhile, *VviGGT* was also considered to be involved in the conversion of Glut-3SH to Cys-3SH (Kobayashi et al., 2010). The expression profile of these genes was determined in two varieties at different developmental stages (Figure 8). Their expression was evaluated relative to the expression of two endogenous reference genes used as internal standards *VviEF1* and *VviUBIQUITIN*.

The expression pattern of *VviOPT1* differed between the two varieties. The expression level was always higher in SB berries than in CS. Elevated temperature did not affect *VviOPT1* expression levels in CS berries, but resulted in a longer increasing period in SB berries (figure 8). The expression level of *VviOPT1* increased till veraison in control berries, but it increased up to the peak at MR stage. Elevated temperature only decreased the relative transcript abundance of *VviOPT1* at BC and MV stage in SB berries.

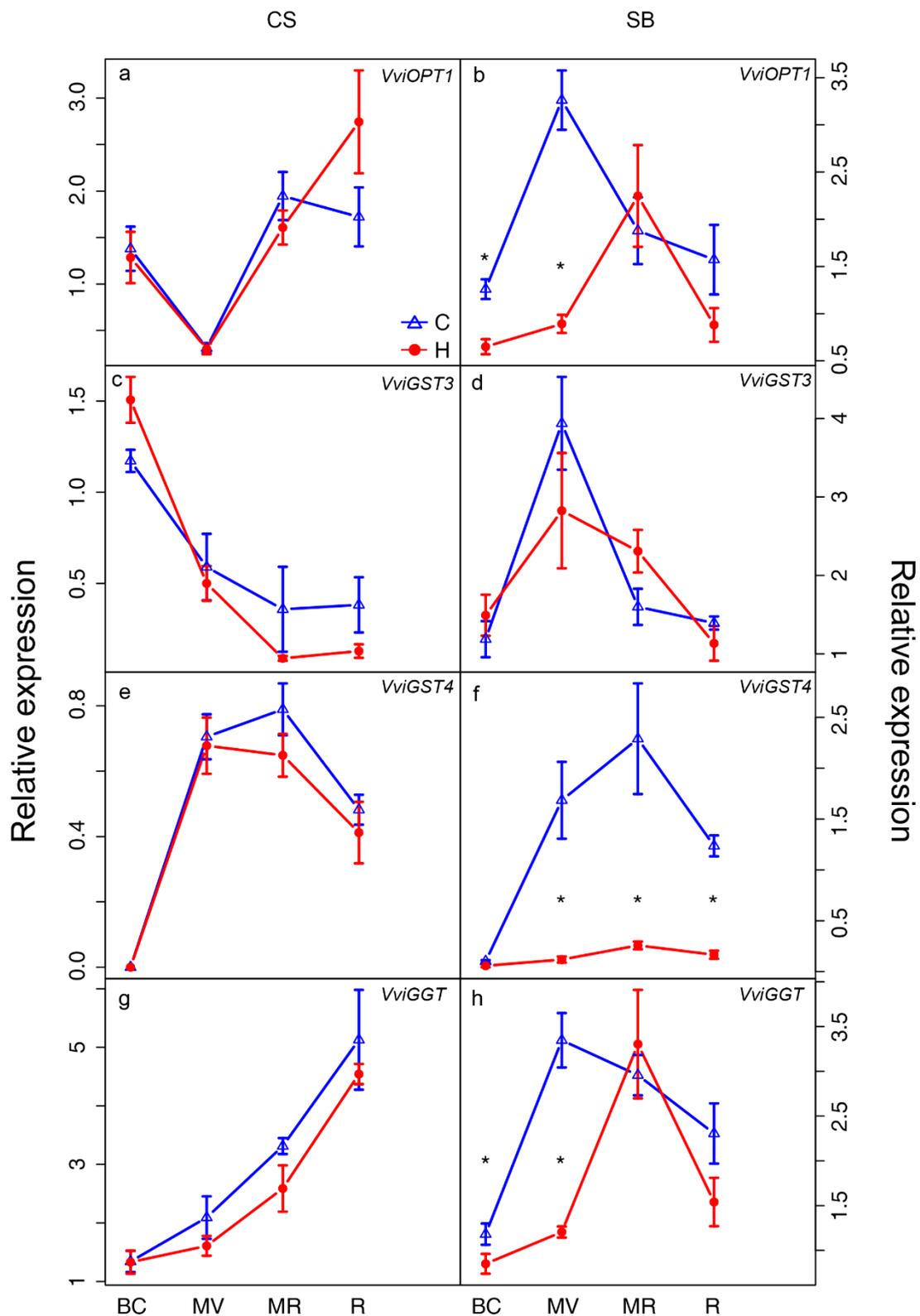


Figure 8: Changes in relative transcript levels of gene of *VviOPT1*, *VviGST3*, *VviGST4* and *VviGGT* in CS (left) and SB (right) berry development in 2015.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$).

The transcript abundances of *VviGST3* and *VviGST4* were also studied during berry development (figure 8). In CS berries, the transcript abundance of both GST genes was down-regulated by elevated temperature only at BC stage. *VviGST3* transcripts amounts decreased during berry ripening, but *VviGST4* transcripts increased till MV and MR stage in control and warmed berries respectively, then it decreased to harvest. In SB berries, elevated temperature did not influence the transcript abundance of *VviGST3* during berry development, but significantly repressed *VviGST4* expression. Transcript amounts of *VviGST4* in control berries were almost 10-fold higher compared to warmed berries. Over development, *VviGST3* and *VviGST4* increased from BC to MV stage and decreased from veraison onwards. *VviGGT*, encoding a gamma glutamyl-transpeptidase, regulates the synthesis of S-(3-hexan-1-ol)-L-cysteinylglycine, an intermediate between Glut-3SH and Cys-3SH. The expression profile of this gene differed between the two varieties studied (figure 11). In CS berries, the transcript level increased till to harvest and was not affected by temperature. In SB berries, the expression pattern was similar with *VviOPT1*, peaking at MV and MR stage in control and treated berries respectively. Elevated temperature only statistically repressed *VviGGT* expression at BC and MV stage.

Combined with RNA-seq results, three genes coding for glutathione transferase activity: *VIT_08s0007g01420*, *VIT_15s0024g01650* and *VIT_14s0060g02170*, was altered by temperature. Among them, *VIT_08s0007g01420* (*VviGSTU18*) was down-regulated in all warmed samples, but only showed a significantly lower expression level with a log₂ (heated/control) value around -2 at MR stage in both varieties. Down-regulated *VviGSTU18* by temperature was corresponded with negative influence on 3SH precursors. *VviGSTU18* could be a potential candidate gene encoding enzyme involved in the biosynthesis pathway of 3SH precursors. In addition, Helwi et al. (2016) mentioned that *VIT_19s0015g00860* sequence had a high (69%) similarity with *ATGSTU25* in *Arabidopsis* which can catalyse the addition of glutathione to trans-2-hexenal. In our study, no different expression was found about this gene.

III.4 Conclusion

This work provides the first molecular insights into the understanding of the effect of elevated temperature in actual vineyard conditions for Cabernet Sauvignon and Sauvignon Blanc, and brings data describing the response of certain important metabolites to temperature. Increasing ambient mean temperature by 0.5-1.6 °C from fruit setting to harvest, similar with

projected global warming, remodelled the berry transcriptome, decreased anthocyanins content in Cabernet Sauvignon berry skin and precursors of 3-mercaptohexanol in both varieties, but did not affect tannin levels. Based on the enrichment of Gene Ontology, temperature mainly regulated four GO categories: microtubule, cell wall, extracellular region and transcription factor activity. 22 downregulated transcripts encoding biosynthetic enzymes of phenylpropanoid and flavonoid pathways were found based on RNA-seq data, which could explain the decreased concentration of anthocyanins in Cabernet Sauvignon berry. *VviGST4* expression profile correlated tightly with the accumulation precursors of 3-mercaptohexanol. Meanwhile, *VIT_08s0007g01420 (VviGSTU8)* was also selected as a potential candidate gene involved the biosynthesis of precursors of 3-mercaptohexanol. Finally, the data provide a rich transcriptomic and metabolomic resource for further studies about plant responses to global warming.

**Chapter IV: Effect of moderately elevated
temperature on 2-methoxy-3-isobutylpyrazine in
berries of Cabernet Sauvignon and Sauvignon
Blanc**

ABSTRACT

Methoxypyrazines (MPs) are a group of nitrogen heterocycle compounds, which are responsible for the very characteristic “green, herbaceous, or vegetative” aromas. 2-methoxy-3-isobutylpyrazine (IBMP) is the major MPs component in grape berries and wine, and its concentration is reported to be highly affected by temperature. The present study investigated the effects of elevated temperature on berry IBMP content under actual vineyard conditions. A passive open-top heating system was used to increase temperature from fruit setting to over-maturity by 0.5-1.6°C at bunch zone. However, final IBMP concentrations in berries were not affected by elevated temperature in two vintages (2015 and 2016) and two varieties (Cabernet Sauvignon and Sauvignon Blanc). The elevated temperature only significantly reduced IBMP content and expression level of *VviOMT3* in Cabernet Sauvignon berries at bunch closure stage in both vintages. On the other hand, the elevated temperature reduced the expression level of *VviOMT3* at bunch closure stage without affecting IBMP concentration. This limited and genotype-dependent effect of elevated temperature suggest that a moderate temperature elevation (0.5-1.6 °C) may not be sufficient to modify IBMP at maturity, or other environmental factors (e.g. light) play dominant role in regulating IBMP than temperature. In addition, IBMP concentration in mature berry was higher in 2015 than in 2016 in both varieties, while the mean temperature and thermal degree day were similar between both vintages. More detailed analysis showed that 2015 was warmer at pre-veraison but cooler at post-veraison than 2016, which may lead to a lower degradation in 2015 and therefore a higher IBMP content. Therefore, it is most likely that a separate analysis of the effect of pre- and post-veraison temperature conditions on IBMP levels may be more suitable for predicting IBMP concentration in mature berries.

KEYWORDS: 2-methoxy-3-isobutylpyrazine, aroma, IBMP, temperature, global warming, *Vitis vinifera*, *VviOMT*

IV.1 Introduction

The characteristics and quality of a wine are an overall impression which composes of appearance (colour, transparency etc.), smell (olfactory) and taste (gustatory). Particularly, olfaction plays a “dominant” role in the tasting of food (Spence, 2015). In wine, olfactory quality is influenced by a complex mixture of volatile aroma compounds which are determined by many variables, including grape variety, environmental conditions, viticultural and oenological practices (Lacey et al., 1991; Swiegers and Pretorius, 2005; Pickering et al., 2006). Varietal aromas explain the unique flavours of wines made with different types of grapes. They are considered as key factors to recognition of a typicality or sensory identity of wine, such as methoxypyrazines, monoterpenes and thiols.

Methoxypyrazines (MPs) are a group of nitrogen heterocycle compounds, widely found in plant kingdom including grapevine (Bayonove et al., 1975; Murray and Whitfield, 1975). They are responsible for the very characteristic “green, herbaceous, or vegetative” aromas of Sauvignon Blanc, Cabernet Sauvignon and some other Bordeaux variety wines (Lacey et al., 1991; Noble et al., 1995; Ryona et al., 2008). Five MPs were identified in grape berries and wines. Based on previous studies (Allen and Lacey, 1998; Godelmann et al., 2008; Darriet et al., 2012) (Table 1), three of them are highly odorous with very low odour thresholds in water: 2-methoxy-3-isobutylpyrazine (IBMP), 2-methoxy-3-isopropylpyrazine (IPMP) and 2-methoxy-3-*sec*-butylpyrazine (SBMP). IBMP is the major MPs component in grape berries, juice and wine. The olfactory perception threshold of IBMP in water is very low, below to 1ng/L. The recognition threshold in the wine, in range of 2-6 ng/L, is quite different from those determined in water and is variable due to the different composition from one wine to another (Roujou de Boubée et al., 2000; Pickering et al., 2007). An excessive level of IBMP concentration (≥ 15 ng/L) in wine has a negative effect as an obvious herbaceous off-flavours (Roujou de Boubée et al., 2000), but a concentration near its detection threshold can contribute to pleasant varietal aromas in Sauvignon Blanc wines (Allen et al., 1991). Therefore, controlling IBMP concentration at a suitable level is very important to wine quality. Based on previous studies, IBMP concentration in wine is highly correlated with its concentration in berry at harvest. It is easily extracted during maceration and difficult to reduce by oenological practice (de Boubée et al., 2002; Darriet et al., 2012).

Table 1: 2-methoxy-3-alkylpyrazines identified in grapes and wines

Name	Abbreviations	Structure	Detection threshold (ng/L)	Levels in wine (ng/L)	Aroma descriptors
2-methoxy-3-isobutylpyrazine	IBMP	R: CH ₂ (CH ₃) ₂	1	2-60	Bell pepper / Herbaceous/Pea pod
2-methoxy-3-isopropylpyrazine	IPMP	R: CH (CH ₃) ₂	2	< 10	Bell pepper/Asparagus/rotten vegetable
2-methoxy-3-sec-butylpyrazine	SBMP	R: CH (CH ₃)CH ₂ CH ₃	1	< 10	Bell pepper/Galbanum oil/earthy
2-methoxy-3-ethylpyrazine	EMP	R: CH ₂ CH ₃	425	<40	Earthy/Potato-like
2-methoxy-3-methylpyrazine	MEMP	R: CH ₃	4000	<1	

The biosynthesis pathway of MPs has not yet been well determined, in not only grapes, but in any plant species (Dunlevy et al., 2010). MPs formation has been hypothesised to begin with an amino acid (Glycine, leucine, isoleucine, valine) (Figure 1). After transforming into an amide and reacting with a 1,2-dicarbonyl compound, 3-alkyl-2-hydroxypyrazine (HP) is formed. The last reaction is enzymatic methylation from HP to methoxypyrazine (MP) by an *O*-methyltransferase enzyme (OMT) (Dunlevy et al., 2010; Darriet et al., 2012; Dunlevy et al., 2013). In recent studies, four OMTs (*VviOMT1*, *VviOMT2*, *VviOMT3* and *VviOMT4*) which are able to methylate HP to MP have been identified (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013). *VviOMT1* and *VviOMT2* showed a higher catalytic activity against flavanol quercetin, while *VviOMT3* and *VviOMT4* showed a high specificity and efficiency for methylated reaction during MPs formation (Dunlevy et al., 2013; Guillaumie et al., 2013).

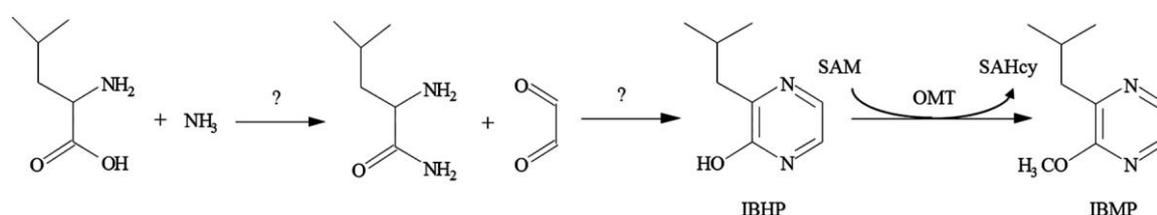


Figure 1: Hypothetical biosynthesis pathway of IBHP and IBMP from valine respectively (Helwi et al., 2015).

It has been found that MPs accumulate in grape berries before veraison and degrade thereafter (Darriet et al., 2012). Their concentrations are affected by genotype and an interactive ecosystem (including climate, soil and human factors) as well as the interaction between genotype and the ecosystem (Allen and Lacey, 1993; Ryona et al., 2008; Mendez-Costabel et

al., 2014; Helwi et al., 2015). In general, high temperature has been reported to reduce IBMP level (Lacey et al., 1991; Allen and Lacey, 1998; Marais et al., 1999; Falcão et al., 2007). However, these results were often obtained by comparing wine-growing regions or vintages that have relatively different climate conditions such as geographic variation between regions and precipitation between vintages, making the temperature effect confounded with effects of other environmental factors. According to a recent report (Power et al., 2017), global annual average temperature has increased by more than 1.2°C since 1900 (through 2012) and will continue to warm up. In France, mean temperature is projected to rise by 0.6 to 1.3 °C by the mid-21st century (2021-2050) (Ouzeau et al., 2014). Therefore, it is necessary to understand whether the projected global warming has a direct effect on MPs synthesis and degradation.

The present paper, in which the heating effect manipulated in realistic vineyard using an open-top heating system with minimizing influence on other environmental factors, has investigated the effect of a 0.6-1.5°C increase in mean temperature on IBMP concentration during berry development in the two representative red and white varieties of Bordeaux region, Cabernet Sauvignon and Sauvignon Blanc. Meanwhile, as some genes involved in the biosynthesis of IBMP are known, their expression levels were also studied.

IV.2 Materials and methods

IV.2.1 Site and vines

Experiments were conducted in 2015 and 2016 in Bordeaux vineyard (44° 46' 46'' N, 00°34' 01'' W) with the varieties *V. vinifera* cv. Sauvignon Blanc (SB) and Cabernet Sauvignon (CS). They were planted in an experimental vineyard “VitAdapt” growing in level ground with no slope or geospatial variations. The field-grown grapevines were 8 years old, spur pruned, with a spacing of 1.6m between rows and 1m between plants. Vineyard management followed the local standards.

IV.2.2 Heating system

The passive open-top heating system was used to elevate temperature from fruit setting to over-maturity (14 days after harvest). Full details of the heating system, its performance and limitations are in chapter II.

One hundred fresh berries were randomly sampled from each replicate at four different harvest stages: bunch closure (BC), mid-veraison (MV), mid-ripening (MR) and ripening (R). All samples were ground into powder in liquid nitrogen using a ball grinder MM200 (Retsch, Haan, Germany), and stored at -80°C for later analysis.

IV.2.3 Extraction and analysis of IBMP

IBMP was quantified in whole grape berries by a stable isotope dilution assay using headspace solid phase micro-extraction coupled to a gas chromatograph and a mass spectrometer (SIDA-SPME-GC-MS) adapted from Guillaumie et al. (2013). Samples preparation involved weighing 1 g of ground frozen berries dissolved in 6 mL of deionized water into 20 mL SPME vials along with 4 g of sodium chloride (NaCl). An internal standard, [²H₃]-IBMP, was also added to yield a final concentration of 100 ng L⁻¹.

The IBMP extraction and assay method was adapted from Guillaumie et al (2013). Samples were submitted to agitation (500 rpm for 5 s, stop for 2 s) for 5 min at 50 °C and then to the extraction with the SPME fiber for 40min at 50 °C. A three-phase divinylbenzene/ carboxen/ polydimethylsiloxane fibre (DVB/CAR/PDMS 50/30 µm thickness, 24 gauge, Supelco Bellefonte, PA, USA) was inserted, and the vial was agitated at 500 rpm for 40 min at 50 °C. SPME injection was then implemented in splitless mode for 10 min with a desorption temperature of 240 °C. Automated GC-MS analysis was carried out on a 6890 N gas chromatograph (Agilent Technologies) equipped with a Combi PAL autosampler (CTC Analytics). The GC was coupled to an HP 5973N mass selective detector (Agilent Technologies) functioning in electron impact mode at 70 eV. The analyses were performed on a Carbowax 20 M capillary column (BP20, 50 m, 0.25 mm internal diameter, 0.2µm film thickness, Scientific Glass Engineering). Helium N60 (Air Liquide) was used as a carrier gas at a flow rate of 0.9 mL min⁻¹. The temperature program was as follows: initial hold for 5 min at 45°C, followed by a 3°C min⁻¹ ramp to 140°C and then a ramp at 30°C min⁻¹ to 240°C, and a 10 min hold. The injector port was at 240°C. During the elution of the methoxypyrazine, the GC-MS was switched to single-ion monitoring mode and tuned to measure m/z values of 127, 94, and 154 for ²H³-IBMP and 124, 94, and 151 for IBMP. Data processing was carried out by MSD Chemstation software (5973n Data Analysis, Agilent Technologies). Results were reported on a per weight basis of nanograms per kilogram of fresh weight for berries.

IV.2.4 RNA extraction and gene expression analysis

Total RNA from 1 g powder of each sample was isolated according to the previous described method (Reid et al., 2006). RNA isolation was followed by DNase I treatment (Turbo DNA-free™ kit, Ambion, Austin, TX, USA). Afterward, total RNA was quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, DE), and the RNA quality was evaluated on 1% agarose gels. First-strand cDNA was synthesized from purified RNA using Superscript III enzyme (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA obtained was diluted (target concentration: 0.01µg/µl) in distilled water for later analysis. Quantitative real-time PCR expression analysis was carried out using the CFX96 Real-Time PCR Detection system (Bio-Rad). PCR reaction mix and specific oligonucleotide primer pairs were those from Guillaumie et al (2013). PCR conditions were 3 min at 95°C, 40 cycles of 10 s at 95°C, and 15s at 60°C. The amplification efficiencies were determined by serial dilution. Melting curves to control the annealing specificity of oligonucleotides were recorded after each run. All experiments were performed with three biological replicates and two technical replicates. Normalized expression of each gene was calculated using Bio-Rad CFX Manager software. Gene transcripts were quantified upon normalization to *VviEF1* and *VviUBIQUITIN* as internal standards by comparing the cycle threshold of the target gene with those of standard genes.

IV.2.5 Statistical analysis

Data were analysed with multivariate analysis methods using the R statistics Environment R CORE TEAM (TEAM, 2010). The Student's t-test was used to test the significance of differences between berries under control and elevated temperature at $P < 0.05$ level. Dynamic profiles of IBMP and gene relative expressions were drawn using Sigmaplot 11.0 (Systat Software Inc.).

IV.3 Results

IV.3.1 Heating system performance and grape berry response

All details were shown in chapter II. Briefly, the open-top heating system achieved to increase mean bunch zone air temperature 0.5-1.6 °C. The system maintained daily temperature dynamic, slightly increased relative humidity and increased vapor pressure deficit. Soil and plant water status were not affected by this level of temperature increment. Through the two

years and two varieties, neither berry weight nor sugar content were influenced by temperature at harvest. Only significant lower concentration of malic acid and higher concentration of tartaric acid in CS berries in 2016 were found.

IV.3.2 IBMP content in grape berries and *VviOMT* expressions

IBMP profiles of CS and SB berries under two moderately contrasted temperature regimes were determined during two successive years, 2015 and 2016 (Figure 2). For both varieties, IBMP accumulation profile was similar regardless of vintage and treatment. As expected, maximum values of IBMP were found at BC stage, and then IBMP content decreased dramatically till to harvest. No significant difference was observed between control and treated berries at harvest as well as at MV and MR of both varieties. Only CS berries contained a statistically lower IBMP under warming conditions at bunch closure, 20.3% less in 2015 and 16.4% in 2016. Meanwhile, the concentration of IBMP slightly increased or kept stable from BC to MV in warmed CS berries. SB berries had less concentrated IBMP at harvest compare to CS berries, 19.1 *versus* 30.3 ng/kg FW in 2015 and 14.5 *versus* 19.1 ng/kg (FW) in 2016. Comparison between two vintages, the monthly mean temperature and the average growing degree accumulation (°C) were similar (Table 2), IBMP contents in both varieties were higher in 2016 at bunch closure than in 2015. Conversely, final concentrations in CS were lower in 2016 than in 2015, SB berries had similar final concentrations in both years.

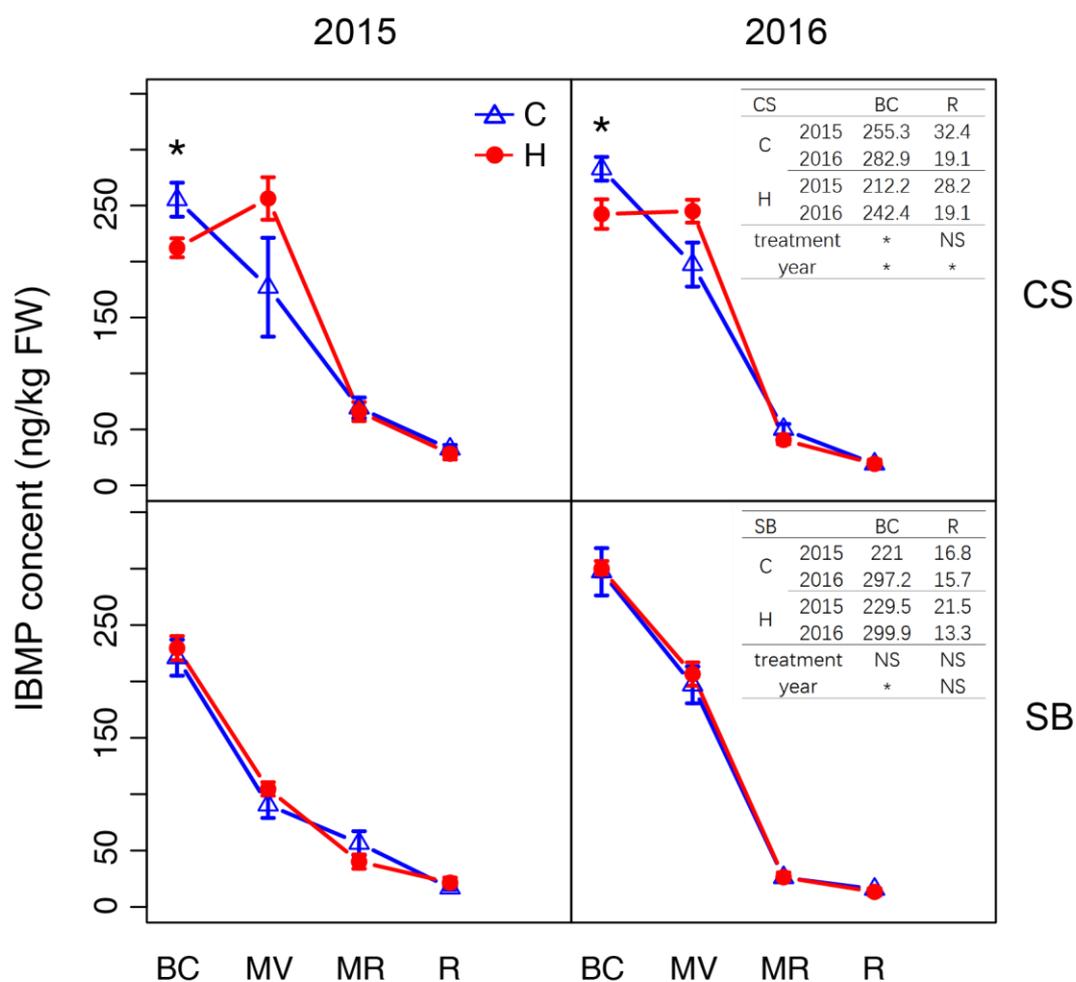


Figure 2: Seasonal variation of IBMP concentration in Cabernet Sauvignon and Sauvignon Blanc in 2015 and 2016. Insert tables are concentrations in BC and R stages and for the effect of vintage and treatment by two-way ANOVA.

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$)

Table 2: Comparison of weather conditions (average monthly temperature, accumulation of growing degree days (GDD), and monthly precipitation) between June and September in 2015 and 2016^a

	av. monthly temp (°C)		sum. GDD (°C) ^b		monthly precipitation (mm)	
	2015	2016	2015	2016	2015	2016
June	21.3	19	345	274.3	38.8	74.5
July	23	21.6	420.2	370.4	26	11.5
Aug	21.8	22.6	380.7	400	109.5	10
Sept	17.4	20.4	235.6	327.2	24.5	48
average	20.9	20.9	345.4	343	49.7	36

^a Data provided by the INRA weather station, La Grande Ferrade-Villenave d'Ornon

^b Growing degree days (GDD) were determined as $GDD = [(maximum\ daily\ temperature + minimum\ daily\ temperature)/2] - 10$

Transcript levels of two genes from methyltransferases family, *VviOMT3* and *VviOMT4* which have a high specificity of MPs formation (Guillaumie et al. 2013), were analysed by qPCR (Figure 3 and 4). Their expression patterns followed the same trend as IBMP concentration in grape berries, which were high at BC stage, and almost undetectable from veraison to mature stages. In CS berries of the two vintages, significantly higher expression level of *VviOMT3* and relative higher expression level of *VviOMT4* were observed at BC stage in control berries. These results are consistent with those obtained for IBMP content in CS berries at the same stage. In SB berries, these two OMT genes were more expressed in control than in heated berries at BC stage, and expression of *VviOMT3* in 2015 and *VviOMT4* in 2016 differed significantly. However, the concentrations of IBMP did not differ between control and elevated temperature conditions.

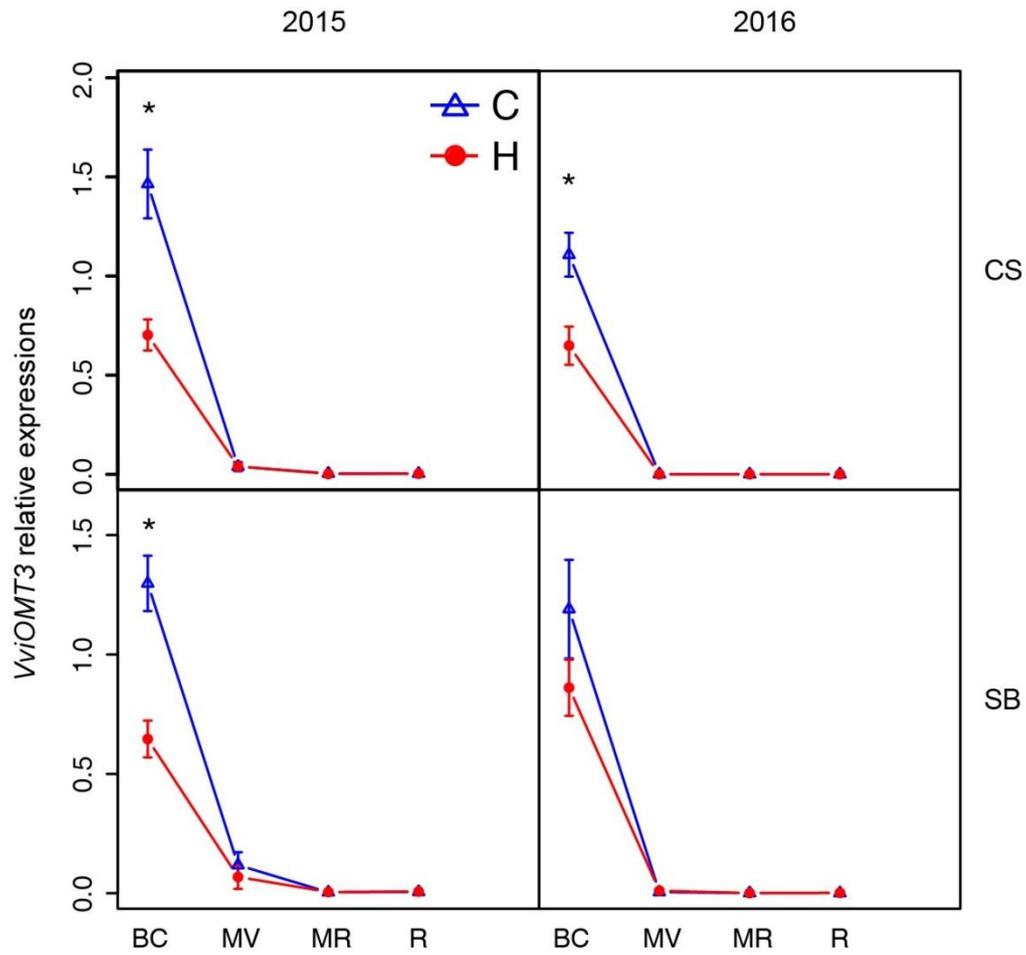


Figure 3: Expression profiles of *VviOMT3* genes in CS and SB berry development in 2015 and 2016

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$)

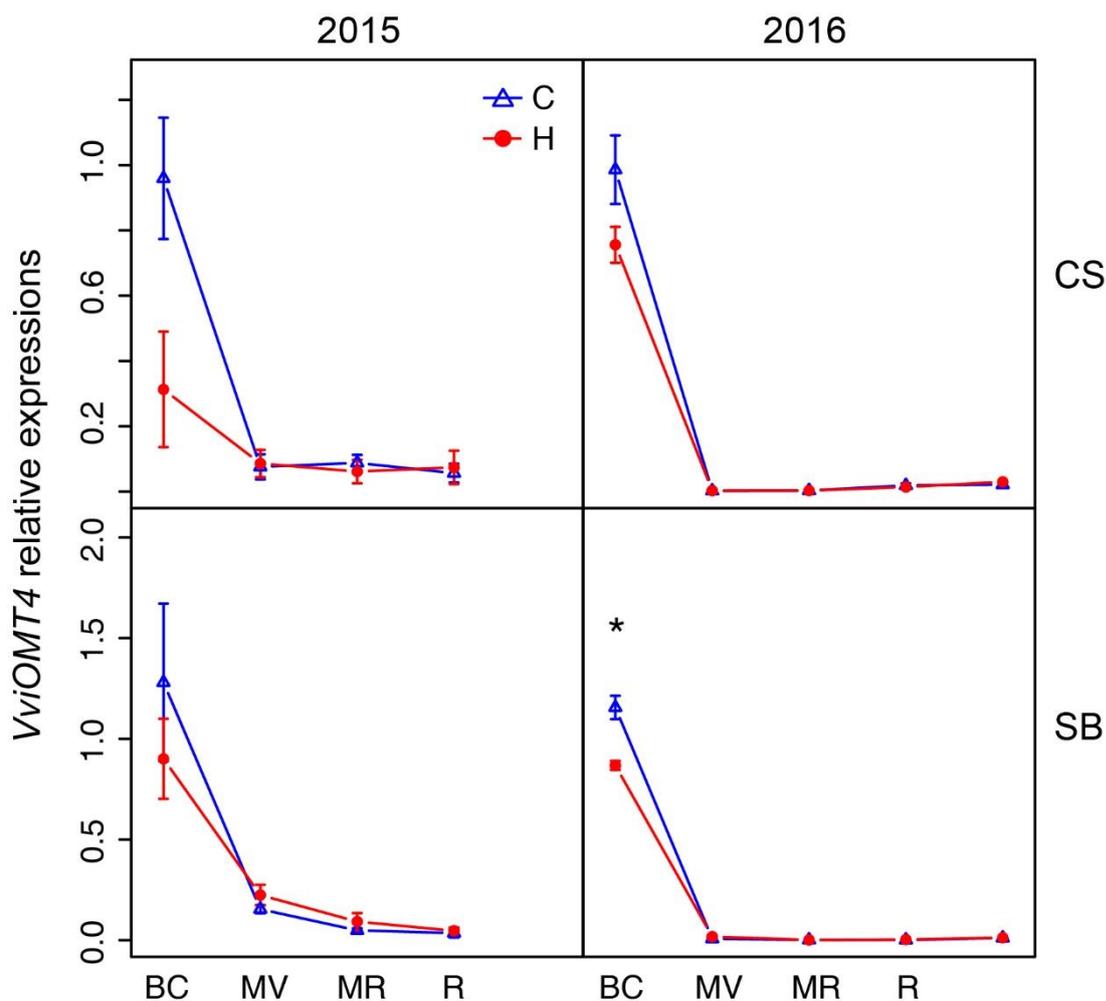


Figure 4: Expression profiles of *VviOMT4* genes in CS and SB berry development in 2015 and 2016

Error bars indicate standard error. Each point is the mean of five replicates. * Significantly different from control (independent t-test, $p < 0.05$)

IV.4 Discussion

Methoxypyrazines, particularly the IBMP, play an important role in shaping the aromas of both juice and wines. The magnitudes of IBMP concentration quantified in our study in CS and SB are in agreement with previous studies (Allen et al., 1991; Lopez et al., 1999), which established that CS and SB are two varieties known for producing higher levels of IBMP than other varieties. Concentrations of IBMP were maximum before veraison and decreased rapidly thereafter in both varieties and vintages, also in line with literatures (Scheiner et al., 2010; Helwi et al., 2015). Moreover, the IBMP concentrations at harvest were above the reported odour detection threshold, indicating its potential influence on wine aroma.

Therefore, sensorial and chemical analysis of wines made by grape berries from experimental plots would be valuable.

Temperature has been considered as one of the key factors affecting the level of MPs in grape berries. Comparisons of different growing regions or vintages showed higher IBMP concentrations in grape berries and wine under cooler growing conditions (Allen et al., 1991; Falcão et al., 2007; Mendez-Costabel, 2012; Helwi et al., 2015). In the present study, the average growing degree day (GDD) accumulation ($^{\circ}\text{C}$ day) from June to September in 2015 and 2016 was similar, with 345 $^{\circ}\text{C}$ day and 343 $^{\circ}\text{C}$ day respectively. However, IBMP concentrations in mature grapes of CS were higher in 2015 than in 2016, which is contradictory with the assumption of cooler conditions coupled with higher IBMP level. Moreover, lower IBMP concentrations at bunch closure were found in 2015 than 2016 in both varieties. This observation also contrasts to the study of Ryona et al. (2008), where a positive correlation was observed between IBMP concentrations at pre-veraison and at harvest. The contrary results observed in the present study may be explained by the monthly temperature differences between the two vintages (Table 2). In 2016, a lower average growing degree accumulation from fruit set to veraison (June and July) resulted in a hyper-accumulation of IBMP, but a higher average growing degree accumulation after veraison to harvest (August and September) accelerated IBMP degradation. Accordingly, the degradation rate in 2016 was indeed higher than in 2015, with IBMP concentrations in CS and SB berries decreased by 92.7% and 95.1% in 2016 versus 87% and 91.5% in 2015, respectively. Therefore, the well accepted conclusion that cooler growing conditions produce more IBMP is oversimplified. Our results may also provide clues to understand why temperature was not a good predictor for IBMP accumulation and degradation in a multisite partial least squares regression to model IBMP concentrations by several viticultural and environmental factors (Scheiner et al., 2010). We argue that considering the relative contributions of the effects of pre- and post-veraison temperature conditions may be more reliable for predicting final IBMP concentrations in grape.

In addition to the differences between vintages, our heating system affected IBMP concentration in a genotype and developmental stage dependent manner. It decreased IBMP concentration in CS berries at BC stage in both vintages, but had no effect at mature stage in CS and no effect at all stages in SB berries. The limited effect of a moderate temperature elevation (0.5-1.6 $^{\circ}\text{C}$) on IBMP in our study seems to be contrasted with other reports, where

negative effect of high temperature and light exposure on IBMP accumulation was concluded based on experiments of leaf removal or un-shading conditions (Marais et al., 1999; Scheiner et al., 2010; Šuklje et al., 2012). However, two confusing points may hamper the comparison between those studies and ours. First, leaf removal may significantly change the leaf area/crop weight ratio, which is one of the determining factors for berry composition and quality (Kliwer and Dokoozlian, 2005), and it also decreases the whole-vine photosynthesis (Petrie et al., 2003). The modified berry composition and vine growth rate may also affect IBMP content. Second, other environmental factors may be changed by leaf removal, not only temperature and light but also wind speed, canopy density, vapour pressure deficit etc. (English et al., 1989). In our study, no part of grapevine aerial canopy was manipulated. A 0.5-1.6 °C mean temperature increase in the bunch zone achieved by an open top heating system in which temperature can be considered as the largely predominant changed environmental factor. This magnitude and duration of warming did not change final IBMP concentrations in mature berries, regardless of varieties and vintages, although the concentrations at BC stage responded to elevated temperature in CS but not SB. These results suggest that a moderate temperature elevation (0.5-1.6 °C) may not be sufficient to modify IBMP at maturity, or other environmental factors (e.g. light) play dominant role in regulating IBMP than temperature.

To understand the different response of IBMP concentrations to warming conditions between varieties, the expression of *O*-methyltransferase genes, *VviOMT3* and *VviOMT4*, was analysed. Until now, only a single enzyme family involved in MPs biosynthesis has been identified: *VviOMT1*, *VviOMT2*, *VviOMT3* and *VviOMT4*. All of them are able to methylate hydroxypyrazine intermediate. Among them, *VviOMT3* shows a higher specificity for IBMP methylation (Dunlevy et al., 2010; Dunlevy et al., 2013; Guillaumie et al., 2013; Battilana et al., 2017). In both varieties, *VviOMT3* and *VviOMT4* genes expression followed the expected pattern, as has been described in literatures. In heated berries of CS at bunch closure, *VviOMT3* expression level and IBMP accumulation were all significantly repressed, but *VviOMT4* did not differ significantly. Based on their differential expressions, *VviOMT3* showed a higher specification to IBMP biosynthesis than *VviOMT4*. This result confirms the conclusion of Guillaumie et al. (2013). Unexpectedly, these two OMT genes were down-regulated in treated SB berries, while IBMP levels were similar between control and treated berries at BC stage, suggesting that other regulators than OMTs may also play a role in

regulating IBMP, such as the alteration of precursor concentration and/or IBMP degradation by elevated temperature (Guillaumie et al. 2013).

IV.5 Conclusion

Understanding the effect of temperature on chemical and biological differences in grape berries can help vines growers and winemakers to manage grape and wine quality under the global warming context. In the current study, final IBMP concentrations in berries of CS were not affected by a warming condition of 0.6-1.5 °C mean temperature increase during berry development, but this magnitude of warming treatment significantly decreased IBMP content and *VviOMT3* expression level at bunch closure in CS berries. In contrast, IBMP content in SB berries was not altered at any stages and in any vintages, although two OMTs genes expression level were lower under elevated temperature at bunch closure. The unchanged IBMP content with changed OMT expression may be caused by the alteration of its precursors and/or degradation rate by temperature. So far, very little is known about the MPs degradation. IBMP concentration in mature berry was higher in 2015 than in 2016 in both varieties, while the mean temperature and thermal degree day were similar between both vintages. More detailed analysis showed that 2015 was warmer at pre-veraison but cooler at post-veraison than 2016, which may lead to a lower degradation in 2015 and therefore a higher IBMP. Therefore, we argue that a separate analysis of the effect of pre- and post-veraison temperature conditions on IBMP levels may be more suitable for predicting IBMP concentration in mature berries. Further investigation on IBMP degradation as well as biosynthesis pathway may allow us to explain the different responses of IBMP to temperature. Moreover, longer duration of elevated temperature treatments (not only during berry development) in realistic vineyard for several years could better simulate global warming impact and give more insight plant acclimation ability.

**Chapter V: Effect of moderately elevated
temperature on free amino acids, carotenoids and
C₁₃-Norisoprenoids in berries of Cabernet
Sauvignon in Barossa Valley**

ABSTRACT

In Australia, almost four fifths of total grape production is employed for wine production. Berry quality, a major determinant of wine quality, depends on the composition and concentration of primary and secondary metabolites, which are influenced by temperature. In the context of global warming, temperature elevation could become a threat to viticulture. A passive open-top heating system was applied in Cabernet Sauvignon vines grown with standard practice in the Barossa Valley, Australia to increase the bunch zone mean temperature by around 0.6 °C. This magnitude of temperature elevation was commensurate with the projected warming for the following forty years in Australia. The moderately elevated temperature maintained yield, total soluble solid and titratable acid, but decreased pH value at harvest. Heating treatment increased total amino acid concentrations during berry development, but no significant difference was observed in mature berries. Although some individual amino acids showed different responses to elevated temperature depending on stages, most individual amino acid concentrations were higher in heated mature berries, except Asp, Cys and Glu. Total carotenoid concentrations and two most predominant carotenoids: lutein and β -carotene, were not altered by elevated temperature. Zeaxanthin was reduced by elevated temperature and was significantly less concentrated in heated mature berries than in control. This lower concentration may limit the biosynthesis of β -damascenone and explain the observation that lower β -damascenone in post-ripen berries in the present study.

KEYWORDS: Amino acids, carotenoids, zeaxanthin, C₁₃-norisoprenoids, β -damascenone, fruit quality, ripening, temperature, *Vitis vinifera*.

V.1 Introduction

Grapevine is one of the most valuable fruit species worldwide because of its multi-usage, such as fresh fruit, raisin, juice, wine and liquor. In 2016, total world area under vines was 7.6 million hectares and global grape production was 7.8 million tons, among which around 50% were used for wine production (OIV, 2017). Moreover, this percentage reached 78% in Australia (OIV, 2017).

Berry quality, a major determinant of wine quality; depends on the composition and concentration of primary and secondary metabolites, which are influenced by complex interactions between genotypes, environment and human factors (Seguin, 1986; Van Leeuwen et al., 2004; Deluc et al., 2007). Among environmental factors, temperature is important to regulate grapevine phenology, alter berry quality, and even threaten the viticulture sustainability (Bergqvist et al., 2001; Azuma et al., 2012; Fraga et al., 2016). Global climate change is a worldwide phenomenon and global annual average temperature of 2012 was more than 1.2°C higher than that of 1900 (through 2012) (IPCC, 2014a) (Power et al., 2017). Although most wine grapes (*Vitis Vinifera* L.) display large variation in terms of tolerance to excessive temperature (Palliotti and Poni, 2015), earlier harvest dates and higher potential alcohol levels at harvest associated with a significant increase in temperature were observed in the past several decades (Chuine et al., 2004; Duchêne and Schneider, 2005; Stoll et al., 2012). These modifications induced by elevated temperature may modify wine typicity and threaten viticulture sustainability in the future.

In general, accelerating the sugar accumulation is often associated with thermal increase, as indicated by the trend observed in the last decades (Coombe, 1986; Petrie and Sadras, 2008; Rienth et al., 2016). However, recent experiments with precise temperature regulations established that the effects of temperature on grape quality depend on the intensity of temperature elevation, stage of high temperature treatment, and grape genotypes. Two weeks treatment of 2-4°C increase in maximal temperature at different phenological stages did not increase sugar concentration in Shiraz berries (Sadras and Moran, 2012; Sadras et al., 2013; Sweetman et al., 2014). Moreover, Lecourieux et al. (2017) showed that bunch zone

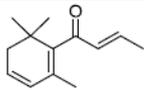
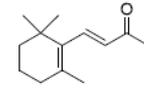
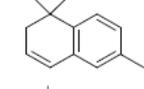
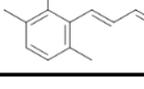
high temperature imposed pre-veraison or during veraison did not change sugar concentrations. Contrast with the general consideration of negative correlation between acid concentrations and temperature increment, variety-dependent thermal response of acidity was observed (Bergqvist et al., 2001; Sadras et al., 2013). Moreover, Rienth et al. (2016) found that higher day temperature brought forward malic and tartaric acid accumulation by 5.5 days without changing their maximal accumulation rates. Meanwhile they observed that higher temperature desynchronized sugar accumulation and organic malate breakdown.

In grape, free amino acids are the major nitrogenous compounds and can serve as an important nitrogen source for yeast during winemaking (Marcy et al., 1981). Meanwhile, several amino acids are involved in the biosynthesis of many important secondary metabolites, such as phenylalanine, glutathione and leucine (Hashizume et al., 2001; Thibon et al., 2016; Guan et al., 2017). In greenhouse-grown grape, higher temperature (+8°C) applied to the bunch zone during veraison or ripening stages significantly increased 7 amino acids (Thr, Arg, The, Cys, Lys and GABA) (Lecourieux et al., 2017). In field-grown Riesling grape, leaf removal associated with higher temperature negatively affected the accumulation of amino acids (Friedel et al., 2015). However, Pieri et al (2016) found that south-exposed Merlot berries in actual vineyard conditions with higher temperature (+5°C) and solar radiation had similar amino acid concentrations, compared with north-exposed berries.

Fruit-derived flavours are important contributors to wine typicity. Most of them present in grape as odourless precursors and are released during alcohol fermentation and aging process, such as monoterpenes, thiol and C₁₃-norisoprenoids (Sefton et al., 1994; Pinu et al., 2012). Among them, the C₁₃-norisoprenoids are responsible for several kinds of aromas in wine depending on their chemical structures and C₁₃-norisoprenoids are widely identified in wine (Darriet et al., 2012) (Table 1). TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) is considered to be responsible of petrol/kerosene-like aroma in Riesling wine (Black et al., 2012). TPB (1-(2,3,6-trimethylphenyl)buta-1,3-diene) gives aroma of geranium leaf and is more concentrated in white wine than in red wine (Cox et al., 2005). β -damascenone and β -

ionone have been identified as “floral” aromas and widely exist in red and white wines (Ferreira et al., 2000; Gomez-Miguez et al., 2007; Ristic et al., 2010; Darriet et al., 2012).

Table 1: Characteristics of important C₁₃-norisoprenoids in grapes and wines. (Darriet et al., 2012)

Name	Structure	descriptors	detection threshold (ng/L)	Concentration range in wine (ng/L)
β-damascenone		apple sauce rose	60/2 ^a	100-2500
β-ionone		violet raspberry	800/120	nd-2415
TDN		kerosene petrol	20000/3000	nd-30000
TPB		geranium leaf	24/20	nd-120

^a Detection threshold in model solution and water, respectively.

nd: no detected.

C₁₃-norisoprenoids are thought to be formed as biodegradation products of carotenoids, which are C₄₀ tetraterpenoid compounds with an important role in biochemical hormone regulation and the formation of numerous biologically active cleavage products such as aroma compounds, vitamins and phytohormones (Choudhury and Behera, 2001; Winterhalter and Rouseff, 2002). Of all the carotenoids detected in grapes, β-carotene and lutein are present in the highest concentration representing nearly 80% of the total carotenoids, with the xanthophylls neoxanthin, violaxanthin, zeaxanthin and flavoxanthin making up the bulk of the remainder (Mendes-Pinto, 2009). These carotenoids are usually located in chloroplasts and can be found in a wide range of fruits and vegetables such as mango and tomato (Medlicott et al., 1986; Dumas et al., 2003). The biosynthetic pathway proposed for the formation of C₁₃-norisoprenoids from carotenoids combines three steps (Figure 2). Initially, the bio-oxidative cleavage of the carotenoid gives rise to primary degradation products, followed by enzymatic conversion to the aroma precursor involving glycosylation (catalysed by glycosyltransferases) of those norisoprenoids with a hydroxyl

group. At harvest, C₁₃-norisoprenoids are present in the grape as non-volatile glycosidically bound precursors. The aglycones are released from the carbohydrate moiety (mono- and disaccharide) during maturation, storage, processing or aging through the action of enzymes, acid, or heat and undergo further transformation and rearrangement to result in the final aroma-active compound (Figure 1) (Marais et al., 1992; Winterhalter and Rouseff, 2002; Mendes-Pinto, 2009; Winterhalter and Gök, 2013).

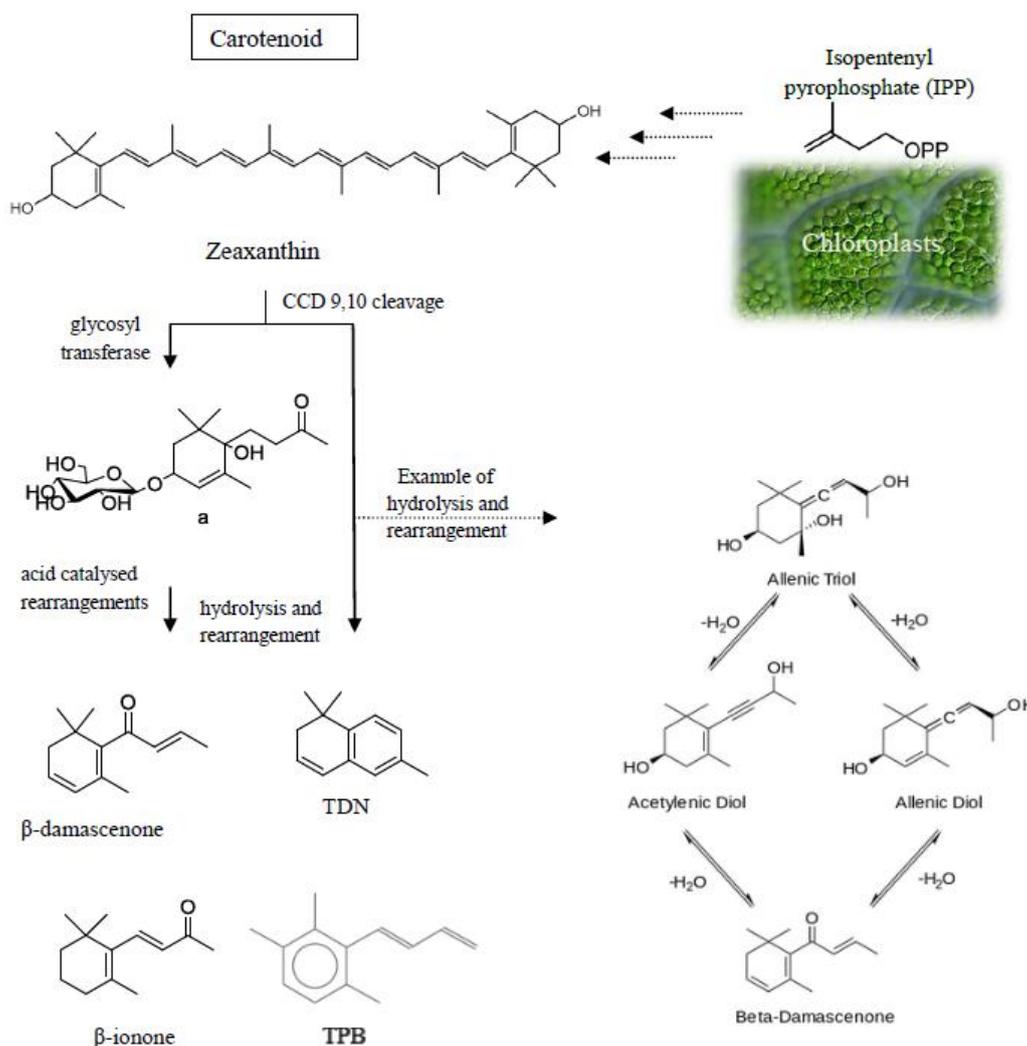


Figure 1: Putative pathway for the formation of important C₁₃-norisoprenoids, in detail for β-damascenone (Mendes-Pinto, 2009; Winterhalter and Gök, 2013).

(a): 3,6-dihydroxymegastigm-4-en-9-one 3-O-glucoside.

A linear relationship between the concentration of carotenoids and C₁₃-norisoprenoids was observed under various environmental conditions (Bindon et al., 2007; Kwasniewski et al.,

2010). Temperature is known to affect the carotenoids concentration in many plants. For example, current-year needles of Douglas-Fir seedlings had a higher carotenoid concentration at elevated temperature (Ormrod et al., 1999), but total carotenoid content in Cavendish banana peels remained constant under different thermal conditions (Thomas and Janave, 1992). More detailly, Tran and Raymundo observed that α -carotene, α -cryptoxanthin, β -cryptoxanthin, zeaxanthin and violaxanthin in bitter melon was not significantly affected at high temperature while β -carotene and the total carotenoids increased (Tran and Raymundo, 1999). In grape berries, investigating the effect of temperature on carotenoids and C₁₃-norisoprenoids was always associated with alteration of light conditions. In light exposure Sauvignon Blanc berries, total carotenoids concentration were significantly lower, but total norisoprenoids concentrations were higher (Young et al., 2016). Moreover, higher concentration of C₁₃-norisoprenoidic glycosides was found under light exposure conditions in Syrah and Pinot noir (Bureau et al., 2000; Young et al., 2016). Conversely, Marais et al (1992a) found a higher TDN concentration but an unchangeable β -damascenone concentration in light exposed berries of Riesling and Chenin Blanc. Besides, higher altitude combined with lower temperature resulted in a higher carotenoids concentration in berries in Dauro Valley (Oliveira et al., 2004), but Falcão et al (2007) found that no significant relation was observed between α - and β -ionone and β -damascenone in wine with the vineyard's altitude in southern Brazil.

In summary, most studies mentioned above either confounded with other environmental factors or focused on abrupt thermal-shock stress; therefore, the obtained results have limitations to extrapolate the effects of elevated temperature on berry compositions in the climate change context. Whether a modest but long-term temperature elevation influences the accumulation and composition of free amino acids, carotenoids and C₁₃-norisoprenoids remains an open question. In 2009, Sadras and Soar created a new and inexpensive open system to increase temperature in realistic vineyard conditions, a passive open-top heating system. This system gently increased maximum bunch temperature by 2.3-3.2°C and maintained daily temperature cycle with little effect on other climate factors, therefore providing a suitable way to investigate the effects of future temperature elevation.

Using the open-top heating system in realistic vineyard conditions, the current study aimed to explore the effect of a modest but long-term temperature elevation on free amino acids, carotenoids and hydrolytically derived C₁₃-norisoprenoids in Cabernet Sauvignon, one of the most cultivated varieties worldwide, including the South Australia region.

V.2 Material and methods

V.2.1 Site and vines

Experiments were conducted in the 2015-2016 growing season with *V. vinifera* cv. Cabernet Sauvignon (CS) at Nuriootpa Research Station in the Barossa Valley of the South Australian Research and Development Institute (34°S, 139°E). Gladstones (1992) and Dry and Coombe (2004) described the climate and viticultural practices of the region. Vines were own-rooted and planted in 1989. They are arranged with a row by vine spacing of 3.50-2.25 m; cordon trained to a single-wire trellis, have been spur-pruned and managed with irrigation since planting using a single 4 L/h dripper per vine.

V.2.2 Heating system and measurements

The passive open-top heating system (Figure 2) was used to increase temperature from fruit setting to over-maturity (two weeks after harvest). The system, as described in Sadras and Soar (2009), consists of modular rectangular polycarbonate units (158 cm high×151 cm wide) each supported by a pair of fold-out legs (870 mm tall) hinged 125 mm below the top of the panel face. The frame was made from 25 mm square tube steel (Stratco, Australia) and the unit face was made from solid Standard-Clear-Greca polycarbonate sheeting fastened to the steel frame (Suntuf, Australia). Consecutive units were fastened together during vineyard installation using plastic “zip” cable ties and each unit was independently anchored to the ground using 30 cm pegs.

The treatment was conducted with four replicates of five vines each. To avoid boundary effects, measurements and sample collections were made only in the central three vines within each treatment. Ambient temperature and relative humidity at bunch height were

recorded at 15 min intervals using TinyTag Ultra2 loggers (Hastings Dataloggers, Port Macquarie, Australia) which were shielded in Stevenson-type screens. Vapour pressure deficit was calculated as a function of temperature and relative humidity.

One hundred berries were randomly sampled from each replicate at five different harvest stages: bunch closure (BC), mid-veraison (MV), mid-ripening (MR), ripe (R) and post-ripe (PR). All samples were ground into powder in liquid nitrogen using an Ultra-Turrax T 25 (IKA Labortechnik, Staufen, Germany). The fine powders were stored at -80°C for free analysis of amino acids, carotenoids and β -damascenone.



Figure 2 : Passive open-top system for increasing temperature in vineyard.

V.2.3 Total soluble solids, titratable acidity and pH measurement

Additional fifty berries were taken on each sampling date for sugar and acids analysis. Berries were weighted, and then crushed with a manual press. The free-run juice was decanted into a 50-mL centrifuge tube and spun at 2000 \times g for 10 min. Total soluble solids (TSS), titratable acidity (TA) and pH were measured on the clarified juice sample using a digital refractometer (HI 96801, HANNA, Woonsocket, Rhode Island, USA) and an autotitrator (CRISON, Barcelona, Spain) for TA and pH.

V.2.4 Extraction and analysis of free amino acids

Quantification of amino acids was performed using a derivatisation technique with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by liquid chromatography (LC) tandem mass spectrometry (MS/MS) in ESI positive ionization mode, adapted from Bonghton et al (2011). Sample preparation involved weighing 50mg freeze dried powder of whole berries dissolved in 0.5 mL de-ionized water and vortexed in 1 min. the centrifugal supernatants were diluted 1:100 with Milli-Q water to be in the appropriate concentration range. An Agilent 1290 Infinity LC system coupled with Agilent 6490 triple quadrupole LC/MS system with iFunnel technology (Forest Hill, VIC, Australia) were used for qualify and quantify for free amino acids. Calibration standard was prepared with 12 calibrant levels, ranging from 0.0005 μM to 200 μM . 1-Valine- $^{13}\text{C}_5$, ^{15}N was used as internal standard (250 μM). The LC-MS conditions were as follows: 1 μL of sample was injected at a flow rate of 0.8 mL/min. Solvent A was 0.1% formic acid in Milli-Q-Water, and solvent B was 0.1% formic acid in acetonitrile. Separation was achieved using an Agilent Zorbax Eclipse Plus C18 RRHD (2.1 \times 100 mm 1.8 μm , Forest Hill, VIC, Australia) column, held at 60 $^\circ\text{C}$. The solvent ramp was as follows: 0 min to 0.5 min (1% B), 3.5 min (10% B), 6 min (15% B), 6.5 min (20% B), 6.6 min to 7.5 min (75% B), 7.6 min to 10 min (1% B). The MS conditions were as follows: gas temperature 315 $^\circ\text{C}$, gas flow 14 L/min, nebulizer pressure 40 psi, sheath gas heater 400 $^\circ\text{C}$, sheath gas flow 11 L/min, capillary voltage 3800 V, nozzle voltage 1500 V, start time 0 min, with dynamic multiple reaction monitoring scan type.

V.2.5 Extraction and analysis of carotenoids

The extraction procedure for carotenoids was adapted from Bindon et al. (2007) and Pop et al. (2014). Total carotenoids were extracted from 10.0 g finely ground powder of whole berries two times using 10ml methanol/ethyl acetate/petroleum benzine (1:1:1, v/v/v) containing 0.2% (w/v) butylated hydroxytoluene (BHT) as an anti-oxidant in the dark for 10 mins on an orbital shaker. The two upper organic phases were combined and spiked with a known amount of β -apo-8'-carotenal (Sigma-Aldrich, Germany) as an internal standard. The extracts were dried with around 1.5 g of anhydrous magnesium sulphate, and then

evaporated using a vacuum rotary evaporator. The extract was dissolved in 1 mL of methanol/ Methyl tert-butyl ether (MTBE) (1:1, v/v) and filtered through a 0.45 μm PTFE-filter into amber glass vials.

Carotenoids were identified and quantified using the HPLC system Agilent 1260 Infinity. Samples were injected onto a C30 reversed phase column YMC carotenoid (250 mm x 2.1 mm i.d., 3 μm particle size). Total run time was 60 min with a flow rate of 0.3 mL \cdot min⁻¹, and a column temperature of 25 °C. The injection volume was 10 μL . Mobile phases consisted of methanol/ MTBE /water 93:5:2 (v/v/v) for solvent A, and 8:90:2 (v/v/v) for solvent B. The following elution profile was used: isocratic at 100% A for 2.5 min, from 100% to 40% A in 37.5 min, from 40% to 0% A in 1 min, isocratic at 0% A for 9 min, back to 100% A in 0.5 min, and for re-equilibration isocratic at 100% A for 9.5 min. Individual carotenoids were quantified by in-line UV/Vis at 450nm and additional UV/Vis were recorded in the range from 200 to 640 nm. The calibration curve for pure β -carotene, lutein, and zeaxanthin were prepared. Remaining carotenoids were quantified using the calibration of lutein.

V.2.6 Extraction and analysis of total C₁₃-norisoprenoids

The analysis of total C₁₃-norisoprenoids was based on a solid-phase extraction (SPE) protocol described by Kwasniewski et al. (2010). 20 g of frozen powder was thawed at room temperature, and then centrifuged and filtered through Whatman filter paper to get 10 mL juice. Juice was spiked with 20 μL deuterated standard mix solution (d_4 - β -Damascenone, d_3 - β -ionone and d_8 -Naphthalene with a concentration of 10.0 mg/L in ethanol) and hydrolysed for 60 min in a 25 mL sealed pressure vessel at 100 °C at pH 1 adjusted by 2 M HCl. Due to the formation of a haze after cooling, juice samples were re-filtered through Whatman filter paper prior to loading onto preconditioned LiChrolut EN SPE cartridges (Merck, Germany), preconditioned with dichloromethane (5 mL), methanol (5 mL) and Milli-Q water (10 mL). Following sample loading, cartridges were rinsed with Milli-Q water (4 mL) to eliminate sugars, acids and other water-soluble compounds prior to elution of the analytes with dichloromethane (4 mL). The eluent was concentrated under N₂

gas using a TurboVap® LV evaporator (Zymark) at 40 °C to a final volume of approximately 100 µL. Instrument parameters, acquisition ions and retention times were described in tables 2 and 3.

Table 2: GC setting for total C₁₃-norisoprenoids analysis

Instrument	Agilent 7890 series gas chromatograph with Agilent 5975C mass spectrometer
GC Column	VF-WAX column, 30 m x 250 µm ID x 0.25 µm FT (Agilent J&W GC columns, CP9205)
Carrier gas	Helium, constant flow, 1.2 mL/min
Syringe	10 µL – needle: 0.64mm OD, 52.2 mm length, conical tip type C, Gerstel Art. Nr. 009970-024-00
Injection	Splitless, 2 µL (volume)
Inlet temperature	200 °C
MS transfer line	250 °C
Oven program	60 °C held for 1 min then 10 °C/min to 250 °C for 10 min
Injection pulse pressure	34 psi until 0.5 min
Purge flow to split vent	50 mL/min at 0.6 min
Gas saver	OFF
Solvent delay	5 min
Run time	30 min
Acquisition Mode	scan m/z 40–220, SIM listed in Table 3
Data acquisition	MSD ChemStation Data Analysis Application (Agilent Technologies, USA)

Table 3: MS Acquisition ions and retention times of analytes under method conditions for total C₁₃-norisoprenoids analysis – SIM parameters

Analyte	Target Ion	Qualifier Ion I	Qualifier Ion II	RT (mins)
TDN	142	157	172	15.5
d ₈ -naphthalene	136	108	68	15.5
β-damascenone	190	175	69	16.26
d ₄ -damascenone	194	179	73	16.00
β-Ionone	177	192	178	17.35
d ₃ -ionone	180	195	181	17.32

RT: retention time

V.3 Results

V.3.1 Heating system performance

The seasonal dynamics of maximal, average and minimal bunch zone temperatures in control (Figure 3a) and the elevation of temperature in treatment (Figure 3b) were evaluated. Over the growing season, the heating system increased the maximal temperature at bunch zone by 1.23 ± 0.64 °C and the minimal temperature by 0.28 ± 0.27 °C (Figure 4). The mean temperature of treated plots was 0.6 ± 0.29 °C higher than in the control (Figure 4).

Under prevailing conditions of this experiment, the heating system maintained the daily cycle of temperature and relative humidity with a very slightly increase of vapor pressure deficit (VPD) (Figure 5). Considering vineyard was drop-irrigated during the whole season, stem water potential was randomly measured two times during berry development and no difference between control and heated vines (data not shown).

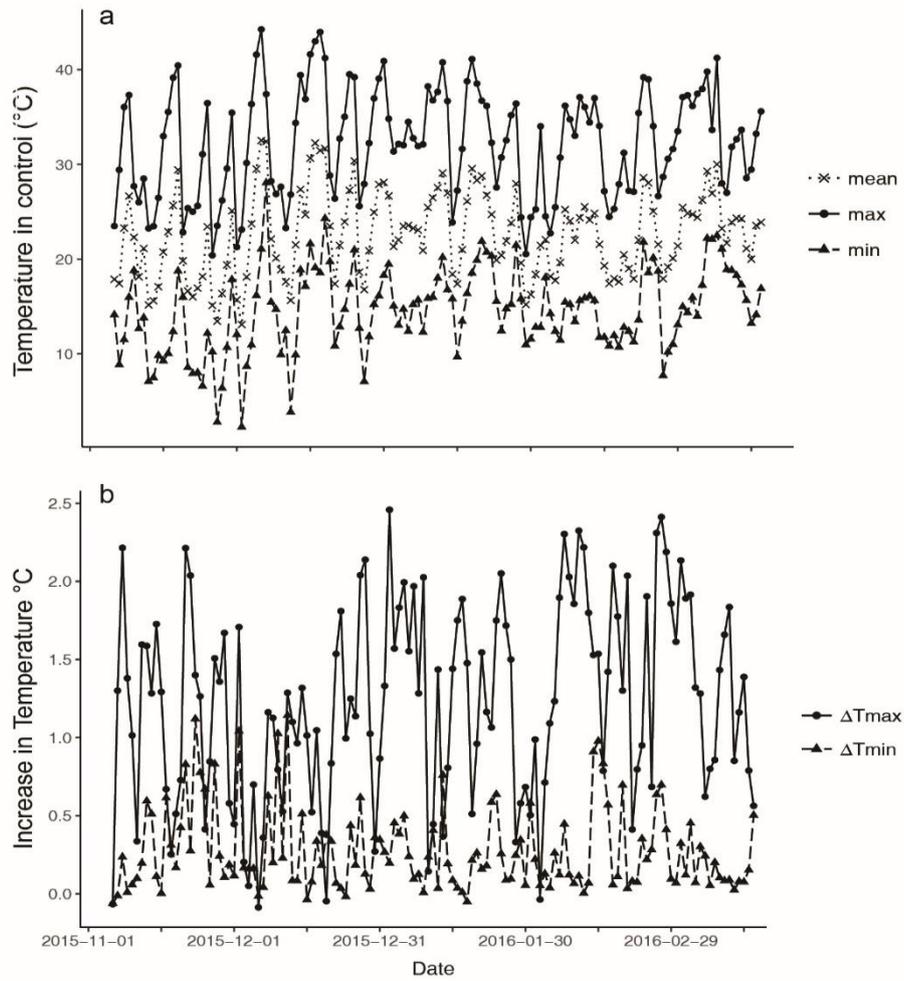


Figure 3: (a) Seasonal dynamics of maximal, average and minimal bunch zone air temperature in controls. (b) Differences of maximum and minimum temperature between heated (H) and control vines (C).

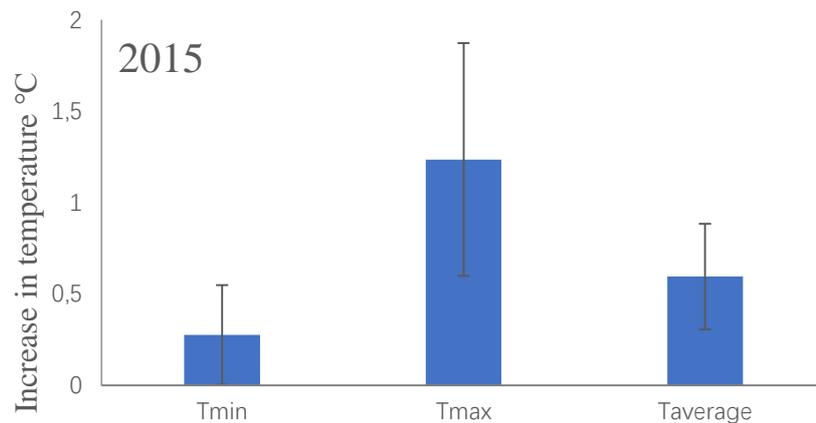


Figure 4: Increment of minimal, maximal and average air temperature in bunch zone area during berry development in season 2015-2016.

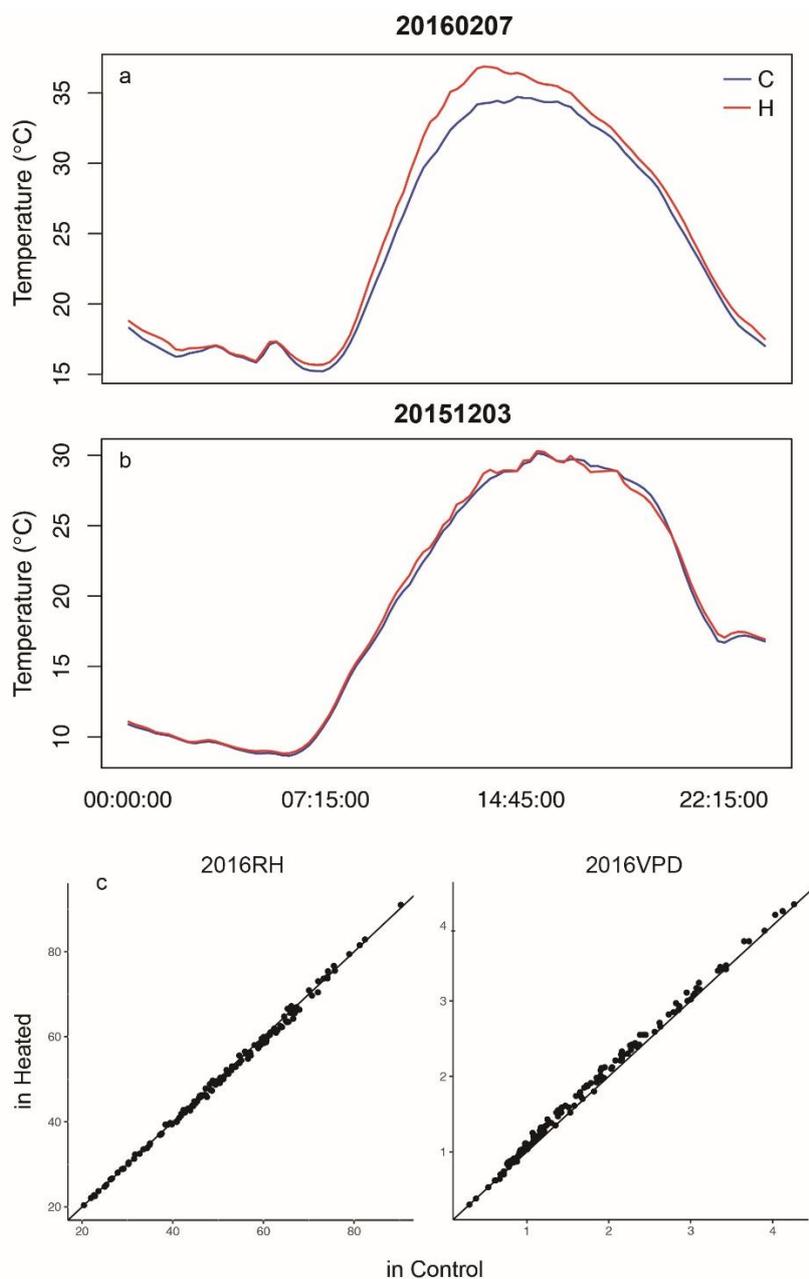


Figure 5: Daily temperature dynamics of representative sunny days (a) and cloudy days in each year (b). Regression of relative humidity (RH) (c) and vapour pressure deficit (VPD) (d) between control and heated.

The solid line is the 1:1 line.

V.3.2 Response of technical maturity to elevated temperature

Berry weight, total soluble solids (TSS), pH and titratable acidity (TA) were determined in berries for assessing whether elevated temperature affected these berry ripeness parameters (Table 4). CS vine maintained yield at around 7-8 kg/vine in response to elevated

temperature. At harvest, the heated berries had a significantly lower pH than the control. At PR stage, heated berry weight was statistically higher and other parameters did not show any differences.

Table 4: Influence of elevated temperature on berry weight, yield, total soluble solids (TSS), titratable acidity (TA) and pH at harvest and post-ripen stages in Cabernet Sauvignon berries in the Barossa Valley of Australia

Stage	Treatment	Berry weight (g)	Yield (kg/vine)	TSS (°Brix)	TA (g/L)	pH
Ripen	control	0.66 ^a	7.26 ^a	25.13 ^a	7.34 ^a	3.22 ^a
	Heated	0.68 ^a	7.99 ^a	24.95 ^a	7.98 ^a	3.13 ^b
Post-ripen	control	0.68 ^a	nd	25.35 ^a	6.97 ^a	3.45 ^a
	Heated	0.74 ^b	nd	25.1 ^a	6.54 ^a	3.55 ^a

Values are mean of four replicates. Different letters within the same parameter indicate statistically significant differences between heated and control berries as determined by Student's t test (P value < 0.05). FW for fresh weight; nd for non-detect.

V.3.3 Developmental changes of amino acid concentration in response to elevated temperature

The LC/MS method allowed us to identify and quantify twenty-two free amino acids in the berries. The total amino acid concentration was calculated by summing all 22 amino acids and dividing by berry fresh weight. Total amino acid concentration had the similar developmental profile between control and heated conditions, maintaining at low level between BC and MV and increasing continuously thereafter (Figure 6). The elevated temperature consistently increased total amino acid concentration at all stages, although significant differences were only found at BC and MR stages. At harvest, elevated temperature did not affect total amino acid concentration (Figure 6).

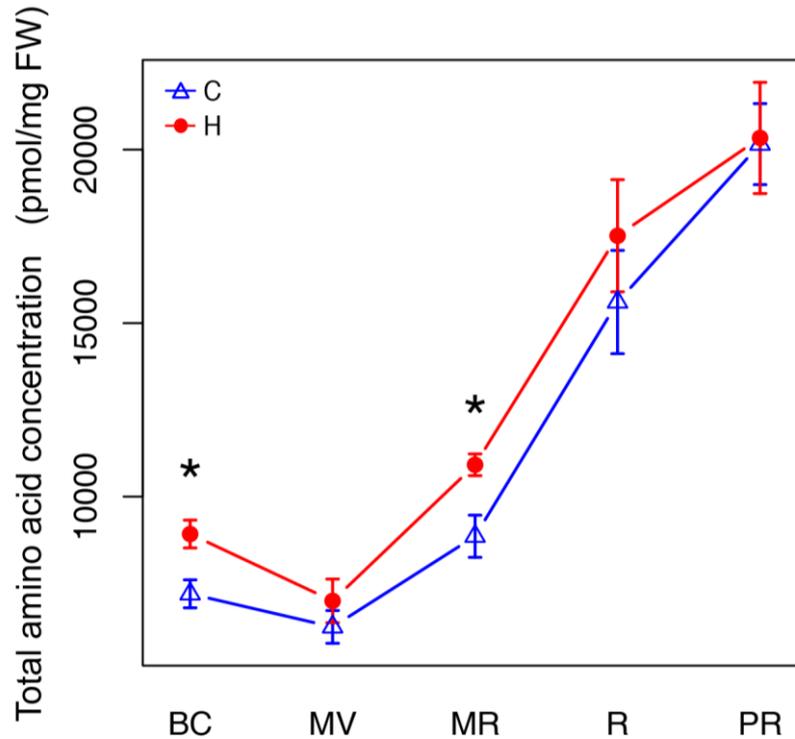


Figure 6: Seasonal variations of total amino acid concentrations in Cabernet Sauvignon under heated and control conditions.

Error bars indicate standard error. Each point is the mean of four replicates. * Significantly different from control (independent t-test, $p < 0.05$)

According to their biosynthetic pathway, the 22 amino acids are separated into six groups based on their metabolic precursor: glutamate family (Glu, Gln, Arg, Pro and GABA), aspartate family (Lys, Asp, Asn, Met, Thr and Ileu), serine family (GSH, Ser, Gly and Cys), Pyruvate family (Ala, Leu and Val), aromatic amino acid family (Trp, Tyr and Phe) and histidine family (only His) (Figure 8). The proportion of glutamate family increased continuously during berry development and was the highest proportion (56.9-89.8%) (Figure 7e). The proportion of serine family decreased gradually during the whole season from 5% to 1.5%. The rest four families increased till MV then decreased to lower or similar levels comparing with initial levels. Only the proportion of histidine family was significantly increased by the elevated temperature after veraison, the proportion of other amino acid families was conserved between heated and control berries during berry development (Figure 7).

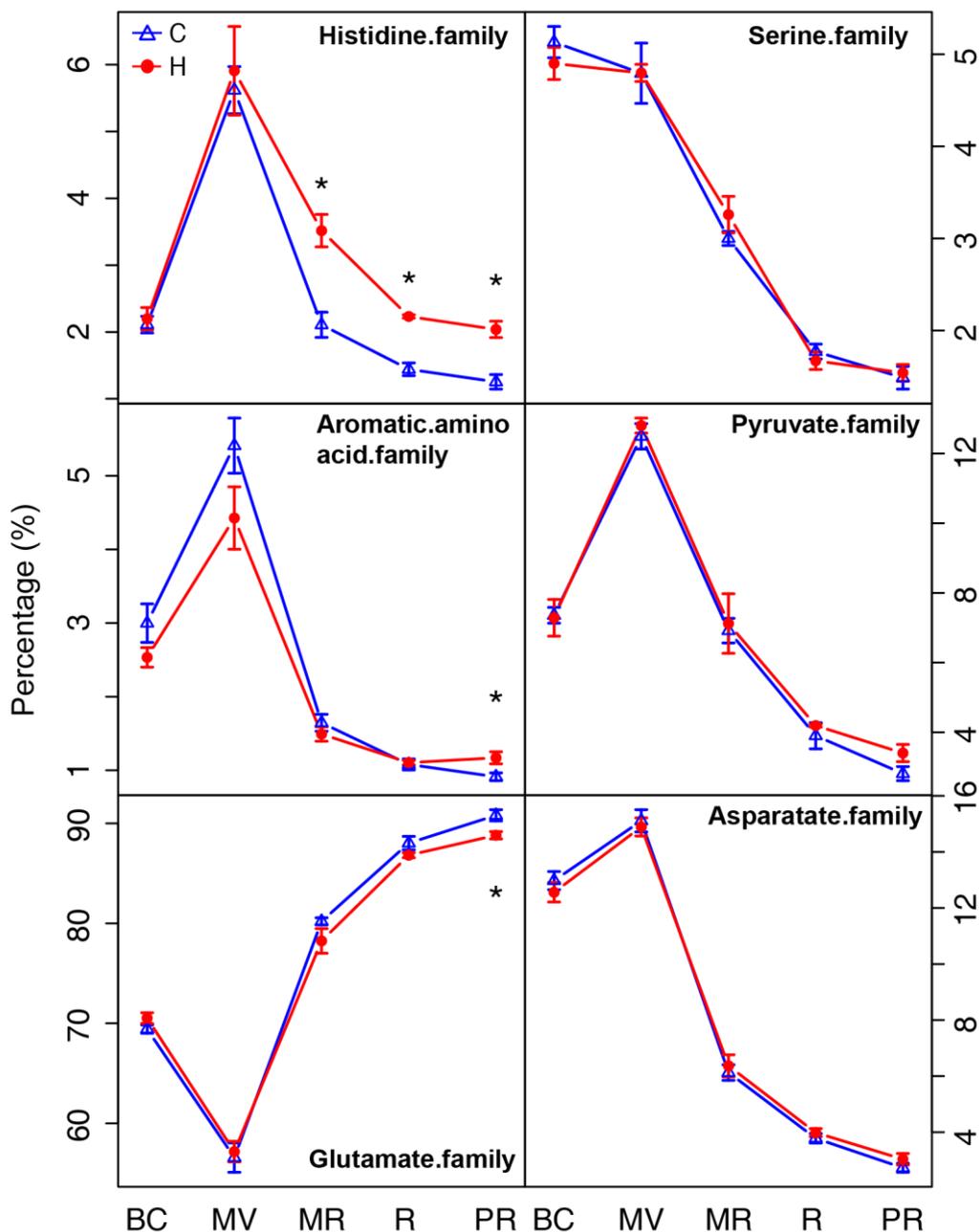


Figure 7: Seasonal variations of the proportion of six amino acid families in Cabernet Sauvignon berries under heated or control conditions.

Error bars indicate standard error. Each point is the mean of four replicates. * Significantly different from control (independent t-test, $p < 0.05$)

The dynamic changes of each amino acid are presented in detail according to their position in the biosynthesis pathway (Figure 8). To further clarify the differences between treatment and control of individual amino acid along berry development, heatmaps (Figure 9) were performed on the normalized ratio between heat treatment and control (H/C). Although the elevated temperature mainly showed a positive effect on amino acids during berry

maturation (Figure 9), except Cys and GSH, it did not alter free amino acid accumulation profiles (Figure 8). At the early stage (BC to MV), Arg, Gln and Glu were dominant in CS with a concentration between 800-4000 pmol/mg FW, ranging from 10-40% of total amino acids; then decreased to 200-400 pmol/mg FW at harvest. These three amino acids were more concentrated in heated berries than in control before harvest, but no significant effect of elevated temperature was found at R and PR stages. In contrast, Pro concentration increased sharply from 1% to 80% of total amino acid at harvest with a concentration around 13000 pmol/mg FW, and it was not affected by elevated temperature during berry ripening.

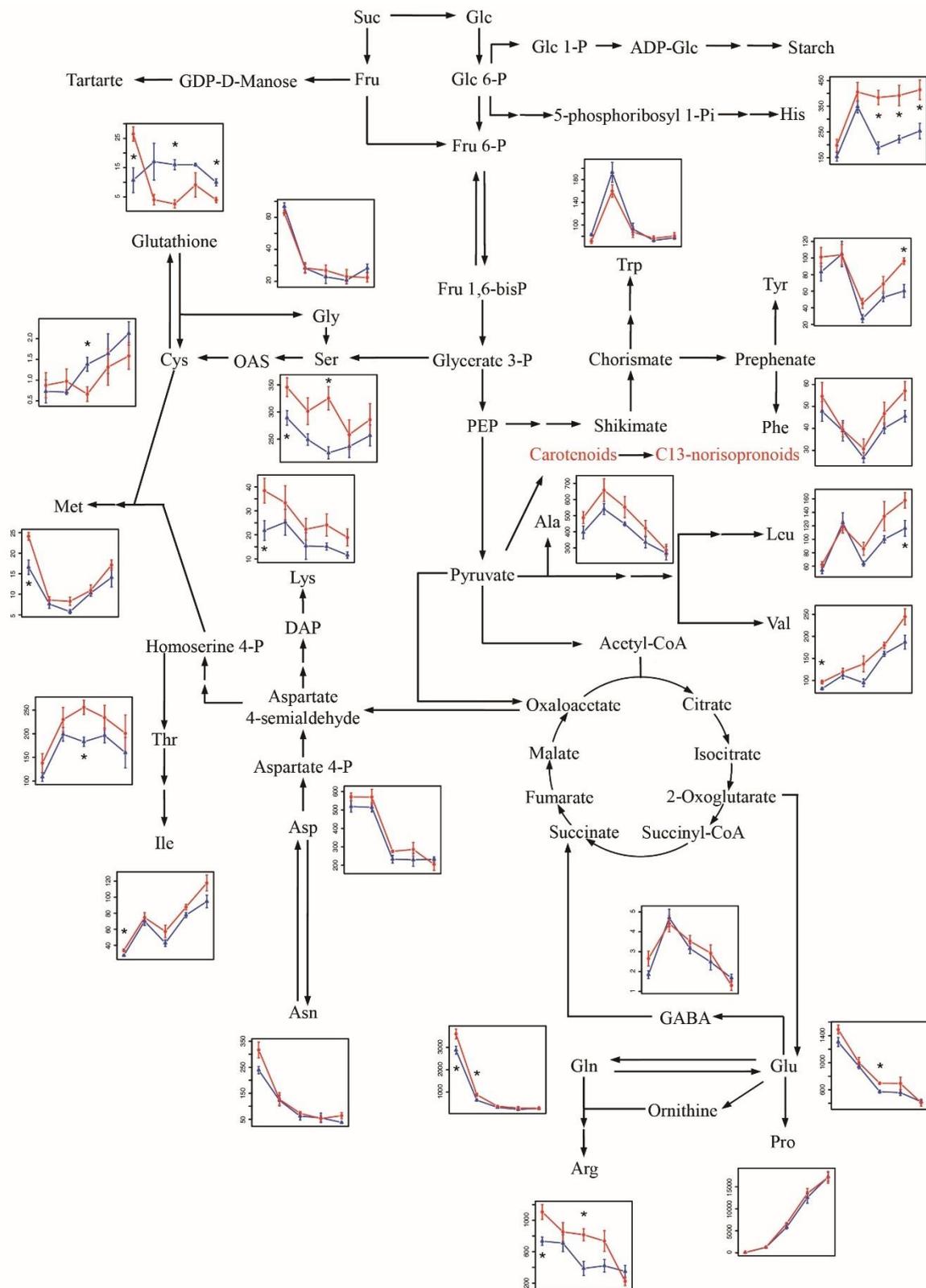


Figure 8: Sugar-amino Acid metabolism pathways. The graphs demonstrate the dynamic changes of each amino acid concentration (pmol/mg FW).

Closed red circles represent heated berries and open blue circles represent control berries. Error bars indicate standard error. Each point is the mean of four replicates. * Significantly different from control (independent t-test, $p < 0.05$)

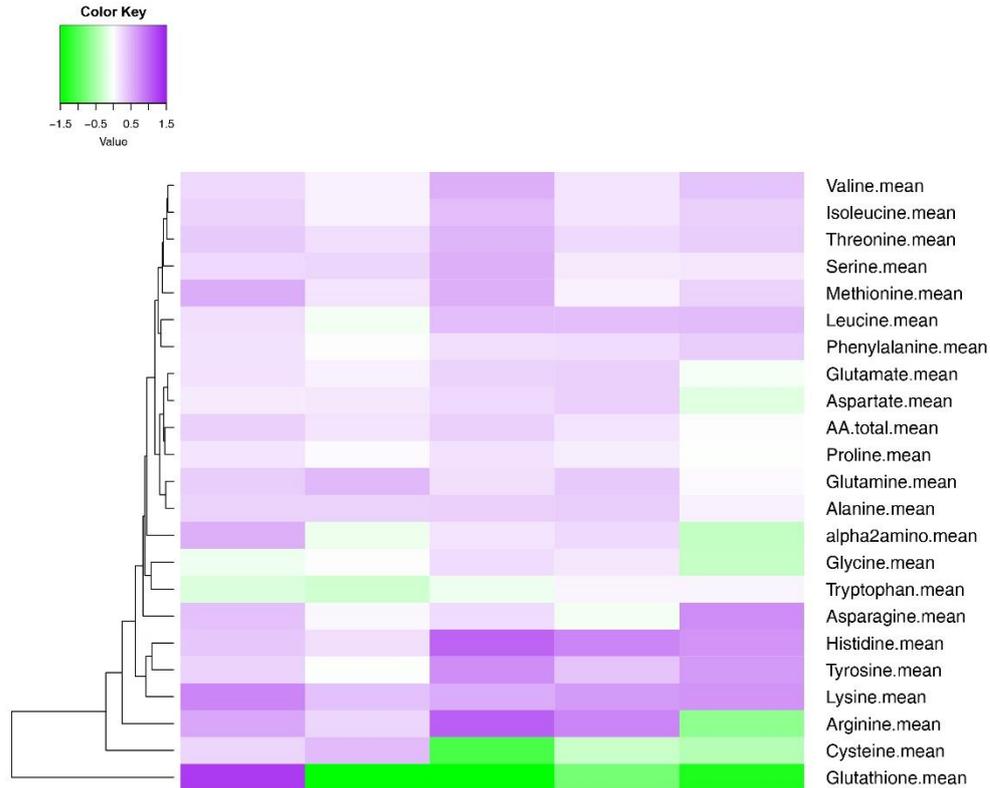


Figure 9: Heatmaps of temperature influence on each amino acid during berry development using the normalized ratio between heated and control berries ($\log_2^{H/C}$) in Cabernet Sauvignon berries.

Each column represents a developmental stage (from left to right: bunch closure to post-ripen stage). The ratios of each compound concentration between treatment and control were represented as false colour with violet for higher concentration in heated berries and green for lower as illustrated in the colour key.

V.3.4 Developmental changes of carotenoids in response to elevated temperature

The HPLC method allowed us to identify and quantify eleven carotenoids in the berries. The total carotenoid concentration was calculated by summing all carotenoids identified and dividing by berry fresh weight (Figure 10). Total carotenoids declined sharply from BC to MR and thereafter declined slowly to PR (from 20 $\mu\text{g/g}$ FW to 5 $\mu\text{g/g}$ FW at harvest). Heated berries had similar concentrations with control berries throughout berry development. Lutein was the most predominant carotenoid which represented 50% of the total carotenoids followed by β -carotene (15%) in berries. Their proportions were

maintained but their concentrations decreased from BC to PR stage. Zeaxanthin, considered as the precursor of C₁₃-norisoprenoids (Deluc et al., 2009), was the third abundant carotenoid (10%) and continuously decreased from BC to PR stage with some fluctuations. Its concentrations were lower in heated berries except for PR stage, but only at R stage the difference was significant. The rest 7 carotenoids: violaxanthin, neoxanthin, neochrome, flavoxanthin, chrysanthemaxanthin, auraxanthin, and luteoxanthin, totally accounted 25% of the total and decreased during berry development without significant differences between heated and control berries (Figure 11).

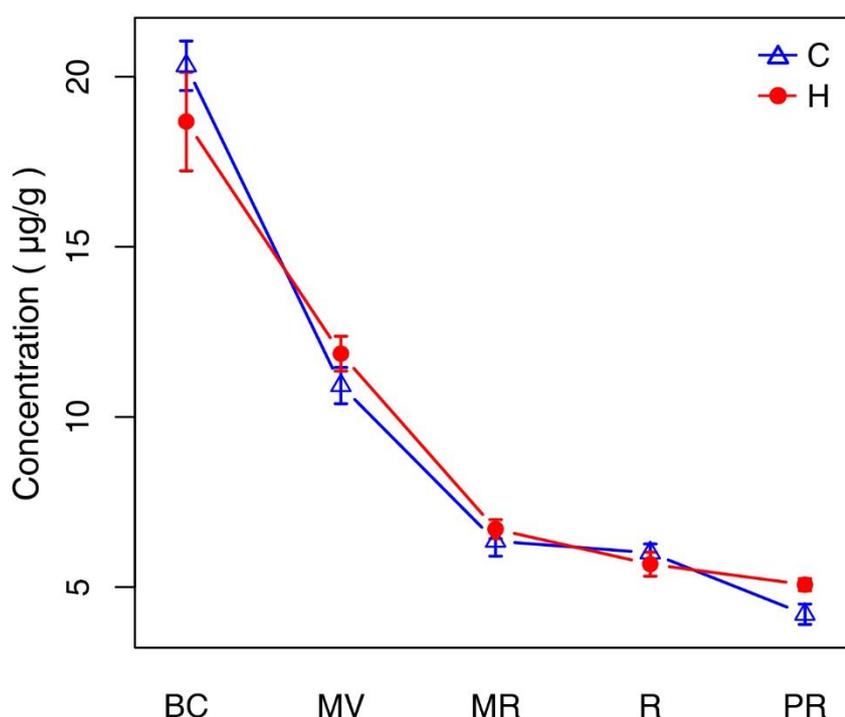


Figure 10: Seasonal variations of total carotenoids concentrations in Cabernet Sauvignon under heated and control conditions.

Closed red circles represent heated berries and open blue circles represent control berries. Error bars indicate standard error. Each point is the mean of four replicates.

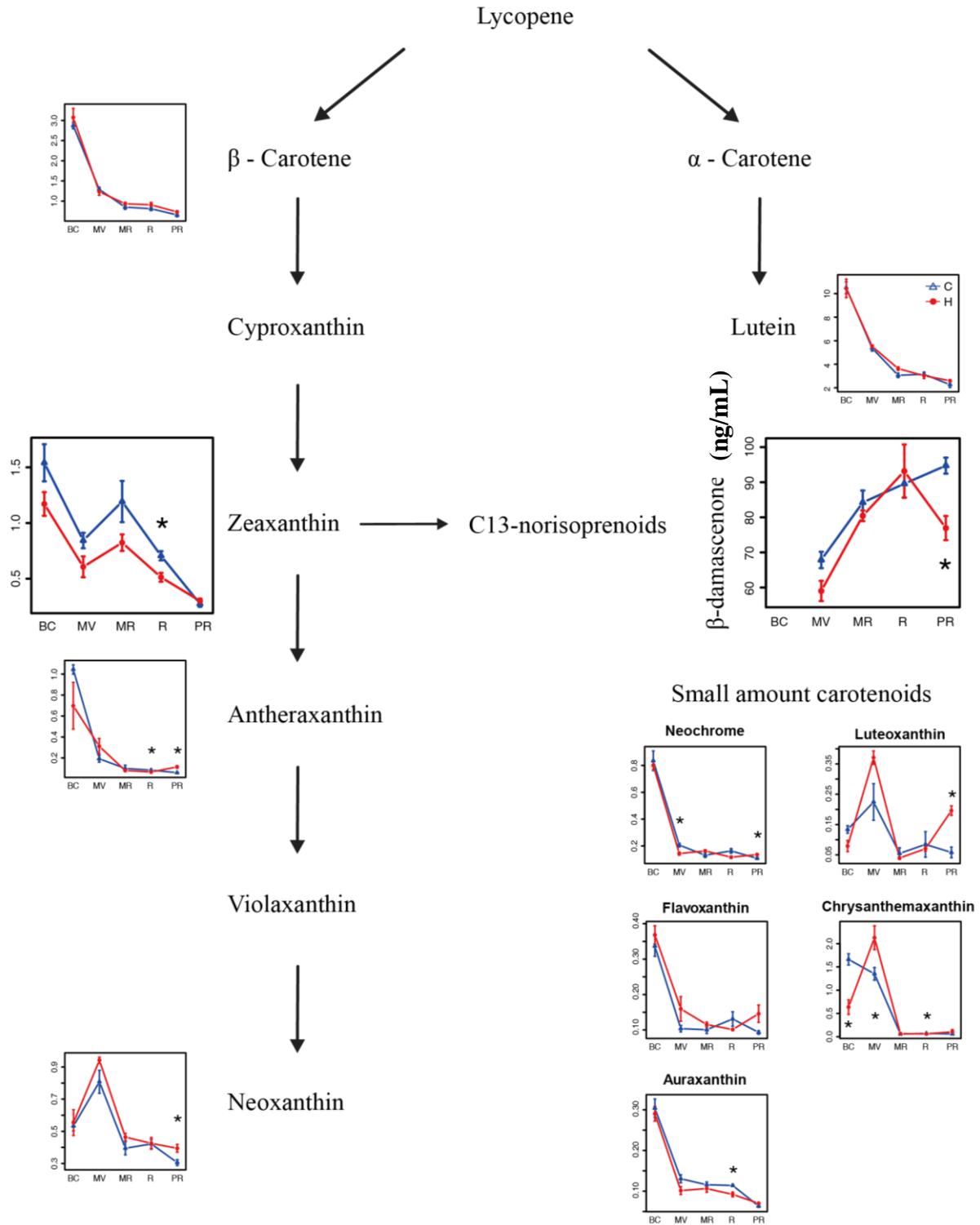


Figure 11: Seasonal variations of individual carotenoid and β -damascenone concentrations in Cabernet Sauvignon under heated or control conditions.

Error bars indicate standard error. Each point is the mean of four replicates. * Significantly different from control (independent t-test, $p < 0.05$)

V.3.5 Developmental changes of C₁₃-norisoprenoids in response to elevated temperature

Based on the GC/MS method for C₁₃-norisoprenoids, β -damascenone, β -ionone and TDN were detected in all samples, but only β -damascenone concentrations were above the limitation of quantification. Because of the limitation of sample quantity, BC samples were not analysed. β -damascenone was accumulated from MV to R stage in both control and heated berries (Figure 11). Meanwhile, during the same developmental period, no significantly different concentrations were observed between control and heated berries. During PR stage, β -damascenone concentration continued increasing to 90 ng/mL (juice) in control berries, but it decreased to 77 ng/mL (juice) in warmed berries, which was significantly lower than that in the control.

V.3.6 PCA analysis

To gain an overview on the effect of elevated temperature on berry biochemical compositions, a principal component analysis (PCA) was performed (Figure 13). Principal component 1 (PC1), which explained the differences between developmental stages, accounted for 52.5% of the variance (figure 13a). It mainly correlated with glutamate and serine family as well as three predominant carotenoids. PC2 (23.3%) almost separated treatments, strongly based on aromatic amino acid family, pyruvate family, His, Met, and several low concentrated carotenoids.

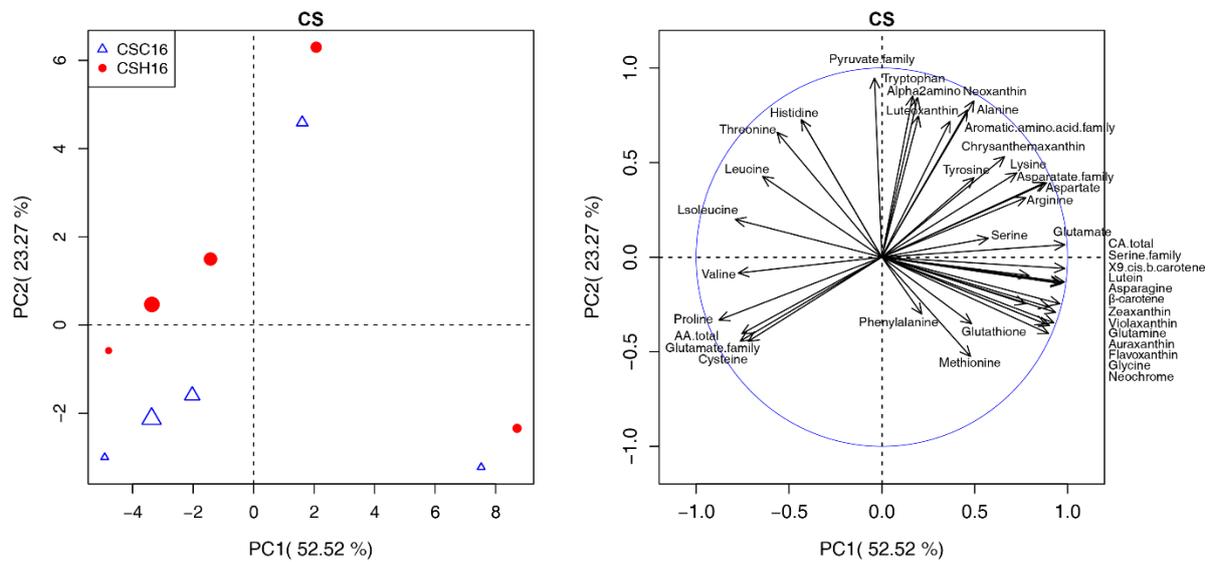


Figure 12: Principal component analysis of amino acid and carotenoid concentrations in Cabernet Sauvignon under heated and control conditions. a discriminate treatment and developmental stages.

Berry size is represented by the size of the symbols. b load plots of amino acid and carotenoid for the first two principal components

V.4 Discussion

V.4.1 Global warming context and heating system performance

In many of the Australian grape-growing regions, projected increase in annual average temperature is 0.4 to 2.6 °C in the next forty years (Webb, 2006). Simulating a similar temperature increase under realistic vineyard conditions can better assess and help understand the potential impact of warming climate on grape berry development and quality. In our study, the open-top heating system achieved to increase mean temperature during berry development by 0.6 °C, which was commensurate with the projected warming for the next decades in Australia. The mean minimal temperature also had an increase of 0.3 °C by using this system, and the relative humidity and VPD were not altered, partially in accordance with the results of Sadras and Soar (2009), which showed an unchanged minimal temperature and relative humidity with a distinct increase of VPD under heated conditions. Although we applied the system as used by Sadras and Soar (2009), the different

performances of the heating system were found between the two studies. The different durations of treatment may explain this difference. Over all, the open-top heating system effectively increased temperature during berry development with little effect on other environmental factors.

V.4.2 Response of technical maturity

Many previous studies found that an increased sugar concentration and a reduction of TA coupled with increase in pH were associated with high temperature (Keller, 2010). However, these studies were often based on indirect methods to modulating temperature by comparison between years and/or regions and therefore may lead the conclusions about elevated temperatures confounded with other environmental factors (Coombe, 1986; Duchêne and Schneider, 2005; Petrie and Sadras, 2008). In our experiment, temperature was not the only but the most predominantly changed environmental factor. Yield, TSS and TA did not response to elevated temperature in CS berries at R stage. This finding matched previous studies which showed undetectable treatment effect on Shiraz berry TSS, TA and vine yield at harvest (Sadras and Soar, 2009; Sadras et al., 2013). Interestingly, although TA was not affected, pH was lower in heated berries at R stage. This suggests that the elevated temperature may have also influenced other components that could influence must pH, such as Na^+ and K^+ (Iland et al., 2011) and further investigations are needed.

For understand more about temperature effect, post-ripening berries were collected after two weeks after standard harvest. Brix continued increasing from R to PR stage and did not show any difference between two thermal regimes. This observation accorded with the results of Mccarty (1999) that Brix was more closely correlated with days after flowering than with temperature summation. This further increase in Brix seems not a result of berry dehydration, because berry weights were higher at PR than those of R stage. On the other hand, the higher berry weight in heated conditions at PR stage may dilute TA and result in similar pH values between the two conditions.

V.4.3 Amino acid response

To our best knowledge, nowadays no published work allows a direct assessment of season long warming effect on free amino acids in grapevine berries under realistic vineyard conditions. The present study showed no significant difference of total amino acid concentrations between control and heated berries at harvest, although they were higher in heated berries than in control during all berry development stage except PR. This is mainly caused by unchanged concentrations of glutamate family, especially Pro under heated conditions, which was the most predominant amino acid family. Similar results were obtained in our experiments in Bordeaux (data in Chapter II) that total amino acid concentrations in CS mature berries was not affected by elevated temperature. Pro accounted almost 80% of total amino acids in mature CS berries, and this consisted with the results obtained in Bordeaux. Moreover, Pro was also confirmed as the most predominant amino acids in CS berries in many other studies (Kliewer, 1968; Stines et al., 2000; Hernández-Orte et al., 2002). Significant differences of Pro concentration in CS berries were observed neither in Adelaide nor in Bordeaux. This high stability of Pro to temperature elevation was also found by Lecourieux et al. (2017), who showed that a short heat treatment (+8 °C) applied in bunch zone at pre-veraison and veraison did not influence Pro concentration in CS berries. This high resistance of Pro to high temperature was also confirmed in other plants, such as in tobacco and Arabidopsis plants (Rizhsky et al., 2004; Dobra et al., 2010).

In the present study, the elevated temperature had a general positive effect on amino acid concentrations. Only GSH and Cys were significantly decreased in heated berries. GSH, a non-protein thiol compound, plays a critical role in protecting organism against oxidative stress (Masella et al., 2005). Tolerance to high temperature stress in crop plants have been reported to be associated with an increase in antioxidant enzymes activity included GSH reductase (Rui et al., 1990; Badiani et al., 1993). Although a 0.6 °C increase of mean temperature could hardly cause an actual heat stress, the high sensitivity to temperature of GSH may explain its significantly lower concentrations in heated berries. Cys, a sulphur-

containing amino acid, involves in dimethyl sulphide synthesis by yeast during alcohol fermentation (Moreira et al., 2002). Its lower concentration may change wine aromas. Overall, free amino acid compositions were altered by elevated temperature. 19 out of 22 identified amino acids had a higher concentration in heated mature berries than in control. Although most of them presented at such a low level, they are involved in the biosynthesis of many important secondary metabolites such as sulfur volatile compounds, flavonoids and anthocyanins (Dai et al., 2014). Further studies are needed to investigate whether those amino acid concentrations could result in alternation of aromas and aroma precursors in berry and wines.

V.4.4 Carotenoids and β -damascenone response

Carotenoids are a heterogeneous group of plant isoprenoids primarily involved in photosynthesis and their degradations lead to the formation of apocarotenoids with functions ranging from phytohormones (i.e. abscisic acid and strigolactone) to volatile aroma compounds (C_{13} -norisoprenoids) (Mendes-Pinto, 2009; Young et al., 2012). During grape berry ripening, the levels of 11 carotenoids and total β -damascenone (the only quantified C_{13} -norisoprenoid in the present study) were found to change in opposite patterns. The carotenoids had the highest concentrations at the earliest stage and decreased gradually throughout berry development, while β -damascenone increased during the whole season. Carotenoids showed the expected decline in concentration during CS berry development, which was also found in different cultivars and also in other plants, such as olives (Razungles et al., 1988; Minguez-Mosquera and Garrido-Fernandez, 1989; Oliveira et al., 2004; Bindon et al., 2007). Many previous studies showed that lutein and β -carotene are two major carotenoids in grapes (Razungles et al., 1987; Razungles et al., 1996; Marais et al., 1999). This observation was also confirmed in the present study. Elevated temperature did not alter total and two major carotenoids in CS berries in our conditions. In literature, the results about carotenoids response to environmental factors were lack of consistency. Higher carotenoids level in Riesling and Chenin Blanc were found in hotter regions (Marais et al., 1991); lower altitude combined with higher temperature resulted in a lower berry

carotenoids concentration in Dauro Valley (Oliveira et al., 2004); different timings of leaf removal in the fruiting zone, which generally increased temperature around berries, did not alter total carotenoids in Riesling at harvest (Kwasniewski et al., 2010); carotenoid contents were consistently higher in grapes exposed to shade than in those exposed to direct sunlight (Oliveira et al., 2004). All studies mentioned above did not have a direct conclusion about temperature effect on carotenoids, because many other environmental factors were also changed. In the present study, temperature can be considered as the most predominantly modulated variable among all environmental factors and there was no significant correlation between elevated temperature and carotenoids concentrations. This may indicate that other factors, such as radiation, soil and leaf-fruit ratio, may play more important roles in carotenoid accumulation than temperature.

Zeaxanthin can be cleaved into C₁₃-norisoprenoids and C₁₄-dialdehyde by a carotenoid cleavage dioxygenase (CCD) (Mathieu et al., 2005; Deluc et al., 2009). Zeaxanthin concentrations were always relatively lower in heated berries. It only showed a significantly lower level at harvest while β -damascenone concentrations had no difference, but a significantly lower concentration of β -damascenone was observed at PR stage. Earlier acceleration of zeaxanthin degradation in heated berries by elevated temperature may have contributed to the later lower total β -damascenone concentration. In the present study, total β -damascenone concentration was not affected by elevated temperature from veraison to harvest. Unaffected total β -damascenone concentrations were also found between different vineyard altitudes (Falcão et al., 2007) and between different light exposures (Marais et al., 1992; Kwasniewski et al., 2010). However, a positive correlation between β -damascenone and the bunch light exposure was observed in Syrah and Pinot noir (Bureau et al., 2000; Young et al., 2016). Combining with our results, the repression of β -damascenone accumulation could be better explained by the limitation of the precursors rather than a direct influence of temperature.

V.5 Conclusion

Bunch zone mean temperature increasing by around 0.6 °C during whole berry development did not alter the yield, TTS and TA in berries of Cabernet Sauvignon vines grown with standard practice in the Barossa Valley. Only a significant lower pH was found in heated berries at harvest. Heating treatment increased total amino acid concentrations during berry development, but no significant difference was observed in mature berries. Although some individual amino acids showed different responses to elevated temperature depending on stages, all individual amino acid concentrations were higher in heated mature berries, except Asp, Cys and Glu. The concentrations of total carotenoids and two most predominant carotenoids: lutein and β -carotene, was not altered by elevated temperature. Zeaxanthin showed a negative correlation with elevated temperature and was significantly less concentrated in heated mature berries than in control. Its lower concentration may limit the further biosynthesis of β -damascenone and explain the observation that lower β -damascenone in post-ripening berries in the present study.

Chapter VI : General conclusions and perspectives

A recent report shows that global annual average temperature has increased by more than 1.2°C since 1900 (through 2012). In the context of global warming, many studies focus on the elevated temperature impacts on viticulture including grapevine phenology, berry development, and even re-distribution of wine grape-growing regions. Although no large-scale change of viticultural regions has been realized to-date, earlier harvest dates and higher potential alcohol levels at harvest associated with increases in temperature were observed worldwide in the past several decades. In this work, both metabolomic and transcriptomic analysis were used to investigate the effects of moderately elevated temperature on berry quality in realistic vineyard conditions.

The experiments were conducted in two different places: Bordeaux, France and Barossa Valley, Australia, which are two of the most reputable and representative wine regions in the world. Meanwhile, they represent two different climatic types (Figure 1). Firstly, Bordeaux is temperate oceanic climate and Barossa valley is classified as hot-summer Mediterranean climate, based on Köppen climate classification (Köppen et al., 2011). Secondly, because of the different precipitations, irrigation is required in Barossa Valley, but it is forbidden in Bordeaux AOC vineyards. Thirdly, all grapevines used in Barossa Valley are own-rooted, but grapevines from Bordeaux are all grafted to rootstocks. The three above-mentioned points are the most noteworthy ones but not the only differences between two regions. Thus, our results may provide wider views on elevated temperature effects on grape berries.

The applied open-top heating system effectively increased the mean bunch zone air temperature by 0.5-1.6 °C. Comparing with the previous temperature-related studies reported in literature, this system has several advantages. First of all, the magnitude of temperature increase was similar with the projected global warming for the next thirty years. Furthermore, the system was applied in the realistic vineyard conditions, from which the results can be better extended to estimate the effect of global warming on berry quality than those studies conducted under artificial environment such as phytotron. Although we are not entirely sure that temperature was the only altered factor in our system, it can be considered as the most predominant independent variable among potential sources of variation.

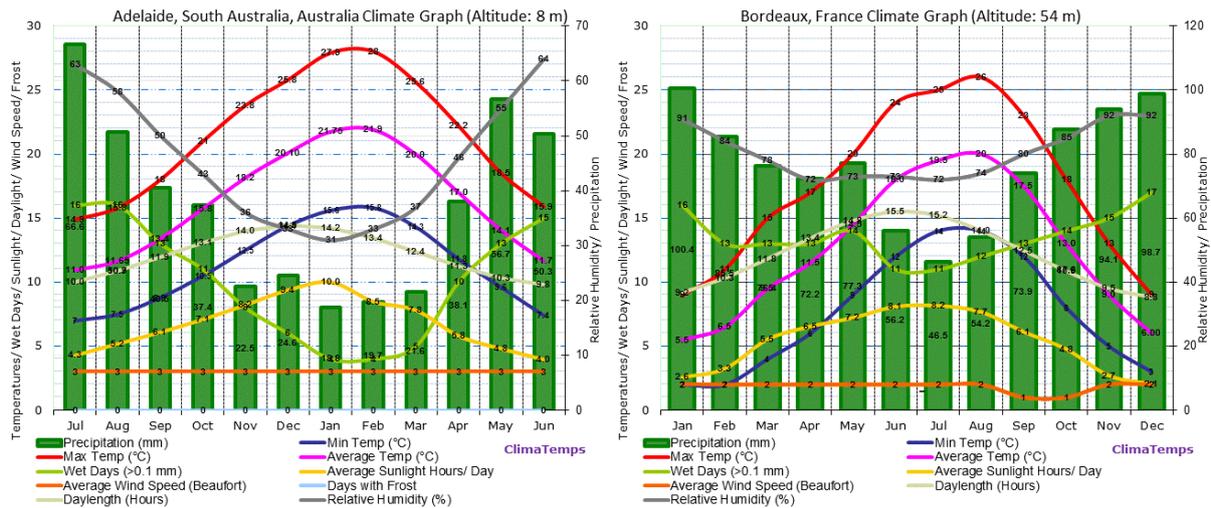


Figure 1: Adelaide (Barossa Valley) and Bordeaux monthly climate graph

(www.climatemprs.com)

The primary and secondary metabolite contents in Cabernet Sauvignon (CS) and Sauvignon Blanc (SB, only in Bordeaux) berries were analyzed at different developmental stages in two vintages in Bordeaux (2015 and 2016) and only one vintage in Barossa Valley (the 2015-2016 growing season). Besides metabolite analysis, q-PCR and RNA-seq analysis were conducted to obtain a comprehensive view of berry responses to moderately elevated temperature.

EFFECT ON PRIMARY METABOLITES

The moderate rise of temperature hardly affected the concentrations of sugars and acids in all vintages, cultivars, and locations. It neither affected the developmental profiles of individual amino acids nor the total amino acid concentration at harvest, except Sauvignon Blanc in 2015 at Bordeaux (higher in control), but it altered free amino acid composition depending on varieties, vintages and locations. Sugars variations depend more on leaves activity than on grape temperature. This small sugar variation may indicate that the heating system had no bias linked to primary metabolism at leaf level.

In the past several decades, higher potential alcohol levels at harvest associated with increases in temperature were observed. Conversely with our results, it may indicate that vintage effects on primary metabolites are more important than the effects of a moderate temperature elevation in the bunch zone, as imposed by our open-top system. Moreover, designing a heating system which could heat all along the year, may better simulate interactions between primary and secondary metabolism. Also, for a clear understanding of the effects of global

warming, long-term multi-year experiments may be more valuable since taking into account the acclimation of grapevines.

EFFECT ON BERRY METABOLITES THROUGH RNA-SEQ

A total of 357 genes were differentially expressed in response to the moderately increased temperature in Bordeaux samples in 2015. Among them, 11 down-regulated DEGs (differentially expressed gene) were shared between CS and SB at Mid-ripe (MR) stage and three of them were also down-regulated at bunch closure (BC) stage in SB berries. Enrichment analysis of Gene Ontology showed that temperature mainly regulated four GO categories, including microtubule, cell wall, extracellular region, and transcription factor activity. To a less extent, four GO terms about metabolic process and one term related to biotic stimulus response were enriched. Thirty DEGs were related to the functional category “Cell wall” and mainly belonged to gene encoding cell wall degrading enzymes. 11 DEGs encoding microtubules were down regulated by elevated temperature mainly at the BC stage. Twenty-four DEGs were related to transcriptional factors and mainly belonged to “ethylene-responsive transcriptional factors” and “Basic leucine zipper/ HomeoDomain leucine zipper transcriptional factors”.

The RNA-seq was only analyzed with the samples from bunch closure and mid-ripening of two varieties in 2015. The interesting candidate genes selected through RNA-seq analysis should be checked by q-PCR in 2016 samples. This will provide more details about gene expression at different vintages and further validate the current results.

EFFECT ON IBMP

The IBMP concentrations in mature berries were not affected by elevated temperature in two vintages and two varieties, which seems to contrast with the conclusion of a negative correlation between thermal increase and final IPMP concentrations from previous studies in literature. The most likely explanation for this contrast is that the previous studies investigated temperature effect on IBMP concentration mostly based on indirect comparisons between vintages and/or regions. These comparisons generally confounded the temperature effects with other environmental factors. Here, interestingly, the elevated temperature significantly reduced IBMP content and expression level of *VviOMT3* in Cabernet Sauvignon berries at bunch closure stage in both vintages, but it reduced the expression level of *VviOMT3* at bunch

closure stage without affecting IBMP concentration in Sauvignon Blanc berries. This limited and genotype-dependent effect of elevated temperature suggests that a moderate temperature elevation (0.5-1.6 °C) may not be sufficient to modify IBMP at maturity, or that other environmental factors (e.g. light) play a dominant role in regulating IBMP rather than temperature. In addition, IBMP concentration in mature berry was higher in 2015 than in 2016 in both varieties, while the mean temperature and thermal degree day accumulation were similar between the two vintages. More detailed analysis showed that 2015 was warmer at pre-veraison but cooler at post-veraison than 2016, which may lead to a lower degradation in 2015 and therefore a higher IBMP concentrations. Therefore, it is most likely that a separate analysis of the effect of pre- and post-veraison temperature conditions on IBMP levels may be more suitable for predicting IBMP concentration in mature berries. Further experiments increasing bunch zone temperature separately pre- and post-veraison should be helpful to verify this hypothesis.

EFFECT ON PENOLIC COMPOUNDS

The phenolic compounds were only analyzed in Cabernet Sauvignon berries in 2015. Significantly reduced anthocyanin concentrations were observed in warming conditions. Based on the RNA-seq results, three anthocyanidin UDP-glycosyltransferase genes and two *O*-methyltransferase gene expressions were repressed at MR stage by heating treatment. This could explain, at least in part, that elevated temperature decreased anthocyanins concentration through down-regulation of related structural genes. Furthermore, the decrease in anthocyanin concentration at high temperature may be due to the down-regulation of genes encoding for transporters mediating the transport of anthocyanins into the vacuole. Here, two MATE efflux proteins were also down-regulated by elevated temperature.

On the other hand, monomer and dimer tannin concentrations increased from BC to MV and thereafter gradually decreased towards maturity. Elevated temperature resulted in a higher monomer and dimer tannin concentrations at MV stage, but showed no effect on the concentration at maturity. Therefore, elevated temperature resulted in a larger decrease (66%) in tannin concentration from MV to maturity than that under control condition (50%). Decreases in tannin concentrations during berry development are generally considered as a result of decrease in tannin extractability rather than degradation or turnover. In warmed berries, a gene (*VIT_05s0062g01160*) encoding for pectinesterase was down-regulated at mi-maturation that may result in a higher proportion of pectic polysaccharides (greater affinity

than other cell wall compositions) in cell wall and, in turn, lead to a lower tannin extractability. This lower tannin extractability at maturity stage in heated berry may counteract the higher tannins observed at MV stage and result in a higher decrease from MV to maturity. An alternative possibility for the higher decrease in tannin monomer and dimer concentrations from MV to maturity might be that more tannin monomers and dimers have been transformed into high tannin polymers in heated berries. These hypotheses deserve further investigation by analysing tannin extractability, tannin polymers, tannin polymerization degree, in combination with analysing key genes involved in tannin biosynthesis.

EFFECT ON 3SH PRECURSORS

3SH is a major aromatic compound in CS and SB wine which has a smell of grapefruit or passion fruit, and is present under its odorless precursor form in berries. Using a direct extraction method of 3SH precursors from frozen powder of whole berries, Glut-3SH-Al was found to be much more concentrated than Glut-3SH and Cys-3SH. Reduced Glut-3SH-Al and Cys-3SH was associated with a significantly lower expression level of *VviGST4* (a proposed key gene for 3SH precursors biosynthesis) in heated Sauvignon Blanc berries in 2015. Meanwhile, similar *VviGST4* expression patterns were confirmed with 2016 samples. Besides that, *VIT_08s0007g01420*, a putative GST homologue, was also down-regulated by elevated temperature and might be a potential candidate gene involved the biosynthesis of precursors of 3SH.

It has to be mentioned that the biosynthetic pathways of 3SH precursors are still far from being fully understood. Glut-3SH-Al has been identified as 3SH precursor recently, its concentration in berries through a direct extraction method should be checked in more varieties to confirm its role in the 3SH metabolism pathway. The function of genes which showed a high correlation with 3SH precursors, such as the novel candidate gene (*VIT_08s0007g01420*), also needs to be verified in more varieties and to be confirmed enzymatically.

EFFECT ON CAROTENOIDS AND β -DAMASCENONE

In Cabernet Sauvignon from Barossa Valley, the concentrations of total carotenoids and two major individual carotenoids, lutein and β -carotene, were not altered by the increased

temperature. In contrast, a minor component of carotenoids, Zeaxanthin, showed a negative trend with elevated temperature and was significantly less concentrated at harvest in heated berries. Moreover, it is considered as the precursor of C₁₃-norisoprenoids, including β -damascenone. This lower concentration may limit the biosynthesis of β -damascenone and explain the observed lower β -damascenone in post-ripen berries under elevated temperature.

In summary, a moderate bunch zone temperature increase in realistic vineyard conditions hardly affected primary metabolite concentrations but altered their compositions. For secondary metabolites, this heating treatment showed different effects:

- i. decreased the initial IBMP concentrations in CS but unaffected the final concentrations in the two varieties;
- ii. increased Glut-3SH-Al concentrations in SB but not in CS;
- iii. decreased total anthocyanins;
- iv. unaffected final tannins concentrations but increased them at mi-veraison;
- v. unaffected total carotenoids and β -damascenone at harvest, but decreased zeaxanthin coupled with lower concentration of β -damascenone at post-harvest.

Overall, our results provide a better understanding of global warming effects on metabolite changes during berry development and gain novel molecular insights into the responses of grape berry in vineyard. Furthermore, our observations may constitute an advantage for the design of adaptive strategies to preserve high quality viticulture in the context of global warming.

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