Grapevine Plasticity in Response to an Altered Microclimate: Sauvignon Blanc Modulates Specific Metabolites in Response to Increased Berry Exposure[1]

Philip R. Young, Hans A. Eyeghe-Bickong, Kari du Plessis, Erik Alexandersson[2], Dan A. Jacobson[3], Zelmari Coetzee[4], Alain Deloire[4], and Melané A. Vivier*

Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

ORCID IDs: 0000-0002-6488-4859 (P.R.Y.); 0000-0002-6822-9157 (H.A.E.-B.); 0000-0001-6320-2492 (E.A.); 0000-0003-2776-955X (Z.C.); 0000-0003-0376-9301 (A.D.); 0000-0001-6656-9353 (M.A.V.).

In this study, the metabolic and physiological impacts of an altered microclimate on quality-associated primary and secondary metabolites in grape (*Vitis vinifera*) 'Sauvignon Blanc' berries was determined in a high-altitude vineyard. The leaf and lateral shoot removal in the bunch zones altered the microclimate by increasing the exposure of the berries. The physical parameters (berry diameter and weight), primary metabolites (sugars and organic acids), as well as bunch temperature and leaf water potential were predominantly not affected by the treatment. The increased exposure led to higher levels of specific carotenoids and volatile terpenoids in the exposed berries, with earlier berry stages reacting distinctly from the later developmental stages. Plastic/nonplastic metabolite responses could be further classified to identify metabolites that were developmentally controlled and/or responded to the treatment in a predictable fashion (assessed over two consecutive vintages). The study demonstrates that grapevine berries exhibit a degree of plasticity within their secondary metabolites and respond physiologically to the increased exposure by increasing metabolites with potential antioxidant activity. Taken together, the data provide evidence that the underlying physiological responses relate to the maintenance of stress pathways by modulating antioxidant molecules in the berries.

[1] This work was supported by the National Research Foundation, the Technology and Human Resources for Industry Programme, and the Wine Industry Network for Expertise and Technology.

[2] Present address: Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Box 102, SE–230 53 Alnarp, Sweden.

[3] Present address: Biosciences Division, Oak Ridge National Laboratory, P.O. Box 2008, MS 6420, Oak Ridge, Tennessee 37831–6420.

[4] Present address: National Wine and Grape Industry Centre, Charles Sturt University, Boorooma Street, Locked Bag 588, Wagga Wagga, New South Wales 2678, Australia.

* Address correspondence to mav@sun.ac.za.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Melané A. Vivier (mav@sun.ac.za).

M.A.V. and A.D. conceptualized the initial study; P.R.Y., E.A., D.A.J., and M.A.V. were involved in the experimental layout; Z.C., P.R.Y., and A.D. implemented and monitored the vineyard and viticultural treatments and maintenance; P.R.Y., E.A., Z.C., and D.A.J. did the field sampling; Z.C. did the climatic calculations and stem water potential measurements; P.R.Y. and Z.C. processed and analyzed the temperature and light data; E.A. and P.R.Y. processed the grape samples; E.A. characterized the berries; P.R.Y. performed the pigment extractions; H.A.E.-B. performed the ultra-high-performance liquid chromatography analysis; P.R.Y. created the pathway visualization; D.A.J. provided statistical and computational support for the study; P.R.Y. and H.A.E.-B. processed the samples for sugar and organic acid extractions and performed the HPLC analysis; K.d.P. performed the RNA extraction and transcriptome analyses; P.R.Y. and M.A.V. drafted the initial article; all authors contributed to discussion of the results, reviewing of the article, and approved the final article.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01775

Vineyards are highly variable environments where the plant must respond to changes within and across seasons. Grapevine (*Vitis vinifera*) berry ripening occurs over months, and the final berry composition is the expression of the interaction between the specific genotype (cultivar) and the environment over time (vintage). The grape and wine industries rely on cultivars and clones that have been purposefully selected and domesticated for thousands of years based on predominantly observable phenotypes (color, flavor/aroma, and/or survival [i.e. resistance to biotic/abiotic stresses]; Terral et al., 2010; Bouby et al., 2013). The genetic basis of these traits obviously underpins a biological function in the plant, but these functions and underlying mechanisms are still relatively poorly studied in grapevine.

The mechanism of phenotypic plasticity, defined as the capacity of a genotype to modulate its phenotypes under variable environmental conditions, is of specific interest in plant physiology. The observed phenotypic variations are due to differential regulation of the expression and/or function of genes involved in so-called plastic traits by the environment (Schlichting, 1986; Schlichting and Smith, 2002; Via and Lande, 2013). Transcriptomic plasticity has been demonstrated previously in grapevine 'Corvina', and candidate genes potentially involved in phenotypic plasticity have been putatively identified (Dal Santo et al., 2013). Those authors demonstrated that specific candidate plastic transcripts were associated with groups of vineyards

(i.e. a single genotype, cv Corvina) sharing common viticulture practices and/or environmental conditions, and plastic transcriptome reprogramming was more intense in the years characterized by extreme weather conditions. In a follow-up study, the variability in the observed metabolic plasticity of cv Corvina berries was illustrated in a comprehensive multiple vintage study (Anesi et al., 2015). Berry metabolites displaying terroir-specific signatures (and not year-to-year/vintage variation) were identified. The metabolites characterizing each of the macrozones included specific stilbenes, flavonoids, and anthocyanins (Anesi et al., 2015).

These studies and results further suggest that human intervention (e.g. via viticultural manipulations) combined with the prevailing environmental condition indelibly affects berry composition through changes in transcription that subsequently affect enzyme activity and/or the kinetics of biochemical reactions in the developing berry. Berry composition is not static and can be differentially modulated, thereby providing scope for human intervention in influencing and directing berry metabolism. Linking specific treatments conclusively to physiological mechanisms and metabolic impacts is required to address the questions of what, how, and, most importantly, why these changes occur to identify their underlying biological relevance.

In viticulture, one of the commonly used industrial practices involves canopy manipulations, such as leaf removal. It is not unique to grapevine and is used in many cultivated fruit crops for a variety of reasons that include (1) balancing vegetative growth and fruit production (crop load; Gordon and Dejong, 2007), (2) facilitating fruit collection (via training/trellising), (3) maximizing light incidence (via trellising/training and/or leaf removal; Stephan et al., 2008), and (4) pest control (by improving air flow and light penetration in the canopy; O'Neill et al., 2009).

Leaf removal has been used for diverse purposes, usually with a predisposed viticultural and/or oenological outcome, for example: (1) crop reduction (via early prebloom leaf removal) in high-yield cultivars (Reynolds and Wardle, 1989; Palliotti et al., 2012); (2) improving the quality of grapes (where quality is defined as acid balance and lower pH juice [via a predominantly higher tartrate content]; Hunter and Visser, 1990; De Toda et al., 2013); (3) decreasing fungal infection (usually *Botrytis* spp.) by improving air flow (in this context, healthy grapes are associated with quality; English et al., 1989; Gubler et al., 1991; Staff et al., 1997); (4) improving the sensory perception of the resultant wines (typically described as a reduction in the perception of the green character in both white [e.g. cv Sauvignon Blanc] and red [e.g. cv Cabernet Sauvignon] wines, or as an increase in tropical attributes [typically in white cultivars, such as cv Sauvignon Blanc]; Staff et al., 1997; Tardaguila et al., 2008; Šuklje et al., 2014); and (5) improving the color stability of wines from red cultivars (Chorti et al., 2010; Sternad Lemut et al., 2011; Lee and Skinkis, 2013). Typically, however, these studies report a vintage effect (i.e. an inconsistent/irreproducible effect and/or unclear results, referred to as slightly significant effects and/or tendencies, between consecutive years of experimentation), or conflicting data are obtained from different cultivars or the same cultivar in different geographical locations (for review, see Kuhn et al., 2014).

Although this specific viticultural treatment is widely used in viticulture, it has not yet conclusively been linked to a physiological mechanism(s) and metabolic impacts in grapevine berries. Our aim with this study was to apply a field-omics workflow (seeking a causal relationship between a viticultural treatment, the microclimate, and metabolic responses at different stages of berry development) to characterize the physiological outcome(s)/mechanisms of a targeted leaf removal in the bunch zone. The principles and benefits of this type of approach are outlined by Alexandersson et al. (2014). The impact of the leaf removal treatment, performed at an early phenological stage, was characterized by quantifying the abiotic (environmental) variables in the bunch zone (i.e. microclimate) in a characterized commercial experimental vineyard. The consequent impact on berry composition was measured by focusing on the primary and secondary metabolites typically associated with quality parameters, namely (1) sugars and organic acids, (2) carotenoids, and (3) volatile terpenoid-derived flavor and aroma compounds (predominantly monoterpenes and norisoprenoids). The results showed that pools of specific metabolites were under comparatively strict developmental control (e.g. sugars, organic acids, chlorophylls, and the major carotenoids), whereas other metabolites (e.g. specific xanthophylls, monoterpenes, and norisoprenoids) responded to the altered microclimate (i.e. increased exposure) differentially and displayed developmental stage-specific phenotypic plasticity. Pathway analysis of the genes and metabolites involved in the carotenoid metabolic pathway was subsequently performed to verify the observed metabolic response(s). This study led to a proposal that the impact of the treatment can be explained by a mechanism of antioxidant homeostasis maintenance in the berries experiencing increased exposure.

RESULTS

Quantitative Characterization of the Macroclimate in the Model Vineyard

An overview of the research methodology is outlined in Supplemental Figure S1. The Elgin region and vineyard site were classified according to viticultural climatic indices based on weather station data (i.e. regional macroclimatic) and mesoclimatic data (i.e. local vineyard). The indices selected for characterization are typically used to categorize the climatic potential of a region or vineyard (for grape growing) and, therefore, are indirectly linked to the characteristics and qualitative potential of grapes (Tonietto and Carbonneau, 2004). The various classification indices characterize the Elgin region as a temperate region with moderate to cool

nights (Supplemental Table S1). At more than 250 m above sea level, Elgin is a high-altitude wine-grape-growing region in South Africa. This elevation and the proximity to the cold Atlantic Ocean (and subsequent exposure to the cooling sea breeze) make it the fourth coolest wine-grape-growing region in South Africa. This site was chosen as a typical moderate climatic site for the production of a commercially desirable style of cv Sauvignon Blanc wine. The altitude and moderate climate minimized the potential for sunburn damage of berries in the leaf removal-treated vines.

Quantitative Characterization of the Microclimate in the Bunch and Canopy Zones Confirmed Increased Exposure for the Treated Berries

Leaf removal is typically used in viticulture to increase the photosynthetic active radiation (PAR) reaching the bunch zone and/or to decrease humidity at the fruit level. The light exposure in the bunch zone was strongly modified by the leaf removal treatment, with average light intensity (PAR) values of 52% ± 14% (average percentage PAR relative to the ambient, full sunlight [100%] at the date and time of sampling) for all cloudless sampling dates (Fig. 1). Conversely, the control bunches intercepted significantly less incoming radiation (PAR values of 4% ± 2%, relative to 100% ambient, full sunlight). Bunches in the exposed panels, therefore, received significantly more (seasonal average of more than 10 times higher) light than the shaded control bunches.

The daily average temperatures in the bunch zones of the respective treatments for the growth period (season) were not statistically significantly different when the data were considered on a daily mean hourly basis across the complete season (Fig. 2A). The temperature differences within the bunches of the treatments were insignificant throughout the entire season, ranging from a daily minimum of 11.4°C to a daily maximum of 38.2°C with a mean of 21.5°C ± 5.3°C for the exposed bunches, versus a range of 11.5°C to 37.7°C with a mean of 21.5°C ± 5.2°C for the bunches in the control treatments. Interestingly, the temperature in the canopy (above the bunch zone) of the exposed treatments was higher than that from the canopy of the control vines (Fig. 2B). Significant differences, however, could only be seen in the nighttime canopy temperatures (i.e. from sunset to sunrise), with the exposed canopies displaying higher temperatures than the control canopies, possibly indicating increased reflectance from the soil. This result was also shown in the seasonal thermal unit accumulation for the canopy and bunch temperatures, with significant seasonal differences only in the canopy temperatures (Fig. 2C). No significant differences in daytime canopy temperatures or bunch temperatures (per treatment) were found (Fig. 2).

It is understandably difficult to separate the effects of light from temperature in field experiments, since exposure to sunlight invariably results in increased temperatures. ANOVA and statistical testing were used to evaluate light and temperature as environmental factors potentially altered by the treatment. Supplemental Figure S2 shows the contribution of canopy temperature, bunch temperature, and light to the observed variance.

Leaf Removal Did Not Affect the Berry Physical Characteristics or the Ripening Dynamic of the Berries

Berry weight and diameter were measured for all the berries sampled for metabolite analyses. The relationship between berry weight and diameter showed a positive linear relationship (r^2 = 0.99) across all developmental stages, irrespective of the treatment. There were no significant differences between the control and exposed berries (Supplemental Fig. S3). Major sugars (Glc and Fru) and organic acids (tartaric acid, malic acid, and succinic acid) concentrations in berries were measured at five developmental stages (Supplemental Fig. S4, A–E). In berries, the changes in major sugars and organic acids are well described, with the sugar concentrations accumulating as ripening progresses and the total organic acid concentrations decreasing. Glc was the most abundant hexose in the earlier stages of development (Eichhorn-Lorenz [EL] stages EL31 and EL33), but from véraison (EL35) until harvest (EL38), Glc and Fru were present in approximately equal ratios.

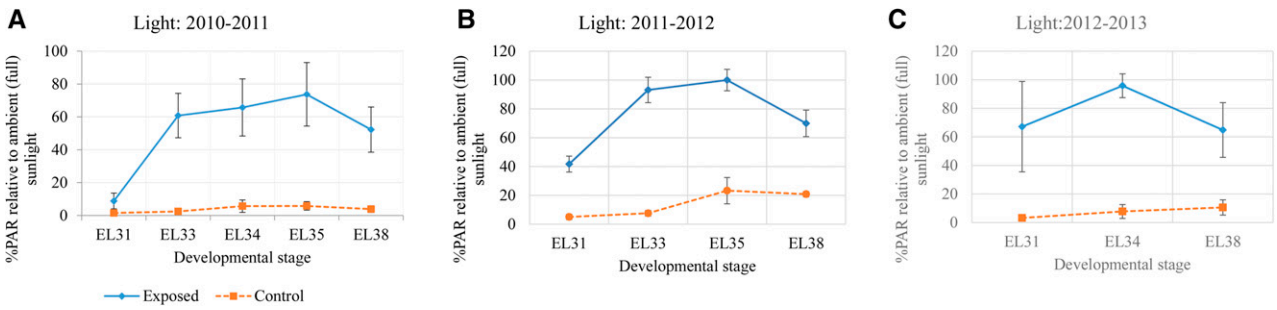


Figure 1. Characterization of the microclimate: light. PAR is shown in the bunch zone at the time of sampling for the respective sampling days for 3 consecutive years (vintages): A, 2010-2011; B, 2011-2012; and C, 2012-2013. Only cloudless days are represented.

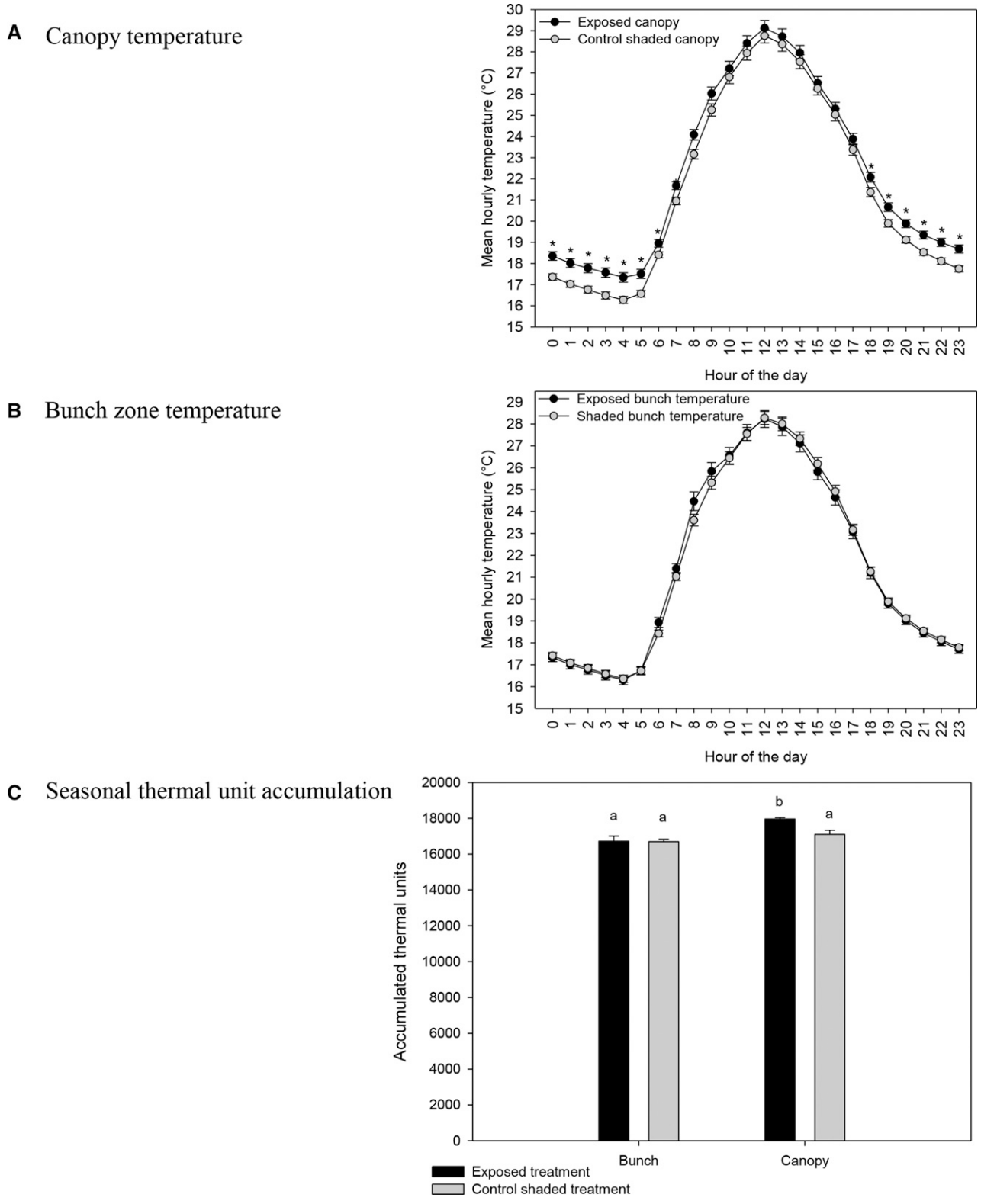


Figure 2. A and B, Hourly average temperature data in the canopy (A) and the bunch zone (B) in the exposed and control vines. Hours that are statistically different ($P \leq 0.05$) are indicated with asterisks. C, Mean thermal unit accumulation for the canopy and bunches. Different letters indicate significant differences (calculated with Fisher's LSD) between treatments, where $P = 0.05$.

The individual sugars and organic acids were not significantly affected by the leaf removal treatment in all but the EL38 developmental stage, in which a slight difference was shown (Supplemental Fig. S4E).

Developmental and Treatment-Specific Patterns of Metabolites Were Evident

Principal component analysis (PCA) and hierarchical clustering analysis were two of the data-mining tools used to reduce the complexity of the metabolite data. Metabolite analysis of field samples is typically hampered by inherent biological variation. Each panel analyzed in this study represents a unique biological entity, standard data interpretation potentially results in the loss of biologically relevant data (e.g. due to averaging), and potential correlations to the measured environmental variables can be blurred. Multivariate data analysis (e.g. PCA) reduces data complexity and can be used to identify the variables (metabolites in this study) that contribute the most to the optimal model. Unsupervised PCA plots were used to visualize the metabolite data (Supplemental Fig. S5), and separation was observed for developmental stages (EL31–EL38; PC1 on the horizontal axis) as well as treatment (exposed versus control samples; PC2 on the vertical axis). The increase in Glc and Fru, and inversely the decrease in chlorophylls (chlorophyll *a* and *b*) and the majority of the photosynthetic carotenoids (i.e. β-carotene, lutein, and neoxanthin), during ripening drove the developmental stage separation (considering PC1). The compositional differences in specific carotenoids (most notably the xanthophylls zeaxanthin, antheraxanthin, and lutein epoxide) and specific monoterpenes were predominantly responsible for the treatment separation on PC2 (Supplemental Fig. S5).

PCA is particularly useful for simplifying and visualizing data sets and helps to identify potential correlations in the underlying data sets. The associated scores and loadings plots are then used to identify correlations. The loadings plot relates to the variables and is used to explain the positions of observations in the scores plot. The scores plot relates to the observations, separates signal from noise, and is used to observe patterns and clustering in the observations. Whereas PCA models are unsupervised and find the maximal variation in the data, orthogonal partial least squares (OPLS) models are supervised prediction and regression methods. Orthogonal partial least squares-discriminant analysis (OPLS-DA) is used to analyze the relationship between the quantitative data matrix, x (i.e. the measured variables [e.g. metabolite concentration and/or transcript levels]), and a vector, y, containing qualitative values (i.e. the data descriptors or classes [e.g. developmental stages {EL31–EL38} or treatment {control or exposed}]). Separate OPLS models were generated to analyze the developmental and treatment class separations to identify the variables statistically contributing to the optimal models for (1) developmental stage discrimination (Fig. 3) and (2) treatment discrimination (Fig. 4).

The metabolites contributing the most to the model for developmental discrimination (Fig. 3) were the organic acids malic acid and succinic acid (and the associated total organic acid pool) and the monoterpenes trans-linalool oxide and eucalyptol (and the associated total monoterpene pool). The metabolites contributing the most to the model for treatment (exposure; Fig. 4) discrimination were the xanthophylls zeaxanthin and antheraxanthin (and the associated De-epoxidation state ratio and total xanthophyll pool) and the norisoprenoids geranylacetone and 6-methyl-6-hepten-2-one (MHO; and the associated total norisoprenoid pool).

Hierarchical cluster analysis was subsequently used to identify profiles (clusters) with similar trends between the analyzed metabolites (Fig. 5). A number of clusters were of particular interest: (1) metabolites showing a predominant developmental trend (Fig. 5, clusters 2, 4, and 6); (2) metabolites showing a predominant treatment effect (Fig. 5, clusters 1, 3, and 7); and (3) metabolites showing both developmental and treatment effects (Fig. 5, clusters 1, 2, 3, 5, and 6). The responses of the measured metabolites typically varied between the different developmental stages, with the early stages (EL31 and EL33) and the later stages (EL35 and EL38) generally responding similarly and with véraison as a transition stage (between the early/green and late/ripe stages).

Metabolites showing the developmental trend (Fig. 5, clusters 1 and 2) could be further subgrouped into metabolites that increased with development progression (Fig. 5, cluster 6) and metabolites that decreased with development progression (Fig. 5, clusters 2 and 4). The major sugars (Glc and Fru), MHO, and three monoterpenes (geraniol, linalool, and nerol) increased with developmental stage (similar to berry weight and diameter in the same cluster). It is important to note that hierarchical cluster analysis relies on Pearson correlation coefficients to match trends and does not discriminate similar trends that differ in amplitude. This is evident in the line graphs of geraniol, linalool, and nerol (Fig. 6), where both the control and exposed display upward developmental trends but the absolute values of the respective metabolites in the exposed berries were significantly higher (than the control). Chlorophylls *a* and *b* and the major carotenoids (e.g. lutein and β-carotene, representing approximately 80% of the total carotenoids), however, decreased concomitantly throughout development (Fig. 6A). The major organic acids (i.e. malic acid, succinic acid, and tartaric acid), as well as the xanthophyll neoxanthin and the norisoprenoid (apocarotenoid) β-ionone, displayed a similar developmental decrease (Fig. 5, clusters 2 and 4).

A cluster of three carotenoid-derived apocarotenoids (i.e. norisoprenoids; pseudo-ionone, β-damascenone, and geranylacetone) were characterized by an early-stage (EL31 and EL33) developmental pattern followed by a treatment-related response (from EL34/véraison),

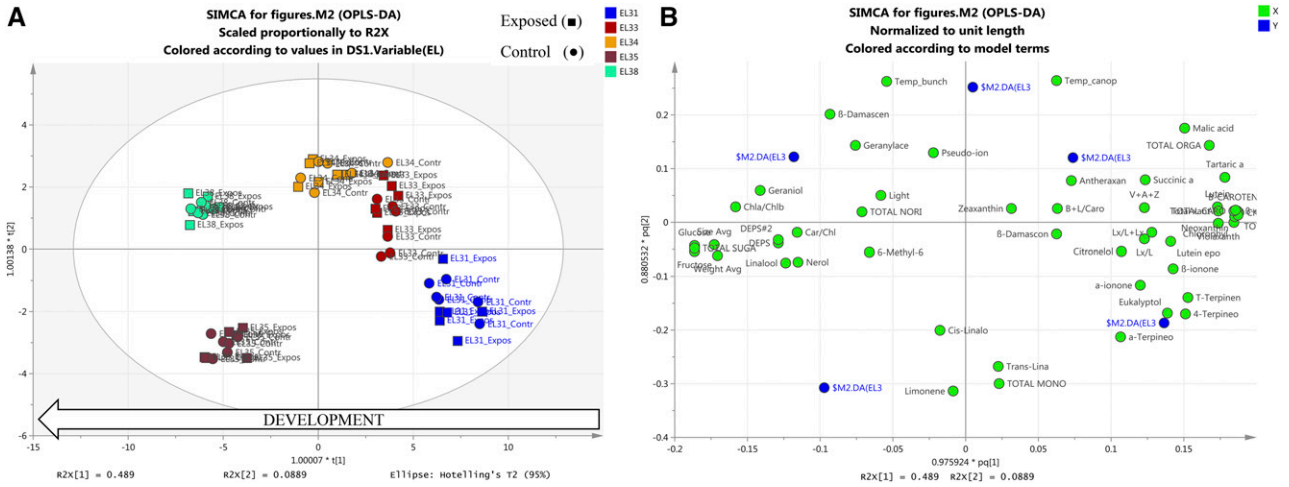


Figure 3. Supervised (developmental stage) OPLS of all metabolites from all stages. A, Scores plot for the respective samples. Samples are colored by developmental stage; control samples are indicated by circles and exposed samples by squares. B, Loadings plot for the measured variables in green and discriminant classes/categories in blue.

with higher levels in the samples from the exposed versus control bunches and positively correlated to the bunch temperature (Fig. 5, cluster 5).

The monoterpenes α-terpineol and trans-linalool oxide displayed a biphasic treatment effect, with higher levels in both the exposed berries (versus the control berries) in the early (EL31) and late (EL35 and/or EL38) stages, with insignificant differences in the midripening stages (EL34 and/or EL35; Fig. 5, cluster 8). The xanthophylls antheraxanthin and zeaxanthin showed a clear treatment effect, with higher levels in the exposed berries (versus the control) in all developmental stages (EL31–EL38). The treatment effect was significantly greater in the early stages (EL31 and EL33) versus the later stages (EL34, EL35, and EL38; Fig. 5, cluster 7).

Sugars and Organic Acids Are Predominantly Developmentally Regulated

It is interesting that the Glc and Fru concentrations in the berries were present in equal proportions (Glc:Fru ratio approximately 1) only from véraison (EL35) onward (Supplemental Fig. S4D). In the earlier stages, however, Glc is the dominant hexose. In the EL31 stage, no Fru could be detected. A Glc:Fru ratio of approximately 1 illustrates that Glc and Fru in the berries are

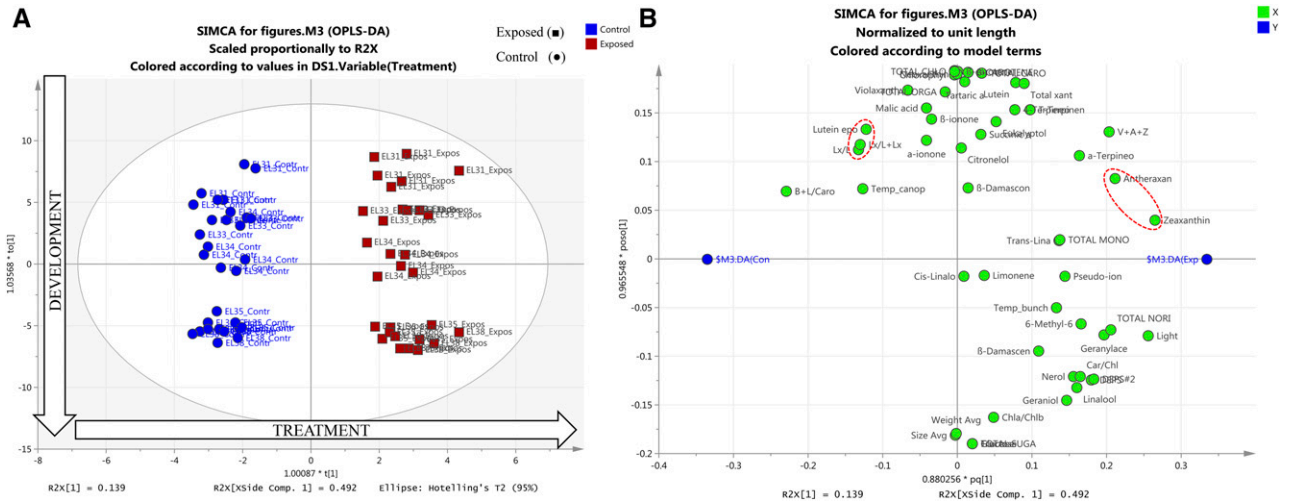


Figure 4. Supervised (treatment) OPLS of all metabolites from all stages. A, Scores plot for the respective samples. Samples are colored by treatment; control samples are indicated by circles and exposed samples by squares. B, Loadings plot for the measured variables in green and discriminant classes/categories in blue. Compounds significantly contributing to the models are circled in red.

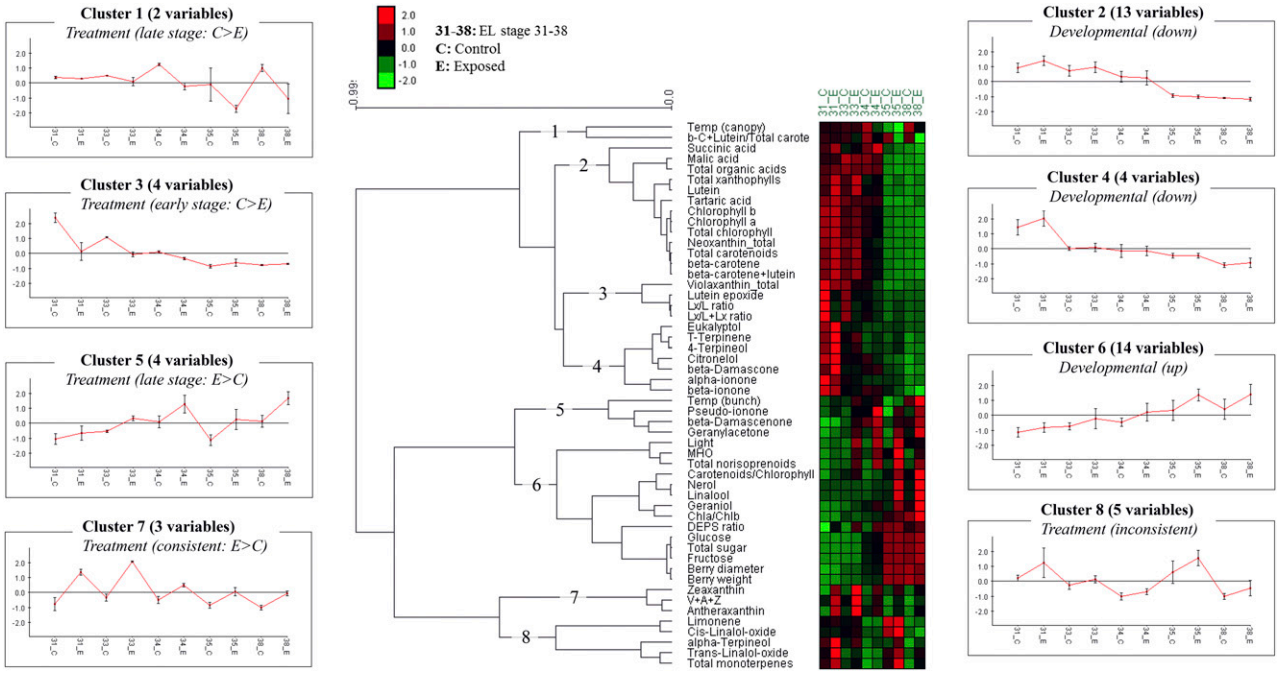


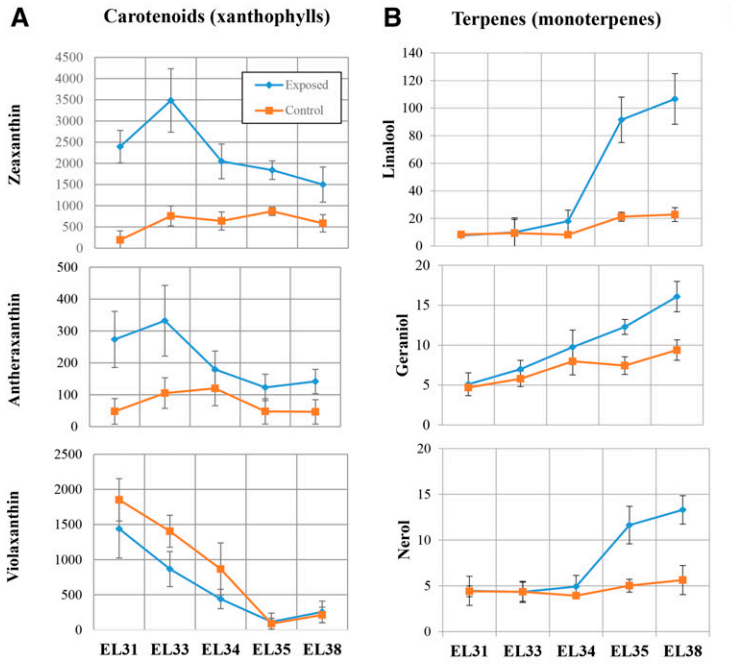
Figure 5. Hierarchical cluster analysis of all variables from all stages, with line graphs of representative clusters.

derived from the hydrolysis of Suc (as expected in a sink organ).

Although the absolute concentrations of the individual organic acids were not significantly affected by the leaf removal treatment across all developmental stages (all but EL38), interesting trends could be seen in the ratio of tartaric acid to malic acid (Supplemental Fig. S6). This ratio, referred to as the β-ratio (proposed by Shiraishi [1995]), has been used previously to evaluate the organic acids from *Vitis* spp. germplasm collections. Until véraison, the β-ratio remained relatively constant (approximately 1) for the exposed and control berries, but from EL35, the ratio increased in both the exposed and control berries. At harvest (EL38), the exposed berries had a β-ratio of 4, double that of the control berries (with a β-ratio of 2). This phenomenon is due to the combination of a slight (but statistically significant) increase in tartaric acid concentrations and a concomitant decrease in malic acid concentrations (relative to the control berries; Supplemental Fig. S4E). Across all stages, the percentage of tartaric acid and malic acid (relative to total organic acids), however, remained relatively constant (approximately 85%–90% of total acids) for both the exposed and control berries. Succinic acid levels were similar in the exposed and control berries and fluctuated from 5% to 15% of total organic acids (Supplemental Fig. S6B). In grapes, malate levels have been shown to be more susceptible to temperature-induced degradation than tartrate, but since the bunch temperatures were not significantly different between the treatments, it is not possible to link bunch temperature to this observation (Sweetman et al., 2014). The canopy temperature of the exposed vines, however, was significantly higher than the control canopy temperature during the night, and it is possible that differences in photorespiration in the leaves, for example, affected the organic acid levels in the berries. The mechanism for this is not known and deserves further investigation.

Major Carotenoids and Chlorophylls Were Predominantly Developmentally Regulated, But the Xanthophylls Responded to the Treatment

Pathway analysis was used to analyze the metabolism of the carotenoids (Fig. 7). For carotenoid metabolism (biosynthesis and catabolism), the pathway described by Young et al. (2012) was used to provide an overview of the relative changes and flux of the related metabolites over time. The regulated catabolism of chlorophylls and the concomitant decrease in total carotenoid concentration are well described for grape berry development (Razungles et al., 1996; Young et al., 2012). The ratio of chlorophyll *a* to chlorophyll *b* increased from 2.5 (EL31) to 3.5 (EL38), with no significant differences between the ratio in exposed berries versus control berries (Supplemental Fig. S7). Until véraison, grapevine berries are photosynthetically active, albeit at much lower levels (1%–10%) than photosynthetically active leaves (Goodwin, 1980). The decrease in the more abundant carotenoids (i.e. lutein and β-carotene, representing approximately 80% of the total carotenes in a grape berry) followed the trends of chlorophylls *a* and *b* in both the control and exposed berries and was generally associated with the developmental stages of berries, with the



C

Exposed:Control (log_2 ratio)	EL31	EL33	EL34	EL35	EL38
Zeaxanthin	3.6[c]	2.2[c]	1.7[c]	1.1[c]	1.4[c]
Antheraxanthin	2.5[c]	1.7[c]	0.6	1.4[b]	1.6[b]
Trans-Linalol-oxide	1.1[b]	0.7	0.6	0.5	1.1[a]
α-Terpineol	0.8[b]	0.5	0.6[a]	0.9[b]	1.3[b]
Eucalyptol	0.7[a]	0.0	-0.2	0.2	0.7
4-Terpineol	0.6[b]	0.4[a]	0.3	0.4	0.2
Lutein	0.6[c]	0.5[c]	0.1	0.1	0.1
γ-Terpinene	0.6[a]	0.4[a]	0.5	0.5	1.0[b]
Succinic acid	0.3[b]	0.2	0.4[a]	-0.4	-0.4
Geranylacetone	0.3[a]	0.5	0.7[a]	1.0[b]	0.8[c]
6-Methyl-6-hepten-2-one (MHO)	0.2	0.6	0.5[a]	0.7[a]	0.5[b]
ß-carotene	0.2[a]	0.1[b]	-0.3	-0.4[a]	-0.5
ß-Damascone	0.2	0.0	0.0	0.0	0.0
Limonene	0.2	0.2	0.4	0.1	0.7
ß-Damascenone	0.2	0.5[b]	0.3	0.5	0.4[b]
Neoxanthin_like	0.2	0.1	-0.2	-0.1	-1.3
Neoxanthin	0.1	0.0	-0.3	-0.4	-0.7
Pseudo-ionone	0.1	0.2	0.4	0.6[a]	0.4[c]
Tartaric acid	0.1[c]	-0.1	0.0	0.0	0.2[c]
Geraniol	0.1	0.3	0.3	0.7[c]	0.8[c]
Chlorophyll a	0.1	0.1[a]	-0.2	-0.3[a]	-0.5
Chlorophyll b	0.1	0.2[a]	-0.3	-0.4[a]	-0.7
Citronellol	0.1	0.0	-0.2	0.0	0.1
Malic acid	0.0	-0.2	-0.2	-0.5[a]	-0.6[a]
Nerol	0.0	0.0	0.3	1.2[c]	1.2[c]
Cis-Linalol-oxide	0.0	0.1	0.0	0.3	-0.9
Glucose	-0.1	0.5	0.2	0.0	0.2[a]
ß-ionone	-0.1	0.0	-0.1	-0.2	-0.3[a]
Linalool	-0.1	0.1	1.1[a]	2.1[c]	2.2[c]
Violaxanthin_like	-0.3	-0.7[b]	-1.0[a]	0.0	0.3
α-ionone	-0.3	-0.1	0.0	0.0	0.1
Fructose	-0.4	0.8	0.3	0.0	0.2[b]
Violaxanthin	-0.4	-0.7[b]	-1.0[a]	1.0	0.2
Lutein epoxide	-2.3[c]	-1.4[c]	-0.8	1.0	2.6[c]

[a]: 0.01<p-value<0.05; [b]: 0.001<p-value<0.01; [c]: p-value<0.001

Figure 6. Bar graphs of selected individual carotenoids (A; ng/g FW) and monoterpenes (B; ng/g FW) as well as a heat map (log_2 fold change) representation of all analyzed metabolites (C). FW, Fresh weight.

earlier stages typically having higher concentrations than the later stages (Figs. 5, cluster 2, 6, and 8). The levels of lutein closely followed the trend of chlorophyll *b*, whereas β-carotene followed chlorophyll *a* degradation (Supplemental Fig. S8).

The responses of specific carotenoids, the xanthophylls (i.e. lutein, lutein epoxide, zeaxanthin, antheraxanthin, and violaxanthin), to light are well described in a host of different photosynthetic organisms (for review, see Cunningham and Gantt, 1998; Jahns and Holzwarth, 2012). Of particular importance in this study were the two xanthophyll cycles: (1) the lutein: lutein epoxide cycle and (2) the zeaxanthin:violaxanthin cycle. These two cycles are functional in plants in response to shade and high light, respectively. The lutein: lutein epoxide cycle is considered taxonomically restricted (predominantly woody plants and not formed in Arabidopsis [*Arabidopsis thaliana*], for example), and it has been proposed that it is involved in the maintenance of photosynthetic performance under limiting light as well as serves a photoprotective function, especially in response to sudden changes in irradiance (Esteban et al., 2009). Lutein epoxide typically accumulates in older leaves that are predominantly in the shade but has been reported in grape berries (Razungles et al., 1996; Young et al., 2012).

The levels of lutein epoxide were significantly lower in the berries from exposed vines (relative to the berries from control vines) in the first two stages of development (i.e. EL31 and EL33; Fig. 8). Lutein epoxide displayed the largest coefficient of variation (135% for exposed versus control) of all the metabolites analyzed (Supplemental Fig. S9). The ratio of lutein epoxide to lutein was 10% that of the ratio of berries from control vines in EL31 (Fig. 8). The lutein epoxide-lutein ratio stayed relatively low and constant in the exposed berries but decreased rapidly in the berries from control vines from the initial high at EL31. From stage EL35 onward, the lutein epoxide-lutein ratio was low (less than 0.01) and not significantly different in the berries from exposed vines (relative to the control berries). Lutein epoxide, and to a lesser extent violaxanthin, decreased in the berries from exposed vines (Fig. 8), and conversely, zeaxanthin and antheraxanthin increased in the berries from exposed vines relative to the control. It is also interesting that the ratio of β-carotene to lutein (as an indicator of flux to the β- and α-branches of the carotenoid metabolic pathway) was lower in the exposed berries relative to the control berries. This was due to lower levels of lutein in the control berries (resulting in a higher β-carotene-lutein ratio). The lutein in the control berries was presumably converted to lutein epoxide in the shaded conditions. Conversely, comparatively low levels of lutein epoxide were found in exposed berries (Fig. 8). Although lower levels of lutein were present in the control berries, it still followed a similar developmental pattern to β-carotene and chlorophylls *a* and *b* (Fig. 8), but the linear relationship between lutein and chlorophyll *b* was lower in the control berries than in

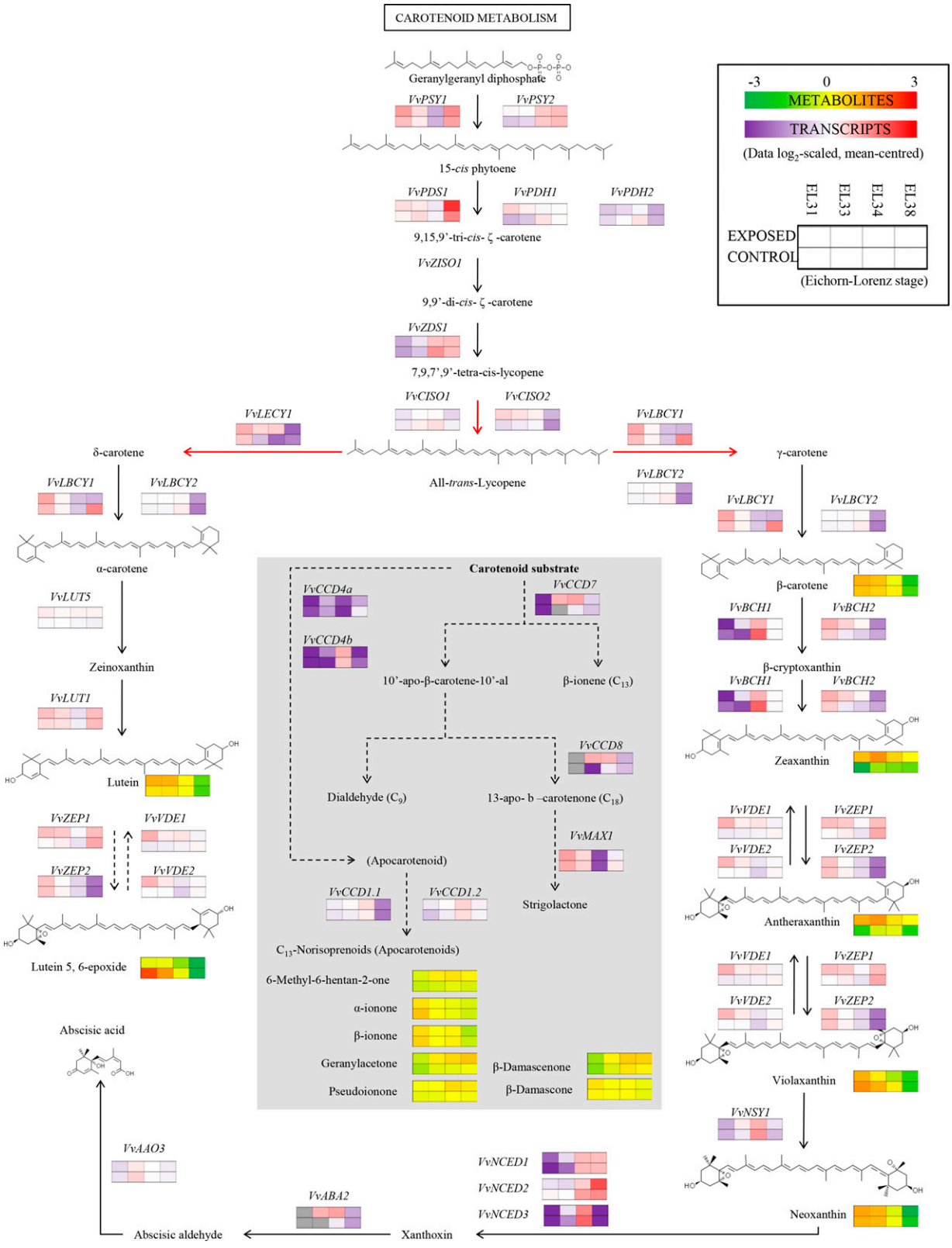


Figure 7. Pathway analysis of genes and enzymes involved in carotenoid metabolism. The heat maps represent the transcript (purple-red) and metabolite (green-red) data (log_2 scaled and mean centered). Reactions that have not been fully elucidated are indicated with dotted lines. Enzymes involved in the branch points in carotenoid metabolism are indicated with red arrows.

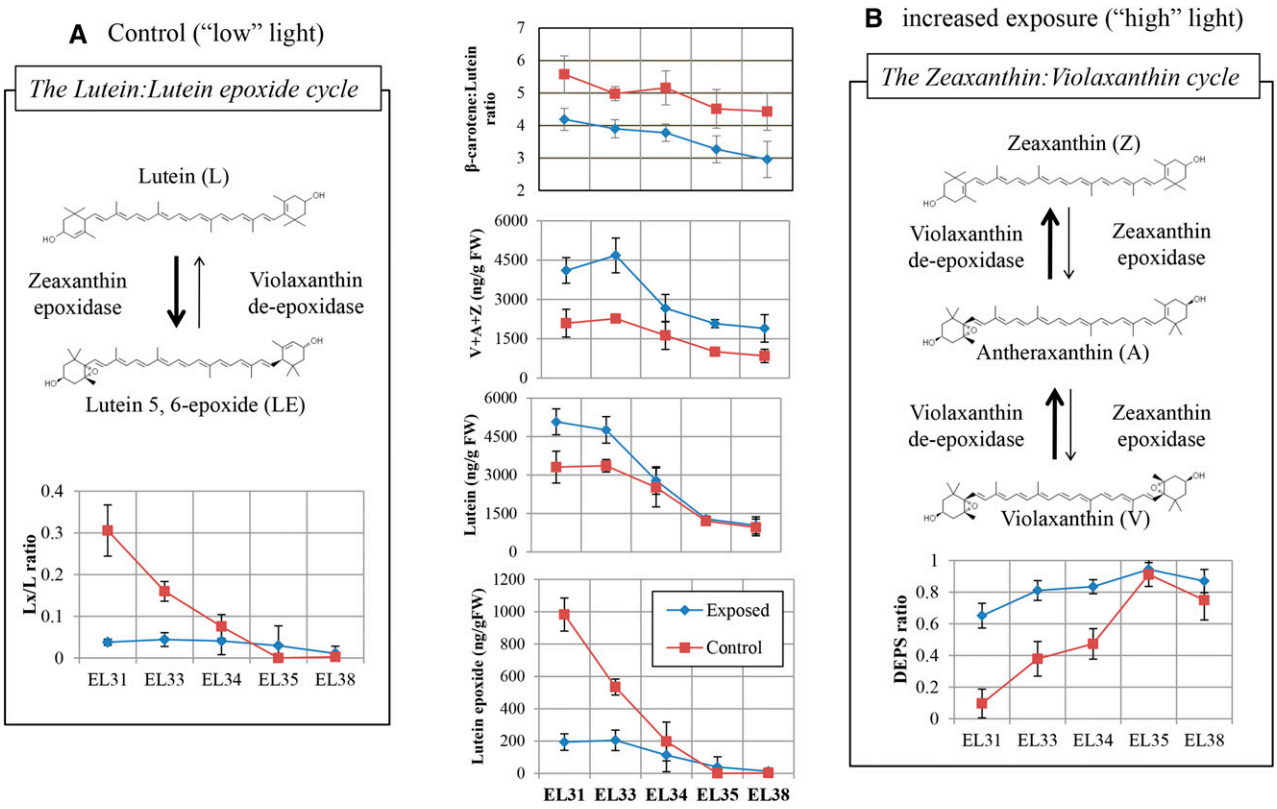


Figure 8. The xanthophyll cycles functional in grapevine and the individual carotenoids involved. A, The lutein:lutein epoxide cycle. FW, Fresh weight. B, The zeaxanthin:violaxanthin cycle. DEPS ratio, The deepoxidation state of the zeaxanthin:violaxanthin cycle [calculated as (Z+E)/(V+A+Z)]; Lx/L, the epoxidation state of the lutein:lutein epoxide cycle (calculated as lutein epoxide/lutein); V+A+Z, violaxanthin, antheraxanthin, and zeaxanthin. Bold arrows indicate increased flux.

the exposed berries (Supplemental Fig. S8). As mentioned, in photosynthetic tissues, a linear relationship was found for major carotenes (β-carotene and lutein) and chlorophylls (chlorophylls *a* and *b*).

The ability to modulate the levels of specific carotenoids by a viticultural treatment is of particular interest, since the carotenoids have been shown to be precursors for the flavor and aroma compounds, the norisoprenoids (apocarotenoids). It has also been shown that carotenoid cleavage dioxygenases catalyze the cleavage of specific C_{40}-carotenoid substrates to specific C_{13}-apocarotenoid cleavage products (Mathieu et al., 2005, 2006; Lashbrooke et al., 2013).

Genes Encoding Specific Xanthophyll Deepoxidation Enzymes, as Well as Branch Point Enzymes in Carotenoid Metabolism, Are Differentially Expressed in Response to the Treatment

In order to determine the contribution of transcriptional regulation to the metabolic plasticity observed in specifically carotenoid and carotenoid-derived metabolites, the transcripts encoding the enzymes involved in carotenoid metabolism were analyzed. Pathway analysis showed that the majority of the genes were not differentially affected by the treatment (across the four developmental stages analyzed for expression: EL31, EL33, EL34, and EL38; Fig. 7). Only five pathway genes were significantly affected by the treatment across the developmental stages ($P \leq 0.05$). The majority of the differentially expressed genes (four of five) were up-regulated in the exposed bunches (versus the control bunches). Three of the up-regulated genes are directly involved in xanthophyll metabolism: *VvVDE1* and *VvVDE2*, encoding violaxanthin deepoxidase that catalyzes the deepoxidation of violaxanthin to zeaxanthin (via antheraxanthin), and *VvLUT5*, a cytochrome P450 gene (CYP97A4) encoding a carotenoid β-ring hydroxylase that catalyzes the conversion of α-carotene to zeinoxanthin and is involved in lutein biosynthesis (Tian and DellaPenna, 2004; Kim et al., 2009). The remaining two differentially affected transcripts encode carotenoid isomerases, *VvCISO1* and *VvCISO2*, and were differentially affected by the treatment. *VvCISO1* was down-regulated in the exposed bunches, whereas *VvCISO2* was up-regulated (Yu et al., 2011).

As was evident in the metabolite data, interesting results can be seen if the developmental stages were analyzed separately (i.e. by treatment per developmental stage). The early developmental stages had the most genes significantly ($P \leq 0.05$) differentially affected by the treatment (exposed versus control

bunches) of the four stages analyzed (Supplemental Table S2). The majority (12 of the 13 genes) in stage EL31 were up-regulated in the exposed bunches (compared with the control bunches), with only *VvBCH1* being down-regulated. *VvBCH1* encodes a β-carotene hydroxylase that catalyzes the hydroxylation of β-carotene (a carotene) to zeaxanthin (a xanthophyll). Conversely, *VvBCH2* is up-regulated. Both *VvVDE1* and *VvVDE2* were similarly up-regulated, as were *VvLUT1* and *VvLUT5*. The net effect of this will hypothetically lead to the accumulation of the deepoxidized xanthophylls lutein and zeaxanthin in the two branches of the carotenoid metabolic pathway in which the violaxanthin and lutein epoxide cycles function (Fig. 8). Flux through the carotenoid pathway should also be increased by the up-regulation of a number of genes involved in the initial reactions of carotenoid biosynthesis: *VvPSY1*, *VvPSY2*, *VvPDS1*, *VvZDS1*, and *VvCISO2* collectively result in lycopene biosynthesis. Lycopene, however, does not accumulate in grape berries and is being converted to predominantly β-carotene and lutein. The up-regulation of *VvCCD1.2* in the exposed berries implicates CCD1 in the maintenance of carotenoid homeostasis in the earlier developmental stages (e.g. EL31; Lashbrooke et al., 2013).

In contrast to the up-regulation of a relatively large number of genes in the early stages of development, the later stages of berry development were characterized by less transcriptional (differential) activity, with the majority of responses being the down-regulation of genes involved in carotenoid catabolism. Of the five transcripts differentially expressed in the exposed versus control berries, only *VvBCH2* was significantly up-regulated at EL38. Of the significantly down-regulated genes, only *VvPSY1* is involved in carotenoid biosynthesis. *VvPSY1* encodes the first dedicated carotenoid biosynthetic enzyme, phytoene synthase. The remaining three genes encode enzymes involved in carotenoid catabolism and were down-regulated: a neoxanthin synthase (*VvNSY1*) and a 9-cis-epoxy carotenoid dioxygenase (*VvNCED2*) involved in abscisic acid metabolism (Frey et al., 2012; Young et al., 2012) and a carotenoid dioxygenase (*VvCCD4a*) involved in C_{13}-norisoprenoid (apocarotenoid) production (Lashbrooke et al., 2013). The decrease in the transcriptional activity of these genes, therefore, followed the overall decrease in their carotenoid substrates.

Volatile Terpenoids Are Increased in Response to Leaf Removal in the Later Stages of Berry Development

The volatile terpenoids measured in this study can be grouped into two major classes: the C_{10}-monoterpenes and the C_{13}-norisoprenoids (or apocarotenoids). The monoterpene content of berries was dominated by the two most abundant monoterpenes: linalool and α-terpineol. The total monoterpene content was affected by the decline in the more abundant linalool in the first three stages (EL31, EL33, and EL34) and then a shift to the increase in α-terpineol in the later developmental stages (EL35 and EL38; Figs. 6B and 9A). A number of monoterpenes were significantly higher in specific stages in the exposed versus the control berries, such as trans-linalool oxide (more than 2-fold in EL31), linalool (more than 2-fold in EL34 and more than 4-fold in EL35), and nerol (more than 2-fold in EL35), but the majority of monoterpenes were typically higher in the exposed berries (versus the control) at harvest (EL38), such as γ-terpinene, trans-linalool oxide, nerol, and α-terpineol (more than 2-fold) and linalool (more than 4-fold; Fig. 6, B and C).

The total volatile norisoprenoids (i.e. α-ionone, β-ionone, pseudo-ionone, geranylacetone, MHO, and β-damascenone) in berries increased until (exposed berries) and EL38 (control berries). MHO and geranylacetone are the two most abundant norisoprenoids, contributing 45% to 60% and 40% to 55%, respectively, to the total norisoprenoid pool in berries. The treatment resulted in higher norisoprenoid content in the exposed berries (relative to the control berries) at the harvest stage (EL38; Figs. 6C, 9, and 10).

Systematic Analysis of the Inherent Variation in the Model Vineyard

Due to the inherent variability of field studies (due to a host of factors), a systematic analysis of the measurable variation between the respective biological repeats (i.e. panels in this study) was undertaken at each sampling time point using all the measured variables (metabolites and microclimatic variables).

Hierarchical cluster analysis of the metabolite concentrations of the samples (per panel) was performed for the entire season and per developmental stages (Supplemental Fig. S10). Based on the variables, hierarchical cluster analysis showed that the separation of the samples across all stages was predominantly on development. Stages EL31, EL33, and EL34 formed a clearly defined early-stage group/cluster, and EL35 and EL38 formed a separate distinct late-stage group/cluster. Within the early stages (EL31–EL35), the samples clustered predominantly by treatment (exposed versus control), whereas in the later stages (EL35 and EL38), the samples clustered predominantly according to their developmental stage and then subclustered within this grouping into their respective treatments (Supplemental Fig. S10). Supplemental Figure S11 shows an unsupervised PCA of the metabolite data of two consecutive seasons (2010-2011 and 2011-2012). Consistent metabolite trends are clear in both years in response to the same leaf removal treatment, showing that, irrespective of vintage, the metabolites showed a consistent response.

DISCUSSION

The field-omics approach provided an analysis of the leaf removal treatment by following metabolite changes during the developmental and ripening stages of the

A

Exposed:Control (Log2 ratio)	EL31	EL33	EL34	EL35	EL38
Total sugars	-0.1	0.6	0.2	0.0	0.2[b]
Total organic acids	0.1[b]	-0.1	-0.1	-0.2	0.0
Total chlorophylls	0.1	0.2[a]	-0.2	-0.3[a]	-0.5
Total carotenoids	0.3[a]	0.3[c]	-0.1	0.0	-0.1
Total xanthophylls	0.5[b]	0.5[c]	0.2	0.5[c]	0.5
Total monoterpenes	0.5[a]	0.2	0.6	0.7[a]	0.9[c]
Total norisoprenoids	0.2[a]	0.5[a]	0.8[a]	0.7[c]	0.6[c]
Chlorophyll a:Chlorophyll b (ratio)	0.0	-0.1	0.0	0.1	0.2[a]
Carotene:Chlorophyll (ratio)	0.2[a]	0.1[b]	0.1[a]	0.3[b]	0.5[c]
β-Carotene+Lutein (B+L)	0.3[a]	0.2[c]	-0.2	-0.3[a]	-0.4
β-carotene+Lutein:Total Carotenoids (ratio)	0.0	-0.1[c]	-0.1[c]	-0.3[c]	-0.3[c]
V+A+Z[1]	1.0[c]	1.0[c]	0.7[b]	1.0[c]	1.2[b]
DEPS (ratio)[2]	2.8[c]	1.1[c]	0.9[c]	0.0	0.2
Lx:L (ratio)[3]	-3.0[c]	-1.9[c]	-0.9		2.1

[a]: 0.01<p-value<0.05; [b]: 0.001<p-value<0.01; [c]: p-value<0.001
[1] Sum of violaxanthin, antheraxanthin and zeaxanthin;
[2] The de-epoxidation state of the zeaxanthin cycle, calculated as: (Z +A)/(V+A+Z)
[3] The epoxidation state of the lutein epoxide cycle, calculated as: Lutein epoxide/Lutein

B

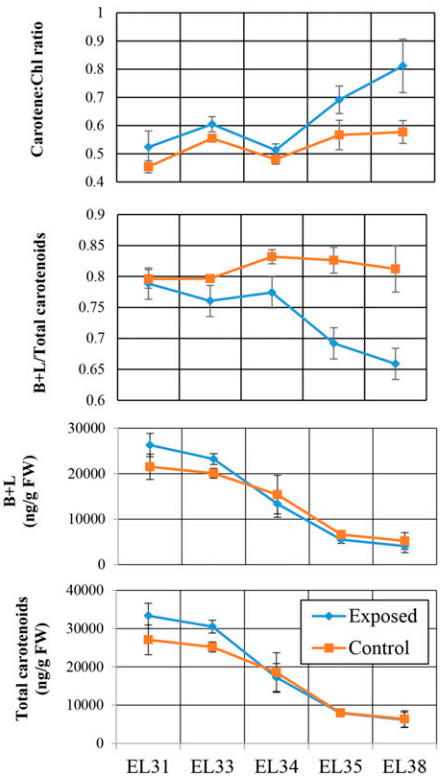
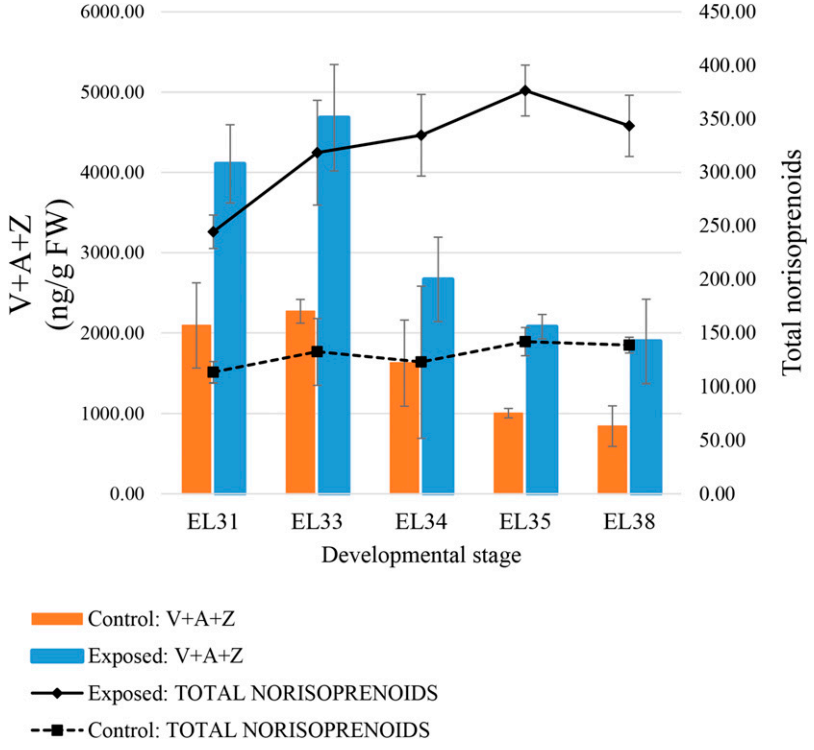


Figure 9. Heat map (log_2 fold change; A) and bar graphs of metabolite pools and selected ratios (per stage; B). FW, Fresh weight.

berry and identified the main berry response to be changes to pigment levels and metabolite pools that have photoprotective and/or antioxidant functions. This logically fits the findings from the environmental profiling that showed an altered (more exposed) microclimate of the treatment. It is possible, of course, that the treatment could have affected other environmental parameters not measured here, but from our

Figure 10. Changes in the norisoprenoid pool and the violaxanthin, antheraxanthin, and zeaxanthin (V+A+Z) pool (in ng/g FW), and in the norisoprenoid pool (in ng/g FW), in the exposed and control berries throughout berry development. FW, Fresh weight.



measurements, statistical analysis confirmed a strong reaction on predominantly light but not bunch temperature.

Compositional Metabolic Plasticity in Grapevine Is Predominantly Due to Stage-Specific Responses in Carotenoids

A number of factors, including variations in the incident light (both quality and quantity), can induce a range of responses that affect plants on multiple levels: from gene transcription to phenotype and from the photosynthetic apparatus to whole-plant architecture. The role of the C_{40}-terpenoid carotenoids in photosynthesis, especially in light harvesting and photoprotection, is well established in numerous photosynthetic organisms, including plant models (for review, see Cunningham and Gantt, 1998). The fate of carotenoids during grape berry development is similarly well documented, with lutein and β-carotene representing the major carotenoids found in grapes (Razungles et al., 1996; Young et al., 2012). The carotenoid concentration in grape berries has been studied in a number of grapevine cultivars, and the total carotenoid levels typically decrease with ripening. Berries until véraison are considered photosynthetically active, carotenes act as light-harvesting antenna pigments, and xanthophylls (oxygenated carotenes) are involved in photoprotection of the plant via the xanthophyll cycles (via lutein:lutein epoxide and zeaxanthin: violaxanthin cycling) in photosynthetic tissues (for review, see Cunningham and Gantt, 1998). Carotenoid concentrations in the grape berries are affected by a number of factors that include the region, the cultivar, exposure to sunlight, and the ripening stage of the berries (Oliveira et al., 2003, 2004; Lee et al., 2007; Song et al., 2015).

From the data presented, it was clear that grapevine berries were capable of more than one response to the altered microclimate. The first response was the modulation of the carotenoid composition in response to the treatment. Most notable was the response of the photoprotective xanthophylls (i.e. zeaxanthin and antheraxanthin; Figs. 6A and 8). Zeaxanthin and antheraxanthin were significantly higher in the exposed berries, and this resulted in a larger xanthophyll pool size (violaxanthin, antheraxanthin, and violaxanthin) and, consequently, an increase in the deepoxidation state of the xanthophylls (deepoxidation state ratio; Fig. 8). Interestingly, the ratio of β-carotene and lutein to the total carotenoid pool remained constant in the control berries but showed a marked decrease in the exposed berries (Fig. 8). Since β-carotene and lutein were unaffected by the treatment (Figs. 6C, 8, and 9), this is due to the total carotenoid pool, especially the xanthophyll pool, increasing in the exposed berries relative to the control berries (Figs. 7–9). Conversely, lutein epoxide levels were significantly lower in the berries from the exposed vines (relative to the berries from control vines; Figs. 7 and 8). The zeaxanthin:violaxanthin cycle is ubiquitous in higher plants, whereas the lutein:lutein epoxide cycle is considered taxonomically restricted, and its occurrence in grapevine berries was only recently shown (Deluc et al., 2009; Crupi et al., 2010b; Young et al., 2012). This resulted in a significantly lower lutein epoxide-lutein ratio in the exposed berries in the early stages (EL31 and EL33; Figs. 7–9). The relationship between lutein epoxide and lutein is markedly different in the exposed and control (shaded) berries (Fig. 8). There is a linear relationship between lutein epoxide and lutein in the exposed berries across the developmental stages (r^2 = 0.98). The relationship between lutein epoxide and lutein in the control berries, however, was not linear (r^2 = 0.75). This could be due to the slow recovery/relaxation of lutein epoxide to lutein in shade conditions, as reported previously (García-Piazola et al., 2007; Esteban et al., 2009; Förster et al., 2011). It is clear that the berries respond to their microclimate utilizing a photoprotective mechanism that is conserved in photosynthetic tissues. Although identified in 1975 in green tomato (*Solanum lycopersicum*) fruit (Rabinowitch et al., 1975), the functionality of the lutein epoxide:lutein cycle in fruit (not leaves) is still relatively unknown. Lutein epoxide has been reported in the petals of flowers (e.g. dandelion [*Taraxacum officinale*]; Meléndez-Martínez et al., 2006) and a minor xanthophyll in squash (*Cucurbita maxima*; Esteban et al., 2009).

Early-Stage-Specific Increases in Carotenoids Result in Concomitant Late-Stage-Specific Increases in Volatile Apocarotenoids

The specific carotenoids formed in grape berries are of particular interest, as their degradation products give rise to the impact odorants, the C_{13}-apocarotenoid/norisoprenoids (Mathieu et al., 2005; Lashbrooke et al., 2013). The norisoprenoids (products) formed are known to be specific to carotenoids, and these degradation products are considered potent varietal flavor and aroma compounds and include α-ionone, β-ionone, pseudo-ionone, geranylacetone, β-damascenone, and vitispirane (Razungles et al., 1996; Baumes et al., 2002; Flamini, 2005; Mendes-Pinto, 2009; Crupi et al., 2010a). Norisoprenoid formation/carotenoid degradation can be catalyzed enzymatically (by the carotenoid cleavage dioxygensase) or physically (by oxidation and/or thermal decomposition; Enzell, 1985; Baldermann et al., 2013).

The increased volatile norisoprenoid concentration in the exposed berries was positively correlated to the increased carotenoid pool (Fig. 10). Previous research has shown that specific carotenoids serve as substrates for carotenoid cleavage dioxygenases, resulting in the formation of volatile C_{13}-norisoprenoids (Mathieu et al., 2005, 2006; Lashbrooke et al., 2013). Lashbrooke et al. (2013) identified and functionally characterized three grapevine carotenoid cleavage dioxygenases (VvCCD1, VvCCD4a, and VvCCD4b). The *VvCCD1*, *VvCCD4a*, and *VvCCD4b* transcripts were detected in all berry developmental stages tested (i.e. green, véraison, and

harvest stages), with *VvCCD4a* having the highest relative expression, peaking at véraison. The different VvCCDs were also shown to have different substrate specificities for their carotenoid substrates and norisoprenoid products formed (Lashbrooke et al., 2013). Here, we have shown an increase in the xanthophyll pool size that potentially serves as a substrate for the chloroplast-localized VvCCD4 enzymes (Figs. 7–9).

From the pathway analysis of carotenoid metabolism (Fig. 7), the expression of the CCD-encoding genes showed interesting differences between the exposed and control bunches: the cytosolic CCD1 was up-regulated in the exposed bunches in the earlier stages of development (from EL31 to EL35/véraison), with *VvCCD10.2* having higher expression levels than *VvCCD10.1*. The cytosolic CCD1 presumably plays an indirect recycling role in maintaining the optimal carotenoid composition in the early berry developmental stages, balancing photosynthesis and photoprotection. Conversely, the chloroplastic CCD4-encoding genes were down-regulated in later stages of development (from EL34 to EL38) in the exposed bunches, *VvCCD4b* typically having higher expression levels than *VvCCD4a*. The increased norisoprenoids, therefore, are not due to increased gene expression (of the CCD4-encoding genes) in the exposed berries but rather due to increased substrate (carotenoid) availability.

The volatile norisoprenoid products were concomitantly increased in the later stages (EL35 and EL38; Fig. 10). With the exception of α-ionone and β-ionone, all the analyzed norisoprenoids (MHO, pseudo-ionone, geranyl acetone, and β-damascenone) were higher in the exposed berries versus the control berries, supporting the findings of Crupi et al. (2010a) linking carotenoids to norisoprenoid content. This analysis also provides evidence of how metabolically interconnected events occurring early (EL31 and EL33) in berry development are: significant changes to photosynthetic pigments carry through to the later stages of berry ripening and, potentially, wine characteristics.

The Monoterpene Pool Is Modulated in the Later Stages of Berry Development in Response to Increased Exposure

The C_{10}-monoterpenes and C_{15}-sesquiterpenes are another class of volatile terpene-derived metabolites that contribute in varying degrees to the flavor and aroma of specific grape cultivars and wine (for review, see Ebeler and Thorngate, 2009). The terpene content of grapes has been well studied in relation to flavor and aroma, predominantly in the aromatic cv Muscat-type varieties. The genome sequence of grapevine (Jaillon et al., 2007) has shown that the genes encoding the enzymes catalyzing the synthesis of these metabolites, the terpene synthases (TPSs), occur in a large overrepresented family in grapevine. Martin et al. (2010) reported 69 predicted TPS-encoding loci in the cv Pinot Noir genome, 39 of which were shown to be functional in in vitro assays.

Volatile monoterpene responses were variable but, collectively, significantly increased in the exposed bunches in the later stages of development (from EL34), with EL38 having double the total monoterpene content (Fig. 9A). Most of the monoterpene levels analyzed were higher in the exposed berries at the later stages of berry development (EL35 and EL38). Linalool, nerol, and α-terpineol were the most significantly affected (Fig. 6B). Only 4-terpineol and cis-linalool oxide decreased with developmental stage, and only cis-linalool oxide was lower in the exposed berries (versus the control) at the harvest stage (EL38; Figs. 5 and 6C). Volatile organic compound (including monoterpenes) emissions are known to increase in response to both biotic (pathogens and herbivory) and abiotic (including temperature and light) stresses (for review, see Muhlemann et al., 2014). In 'Malbec' grapevine, Gil et al. (2013) showed increased monoterpene emissions at the preharvest berry developmental stage, with increased UV-B radiation. Since emissions of volatile terpenoids (monoterpenes [C_{10}] and norisoprenoids [C_{13}]) represent a significant loss of photosynthetic carbon to the plant, it is thought that these compounds must play important physiological and/or ecological roles in the protection of plants from environmental constraints (Loreto and Schnitzler, 2010). It is thought that isoprene (a C_5-hemiterpene) and monoterpenes are capable of stabilizing photosynthetic (chloroplastic) membranes and in so doing protect the photosynthetic apparatus from oxidative damage (Loreto and Schnitzler, 2010). Although the mechanism is controversial and currently not properly understood, the volatile terpenes have been demonstrated to possess antioxidant actions. This coupled with their lipophilic nature implies a potential role in membrane functioning (e.g. stability). Since both carotenoids and monoterpenes were affected by the treatment and both compound groups possess antioxidant activity, one interesting possibility is that the monoterpenes accumulate to compensate for the decrease in carotenoids in the later developmental stages (EL35 and EL38) or that the monoterpenes complement the photoprotection of the carotenoids during abiotic stress conditions (increased light and/or temperature) and in so doing are involved in oxidative stress homeostasis (Carvalho et al., 2015).

Interestingly, Šuklje et al. (2014) also reported elevated levels of linalool in wines made from exposed cv Sauvignon Blanc grapes from the same model vineyard (carotenoids were not analyzed). That study did not evaluate the berries but primarily focused on the wines made from the grapes from the respective treatments. The authors showed that exposed bunches led to an increase in thiols and monoterpenes (most notably linalool) in the resultant wines, which were consequently assigned attributes associated with tropical fruit in sensory evaluation. Conversely, the control wines were assigned green pepper, asparagus, and grassy attributes (Šuklje et al., 2014).

The Physiological Relevance of Compositional Metabolite Changes in Berries in Response to Increased Exposure

Grapevine berries in the early developmental stages respond in the same manner as photosynthetic organs (leaves), albeit at much lower levels. This phenomenon has been reported for a number of crop species, including climacteric and nonclimacteric fleshy fruits (e.g. apples [*Malus domestica*] and grape berries, respectively) as well as dehiscent and indehiscent fruits (e.g. peas [*Pisum sativum*] and cereal grains, respectively), as reviewed by Blanke and Lenz (1989). The data suggest that grape berries possess a pool of carotenoids that are intrinsically linked to photosynthesis (i.e. photosynthesis associated [i.e. β-carotene, lutein, neoxanthin, and to a lesser extent violaxanthin]) and, consequently, decrease during development, in much the same trend as chlorophyll (Fig. 5, cluster 2). There is, however, a second, smaller pool of carotenoids, the xanthophylls (i.e. zeaxanthin, antheraxanthin, and lutein epoxide) with the capacity to respond to the environment by modifying their abundance (e.g. depending on the ambient microclimate). This pool does not follow the developmental degradation trend of chlorophyll or the developmental increase of sugars but instead responds to the microclimate (Figs. 5, clusters 3 and 7, and 8). The individual carotenoids selectively accumulating in response to the higher exposure in exposed bunches were zeaxanthin, antheraxanthin, and lutein, with lutein epoxide accumulating in the less exposed control bunches (Figs. 7–9). For some of the carotenoids (i.e. lutein and lutein epoxide), this response only occurs in the earlier developmental stages (e.g. EL31, EL33, and to a lesser extent EL34) but not in the later stages (e.g. EL35 and EL38; Figs. 5 cluster 3, 7, and 8). The data also show that the increased carotenoid pools from earlier stages result in increased carotenoid-derived norsioprenoids in later berry developmental stages (Fig. 10). This is potentially a way of regulating the carotenoid composition in response to the prevailing/ambient conditions: maintaining photosynthesis under favorable conditions and triggering photoprotection during unfavorable conditions (i.e. shade for lutein epoxide or exposure for zeaxanthin). The temporary shifts in carotenoid pools in response to the microclimate can be subsequently catabolized to volatile C_{13}-norisoprenoids and transported out of the chloroplast, and the carotenoid composition optimal for photosynthesis can then be reestablished (de novo). This is the same metabolism that has been reported in photosynthetic leaf tissue and has been described for Arabidopsis (Lätari et al., 2015) and avocado (*Persea americana*) leaves (Förster et al., 2009).

The CCDs provide potential enzymatic candidates for this regulatory role. They are expressed during berry development, and each has a relatively unique carotenoid substrate specificity, with each carotenoid substrate yielding a different norisoprenoid product (Lashbrooke et al., 2013). Collectively there is a degree of agreement in the up-regulation of genes encoding enzymes involved in flux to carotenoid biosynthesis and the optimal functioning of the xanthophyll (violaxanthin and lutein epoxide) cycles in the earlier developmental stages (i.e. increased zeaxanthin, antheraxanthin, and lutein in the exposed berries). Conversely, the up-regulation of *VvCCD10.2* does not lead to a concomitant increase in the associated C_{13}-apocarotenoids (Fig. 7). The localization of the chloroplastic carotenoids and the cytosolic CCD1 enzyme could be the reason for this disparity, as has been described in Arabidopsis (Auldridge et al., 2006; Floss and Walter, 2009). It is possible that chloroplastic carotenoids are nonenzymatically degraded (due to the treatment) and transported to the cytosol, where they serve as substrates for CCD1. This recycling of carotenoids will ensure that the optimal carotenoid composition is maintained in the chloroplast to either assist photosynthesis or prevent photooxidative damage (Förster et al., 2009).

Although a nonclimacteric fruit, grape berry ripening has been associated with an oxidative burst at the onset of ripening (Pilati et al., 2007, 2014; Rienth et al., 2014). Most stresses result in an oxidative burst, and plants are capable of responding to a diverse array of potentially cooccurring stresses while maintaining active photosynthesis. The up-regulated metabolites described provide metabolite data in support of the hypothesis from a number of grapevine transcriptomic studies (Pilati et al., 2007, 2014; Rienth et al., 2014) that suggests a role in berry oxidative stress homeostasis in ripening grape berries via different antioxidant systems (Carvalho et al., 2015).

We found that the biological basis of the observed phenotypic (metabolic) plasticity is not necessarily in the absolute concentrations of individual metabolites (possibly with the exception of the xanthophylls zeaxanthin and lutein epoxide) but rather the pool size and/or ratio of metabolites within a pool. This is evident in the carotenoid and monoterpene pools and hints at a degree of compensation, possibly linked to their shared antioxidative protective functions (Borges et al., 2014; Kissoudis et al., 2014; Hossain et al., 2015).

We propose that this mechanism of oxidative stress homeostasis then provides the common factor linking the responsive secondary metabolites identified in grapevine leaf removal studies. The biological function of the responsive metabolites is as antioxidants. These include phenolics such as anthocyanins (Neill and Gould, 2003), flavonols (Hernández et al., 2009; Falcone Ferreyra et al., 2012), and stilbenes (for review, see Flamini et al., 2013), ascorbate (Melino et al., 2011), glutathione (Kobayashi et al., 2011), terpenoids (Grassmann, 2005) such as C_{10}-monoterpenoids (Gil et al., 2012), C_{15}-sesquiterpenoids, and C_{40}-tetraterpenoids (for review, see Cunningham and Gantt, 1998), and C_{13}-norisoprenoids (Walter and Strack, 2011). Their presence and associated antioxidant functions, therefore, implicate them in oxidative stress homeostasis observed in ripening grape berries (Pilati et al., 2014; Carvalho et al., 2015) and plant stress responses to, for example, abiotic stresses (Miller et al., 2010; Potters et al., 2010).

CONCLUSION AND FUTURE PROSPECTIVES

The field-omics approach employed in this study showed that the early leaf removal in the bunch zone caused quantifiable and stable responses (over two vintages) in the microclimate, where the main perturbation was increased exposure to light and to a lesser extent temperature, due to the geographical location of the vineyard (high altitude and proximity to the ocean). We showed the physiological impacts on berries in the different developmental stages by studying affected metabolites, providing, to our knowledge for the first time, an explanation for how leaf removal leads to the shifts in grape metabolites typically linked to this treatment (over years). We confirm anecdotal evidence and previous reports that leaf removal treatment at an early stage of berry development affects quality-associated metabolites (monoterpenes and norisoprenoids). Differences in the absolute concentrations of sugars and organic acids were marginal. We show that the main physiological response occurs in the early stages of berry development, when the berry is still photosynthetically active and, therefore, responds to changes to the microclimate in the same way as the major photosynthetically active organs (leaves). This also shows that berries in more shaded conditions activate a different protective system involving the conversion of lutein to lutein epoxide. The compositional changes in the carotenoids in the early stages are carried through to the later stages of berry development (e.g. increased norisoprenoids). This, combined with the increase in monoterpenes observed, implicates redox homeostasis and a degree of plant stress management. This topic has received much attention in grapevine (Carvalho et al., 2015) and in plants in general (Potters et al., 2010; Walter and Strack, 2011; Lätari et al., 2015).

The observation of phenotypic plasticity (metabolic/compositional plasticity) in cv Sauvignon Blanc grape berries, however, does not explain how plasticity is primarily regulated. Analysis specifically of the carotenoid metabolic pathway demonstrated that regulation occurs on both the transcriptional and metabolite levels. Further study of the transcriptome of the berries will provide insights into the transcriptional regulatory networks controlling the observed phenotypic (metabolic) plasticity. It would be interesting to compare the degree of plasticity observed in the transcriptome with that of the metabolome.

MATERIALS AND METHODS

Climatic Classification of the Elgin Model Vineyard Site

The vineyard is located in Elgin within the Overberg region of the Western Cape coastal region of South Africa (34°9′52.19′′S; 19°0′57.48′′E). Climatic classification of the Elgin region and the vineyard site was performed on macroclimatic and mesoclimatic scales according to established climatic indices (Tonietto and Carbonneau, 2004). The Heliothermal Index (Huglin, 1978; Tonietto and Carbonneau, 2004) was calculated for the period October 1 to March 31 (considered to be the biologically relevant period in the Southern Hemisphere) and the Winkler Index from September 1 to March 31. The Cool Night Index was calculated for the final month of ripening in the Southern Hemisphere (February 1–28). Hourly macroclimatic data were collected by the Beaulieu automatic weather station (MCSystems), run by the Institute for Soil, Climate, and Water of the Agricultural Research Council and maintained according to the standards of the World Meteorological Organization (Ehinger, 1993), located 1.55 km east of the experimental site. Hourly mesoclimatic data were collected from a dual-channel internal temperature and relative humidity sensor (MCS 486-TRH logger; MCSystems; maintained by Distell) installed within a Gill screen above the canopy.

Experimental Design, Vineyard/Viticultural Treatments and Management, and Sampling and Sample Processing within a Field-Omics Workflow

Grapevines (*Vitis vinifera* 'Sauvignon Blanc'; clone 316 grafted on 101-14 Mgt) were established in 2004. The vines were planted in a northwest-to-southeast row direction with 2.5-m between-row and 1.8-m in-row spacing. The vines were trellised to a double cordon with a vertical shoot-positioning system and pruned in winter to eight two-bud spurs per running 1 m of cordon. The experimental layout and workflow are outlined in Supplemental Figure S1. The vineyard has a deep shale soil with a high moisture content, so although irrigation was available, the vineyard was managed under dryland conditions, as no water constraints, as determined by stem water potential measurements, were experienced by the vines during the growing season (Supplemental Fig. S12). The treatment involved total leaf and lateral shoot removal in the bunch/fruiting zone (corresponding to removal up to approximately 30–40 cm above the cordon) on the northeast-facing side of the canopy (i.e. the facet of the vine that received morning sunlight exposure in the Southern Hemisphere) at EL29. In the control panels, no leaf removal was performed (Supplemental Fig. S1). The treatment was maintained throughout the season, keeping the fruiting zone exposed through continuous lateral shoot removal. The canopy of the control vines was not manipulated, which resulted in more shaded fruiting zones with reduced exposure. The leaf removal treatment were alternated down two adjacent vineyard rows, creating a checkerboard plot layout with each biological repeat (referred to as a panel) consisting of four consecutive vines (i.e. each row consisted of six panels, and each panel consisted of four healthy consecutive vines; Supplemental Fig. S1). Berry samples were collected (n = 48 berries per sample [i.e. per panel]), with 12 panels per sampling date (representing six exposed and six control panels) at five main phenological stages: green stage (pea-sized berries; EL31), prevéraison (EL33), véraison (EL34), ripening (EL35), and ripe berries at harvest (corresponding to the harvest date; EL38), using a supervised sampling method. The sampling is described as supervised due to the fact that samples were not collected randomly. Bunch positioning within the canopy is typically not uniform; therefore, berries were only sampled from representative bunches from the bunch facet exposed to the outside (northeast facing). All berry samples were collected within 1 h (9–10 AM) on the same day for all five sampling dates. Samples were immediately flash frozen in the field in liquid nitrogen. Seeds were removed, and the frozen tissue was ground in liquid nitrogen and, if not used immediately, stored at −80°C for further analysis.

The experimentation was conducted over three consecutive seasons (2010-2011, 2011-2012, and 2012-2013), but detailed data will only be provided and discussed for the 2010-2011 season. Selected higher order analyses, including supporting data from the additional seasons, will be provided where necessary to confirm repeatability over seasons.

Temperature Measurements

In addition to climatic monitoring to determine the climatic indices, the temperature of the canopy and bunches was monitored on a microclimatic scale. The canopy microclimate was monitored with the use of a dual-channel internal temperature and relative humidity logger (TinyTag TGP-4500; Gemini Dataloggers) and the bunch microclimate via flying lead thermistor probes attached to a dual-channel external temperature logger (TinyTag TGP-4520; Gemini Dataloggers).

Temperature was monitored at two levels, (1) mesoclimatic (i.e. above the canopy; continuously) and (2) microclimatic (i.e. within the canopy and within the bunch zone), using TinyTag data loggers (Gemini Dataloggers) from pea-size stage (EL31) until commercial harvest (EL38). Canopy temperatures were monitored with dual-channel (temperature and relative humidity) data loggers, whereas bunch temperatures were monitored using thermistor flying lead probes connected to a dual-channel external temperature data logger. The thermistor probes were positioned on the surface of the fruit

within representative bunches (for the respective treatments) and within the canopy.

Light Intensity Measurements

PAR was measured between 9:30 and 10:30 AM (before and after berry sampling) with an Accupar ceptometer (model LP-80: Decagon Devices). PAR was measured by positioning the ceptometer parallel to the ground within the bunch zone. Ambient PAR (i.e. full sunlight) was measured before and after each canopy measurement. Relative PAR values were expressed as a ratio relative to the ambient light measurement on the sampling day (i.e. as a percentage relative to full sunlight at the time of sampling).

Midday Stem Water Potential

The water status was determined by measuring the stem water potential according to the method described by Choné (2001) by use of a pressure chamber (Scholander et al., 1965). A single fully expanded, mature leaf per plant was selected for the stem water potential measurements as described by Deloire and Heyns (2011).

Berry Characterization

The weight and diameter for each of the berries sampled per biological repeat (i.e. per panel) were determined before sample processing for metabolite analyses. A sample from each of the six biological repeats per treatment consisted of 48 berries sampled from the exposed facet of a bunch. The 48 berries per sample were weighed individually using a laboratory balance, and the diameters were measured with a digital caliper.

RNA Extraction and Sequencing

Total RNA was extracted from three biological replicates sampled at four developmental stages under both exposed and control conditions according to Reid et al. (2006) from the same deseeded homogenized tissue as described for metabolite analysis. Each of the 24 samples was subjected to DNaseI treatment (Roche) to eliminate contamination with genomic DNA. The concentration and purity of the extracted RNA samples were established using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and the integrity of the samples was confirmed through analysis with the Bioanalyzer Chip RNA 7500 series II (Agilent) according to the manufacturer's instructions. Poly(A) mRNA was prepared for each of the RNA samples and sequenced with an Illumina HiSeq 1000 sequencer according to the supplier's instructions.

Berry Metabolite/Compositional Analyses

Analysis of the Major Sugar and Organic Acid Concentrations in Berries Using Reverse-Phase HPLC

The major sugars and organic acids present in grape berries were extracted from 100 ± 10 mg of frozen, ground berry tissue from the five developmental stages and analyzed by reverse-phase HPLC as described by Eyéghé-Bickong et al. (2012).

Analysis of the Carotenoid and Chlorophyll Concentrations in Berries Using Reverse-Phase Ultra-High-Performance Liquid Chromatography

Carotenoid and chlorophylls were extracted from 250 mg of frozen, ground berry tissue from the five developmental stages as described by Lashbrooke et al. (2010). The analysis of these major pigments was done on a Water Acquity ultra-high-performance liquid chromatography system equipped with a diode array detector. Pigment separation was achieved on a Waters UPLC BEH Shield RP18 (2.1 mm × 100 mm, 1.7 μm) column protected with a Waters UPLC BEH guard cartridge (2.1 mm × 100 mm, 1.7 μm), and the column temperature was set to 20°C. The mobile phases were composed of aqueous 5% acetonitrile in 0.1% (v/v) formic acid (A) and 80%/20% acetonitrile/methanol in 0.1% (v/v/v) formic acid (B). The following gradient program was applied: 0 to 1 min, isocratic 60% B at a flow rate of 0.3 mL min^{-1}; 1 to 12 min, nonlinear (gradient 3) from 60% to 99.8% B at flow rates from 0.3 to 0.5 mL min^{-1}; 12 to 13 min, linear 99.8% to 100% B at a flow rate of 0.5 mL min^{-1}; 13 to 13.1 min, linear 100% to 60% B at flow rates from 0.5 to 0.3 mL min^{-1}; and then for 1.9 min, equilibration with an isocratic 60% B at a flow rate of 0.3 mL min^{-1}. The control of the instrument and the acquisition and processing of the generated data were done using Empower 2 software from Waters, and the injection volume was 5 μL.

The quantification of the major pigments in samples was carried out at 450 nm for xanthophylls and β-carotene, 420 nm for chlorophyll *a*, and 470 nm for chlorophyll *b* using external standard calibration based on standard curves plotted using the peak areas and standard concentrations (in μg mL^{-1}). The concentrations in samples were then normalized to the internal standard amount and the sample fresh weight to obtain the sample amount per berry fresh weight (ng g^{-1} fresh weight). β-Apocarotene was used as the internal standard for all pigments.

Analysis of Berry Volatiles Using Head Space-Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry

Authentic standards for the volatile apocarotenoids (β-damascenone, β-damascone, geranylacetone, α-ionone, β-ionone, pseudo-ionone, and MHO), monoterpenes (eucalyptol, limonene, trans-linalool oxide, cis-linalool oxide, linalool, 4-terpineol, citronellol, nerol, geraniol, fenchone, and α-terpineol), and the internal standard (3-octanol) were purchased from Sigma-Aldrich.

Approximately 500 mg of ground, frozen grapevine berry tissue was weighed into a 20-mL gas chromatography vial, and 2 mL of tartaric acid buffer (2 g L^{-1} tartrate, 2.1 g L^{-1} ascorbic acid, and 0.8 mg L^{-1} sodium azide, pH 3) was added to each vial. The preservatives ascorbic acid and sodium azide (Sigma-Aldrich) were added to the buffer in order to inhibit polyphenol oxidase action and to prevent microbial growth during storage and analysis of the berries, respectively (Flamini and Vedova, 2007). The samples were preincubated for 1 h at 100°C to extract the total volatiles (i.e. the free and bound volatile fractions). If not analyzed immediately, samples were stored at −80°C.

Volatiles were extracted by head space solid-phase microextraction (SPME) using a 50/30-μm divinylbenzene/carboxen/polydimethylsiloxane fiber (gray fiber from Supelco; Barros et al., 2012). Prior to use, the fiber was conditioned at 270°C for 60 min in the gas chromatograph injection port according to the manufacturer's specifications (Supelco).

The samples were equilibrated at 60°C for 5 min in a heating chamber (with constant agitation at 250 rpm). After equilibration, the SPME fiber was inserted through the vial septa and exposed to the sample at 60°C for 30 min with constant agitation at 250 rpm. The bound analytes were thermally desorbed from the fiber in the gas chromatograph injection port. After desorbtion, the fiber was maintained for 20 min in the injection port for cleaning in order to prevent potential carryover between samples.

Gas chromatography analysis was carried out on an Agilent 6890N gas chromatograph coupled to a CTC CombiPal Analytics autosampler and an Agilent 5975B inert XL EI/CI MSD mass spectrometer detector through a transfer line. Analysis was done using an Agilent 122-3263 DB-FF AP capillary column (60 m × 250 μm i.d., 0.5 μm). Desorption of analytes from the SPME fiber was performed in the injection port at 250°C by pulsed splitless mode for 1 min. The purge flow was 1 min at 50 mL min^{-1}. The column operating head pressure was raised from 111 kPa to obtain a pulse pressure of 300 kPa for 1 min. Helium was used as the carrier gas, with a constant flow rate of 1 mL min^{-1}. The oven parameters were as follows: initial temperature of 40°C (2 min), a linear increase to a final temperature of 240°C (at a rate of 5°C min^{-1}), and the temperature was held at 240°C for a final 2 min. The total run time was 44 min. The transfer line temperature was maintained at 250°C.

The mass spectrometry detector was operated in scan and selected ion monitoring modes. The scan parameters were set at mass-to-charge ratio (m/z) ranging from 35 to 350. The dwell time for each ion in a group was set to 100 ms . The software used was MSD ChemStation (G1701-90057; Agilent).

Selected ion monitoring was used to identify compounds according to their elution times and manually integrate their areas. The selected ions monitored were as follows: 3-octanol (internal standard), *m/z* = 83; geranylacetone, *m/z* = 69; eucalyptol, limonene, trans-linalool oxide, γ-terpenene, cis-linalool oxide, linalool, 4-terpineol, α-terpineol, Citronellol, nerol, and geraniol, *m/z* = 93; MHO, *m/z* = 108; β-damascenone, *m/z* = 190; β-damascone, α-ionone, and β-ionone, *m/z* = 177; and pseudo-ionone, *m/z* = 124. The quantification of the volatiles in samples was done using external standard calibration based on standard curves plotted using the peak areas of each standard (total ion count) relative to the peak area of the internal standard versus the standard concentration (μg.L^{-1}) of a nine-point standard dilution series.

Statistical Analyses

Standard statistical analyses were performed using Microsoft Excel (version 14) and Statistica (version 12). Where required, the data statistics (*P* values) were adjusted for false discovery rate by Benjamini-Hochberg correction (adjusted *P* values or *Q* values) as described by Trapnell et al. (2012). Hierarchical cluster analysis of metabolites was performed using Expander (Sharan et al., 2003). All multivariate data analyses were performed using SIMCA (version 13.0.3.0 from Umetrics). For multivariate data analysis, data were normalized and analyzed using PCA and/or OPLS-DA. OPLS-DA was used to analyze the quantitative relationship between the data matrix, *x* (i.e. the variables measured [e.g. metabolite concentration and/or transcript levels]), and a vector, *y*, containing qualitative values (e.g. developmental stages [EL31–EL38] or treatment [control/exposed]).

The reads generated from the RNA sequencing were aligned to the grapevine reference genome (X12) using TopHat (version 2.0; Trapnell et al., 2009). Cufflinks (version 2.0) was subsequently used to assemble transcripts from the generated sequence reads (Trapnell et al., 2010). CuffDiff (version 2.0) was used for differential expression analysis between treatments and/or subsequent developmental stages (Trapnell et al., 2010). The putative carotenoid metabolic genes were obtained from Young et al. (2012).

The data reported (i.e. metabolite and expression data) are provided in Supplemental Table S2A as averages ± SD.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Plot layout and field-omics workflow.

Supplemental Figure S2. Mean seasonal data, where different letters indicate significant differences between treatments ($P \leq 0.05$).

Supplemental Figure S3. Berry characterization.

Supplemental Figure S4. Berry characterization: concentration of the major sugars in grapevine berries.

Supplemental Figure S5. Unsupervised PCA of all variables from the study for all developmental stages.

Supplemental Figure S6. Berry characterization: organic acids.

Supplemental Figure S7. Chlorophyll *a*-chlorophyll *b* ratio in developing berries.

Supplemental Figure S8. Relationship between chlorophyll and the major carotenes.

Supplemental Figure S9. ANOVA of developmental stage × treatment of metabolites showing the highest coefficient of variation.

Supplemental Figure S10. Field-omics: assessment of all late variables for all biological repeats (panels 1–6) across all stages (EL31, EL33, EL34, EL35, and EL38) for the 2010-2011 season.

Supplemental Figure S11. Repeatability of the experiment for two consecutive seasons (2010-2011 and 2011-2012).

Supplemental Figure S12. Midday stem water potential at three developmental stages.

Supplemental Table S1. Climatic indices used to classify the Elgin region.

Supplemental Table S2. Mean and SD of the transcripts ($n = 3$) and metabolites ($n = 6$) reported in this study and significance testing (Student's *t* test) of transcripts and metabolites in the various developmental stages (EL31, EL33, EL34, and EL38).

ACKNOWLEDGMENTS

We thank the following people for their various invaluable contributions to the study: Dr. Liezel Gouws and Varsha Premsagar for assistance with sample processing; Cobus Steyn for assistance with the viticultural treatments and sampling; Mario Pezzotti, Massimo Delledonna, Dr. Marianna Fasoli, and Alessandra Dal Molin for bioinformatics support with the RNA sequencing data; Nina Lawrence for assistance with the implementation of the HPLC method for the analysis of sugars and organic acids; Lucky Mokwena, Malcolm Taylor, and Antonio Ferreira for assistance with the implementation of the head space-SPME gas chromatography-mass spectrometry method for the analysis of volatiles from grapes; Justin Lashbrooke for inputs with the analysis of carotenoids and chlorophylls and the subsequent pathway analysis; Ms Weighill for insights into the correlation analysis; and Dr. Johan Trygg for discussions on multivariate data analysis and providing the license for SIMCA.

Received November 15, 2015; accepted December 1, 2015; published December 1, 2015.

LITERATURE CITED

Alexandersson E, Jacobson D, Vivier MA, Weckwerth W, Andreasson E (2014) Field-omics: understanding large-scale molecular data from field crops. Front Plant Sci **5:** 286

Anesi A, Stocchero M, Dal Santo S, Commisso M, Zenoni S, Ceoldo S, Tornielli GB, Siebert TE, Herderich M, Pezzotti M, et al (2015) Towards a scientific interpretation of the terroir concept: plasticity of the grape berry metabolome. BMC Plant Biol **15:** 191

Auldridge ME, McCarty DR, Klee HJ (2006) Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr Opin Plant Biol **9:** 315–321

Baldermann S, Kato M, Fujita A, Fleischmann P, Winterhalter P, Watanabe N (2013) Biodegradation of carotenoids: an important route to scent formation. *In* P Winterhalter, ed, Carotenoid Cleavage Products. ACS Symposium Series. American Chemical Society, Washington, DC, pp 66–71

Barros EP, Moreira N, Pereira GE, Leite SGF, Rezende CM, Pinho, PG (2012) Development and validation of automatic HS-SPME with a gas chromatography-ion trap/mass spectrometry method for analysis of volatiles in wines. Talanta **101:** 177–186

Baumes R, Wirth J, Bureau S, Gunata Y, Razungles A (2002) Biogeneration of C13-norisoprenoid compounds: experiments supportive for an apocarotenoid pathway in grapevines. Anal Chim Acta **458:** 3–14

Blanke MM, Lenz F (1989) Fruit photosynthesis. Plant Cell Environ **12:** 31–46

Borges AA, Jiménez-Arias D, Expósito-Rodríguez M, Sandalio LM, Pérez JA (2014) Priming crops against biotic and abiotic stresses: MSB as a tool for studying mechanisms. Front Plant Sci **5:** 642

Bouby L, Figueiral I, Bouchette A, Rovira N, Ivorra S, Lacombe T, Pastor T, Picq S, Marinval P, Terral JF (2013) Bioarchaeological insights into the process of domestication of grapevine (*Vitis vinifera* L.) during Roman times in southern France. PLoS ONE **8:** e63195

Carvalho LC, Vidigal P, Amâncio S (2015) Oxidative stress homeostasis in grapevine (Vitis vinifera L.). Front Environ Sci (in press) 10.3389/fenvs.2015.00020

Choné X (2001) Stem water potential is a sensitive indicator of grapevine water status. Ann Bot (Lond) **87:** 477–483

Chorti E, Guidoni S, Ferrandino A (2010) Effect of different cluster sunlight exposure levels on ripening and anthocyanin accumulation in Nebbiolo grapes. Am J Enol Vitic **61:** 23–30

Crupi P, Coletta A, Antonacci D (2010a) Analysis of carotenoids in grapes to predict norisoprenoid varietal aroma of wines from Apulia. J Agric Food Chem **58:** 9647–9656

Crupi P, Milella RA, Antonacci D (2010b) Simultaneous HPLC-DAD-MS (ESI+) determination of structural and geometrical isomers of carotenoids in mature grapes. J Mass Spectrom **45:** 971–980

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol **49:** 557–583

Dal Santo S, Tornielli GB, Zenoni S, Fasoli M, Farina L, Anesi A, Guzzo F, Delledonne M, Pezzotti M (2013) The plasticity of the grapevine berry transcriptome. Genome Biol **14:** r54

Deloire AJ and Heyns D (2011) Leaf water potential: principles, methods and thresholds. Wynboer, **265:** 119–121

Deluc LG, Quilici DR, Decendit A, Grimplet J, Wheatley MD, Schlauch KA, Mérillon JM, Cushman JC, Cramer GR (2009) Water deficit alters differentially metabolic pathways affecting important flavor and quality traits in grape berries of Cabernet Sauvignon and Chardonnay. BMC Genomics **10:** 212

De Toda F, Sancha JC, Balda P (2013) Reducing the sugar and pH of the grape (*Vitis vinifera* L. cvs. 'Grenache' and 'Tempranillo') through a single shoot trimming. S Afr J Enol Vitic **34:** 246–251

Ebeler SE, Thorngate JH (2009) Wine chemistry and flavor: looking into the crystal glass. J Agric Food Chem **57:** 8098–8108

Ehinger J (1993) Siting and exposure of meteorological instruments. World Meteorological Organization: Instruments and Observing Methods. Report No. 55, WMO/TD-No. 589, Geneva.

English JT, Thomas CS, Marois JJ, Gubler WD (1989) Microclimates of grapevine canopies associated with leaf removal and control of *Botrytis* bunch rot. Phytopathology **79:** 395–401

Enzell CR (1985) Biodegradation of carotenoids: an important route to aroma compounds. Pure Appl Chem **57:** 693–700

Esteban R, Becerril JM, García-Plazaola JI (2009a) Lutein epoxide cycle, more than just a forest tale. Plant Signal Behav **4:** 342–344

Eyéghé-Bickong HA, Alexandersson EO, Gouws LM, Young PR, Vivier MA (2012) Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. J Chromatogr B Analyt Technol Biomed Life Sci **885-886:** 43–49

Falcone Ferreyra ML, Rius SP, Casati P (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci **3:** 222

Flamini R (2005) Some advances in the knowledge of grape, wine and distillates chemistry as achieved by mass spectrometry. J Mass Spectrom **40:** 705–713

Flamini R, Mattivi F, De Rosso M, Arapitsas P, Bavaresco L (2013) Advanced knowledge of three important classes of grape phenolics: anthocyanins, stilbenes and flavonols. Int J Mol Sci **14:** 19651–19669

Flamini R, Vedova AD (2007) Preservation of Cabernet Sauvignon grape must samples destined for chemical analysis: addition of sodium azide, allyl isothiocyanate, octanoic acid and ethyl bromoacetate, and effect of pasteurization. J Food Process Preserv **31:** 345–355

Floss DS, Walter MH (2009) Role of carotenoid cleavage dioxygenase 1 (CCD1) in apocarotenoid biogenesis revisited. Plant Signal Behav **4:** 172–175

Förster B, Osmond CB, Pogson BJ (2009) De novo synthesis and degradation of Lx and V cycle pigments during shade and sun acclimation in avocado leaves. Plant Physiol **149:** 1179–1195

Förster B, Pogson BJ, Osmond CB (2011) Lutein from deepoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for nonphotochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark. Plant Physiol **156:** 393–403

Frey A, Effroy D, Lefebvre V, Seo M, Perreau F, Berger A, Sechet J, To A, North HM, Marion-Poll A (2012) Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. Plant J **70:** 501–512

García-Piazola JI, Matsubara S, Osmond CB (2007) The lutein epoxide cycle in higher plants: its relationships to other xanthophyll cycles and possible functions. Funct Plant Biol **34:** 759–773

Gil M, Bottini R, Berli F, Pontin M, Silva MF, Piccoli P (2013) Volatile organic compounds characterized from grapevine (*Vitis vinifera* L. cv. Malbec) berries increase at pre-harvest and in response to UV-B radiation. Phytochemistry **96:** 148–157

Gil M, Pontin M, Berli F, Bottini R, Piccoli P (2012) Metabolism of terpenes in the response of grape (Vitis vinifera L.) leaf tissues to UV-B radiation. Phytochemistry **77:** 89–98

Goodwin TW (1980) The Biochemistry of the Carotenoids. Chapman & Hall, London

Gordon D, Dejong TM (2007) Current-year and subsequent-year effects of crop-load manipulation and epicormic-shoot removal on distribution of long, short and epicormic shoot growth in *Prunus persica*. Ann Bot (Lond) **99:** 323–332

Grassmann J (2005) Terpenoids as plant antioxidants. Vitam Horm **72:** 505–535

Gubler WD, Bettiga LJ, Heil D (1991) Comparisons of hand and machine leaf removal for the control of *Botrytis* bunch rot. Am J Enol Vitic **42:** 233–236

Hernández I, Alegre L, Van Breusegem F, Munné-Bosch S (2009) How relevant are flavonoids as antioxidants in plants? Trends Plant Sci **14:** 125–132

Hossain MA, Bhattacharjee S, Armin SM, Qian P, Xin W, Li HY, Burritt DJ, Fujita M, Tran LSP (2015) Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. Front Plant Sci **6:** 420

Huglin P (1978) Nouveau mode d'évaluation des possibilités héliothermiques d'un milieu viticole. Comptes Rendus de l'Academie d'Agriculture **64:** 1117–1126

Hunter JJ, Visser JH (1990) The effect of partial defoliation on growth characteristics of Vitis vinifera L. cv. Cabernet Sauvignon. II. Reproductive growth. S Afr J Enol Vitic **11:** 26–32

Jahns P, Holzwarth AR (2012) The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochim Biophys Acta **1817:** 182–193

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C, et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature **449:** 463–467

Kim J, Smith JJ, Tian L, Dellapenna D (2009) The evolution and function of carotenoid hydroxylases in Arabidopsis. Plant Cell Physiol **50:** 463–479

Kissoudis C, van de Wiel C, Visser RGF, van der Linden G (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. Front Plant Sci **5:** 207

Kobayashi H, Takase H, Suzuki Y, Tanzawa F, Takata R, Fujita K, Kohno M, Mochizuki M, Suzuki S, Konno T (2011) Environmental stress enhances biosynthesis of flavor precursors, S-3-(hexan-1-ol)-glutathione and S-3-(hexan-1-ol)-L-cysteine, in grapevine through glutathione S-transferase activation. J Exp Bot **62:** 1325–1336

Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, Gomès E, Li SH, Godoy F, Arce-Johnson P, Delrot S (2014) Berry ripening: recently heard through the grapevine. J Exp Bot **65:** 4543–4559

Lashbrooke JG, Young PR, Dockrall SJ, Vasanth K, Vivier MA (2013) Functional characterisation of three members of the *Vitis vinifera* L. carotenoid cleavage dioxygenase gene family. BMC Plant Biol **13:** 156

Lashbrooke JG, Young PR, Strever AE, Stander C, Vivier MA (2010) The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling. Aust J Grape Wine Res. **16:** 349–360

Lätari K, Wüst F, Hübner M, Schaub P, Beisel KG, Matsubara S, Beyer P, Welsch R (2015) Tissue-specific apocarotenoid glycosylation contributes to carotenoid homeostasis in Arabidopsis leaves. Plant Physiol **168:** 1550–1562

Lee J, Skinkis PA (2013) Oregon 'Pinot Noir' grape anthocyanin enhancement by early leaf removal. Food Chem **139:** 893–901

Lee S, Seo M, Riu M, Cotta JP, Block DE, Dokoozlian NK, Ebeler SE (2007) Vine microclimate and norisoprenoid concentration in Cabernet Sauvignon grapes and wines. Am J Enol Vitic **3:** 291–301

Loreto F, Schnitzler JP (2010) Abiotic stresses and induced BVOCs. Trends Plant Sci **15:** 154–166

Martin DM, Aubourg S, Schouwey MB, Daviet L, Schalk M, Toub O, Lund ST, Bohlmann J (2010) Functional annotation, genome organization and phylogeny of the grapevine (*Vitis vinifera*) terpene synthase gene family based on genome assembly, FLcDNA cloning, and enzyme assays. BMC Plant Biol **10:** 226

Mathieu S, Terrier N, Procureur J, Bigey F, Günata Z (2005) A carotenoid cleavage dioxygenase from *Vitis vinifera* L.: functional characterization and expression during grape berry development in relation to C_{13}-norisoprenoid accumulation. J Exp Bot **56:** 2721–2731

Mathieu S, Terrier N, Procureur J, Vigne D (2006) Vitis vinifera carotenoid cleavage dioxygenase (VvCCD1): gene expression during grape berry development and cleavage of carotenoids by recombinant protein. Dev Food Sci **43:** 85–88

Meléndez-Martínez AJ, Britton G, Vicario IM, Heredia FJ (2006) HPLC analysis of geometrical isomers of lutein epoxide isolated from dandelion (*Taraxacum officinale* F. Weber ex Wiggers). Phytochemistry **67:** 771–777

Melino VJ, Hayes MA, Soole KL, Ford CM (2011) The role of light in the regulation of ascorbate metabolism during berry development in the cultivated grapevine *Vitis vinifera* L. J Sci Food Agric **91:** 1712–1721

Mendes-Pinto MM (2009) Carotenoid breakdown products—the norisoprenoids—in wine aroma. Arch Biochem Biophys **483:** 236–245

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ **33:** 453–467

Muhlemann JK, Klempien A, Dudareva N (2014) Floral volatiles: from biosynthesis to function. Plant Cell Environ **37:** 1936–1949

Neill SO, Gould KS (2003) Anthocyanins in leaves: light attenuators or antioxidants? Funct Plant Biol **30:** 865–873

Oliveira C, Ferreira AC, Costa P, Guerra J, Guedes De Pinho P (2004) Effect of some viticultural parameters on the grape carotenoid profile. J Agric Food Chem **52:** 4178–4184

Oliveira C, Silva Ferreira AC, Mendes Pinto M, Hogg T, Alves F, Guedes de Pinho P (2003) Carotenoid compounds in grapes and their relationship to plant water status. J Agric Food Chem **51:** 5967–5971

O'Neill T, Berrie AM, Wedgwood E, Allen J, Xu XM (2009) Effect of canopy manipulation on cane and fruit *Botrytis* in protected raspberry. Commun Agric Appl Biol Sci **74:** 633–643

Palliotti A, Gardi T, Berrios JG, Civardi S, Poni S (2012) Early source limitation as a tool for yield control and wine quality improvement in a high-yielding red *Vitis vinifera* L. cultivar. Sci Hortic (Amsterdam) **145:** 10–16

Pilati S, Brazzale D, Guella G, Milli A, Ruberti C, Biasioli F, Zottini M, Moser C (2014) The onset of grapevine berry ripening is characterized by ROS accumulation and lipoxygenase-mediated membrane peroxidation in the skin. BMC Plant Biol **14:** 87

Pilati S, Perazzolli M, Malossini A, Cestaro A, Demattè L, Fontana P, Dal Ri A, Viola R, Velasco R, Moser C (2007) Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at vèraison. BMC Genomics **8:** 428

Potters G, Horemans N, Jansen MAK (2010) The cellular redox state in plant stress biology: a charging concept. Plant Physiol Biochem **48:** 292–300

Rabinowitch HD, Budowski P, Kedar N (1975) Carotenoids and epoxide cycles in mature-green tomatoes. Planta **122:** 91–97

Razungles AJ, Babic I, Sapis JC, Bayonove CL (1996) Particular behavior of epoxy xanthophylls during veraison and maturation of grape. J Agric Food Chem **44:** 3821–3825

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol **6:** 27

Reynolds AG, Wardle DA (1989) Impact of various canopy manipulation techniques on growth, yield, fruit composition, and wine quality of Gewürztraminer. Am J Enol Vitic **40:** 121–129

Rienth M, Torregrosa L, Luchaire N, Chatbanyong R, Lecourieux D, Kelly MT, Romieu C (2014) Day and night heat stress trigger different transcriptomic responses in green and ripening grapevine (Vitis vinifera) fruit. BMC Plant Biol **14:** 108

Schlichting C (1986) The evolution of phenotypic plasticity in plants. Annu Rev Ecol Syst **17:** 667–693

Schlichting CD, Smith H (2002) Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. Evol Ecol **16:** 189–211

Scholander PF, Bradstreet ED, Hemmingsen EA, Hammel HT (1965) Sap pressure in vascular plants: negative hydrostatic pressure can be measured in plants. Science **148:** 339–346

Sharan R, Maron-Katz A, Shamir R (2003) CLICK and EXPANDER: A system for clustering and visualizing gene expression data. Bioinformatics **19:** 1787–1799

Shiraishi M (1995) Proposed descriptors for organic acids to evaluate grape germplasm. Euphytica **81:** 13–20

Song J, Smart R, Wang H, Dambergs B, Sparrow A, Qian MC (2015) Effect of grape bunch sunlight exposure and UV radiation on phenolics and volatile composition of *Vitis vinifera* L. cv. Pinot Noir wine. Food Chem **173:** 424–431

Staff SL, Percival DC, Sullivan JA, Fisher KH (1997) Fruit zone leaf removal influences vegetative, yield, disease, fruit composition, and wine sensory attributes of *Vitis vinifera* L 'Optima' and 'Cabernet Franc'. Can J Plant Sci **77:** 149–153

Stephan J, Sinoquet H, Donès N, Haddad N, Talhouk S, Lauri PE (2008) Light interception and partitioning between shoots in apple cultivars influenced by training. Tree Physiol **28:** 331–342

Sternad Lemut M, Trost K, Sivilotti P, Vrhovsek U (2011) Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. J Food Compos Anal **24:** 777–784

Šuklje K, Antalick G, Coetzee Z, Schmidtke LM, Baša Česnik H, Brandt J, du Toit WJ, Lisjak K, Deloire A (2014) Effect of leaf removal and ultraviolet radiation on the composition and sensory perception of *Vitis vinifera* L. cv. Sauvignon Blanc wine. Aust J Grape Wine Res **20:** 223–233

Sweetman C, Sadras VO, Hancock RD, Soole KL, Ford CM (2014) Metabolic effects of elevated temperature on organic acid degradation in ripening *Vitis vinifera* fruit. J Exp Bot **65:** 5975–5988

Tardaguila J, Diago MP, de Toda F, Poni S, Vilanova M (2008) Effects of timing of leaf removal on yield, berry maturity, wine composition and sensory properties of cv. Grenache grown under non irrigated conditions. J Int Des Sci La Vigne Du Vin **42:** 221–229

Terral JF, Tabard E, Bouby L, Ivorra S, Pastor T, Figueiral I, Picq S, Chevance JB, Jung C, Fabre L, et al (2010) Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars. Ann Bot (Lond) **105:** 443–455

Tian L, DellaPenna D (2004) Progress in understanding the origin and functions of carotenoid hydroxylases in plants. Arch Biochem Biophys **430:** 22–29

Tonietto J, Carbonneau A (2004) A multicriteria climatic classification system for grape-growing regions worldwide. Agric Meteorol **124:** 81–97

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics **25:** 1105–1111

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc **7:** 562–578

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol **28:** 511–515

Via S, Lande R (2013) Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution **39:** 505–522

Walter MH, Strack D (2011) Carotenoids and their cleavage products: biosynthesis and functions. Nat Prod Rep **28:** 663–692

Young PR, Lashbrooke JG, Alexandersson E, Jacobson D, Moser C, Velasco R, Vivier MA (2012) The genes and enzymes of the carotenoid metabolic pathway in *Vitis vinifera* L. BMC Genomics **13:** 243

Yu Q, Ghisla S, Hirschberg J, Mann V, Beyer P (2011) Plant carotene cis-trans isomerase CRTISO: a new member of the FAD(RED)-dependent flavoproteins catalyzing non-redox reactions. J Biol Chem **286:** 8666–8676