University of Nevada, Reno

Within the skin: Grape berries during the mature stages of ripening

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
Biochemistry

By

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August, 2015

THE GRADUATE SCHOOL

We recommend that the dissertation
prepared under our supervision by

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Entitled

Within The Skin: Grape Berries During The Mature Stages Of Ripening

be accepted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

A systems biology approach was used to investigate berry skins of three red- (Cabernet Sauvignon, Merlot, Pinot Noir) and two white-skinned (Chardonnay, Semillon) wine grape cultivars. Identical sample aliquots were analyzed for transcripts by a grapevine whole genome oligonucleotide microarray and RNAseq technologies, proteins by nano-liquid chromatography-mass spectroscopy, and metabolites by gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy. Principal components analysis of each of five Omic technologies showed similar results across cultivars in all omics datasets. Comparison of the processed data of genes mapped in both RNAseq and microarray data revealed a strong Pearson's correlation (0.80), but concordance of protein with transcript data was low with a Pearson's correlation of 0.27 and 0.24 for the RNAseq and microarray data, respectively. Integration of metabolite with protein and transcript data produced an expected model of phenylpropanoid biosynthesis, distinguishing red from white grapes, yet, provided detail of individual cultivar differences. The integration of multiple high-throughput Omic datasets revealed complex biochemical variation amongst five cultivars of an ancient and economically important crop species.

Grape berry ripening occurs in the late stages of development with increases in sugar, changes in color, and decreases in malate concentration. In the final stages of ripening, fruit flavors and volatile aromas increase to signal readiness for seed dispersal. To identify the common transcriptional changes in the late stages of berry development in multiple grape cultivars, the

transcriptomic responses of the berry skins of 7 cultivars of grapes that were grown in the same vineyard were determined using RNAseq at four different °Brix levels (20 to 26 °Brix). The abundance of thousands of transcripts changed significantly in the late stages of berry development. Gene set enrichment analysis of functional Gene Ontology terms provided evidence for a complex interplay of many gene ontology categories including those involved in the circadian clock, postembryonic development, photosynthesis, hormone signaling, reactive oxygen species (ROS), DNA methylation and transcriptional regulation. There were 809 transcription factors (TF) differentially expressed with increasing ˚Brix (~4% of all transcripts and ~32% of all TF), belonging to 81 families, including the C3H, MYB, AP2/ERF and bHLH families. Our analyses indicate that the circadian clock and epigenetic modification are major factors regulating transcription in mature berries.

Finally, pathogenesis-related proteins that accumulated in skins of three red-skinned and two white-skinned cultivars: Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay and Semillon, were characterized *in silico*, using protein and transcript data. Large amounts of identified proteins were classified as pathogenesis-related in berry skins, more so than what was previously observed in shoot tips. Several PR-families had numerous protein members in skins, which maybe a tissue specific occurrence. The transcript abundance was well correlated to the protein abundance in thaumatins of PR-05, but not so in the L-ascorbate peroxidases of PR-09. Haze-forming proteins, while well represented, did not accumulate with more specificity in the white cultivars and were mostly

higher in the red cultivar, Pinot Noir. Large accumulations of PR-proteins in skins at harvest provide support for a prolonged and possibly a constitutive defense mechanism that protects a maturing seed within the berry.

ACKNOWLEDGEMENTS

I would very much like to thank my family and friends for their help, friendship and support over the years. To my parents, Mark and Heidi Ghan, you both have been incredibly supportive of me, from my time in the Army to the end of my doctoral education. Your love has been unconditional and I thank you both. To the Bradshaw family, I count you as my oldest and dearest of friends who have treated me like a son and a brother. To Sayuri, thank you for the support and love you have shown me, particularly during this final and trying year of my graduate studies. I would not have wanted to go through this without you.

Special thanks to Grant Cramer for taking a wine volunteer into the lab and helping him to become a researcher. I am so grateful to have you as a mentor and a friend. To past and present members of the Cramer lab, thank you for all the help with my experiments, particularly the long hours peeling grape berries. To Danny Hopper, we shared a great journey in our quest for our doctorates. I have been lucky to have you as a hardworking colleague and friend. I would also like to convey my gratitude to each of my committee members for their help throughout my time as a graduate student, particularly to Karen Schlauch.

Finally, my time as a graduate student, ups and downs included, has been an incredible and multifaceted experience. This has included working a vineyard and experimental winery, learning cutting edge technologies, traveling to faraway places around the world and working with some amazing colleagues. I know my life will be better for it all.

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CHAPTER 1:

Introduction

1.1 A little about grapes

The cultivation of grapes predates history, with cultural significance rooted in the ancient Neolithic era (McGovern, Hartung et al. 1997). A woody flowering and long-lived species, early members of the genus *Vitis* were domesticated 7,000 – 8,000 years ago (McGovern, Glusker et al. 1996). Viticulture likely originated in eastern Anatolia (present day Turkey) and through the South Caucuses (Azerbaijan, Armenia, and Georgia) (Arroyo-Garcia, Ruiz-Garcia et al. 2006, This, Lacombe et al. 2006, Imazio, Maghradze et al. 2013). Early wine may have arisen simply from clusters crushed beneath their own weight, some resident-wild yeasts on the berry and then left forgotten in an animal skin or ceramic jar (Rosini, Federici et al. 1982). Today, grapes are produced on every habitable continent of the world, representing many thousands of cultivars that are grown and consumed as fresh fruit, functioning as the root stocks for vinifera scion, and in the production of a range of wines with distinct and complex flavor profiles (Bisson, Waterhouse et al. 2002, This, Lacombe et al. 2006).

Grapes selected for specific, uniform and stable traits are referred to as cultivars, and this includes clones produced from asexual propagation (Brickell, Alexander et al. 2009). For example, Cabernet Sauvignon and Chardonnay are two of the most commonly produced cultivars in the United States and Australia. Cultivated grapes can be classified based upon use (e.g. table, raisin, wine, brandy), by skin color, (e.g. white, red) or aromatic and volatile profile (Boulton, Singleton et al. 1996). Regional environments often referred to as “terrior”, in conjunction with human selective pressures have shaped the sensory attributes

of many popular cultivar grown and enjoyed today (Tomasino, Harrison et al. 2013)

A rich genetic diversity has been maintained in the various grape cultivars since domestication despite a long history of vegetative propagation, from both green and woody tissues (This, Lacombe et al. 2006, Myles, Boyko et al. 2011). Grape polymorphism frequency is high, occurring in one in every 43 bp within coding regions, maintaining nucleotide diversity values much higher than found in humans (Sachidanandam, Weissman et al. 2001, Lijavetzky, Cabezas et al. 2007) but similar to maize (Tenaillon, Sawkins et al. 2001, Ching, Caldwell et al. 2002). A network of close pedigree relationships has occasionally been disrupted by cross hybridization events or from the somatic mutation propagated by clonal propagation (Myles, Boyko et al. 2011, Carrier, Le Cunff et al. 2012).

1.2 Berry ripening

Ripening in fleshy fruits involves complex metabolic interactions that coordinate physical and molecular changes within plant tissues, including induction of color (Jaakola 2013, Jimenez-Garcia, Guevara-Gonzalez et al. 2013), softening of fruit tissues (Carreño, Cabezas et al. 2014, Moore, Fangel et al. 2014), evolution of volatile compounds (Kalua and Boss 2009, Nieuwenhuizen, Chen et al. 2015), and increases in soluble sugars. The culmination of these physiological and biochemical processes at maturity or peak ripeness produces attractive targets for human, avian and other vectors of seed dispersal. Coinciding with the onset of ripening, known as veraison in France, and the expansion and softening of the berry, pathogenesis-related proteins also

begin accumulating in grape as a constitutive defensive mechanism that persists until harvest (Tattersall 1997, Ferreira, Piçarra-Pereira et al. 2001, Monteiro, Picarra-Pereira et al. 2007). Thus, fruit ripening serves an evolutionary programmed effort for survival and palatability.

Ripening in grapes follows a double sigmoidal growth curve (Fig. 1) that can be divided into three mains stages of development and observed in increasing total soluble sugars (˚Brix), decreasing titratable acidity (g L^{-1}) and widening berry diameters (mm):

Stage 1: From fruit set to bunch closure, berries remain hard and green-colored, and total soluble sugars measured in ˚Brix remain low early in development (Fig. 1a). Organic acids, mainly tartrate and malate, accumulate in high concentrations in the berry with the onset of ripening (Fig. 1b). Cellular division and elongation are mediated by the growth hormones auxin, gibberellin and cytokinin that are in high concentrations before declining towards ripening initiates (Davies and Böttcher 2009, Bottcher, Burbidge et al. 2013, Fortes, Teixeira et al. 2015). Auxin levels also vary in individual berries depending upon seed content (high or low) that can lead to asynchronous ripening initiation (Gouthu and Deluc 2015).

Stage 2: The lag phase is defined by the slowing of growth, measurable in berry diameter (Fig. 1c). The levels of abscisic acid (ABA), a plant hormone, begin to increase during the lag phase. Evidence suggests ABA plays a major role in controlling several ripening-associated processes of grape berry at the beginning of ripening at the veraison stage, including coloration, sugar

accumulation, and softening (Jia *et al.*, 2011). Other hormonal interactions control different aspects of ripening. Low levels of ethylene have been recorded before and during ripening of grapes (Coombe and Hale 1973), with A brief increase of ethylene occurring before (Chervin, El-Kereamy et al. 2004) and also a heightened sensitivity to the hormone (Chervin, Tira-Umphon et al. 2008). Just before veraison (color change) in grape, levels of auxin, an inhibitor of ripening (Davies, Boss et al. 1997), have been reported as low prior to the accumulation of sugars (Coombe and Hale 1973). The application of synthetic auxins causes delays in ripening in grape that result in retarded accumulations of sugars, anthocyanins and altered gene expression of ripening associated transcripts (Davies, Boss et al. 1997, Bottcher, Boss et al. 2011, Böttcher, Boss et al. 2012).

Stage 3: Veraison is the onset of ripening when berries begin to asynchronously change color over a period of approximately 7 – 10 days. The translocation and accumulation of sucrose within fruit is another assessable metric for ripeness besides color change. Total soluble sugars measured in ˚Brix rapidly increase in the berry with the onset of ripening (Fig. 1a). Sugars can transcriptionally regulate gene activity (Bläsing, Gibon et al. 2005, Cordoba, Aceves-Zamudio et al. 2015), which can allow for fine-tuned regulation of metabolism with changing sugar levels (Conde, Silva et al. 2007). Berry diameter (Fig. 1c) also rapidly increases through cellular expansion from sap intake and cell wall modifications, possibly induced by the small ethylene burst during the lag phase (Chervin, El-Kereamy et al. 2004, Chervin, Tira-Umphon et al. 2008). Interestingly, the increase in berry size from cellular expansion during ripening is

an unusual trait to grapes, as many fruits (e.g. tomato) finish expansion prior to ripening initiates (Gillaspy, Ben-David et al. 1993). Organic acids also decrease rapidly (Fig. 1b) by maturity and determine the pH of the juice and eventual wine.

1.3 Phenylpropanoids

During the ripening stages of berry development principal members of the phenylpropanoid pathway are transcriptionally regulated during the cooler evenings (Rienth, Torregrosa et al. 2014), producing many polyphenolic products, like the stilbene (trans-resveratrol) phytoalexins that have anti-microbial and anti-oxidative capabilities (Parage, Tavares et al. 2012). Other phenylpropanoids, besides anthocyanins, maintain distinct cultivar differences in both grapes and wine. For example, genetic and environmental factors account for cultivar-dependent differences in abundance of the flavon-3-ols, catechin and epicatechin, in red wines produced from diverse regions (Goldberg, Karumanchiri et al. 1999). The qualities of bitterness and astringency in wine are attributed to monomeric flavan-3-ols and polymeric proanthocyanidins or condensed tannins (Betés-Saura, Andrés-Lacueva et al. 1996, Kallithraka, Bakker et al. 1997, Lesschaeve and Noble 2005, Mercurio, Dambergs et al. 2010), and have been implicated for their effects upon human health, to include antioxidant and anti-inflammatory properties (Frankel, Waterhouse et al. 1995, Landrault, Poucheret et al. 2001, Mattivi, Zulian et al. 2002, Oizumi, Mohri et al. 2010).

Flavonols (e.g. quercetin, myricetin, kaempferol) contribute to the bitter taste and also are important to quality by affecting color when formed into complexes with anthocyanins (Schwarz, Picazo-Bacete et al. 2005, Hilbert,

Temsamani et al. 2015). Wine and table grapes also differ in their concentrations of both hydroxybenzoic and hydroxycinnamic acids levels, with wine grape content significantly higher (Liang, Owens et al. 2011).

As some fruit ripen, polyphenols and carotenoids signal via their bright colors the health related benefits from consumption, a trait benefiting seed dispersal (Jimenez-Garcia, Guevara-Gonzalez et al. 2013). The color of a grape berry's skin contributes a recognizable cultivar characteristic that differentiates red and white-skinned grapes. Anthocyanins are the purple, blue and red pigments that provide the color associated with the skins and wines from red cultivars, and are extracted from the berry skins during winemaking; they are crucial constituents for quality in high-end wines (He, Mu et al. 2010). White cultivars do not synthesize anthocyanins as a result of two adjacent mutations within the genes of the MYB transcription factors, *VviMYBA1* and *VviMYBA2* (Kobayashi, Ishimaru et al. 2002, Walker, Lee et al. 2007). Human selective pressures from domestication are believed to have maintained this phenotype evident in many of today's popular cultivars (This, Lacombe et al. 2006, Myles, Boyko et al. 2011).

1.4 Volatile and aromatics in wine grapes

Fruit flavors and volatile aromatics, sugars, acids, and tannins all provide the chemistry that contributes to the sensory experience of wine. Cultivar differences extend to subtle variations in specific volatile compound ratios affecting a grapes overall aroma profile (Styger, Prior et al. 2011). Grape

composition at harvest can therefore impact the quality of the juice and finished wine.

The environmental influence of water deficit has been positively correlated with the enhancement of quality attributes such as color, aroma and flavor (Matthews, Ishii et al. 1990, Roby, Harbertson et al. 2004). For example, Deluc et al. (Deluc, Quilici et al. 2009) investigated seasonal water deficit in Cabernet Sauvignon observing 2-fold increases in the accumulation of the five major anthocyanins, as well as significant increases to the MYB transcription factors that regulate the final steps in anthocyanin biosynthesis. Drought tolerance amongst cultivars also varies between grapevine cultivars and species (Padgett-Johnson, Williams et al. 2003, Chaves, Zarrouk et al. 2010). Wine produced from low water status vines had significant reductions in vegetal aroma, but were rated highly for fruity aromas associated with red and black fruit (Chapman, Roby et al. 2005). Water-deficit-treated berries also show significantly induced transcripts involved in fatty acid cleavage or hydroxylation of monoterpenes leading to plant volatile production (Grimplet, Deluc et al. 2007). Severe water deficit can also increase berry nitrogen status (des Gachons, Van Leeuwen et al. 2005) by differentially affecting the transcription of amino acid metabolism, including proline, glutamate and phenylalanine (Deluc, Quilici et al. 2009).

Terpene-derived volatiles are also influential in determining a specific cultivars berry and wine flavor, with 69 putatively functional synthases in grape (Martin, Aubourg et al. 2010). The mevalonate and non-mevalonate pathways produce isoprene units, either in the cytosol or plastids, and are utilized in

downstream volatile isoprenoid, carotenoid and sesquiterpenoid synthesis (Rohmer and Rohmer 1999). Farnesyl diphosphates are one of the precursors for sesquiterpenes, an important class of compounds contributing to the peppery flavor in Shiraz, displaying a decreasing transcriptional profile from young berries to harvest ripe fruit (Chen, Tholl et al. 2011, Sweetman, Wong et al. 2012). Additional aromatic compounds, such as α-ionone, β-ionone and 6-methyl-5-hepten-2-one norisoprenoids (Lashbrooke, Young et al. 2013), are formed from the cleavage of various carotenoids to norisoprenoids by carotenoid cleavage dioxygenase (Mendes-Pinto 2009). The terpene synthase gene family also produce the floral compounds (3S)-linalool, geraniol, or α–terpineol.

Linoleic and linolenic polyunsaturated fatty acids are among the most abundant fatty acids in grape berry skins, and serve as substrates for lipoxygenase enzymes localized within the skin (Miele, Bouard et al. 1993). Lipoxygenases belong to class of non-haem, iron-containing dioxygenase enzymes that facilitate the degradation of fatty acid and esterified lipids (Ramey, Bertrand et al. 1986, Schwab, Davidovich-Rikanati et al. 2008). Catabolism of these fatty acids produces the most abundant class of volatiles in tomato fruit (Goff and Klee 2006). Volatile C6 compounds are formed by the physical crushing of berries during the wine-making process, which destroys the subcellular isolation of enzymes and substrate, and can be enhanced by prolonged skin contact common during the maceration of grape must (Ramey, Bertrand et al. 1986, Kalua and Boss 2010). These include an aromatic thiol, 3-mercaptohexan-1-ol (Kobayashi, Matsuyama et al. 2012), and are modulated by

drought and UV-C exposure (Kobayashi, Takase et al. 2011). Grape lipoxygenase transcript activity varies over the course of berry development, upon wounding, and in response to pathogen infection, with gene expression detected in skin, pulp, and seed tissues (Podolyan, White et al. 2010).

1.5 The genomic era

Tractable model organisms like tomato and *Arabidopsis thaliana* continue to serve as screens for identifying new functions in genes and proteins that offer valuable insights into distantly related species (Chow and Kay 2013). Though, specific biochemical pathways maybe absent in the model organism that limits its direct use. For example, *Capsicum annuum*, the hot pepper is famous for capsaicinoid biosynthesis, a unique secondary metabolite to the *Capsicum* genus whose enzyme, capsaicin synthase, is not synthesized in its close relative tomato (Kim, Park et al. 2014).

Upon first glance, grapes are not the ideal model fruit crop due to its intractability to transformation. Yet, the economic impact associated with grape & wine production has justified its continued interest by researchers and industry counterparts. Fruit harvested from grapevines (Vitis vinifera L.) has an economic impact greater than $162 billion to the American wine and grape industry alone (http://www.ngwi.org).

Experiments based on high-throughput technologies (e.g. DNA and RNAseq) have revealed genetic and epigenetic regulatory mechanisms, crop evolution, and insights into trait variation. For example, the rate of sequenced genomes has accelerated in the post-Next Generation Sequencing era, which

will only continue as sequencing and consumable costs decrease. The frequency of the term, “high-throughput sequencing”, found in the literature from 1990 – 2015 has nearly doubled every two years (Fig. 2). The grapevine genome was the first fruit crop to be sequenced by the French-Italian or Italian-French consortium (Jaillon, Aury et al. 2007). Other fruit genomes include both climacteric and non-climacteric, including apple (Velasco, Zharkikh et al. 2010), banana (D'Hont, Denoeud et al. 2012), cacao (Argout, Salse et al. 2011), cucumber (Huang, Li et al. 2009), kiwi (Huang, Ding et al. 2013), melon (Garcia-Mas, Benjak et al. 2012), papaya (Ming, Hou et al. 2008), peach (International Peach Genome, Verde et al. 2013), pear (Wu, Wang et al. 2013), pepper (Kim, Park et al. 2014), strawberry (Shulaev, Sargent et al. 2011), sweet orange (Xu, Chen et al. 2013), tomato (Tomato Genome 2012) and watermelon (Guo, Zhang et al. 2013). Collective resources within the agricultural & life sciences are advancing our understanding of the ‘ripe’ phenotype in fleshy fruits and will continue to benefit crop improvements (Seymour, Ostergaard et al. 2013). Thus, high-throughput sequencing of nucleic acids has become a tool for generating new hypotheses and answering or clarifying longstanding questions.

Many aspects of designing high-throughput experiments should be considered before the first sample is ever obtained, which greatly assists the downstream statistical analysis. This includes having a sound experimental design that is balanced and contains clear hypotheses to be tested. Sufficient experimental replication, at least three but six experimental replicates would be preferred, increases the power of the RNAseq-based experiment, more so than

greater sequencing depth per sample (Ching, Huang et al. 2014, Chhangawala, Rudy et al. 2015). The researcher should also have clear project goals so that the appropriate sequencing technology, library preparation and downstream workflows are chosen (Zhang, Chiodini et al. 2011, Liu, Li et al. 2012, Loman, Misra et al. 2012, Quail, Smith et al. 2012) that will determine read length options, and may impact the end results (Zhang, Chiodini et al. 2011, Chhangawala, Rudy et al. 2015). Complicating selection is the near availability of nanopore-based sequencing technologies like Oxford nanopores MinIION, which promise an inexpensive technology capable of producing extremely long reads that map complex genomic regions (Goodwin, Gurtowski et al. 2015).

Figures

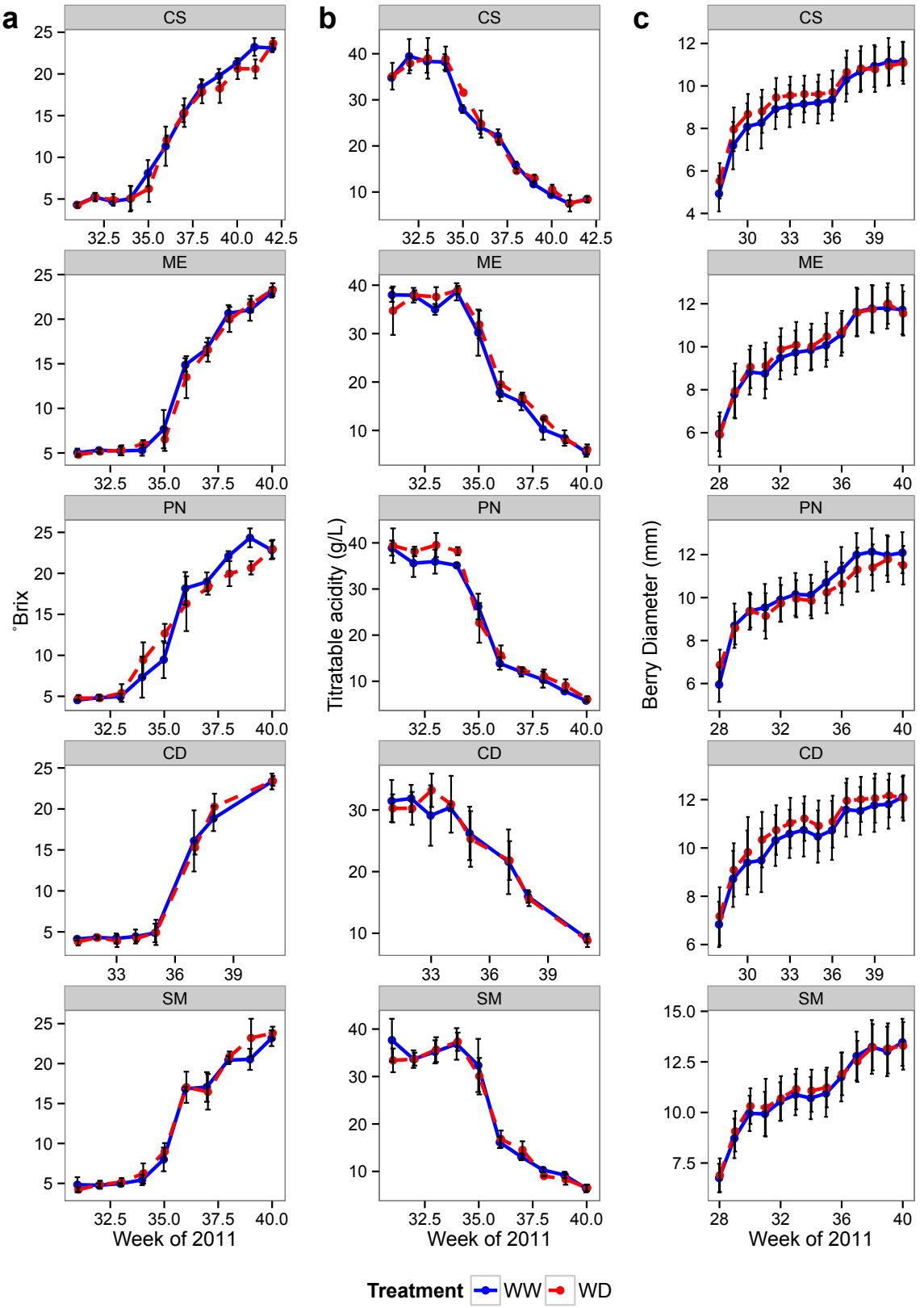
a
b
c
CS
ME
PN
CD
SM
°Brix
Titratable acidity (g/L)
Berry Diameter (mm)
Week of 2011
Treatment
WW
WD

Figure 1. Physiological measurements from ripening grape berries were taken during the 2011 harvest season, from the Nevada Agricultural Experiment Station Valley Road Vineyards. (**a**) ˚Brix, (**b**) Titratable acidity (g/L), and (**c**) berry diameter (mm) were measured weekly from fruit set to harvest in well-watered (WW) and water deficit (WD) vines. Symbols represent mean ± SD, with n = 3 (four clusters per experimental replicate and 15 berries per cluster) for berry diameter and n = 6 for ˚Brix and titratable acidity (TA) measurements, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

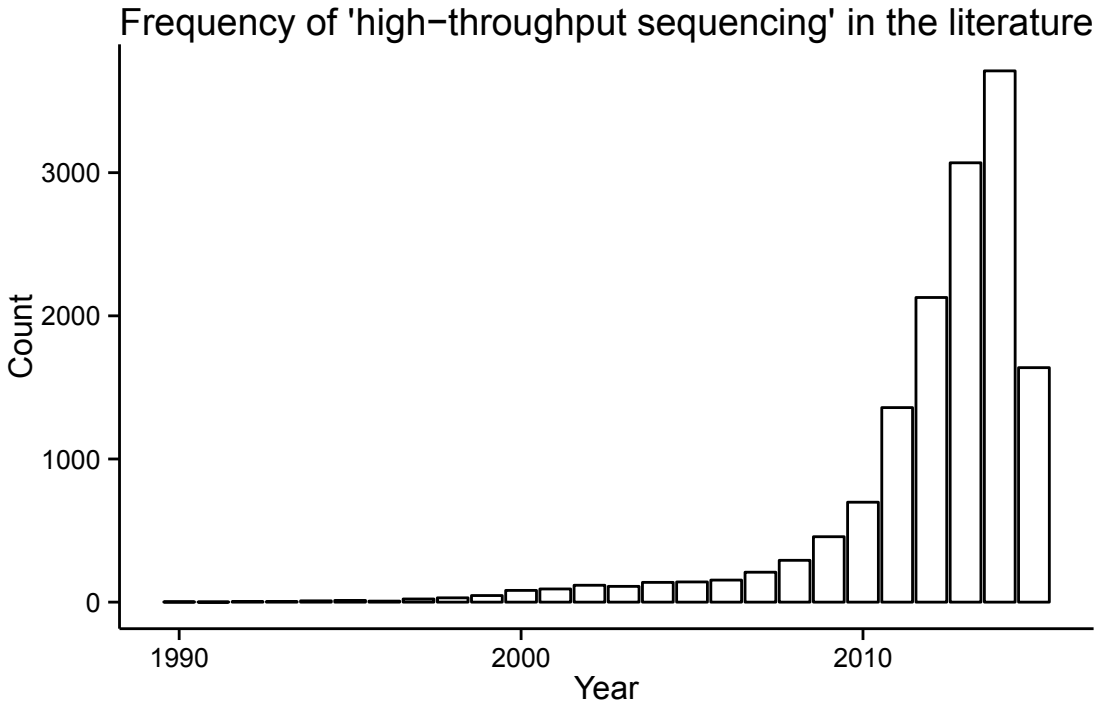

Figure 2. Frequency of the term, “high-throughput sequencing”, found in a PubMed (http://www.ncbi.nlm.nih.gov/pubmed) term search, from 1990 – 2015 has shown massive growth since the mid-2000s.

CHAPTER 2:

FIVE OMIC TECHNOLOGIES ARE CONCORDANT IN DIFFERENTIATING THE BIOCHEMCIAL CHARACTERISTICS OF THE BERRIES OF FIVE GRAPEVINE (*VITIS VINIFERA* L.) CULTIVARS

This Chapter is based on a manuscript that is currently being prepared for submission to *Genome Biology*

Ghan, R., Van Sluyter, S.C., Hochberg, U., Degu, A., Hopper, D.W., Tillett, R., Schlauch, K.A., Haynes, P.A., Fait, A., Cramer, G.R. Five Omic Technologies are Concordant in Differentiating the Biochemical Characteristics of the Berries of Five Grapevine (*Vitis vinifera* L.) Cultivars. *Genome Biology,* In Preparation, (2015).

2.1 Introduction

Fruit harvested from grapevines (*Vitis vinifera* L.) is an economically important agricultural commodity, having an economic impact greater than $162 billion to the American wine and grape industry alone (http://www.ngwi.org). Cultivated grapes are grown and consumed as fresh fruit, used as the root stocks for fruit producing scions, and in the production of a range of wines with distinct and complex flavor profiles (Boulton, Singleton et al. 1996). Grapevines are a long-lived perennial fruit species intertwined within the culture of many countries dating back more than 7,000 years.

There are more than 5,000 distinct cultivars of grapes in the world. Grape production is found on every arable continent around the globe (Bisson, Waterhouse et al. 2002, This, Lacombe et al. 2006). Grapevines have maintained a rich genetic diversity since domestication as a result of vegetative propagation practices that both immortalize existing traits and unknowingly encourages unique phenotypes to arise from clonal cuttings that carry somatic mutations (This, Lacombe et al. 2006, Myles, Boyko et al. 2011). Regional environments often referred to as “terrior”, in conjunction with human selective pressures have shaped the cultivar characteristics associated with many of the popular wines enjoyed presently (Tomasino, Harrison et al. 2013).

The color of a grape berry’s skin contributes a recognizable cultivar characteristic that differentiates red- and white-skinned grapes. Anthocyanins are the purple, blue and red pigments that provide the color associated with the skins and wines from red cultivars, and are extracted from the berry skins during

winemaking; they are crucial constituents for quality in high-end wines (He, Mu et al. 2010). White cultivars do not synthesize anthocyanins as a result of two adjacent mutations within the genes of the MYB transcription factors, in *VviMYBA1* and *VviMYBA2* (Kobayashi, Ishimaru et al. 2002, Walker, Lee et al. 2007). Human selective pressures from domestication are believed to have maintained this phenotype evident in many of todays popular cultivars (This, Lacombe et al. 2006).

Other phenylpropanoids, besides anthocyanins, maintain distinct cultivar differences in both grapes and wine. For example, genetic and environmental factors account for cultivar-dependent differences in abundance of the flavon-3-ols, catechin and epicatechin, in red wines produced from diverse regions (Goldberg, Karumanchiri et al. 1999). Wine and table grapes also differ in their concentrations of both hydroxybenzoic and hydroxycinnamic acids levels, with wine grape content significantly higher (Liang, Owens et al. 2011). The qualities of bitterness and astringency in wine are attributed to monomeric flavan-3-ols and polymeric proanthocyanidins or condensed tannins (Betés-Saura, Andrés-Lacueva et al. 1996, Kallithraka, Bakker et al. 1997, Lesschaeve and Noble 2005, Mercurio, Dambergs et al. 2010), and have been implicated for their effects upon human health, to include antioxidant and anti-inflammatory properties (Frankel, Waterhouse et al. 1995, Landrault, Poucheret et al. 2001, Mattivi, Zulian et al. 2002, Oizumi, Mohri et al. 2010).

Cultivar differences also extend to subtle variations in amino acid composition at harvest (Etiévant, Schlich et al. 1988, Huang and Ough 1991,

Hernández-Orte, Guitart et al. 1999). Ammonia and certain amino acids are the main nitrogen-containing compounds assimilated by yeasts within fresh grape juice or musts before fermentation commences (Henschke and Jiranek 1993). Nitrogenous substances become available to yeasts from pressed berry juice or via extraction from the skins, in the case of fermenting red wines. The assimilable nitrogen levels in grape must also play a role in determining the duration of fermentation, and musts are often amended with ammonium salts (DAP) to ensure efficient fermentation (Henschke and Jiranek 1993). Yeast assimilates free amino acids under anaerobic fermentation conditions, with the exception of proline that stoichiometrically requires oxygen for degradation (Ingledew, Magnus et al. 1987, Huang and Ough 1991). Aroma composition of wines shares a close relationship with must amino acid composition, where volatile compounds such as isoamyl acetate, isobutanol, isobutyric acid and methionol are significantly different among cultivars (Hernández-Orte, Cacho et al. 2002). Grape composition at harvest can therefore impact the quality of the finished wine.

The environmental influence of water deficit has been positively correlated with the enhancement of quality attributes such as color, aroma and flavor (Matthews, Ishii et al. 1990, Roby, Harbertson et al. 2004). For example, Deluc *et al.* (Deluc, Quilici et al. 2009) investigated seasonal water deficit in Cabernet Sauvignon observing 2-fold increases in the accumulation of the five major anthocyanins, as well as significant increases to the MYB transcription factors that regulate the final steps in anthocyanin biosynthesis. Drought tolerance amongst cultivars also varies between grapevine cultivars and species (Padgett-

Johnson, Williams et al. 2003, Chaves, Zarrouk et al. 2010). Wine produced from low water status vines had significant reductions in vegetal aroma, but were rated highly for fruity aromas associated with red and black fruit (Chapman, Roby et al. 2005). Water-deficit-treated berries also show significantly induced transcripts involved in fatty acid cleavage or hydroxylation of monoterpenes leading to plant volatile production (Grimplet, Deluc et al. 2007). Severe water deficit can also increase berry nitrogen status (des Gachons, Van Leeuwen et al. 2005) by differentially affecting the transcription of amino acid metabolism, including proline, glutamate and phenylalanine (Deluc, Quilici et al. 2009).

In the present study, an integrated analysis (transcriptional, translational, and intermediary and end-products of metabolism) is presented to test the uniqueness of three red-skinned and two white-skinned cultivars: Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay and Semillon, respectively. Here, the same berry samples from the same vineyard and climate, free of disease and insect pressures, were sampled and utilized for each Omic analysis. The cultivars were exposed to a mild, seasonal water-deficit treatment from fruit set until harvest in 2011 to provide a more diverse molecular expression that underlies the unique responses of each cultivar. One of the goals of this research was to explore the berry proteome at harvest and analyze them in the context of measurable transcription. Another goal was to assess the platform performance of gene expression profiled by NimbleGen Grape Whole-Genome Microarray and Illumina RNAseq in the five cultivars and under water deficit conditions. In addition to comparing abundance changes of individual proteins and transcripts,

ancillary components of the berry biological system were explored through primary and secondary metabolite analysis using gas chromatography-mass spectroscopy (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS). Interestingly, the cultivars' proteomic, transcriptomic and metabolomic responses to the drought treatment were divergent, reflecting, at the level of the berry skin, unique grape profiles. We aimed to provide a comprehensive assessment of grape berry cultivar differences at harvest. We show that there was concordance between Omics platforms in differentiating each cultivar's uniqueness.

2.2 Methods

2.2.1 Plant material and experimental conditions

Berries from five grapevine (*Vitis vinifera* L.) cultivars, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay and Semillon, were harvested during the fall of 2011 from the University of Nevada, Reno experimental vineyards (Fig. S1). The North Vineyard was divided in half and separated into 15 rows (5-well watered; 10-drought stressed), with Chardonnay on the northern half and Cabernet Sauvignon on the southern half. Each row in the North Vineyard maintained 23 vines of each cultivar. The South Vineyard was divided into six blocks (A-F). Each block contained four rows divided into thirds, with 15 vines of a given cultivar in each third. Merlot, Pinot Noir & Semillon vines were grown in each block. Blocks A, C & D were well watered, and blocks B, E & F were treated with water deficit. Rows in each of the experimental vineyards were planted in a north to south orientation, to achieve nearly maximal daily sunlight exposure. Following fruit set in early July 2011, leaves were removed near the clusters on

the east-facing side of vines in both vineyards to increase fruit exposure to light and air circulation. Vines were drip irrigated with 8 l h^{-1} emitters and grown under well-watered or water deficit conditions post-fruit set. Mid-day stem water potentials were measured weekly with a pressure chamber (3005 Plant Water Status Console, Soil Moisture Corp., Goleta, CA, USA), as in (Grimplet, Deluc et al. 2007), on fully mature leaves to assess plant water status throughout the growing season (Shackel, Ahmadi et al. 1997, Choné, Van Leeuwen et al. 2001); stem water potential measurements were averaged across cultivars, because no significant differences in stem water potentials amongst the cultivars could be detected. Following weekly measurements, water was either applied or withheld in an effort to maintain a mild water deficit treatment at ~ -0.8 MPa and -0.6 MPa for control vines. Titratable acidity (TA) and °Brix (total soluble solids) were assayed from juice crushed from a minimum of two whole berry clusters collected from different vines. The TA (g l^{-1}) measurements were performed with an automatic titrator (HI 84102, Hanna Instruments, Woonsocket, RI, USA). The automatic titrator was standardized daily with tartaric acid (6.4 g l^{-1}), with 0.5 N NaOH utilized as a titrant to an endpoint of a pH of 8.2 for both standard and juice measurements. ˚Brix was measured with a digital refractometer (HI 96811, Hanna Instruments, Woonsocket, RI, USA) that was calibrated with deionized water before each measurement. Daily precipitation, Penman evapotranspiration and temperature measurements (Fig. 1) from the experimental vineyards were collected from the Desert Research Institute's (DRI) Western Regional Climate Center (2011)(2011)(2011)(2011)(2011)(2011). DRI calculates

evapotranspiration using the 1982 Kimberly-Penman equation (Wright 1982). Berry diameter measurements were taken weekly with a digital caliper (General Ultratech No. 147, New York, NY, USA), beginning after fruit set until the week of cultivar harvest. Berry diameter measurements consisted of measuring 15 randomly selected berries per cluster from the same four labeled clusters (technical replicates) on a single vine (biological replicate). Three biological replicates per cultivar and treatment were used to compute diameter means. Six biological replicates, comprised of ≥ 2 whole berry clusters were harvested in early to late October 2011. Sampling dates for berry skin material varied between cultivars in order to achieve similar ˚Brix and TA concentrations in berries, but WW and WD treatments were gathered on the same day (Fig. 1; Table 2). To avoid edge effects, berry clusters were harvested from vines away from the ends of the trellised rows. Each of the six biological replicates was utilized for metabolomic extractions and analysis, five biological replicates were selected for microarrays, and three of the six biological replicates were randomly selected for proteomic and RNAseq analysis. Berry skin tissue for all analyses was separated from the seeds and pulp prior to being flash frozen with liquid nitrogen and finely ground using a RETCH-mill (Retsch MM301, Newtown, PA, USA) with pre-chilled steel holders and grinding beads.

2.2.2 Protein extraction and LC-MS/MS analysis

Proteins were extracted from the frozen, finely-ground skin samples using a modified phenol-based extraction protocol commonly utilized in the Cramer lab (Vincent, Wheatley et al. 2006, Chapman, Castellana et al. 2013). Isolated

protein pellets were prepared similarly to Cramer *et al.* (Cramer, Van Sluyter et al. 2013) for label-free shotgun proteomics by Lys-C- and trypsin-digestion using a modified method of the Filter-Aided Sample Preparation (FASP) methods (Manza, Stamer et al. 2005, Wisniewski, Zougman et al. 2009), using trifluorethanol (TFE/FASP) (Chapman, Castellana et al. 2013). LC-MS/MS spectra were acquired from three biological replicates per treatment by a sample-optimized gas phase fractionation (GPF) method on a LTQ Velos Pro mass spectrometer (Thermo). Chromatography was performed on an Easy-nLC II (Thermo) at 40˚ C, 0.1 x 300 mm Magic 3 µm, 200 Å C18AQ column (Michrom Bioresources, Auburn, CA, USA) interfaced with the mass spectrometer by an Advance captive spray source (Michrom Bioresources). Samples were analyzed in three 220 min LC-MS/MS gas phase fractions run at 0.5 µL min^{-1}. The m/z ranges of each gas phase was optimized empirically by analyzing a mixture of pooled samples from m/z 400-2000, then creating GPF fractions to approximate an even distribution of peptide observations among the three fractions.

A protein database was compiled from three sources: 1) all reviewed *V. vinifera* protein entries in UniProt, "Taxonomy:29760 AND reviewed:yes" (164 sequences); 2) *V. vinifera* proteins predicted by the International Grape Genome Program, "Taxonomy:29760 AND author:vitulo AND reviewed:no" (29803 sequences); 3) mitochondrial proteins associated in UniProt (81 non-redundant sequences). Spectrum-peptide matching was performed with X!Tandem and the GPM Cyclone (www.thegpm.org) in automated mode using MudPit merging as in Cramer *et al.* (Cramer, Van Sluyter et al. 2013). The GPM Cyclone XE and

X!Tandem Cyclone version 2011.12.01.1 were used. Default ion trap parameters were used with the exceptions of MS error (+3, -1 Da), the inclusion of reversed sequences, and a protein expected value of -1. Approximately 50,000 spectra per sample were assigned to peptides. Protein identifications were filtered and protein and peptide FDRs were calculated, respectively, using reverse database searching. Each protein had to meet two criteria to be considered a valid identification. First, all biological replicates had a minimum of 1 spectral count (≥ 6 total spectral counts) within one sample set; a sample set refers to all biological replicates for the two treatments. Second, in the event that a specific cultivar's treatment (e.g. WW Chardonnay) did not have ≥ 1 spectral counts across each of the 3 biological replicates, but the other treatment for the cultivar (e.g. WD Chardonnay) had ≥ 1 counts for all 3 biological replicates and the sum of spectral counts was ≥ 6, then the protein was considered identified for the cultivar. While the protein would be considered 'identified', no quantification or abundance ratios would be made for that protein because it did not meet the ≥ 1 count in each sample set's replicates. Protein abundance was estimated as normalized spectral abundance factor (NSAF), with a suite of R modules known as the Spectral Counting Reporting Analysis Program (Scrappy) (Neilson, Keighley et al. 2013).

2.2.3 RNA extraction

Total RNA was extracted from ~250 mg of finely ground skin tissue using a modified CTAB extraction protocol based on (Chang, Puryear et al. 1993, Jaakola, Pirttila et al. 2001, Tattersall, Ergul et al. 2005, Gambino, Perrone et al.

2008) followed by an additional on column DNase digestion using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA quality and quantity were assessed with a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA LabChip assays (Agilent Technologies, Santa Clara, CA, USA).

2.2.4 Microarray hybridization and data extraction

Ten µg of total RNA from each sample was used for hybridization onto a NimbleGen microarray 090818 Vitis exp HX12 (Roche, NimbleGen Inc., Madison, WI, USA), which contains probes targeted to 29,549 grapevine genes predicted from the V1 annotation of the 12x grapevine genome (https://urgi.versailles.inra.fr/Species/Vitis/Annotations). cDNA synthesis, labeling, hybridization, and washing steps were performed by MOgene (St. Louis, MO, USA) according to the NimbleGen Arrays User's Guide (version 3.2). Data were processed, normalized and analyzed as in (Cramer, Ghan et al. 2014). As in Cramer *et al.* (Cramer, Ghan et al. 2014), a note of caution should be held when examining the microarray data sets due to the likelihood of cross-hybridization of certain *Vitis* gene families with high similarity and are denoted in pink in Supplemental File 4.

2.2.5 RNAseq library preparation and sequencing

For RNAseq, thirty 50bp single-end, barcoded libraries were constructed and sequenced by the Neuroscience Genomics Core at the University of California Los Angeles (Los Angeles, CA, USA) using Illumina TruSeq RNA library prep kits (Illumina Inc., San Diego, CA, USA) according to manufactures

instructions. The libraries were pooled, multiplexed and run across eight lanes of four 1x50 flow-cells, using Illumina TruSeq chemistry (version 3.0) and a HiSeq2000 sequencer (Illumina Inc., San Diego, CA, USA). Due to multiplexing, individual biological replicates were thus sequenced on each of the four flow-cells to reduce technical variation.

2.2.6 Read quality and mapping pipeline

Quality check and filtering of fastq files was performed with the NGS QC Toolkit (Patel and Jain 2012), prior to merging multiplexed replicate files. The TopHat2 splice alignment software (version 2.0.10) (Kim, Pertea et al. 2013) in combination with the PN40024 *Vitis vinifera* reference genome and annotation (http://plants.ensembl.org/Vitis_vinifera/Info/Index) were used to align the quality filtered reads, with the --b2-very-sensitive option and --transcriptome-index option. Approximately 93% of reads from all libraries were mapped. A count matrix of aligned reads was generated with Samtools (Li, Handsaker et al. 2009) and HTSeq (Anders, Pyl et al. 2015) from BAM alignment files, which outputs counts for each gene feature. Using the “union” mode, HTSeq discarded read counts if they were ambiguous, not assigned to any gene feature, or if the alignment was not unique.

2.2.7 Data analysis

The ANOVA and most data analyses were conducted in R (3.1.2) (R Core Team 2015). RNAseq read count normalization and differential expression analysis were performed with edgeR (3.8.6) (Robinson, McCarthy et al. 2010), counts from each aligned sample library (biological replicate). An experimental

design model was created accounting for cultivar (5 levels), treatment (2 levels) and the interaction between these two effects before fitting generalized linear models to estimate log-fold changes. Contrast coefficients for each factor were selected for significance testing. Moderated log-counts-per-million (Supplemental File 5) were computed with the cpm() function in edgeR for data visualization of RNAseq data.

2.2.8 Gene set enrichment analysis

Functional analysis and enrichment of biological processes was determined with the BinGO (version 3.0.2) (Maere, Heymans et al. 2005) application in Cytoscape (version 3.1.1) (Shannon, Markiel et al. 2003). Multiple testing correction adjusted p-values using the Benjamini & Hochberg False Discovery Rate at a 0.05 threshold. Overrepresented GO terms were visualized with a treemap using REVIGO (http://revigo.irb.hr/) (Supek, Bosnjak et al. 2011) and the treemap R package.

2.2.9 GC and LC/MS metabolite analysis

Metabolite extraction was performed on the same finely ground tissue samples utilized for protein extraction above and kept at -80˚C until further analysis. Briefly, skin samples were freeze dried in a lyophilizer (Labconco FreeZone 18, Kansas City, MS, USA) and extracted from 70 mg of frozen tissue with a pre-chilled methanol:chloroform:water (2.5:1:1 v/v), for parallel metabolite profiling (LC and GC/MS) using a protocol described previously (Degu, Hochberg et al. 2014). GC-MS samples were re-dissolved and derivatized as described previously (Hochberg, Degu et al. 2013). An AS 3000 autosampler, a TRACE GC

ULTRA gas chromatograph, and a DSQII quadruple mass spectrometer (Thermo-Fisher Ltd.) comprised the GC-MS system, with system parameter identical to those described in (Bai, Sikron et al. 2012, Hochberg, Degu et al. 2013). LC-MS analysis was performed on an UPLC-QTOF-MS system equipped with an ESI interface (Waters Q-TOF XEVO, Waters MS Technologies, Manchester, UK), in negative and positive ion mode. An Acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm) was used for chromatographic separation. The MS and solvent gradient program conditions were set as described previously (Hochberg, Degu et al. 2013).

2.2.10 Metabolite data processing

GC-MS spectral searching against the RI libraries from the Max-Planck Institute for Plant Physiology in Golm Germany (http://www.mpimp-golm.mpg.de/mms-library/) was performed in the Xcalibur data software (version 2.0.7), with the National Institute of Standards and Technology (NIST, Gaithersburg, USA) algorithm. These metabolites were normalized by the total metabolites and corrected for the dilution factor as in (Degu, Hochberg et al. 2014). LC-MS data acquisition and UPLC system control was performed with the MassLynxTM software (Waters; version 4.1) as described in (Hochberg, Degu et al. 2013). The verification of metabolite identification was done as described in (Degu, Hochberg et al. 2014).

2.2.11 Availability of supporting data

The mass spectrometry proteomics data have been deposited with the ProteomeXchange (Vizcaino, Deutsch et al. 2014) Consortium via the PRIDE

partner repository with the dataset identifier PXD001661 and 10.6019/PXD001661. The microarray expression data sets are available from the Plant Expression Database, under experiment VV37: Grape berry response to continuous water deficit (http://www.plexdb.org/modules/PD_browse/experiment_browser.php?experiment=VV37). RNAseq data were deposited with the Sequence Read Archive database at NCBI with BioProject identifier PRJNA268857 (Leinonen, Sugawara et al. 2011).

2.3 Results

In this study, we investigated the biochemical characteristics of five wine grape cultivars, by sampling wine grape berry skins harvested at maturity. Five Omic data sets comprising transcripts, proteins, and metabolites and generated from the same harvested skins were used to investigate cultivar differences. An emphasis upon biologically important known molecular compounds of the mature berry that affect color and amino acid metabolism are presented here.

2.3.1 Growth conditions and physiological data

Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, and Semillon were grown at the University of Nevada, Reno's Experimental Vineyard during the 2011 growing season (Supplemental File 1). This vineyard is located at high elevation (1372 m) in a very dry climate. Seasonal precipitation (Fig. 1a) from fruit-set through veraison (July – September) was marginal, totaling 0.501 mm, with daily mean temperatures of 22.5˚C (Fig. 1b). The majority of rain accumulation occurred during the post-veraison period (early October 2011),

which also coincided with a period of cooler daily mean temperatures (8.9˚C) and the harvest dates for Semillon, Pinot Noir, and Merlot. The remaining growing days of the 2011 season maintained warmer temperatures (daily mean 14.3˚C) and an absence of rain. Cabernet Sauvignon fruit were harvested the day prior to the season's first freezing temperatures (-3.3˚C), to avoid potential frost damage to berries.

Grapevines were grown in two adjacent experimental vineyards under independent irrigation controllers. Merlot, Pinot Noir, and Semillon were grown in the experimental south, which had a randomized-block experimental design (15 vines each in six blocks with 4 rows per block for a total of 90 vines for each cultivar out of 1080 total vines in the vineyard) and Cabernet Sauvignon and Chardonnay were grown in the experimental north vineyard (300 vines each in 15 rows). Different rows were under different irrigation controls. Drip irrigation was initiated when stem water potentials of the vines reached their target treatment level, stem water potentials (ψ_w) of -0.6 MPa for control vines and -0.8 MPa for a mild water deficit. Mid-day stem water potentials were monitored weekly for well-watered (WW)- and water-deficit (WD)-treated vines to assess plant water status and to determine the amount of water to be applied to maintain stem water potentials over the season (Table 1; Supplemental File 1). The water potentials of vines were close to target stem water potentials at the time of harvest.

Berries were monitored weekly from fruit set through harvest to assess ˚Brix and titratable acidity (TA) levels by sampling two average clusters per

replicate, cultivar and treatment from two non-adjacent vines. The timing of harvest for each cultivar was determined by berries sampled for a target ˚Brix to TA ratio of 3.5. The average ˚Brix and TA (g L^{-1}) were 23.3 and 7.1, respectively, with a ratio of 3.3. For each cultivar, WW and WD grape berries were harvested on the same day. Mild water deficit treatment had no significant effect upon berry diameter, ˚Brix, or TA at harvest (Table 2), with the exception of a 4% reduction of Pinot Noir berry diameters that was statistically significant at $p \leq 0.01$. Reported physiological measurements and water stress levels were similar to data reported by Grimplet *et al.* (Grimplet, Wheatley et al. 2009) in their proteomic analysis of grape berry tissues under water deficit.

2.3.2 Comparative Omic analyses of grape berry skin

Our comparative Omic analyses focused on the skins, which had been separated from the pulp and seeds of ripe berry clusters at harvest and rapidly frozen in liquid nitrogen. At least two clusters per experimental replicate (six individual vines in total) were harvested in preparation for each sample extraction and analysis. Proteins were extracted from three experimental replicates with a modified phenol-based protocol (Vincent, Wheatley et al. 2006), digested with trypsin and Lys-C and analyzed using nanoflow liquid chromatography-mass spectrometry (nanoLC-MS/MS) (Chapman, Castellana et al. 2013). Peptide spectra analysis, protein identification and abundance, as normalized spectral abundance factors (NSAF), were similarly computed as before (Cramer, Van Sluyter et al. 2013) (see Methods for details). Approximately 50,000 spectra per sample were assigned to peptides matching a total of 2,867 non-redundant *Vitis*

vinifera proteins in the UniProtKB database (**Table 3; Supplemental File 2**). From the non-redundant proteins, 1,211 were shared across all five of the cultivars and had spectra assigned for all replicates (**Supplemental File 3**).

Total RNA was extracted with a modified CTAB protocol (Chang, Puryear et al. 1993, Jaakola, Pirttila et al. 2001, Tattersall, Ergul et al. 2005, Gambino, Perrone et al. 2008). Five biological replicates per condition were used for NimbleGen (Roche NimbleGen, Madison, Wi) Grape Whole-Genome Microarray analysis, with standard microarray processing and data normalization as in Cramer *et al.* (Cramer, Ghan et al. 2014). Microarray analysis profiled 29,549 genes as predicted in 12x V1 annotation of the grape genome (**Supplemental File 4**). The same three biological replicates used for the protein analysis were sequenced with an Illumina HiSeq 2000 sequencing system to determine transcript abundance. Transcript data were generated by aligning quality-filtered sequence reads to the grape genome (Jaillon, Aury et al. 2007), assigning transcript counts to the V1 annotation with the htseq-count tool (Anders, Pyl et al. 2015), and then performing a differential expression analysis with the edgeR (Robinson, McCarthy et al. 2010) R package (**Table 3, Supplemental File 5**). We detected the expression of 27,252 transcripts of the 29,971 transcripts in the V1 annotation.

Metabolites were extracted in parallel from six biological replicates, three additional replicates from the aforementioned, with a protocol previously described (Degu, Hochberg et al. 2014). For metabolite analyses, the peaks of each metabolite were normalized to the total peak area giving a relative

metabolic abundance value. The relative metabolic abundance from berry skins of primary and secondary metabolites (**Table 3, Supplemental File 6**) were analyzed by GC-MS and LC-MS based methods.

Venn diagrams illustrate the distributions of identified (**Fig. 2a**) and quantified (**Fig. 2b**) proteins in the different cultivars. In each case, subsets of proteins were distributed to each cultivar. The majority of transcripts were assessed by both platforms (**Fig. 2c**). Microarrays measured probe fluorescence for 2,481 transcripts that did not receive unique counts by RNAseq. A subset of 1,201 transcripts from both platforms could be paired to the quantified proteins. The majority of metabolites was measured in each cultivar (**Fig. 2d**), with the main metabolite differences attributed to the anthocyanin production in red cultivars.

The most abundant proteins and transcripts from each of the five cultivars were determined. Only proteins detected in all samples (1,211) were assessed, but all transcripts measured were considered for this analysis in both platforms. The top ten most abundant proteins (**Table 4a**) surveyed in each cultivar consisted of only 17 proteins, many of which can be classified as pathogenesis-related (PR). Additionally, three of the proteins were in the top of each cultivar: β-1, 3, glucanase (F6HLL9), major latex protein 22 (A5BAX1), and a peroxiredoxin-5 (D7TBK8). Both transcript platforms were assessed for the degree of concordance in reporting highly expressed transcripts. The top most abundant transcripts by microarray (**Table 4b**) consisted of a common set of 16 uniquely annotated transcripts from the cultivars. Again, several of the top transcripts were

PR protein related including a class IV chitinase, a non-specific lipid-transfer protein and two thaumatins. Five of the transcripts were also ranked in the top ten of each cultivar: invertases/pectin methylesterase inhibitor (Q9M4H8/VIT_16s0022g00960), chitinase class IV (Q7XAU6/VIT_05s0094g00340), putative ripening-induced protein 1 (Q6VEQ6/ VIT_05s0049g00760), photosystem II protein D1 (F6GXB0/VIT_11s0052g01680), and one transcript without a known annotation (F6H8M1/VIT_05s0049g00520). A BLAST search of the unannotated transcript references a putative proline-rich protein in several species including grape. For RNAseq transcripts (**Table 4C**), a common set of 18 uniquely annotated transcripts made up the top 10 from the cultivars. As with the proteins and microarray transcripts, many of the top transcripts were the same PR proteins in the microarrays. Five of the transcripts were also ranked in the top ten of each cultivar: putative ripening-induced protein 1 (Q6VEQ6/ VIT_05s0049g00760), chitinase class IV (Q7XAU6/VIT_05s0094g00340), abscisic stress ripening protein 2 (F6GY46/ VIT_18s0072g00380), allergenic protein Pt2L4 (Q9M4H7/VIT_12s0059g00590), and the same unannotated transcript in the microarrays (Q9M4I2/VIT_05s0049g00520). Microarray transcripts that did not fully correspond with the RNAseq are annotated as containing probesets that potentially cross hybridize with other closely related genes. For example, all four probes that map to the cupin and Photosystem II protein D1 listed in **Table 4b** have the potential for cross hybridization (see Cramer et al, 2014 (Cramer, Ghan et al. 2014) for a full list of genes with potential hybridization).

A multifactorial (5 x 2; cultivar x treatment) experimental design was used for each platform to determine significant differences between treatments and cultivars. ANOVA indicated that the cultivar level contributed the largest amount of significant changes in each of the data sets (**Table 5**). Statistically significant transcript abundance changes were found for both transcript technologies below the adjusted p-value (false discovery rate) of 0.05 (herein referred to as "significant" throughout this paper) for cultivar, treatment and cultivar x treatment effects (Benjamini and Hochberg 1995). Neither a treatment effect nor the interaction of treatment x cultivar effects were statistical significant in the protein or metabolite data, but significant cultivar effects were found within protein and metabolite abundances.

Differential expression analysis of transcripts was similarly performed for both platforms. Standard processing and data normalization of the microarrays was performed. ANOVA indicated transcript abundance of 27,064 transcripts changed significantly with cultivar, the transcript abundance of 195 transcripts changed significantly with treatment, and 1,546 transcripts changed with the cultivar x treatment interaction term. RNAseq data were normalized and modeled with the standard edgeR pipeline. Generalized linear models were fit to a multifactorial design formula (5 x 2; cultivar x treatment) for significance testing, and indicated 15,149 transcripts changed significantly with cultivar; the transcript abundance of 1 transcript changed significantly with treatment; and 241 transcripts changed with the cultivar x treatment interaction term. This was analogous to the aforementioned ANOVA *F*-test done for proteins. Genes found

significant in both platforms were similarly adjusted for multiple testing with the Benjamini and Hochberg procedure.

There was a common set of 1,211 proteins that was quantifiable across each of the cultivars and treatments. This consistent set of proteins was considered for further reliable comparative quantitative analyses. The protein abundance of 832 proteins changed significantly with cultivar (Table 5), but no proteins were changed significantly for either treatment or cultivar x treatment interaction terms. In addition, the relative metabolic content of primary and secondary metabolites (**Supplemental File 6**) changed significantly with cultivar, but no metabolites were changed significantly for either treatment or cultivar x treatment interaction terms (Table 5).

A comparison of Table 3 and Table 5 reveals that the percentage of the transcripts varying with cultivar was substantially different between the two transcriptomic platforms: the microarray platform was 92% and the RNAseq platform was 56%. The percentage of proteins varying with cultivar was approximately 69% and the percentage of metabolites varying with cultivar was approximately 95% for both platforms. Thus, all Omic platforms revealed a large variability in molecular abundance amongst all the cultivars.

To summarize the treatment effects, the ANOVA results indicate that while mild water deficit did induce a significant change in the abundance of a small percentage (< 6%) of transcripts, but the products of translation and further metabolism determined in this study were significantly influenced only by differences associated with the genotype of a specific cultivar.

Biological samples from each platform were analyzed by principal components analysis (PCA) (**Fig. 3**), which reduced the dimensionality of the data to observe the underlying structure. Each PCA biplot showed the directions where there was the most variance in the data. Cultivars separated from one another similarly on the first principal component in each platform providing substantial concordance amongst the different Omic approaches. Generally, red cultivars separated from white, but Pinot Noir samples separated somewhere in between. Biological variability in samples was evident particularly in protein and metabolite biplots. The secondary metabolites were separated along the first component, separating the red cultivars that synthesize anthocyanins, and anthocyanin moieties separated Cabernet Sauvignon and Merlot from Pinot Noir. Water-deficit and well-watered samples at harvest could not be differentiated clearly in PCAs reflecting the results from the ANOVA.

A functional analysis (**Supplemental File 7**) was performed to identify gene ontology (GO) categories for the quantifiable proteins with the BinGO (3.0.2) plugin for Cytoscape (3.1.1), using a custom annotation derived from UniProt (uniprot.org), EnsemblPlants (plants.ensembl.org), and Gramene (gramene.org) (Shannon, Markiel et al. 2003, Maere, Heymans et al. 2005). There were 479 significantly overrepresented GO categories after correcting for FDR (adjusted p-value of 0.05). To aid our analysis, overrepresented GO terms were visualized (**Fig. 4**) with a treemap using REVIGO and the treemap R package that depicts loosely related GO terms by color (Supek, Bosnjak et al. 2011). Rectangles in the treemap are size adjusted to reflect their enriched p-

value. The functional analysis examined the results both by the level of significance and by the number of constituents of each GO category, in an effort to look beyond generic or overly encompassing functional categories (e.g. metabolic process). Some of the major biological process GO categories included organic acid metabolic process, monosaccharide metabolic process, generation of precursor metabolites and energy, alcohol metabolic process, and response to abiotic stimulus.

2.3.3 Correlations between proteomic and transcriptomic data

To investigate the linear relationship of transcript level with protein abundance, we fit linear regression models to the transcript-protein pairs and computed Pearson's correlation. A direct sample-to-sample comparison was performed for the RNAseq using the same biological replicates as were used in the proteomics. The microarray analysis contained two additional biological replicates for each treatment and cultivar preventing a direct one-to-one comparison between replicates. Mean expression (transcript) and abundance (protein) values were then computed for each treatment and cultivar prior to regression analysis. When the transcriptomic and proteomic abundance values were compared for all transcript-protein pairs by a single linear regression, the goodness of fit or coefficient of determination was low (r^2 = 0.07, RNAseq; r^2 = 0.06, microarray), and a small positive correlation between the pairs observed (Pearson correlation coefficient = 0.27 with RNAseq and 0.24 with microarray) (**Fig. 5**). Pearson's correlation of each individual transcript-protein pair revealed a subset of moderate to strong positive and negative relationships, with

abundance levels of some proteins well represented by their regressions by the protein-encoding transcript at harvest (**Supplemental File 8; Fig. 6**). The strength of correlation was much higher for protein-transcript pairs positively correlated with one another in either platform. For example, pathogenesis-related proteins, carboxyesterases and proteins related to phenylpropanoid and flavonoid production were modeled well by linear regression. Generally, protein-transcript pairs grouped together by cultivar and occasionally by skin color. Stronger negative correlations were observed in the microarrays (-0.93) than in RNAseq (-0.68). Protein-transcript pairs with strong negative correlations included a translation initiation factor eIF3 subunit (r^2 = 0.41, Pearson correlation coefficient = -0.67; D7TMG2, VIT_13s0019g03470) and a chlorophyll A-B binding protein (r^2 = 0.46, Pearson correlation coefficient = -0.68; A5BPB2, VIT_12s0028g00320), a constituent of the light-harvesting complex. Other negatively correlated protein-transcript pairs included several heat shock proteins and a putative serine/threonine kinase.

2.3.4 Transcriptomic platform concordance

We measured how similar the two different platforms, both open (RNAseq) and closed (microarray), measured gene expression levels by Pearson correlation and linear regression, on a gene-by-gene basis. In Cramer et al. [41], we cautioned readers about the likelihood of cross-hybridization potential of approximately 13,000 genes on the NimbleGen Grape Whole-Genome microarray, which includes multiple probes with identical oligonucleotide sequences. Many of these transcripts belong to *Vitis* gene families with high

sequence similarity that creates an opportunity for at least one probe from a probeset of four probes to cross-hybridize with probes from another gene on the array. A global comparison of measureable transcripts shared between the methods presented an opportunity to investigate their concordance. In Figure 7, a pairwise comparison of each platform's transcript expression was separated into subsets by the number of probes with the potential for cross-hybridization (0, 1, 2, 3 or 4 probes). Platforms were positively correlated as a whole (Pearson's correlation coefficient 0.80), but the correlation decreased when examining subsets of transcripts based on the number of probes that cross-hybridize (Table 6). In particular, lowly expressed transcripts in the RNAseq dataset had a variable range (high to low) of expression values measured by microarray.

2.3.5 Pathway Omic analyses

To get a better understanding of the biochemical processes in the mature berry skin and to emphasize how differentiated the cultivars were at harvest, we mapped our Omic data sets to two important biochemical pathways for further analysis. We used the quantifiable protein data as a framework for each map and their matching transcripts. Additionally, metabolite intermediaries and final products were also mapped, including amino acids, flavan-3-ols, and anthocyanins. Each pathway summarizes abundance differences depicted as side-by-side heat maps that display the ratio of the individual cultivars average to the overall cultivars average abundance for each data point. The Omic data were overlaid onto customized metabolic pathway maps based upon annotated maps located at KEGG (Ogata, Goto et al. 1999), PlantCyc (Zhang, Foerster et al.

2005), and VitisCyc (Naithani, Raja et al. 2014). Mapped enzymes without heat maps did not contain protein data.

2.3.5.1 Phenylpropanoid and anthocyanin biosynthesis

We primarily observed higher protein abundance in the red cultivars (Fig. 6) for enzymes involved in phenylalanine through anthocyanin biosynthesis, such as flavanone 3-hydroxylase and leucoanthocyandin dioxygenase. Missing spectra within the biological replicates of the white cultivars was evidence of their lesser abundance. Relative to the red cultivars, Chardonnay and Semillon proteins involved in phenylpropanoid and flavonoid were less abundant, though, a chorismate mutase (CM) in Chardonnay was an exception to that observation. Chorismate is an important precursor that interfaces the metabolic synthesis of phenylalanine and tyrosine, tryptophan, folate, and phylloquinone (Maeda and Dudareva 2012). Four phenylalanine ammonia-lyases (PAL; A5BPT8, F6HNF5, F6HR33, F6HS12) were identified only within the red-skinned cultivars. Phenylalanine ammonia-lyases (4.3.1.24) are a multigene enzyme family encoding the first committed step in phenylpropanoid biosynthesis (Camm and Towers 1973). Chalcone synthase (CHS; 2.3.1.74) and stilbene synthase (STS; 2.3.1.95) enzymes both catalyze reactions that condense the substrates 3-coumaroyl-CoA and three malonyl-CoA units in production of flavonoids and stilbenoids, respectively. Three grapevine chalcone synthases (A2ICC5, F6H419, Q8W3P6) were identified within the proteomic data set (Goto-Yamamoto, Wan et al. 2002). UDP glucose:flavonoid 3-O-glucosyltransferase (UFGT; 2.4.1.115) proteins were observed only in the red cultivars. They catalyze the O-

glycosylation of anthocyanidins or anthocyanins that enhance the stability and hydophilicity of anthocyanins *in planta* (Sparvoli, Martin et al. 1994, Boss, Davies et al. 1996, Ford, Boss et al. 1998). Of the proteins quantified in each cultivar, all but 3-dehydroquinate synthase (DHQS; 4.2.35) were significantly different at the cultivar level.

In contrast, the transcripts of differentially expressed genes (DEGs) between cultivars in the phenylpropanoid pathway were generally few and occurring after naringenin chalcone in the pathway. More evident were differences between red and white cultivar DEGs of enzymes that centered on flavonoid and anthocyanin biosynthesis such as chalcone synthase, flavanone 3-dioxygenase (F3H; 1.14.11.9) and UDP glucose:flavonoid 3-O-glucosyltransferase (UFGT, 2.4.1.115). These three enzymes had the most abundant transcripts mapped, and are similar to the gene expression for all cultivars but Merlot (not measured) in Boss *et al.* (Boss, Davies et al. 1996). No members of the multi-gene stilbene synthase family were detected in the proteomic data set, but one stilbene synthase (*VviSTS3*) encoding transcript (in microarrays) was significantly changed under the interaction term (F6HIR8; VIT_10s0042g00880), with Cabernet Sauvignon experiencing a -1.6 fold decrease in expression as a result of water deficit (Parage, Tavares et al. 2012, Vannozzi, Dry et al. 2012). However, *VviSTS3* was lowly expressed in microarrays (1-probe with cross-hybridization potential) relative to other transcripts and contained few counts in RNAseq. Only the UDP glucose:flavonoid 3-O-glucosyltransferase transcript (D7T7R5; VIT_16s0039g02230) was

significant at the treatment level in the microarrays, but each transcript, with the exception of the shikimate dehydrogenase, was significant at the cultivar level.

Both primary and secondary metabolites were measured for each cultivar. Shikimate was among the most abundant metabolites in Cabernet Sauvignon. Aromatic amino acid biosynthesis stems from this intermediary product within the shikimate pathway (Maeda and Dudareva 2012). Phenylalanine, tryptophan and tyrosine amino acids were recovered in each cultivar. Stilbenoids were also recovered to include *cis*- and *trans*-resveratrol, their glucosides and the polymerized δ-viniferin. Catechin and epigallocatechin, two flavan-3-ol monomers, and procyanidin dimers B2 and B3, consist of two molecules of (+)-catechin or (-)-epicatechin respectively. Flavan-3-ols co-localize with anthocyanins in the hypodermal cells of the berry skin, comprising a diverse and highly abundant class of soluble phenolic compounds (Adams 2006). The astringent mouth feel sensations experienced in red wines are derived from these phenolic compounds, with increasing concentrations associated with quality wines (Mercurio, Dambergs et al. 2010).

Given that most observable protein and transcript ratio changes were centered at the end of anthocyanin biosynthesis, we present their relative abundance of these metabolites for the three moieties of anthocyanins that were determined (Supplemental File 9). The importance of color to the sensory experience of red wines is derived from the red, purple and blue anthocyanin pigments produced in the berry skin. Observable differences of anthocyanidin content and their glycosylated, acetylated and coumaroylated moieties amongst

the red cultivars were strongly cultivar dependent. All metabolites were significantly different at the cultivar level except malvidin 3-O-(6-p-coumaroyl)glucoside and petunidin 3-O-(6-acetyl)glucoside. Malvidin 3-glucoside had the largest relative abundance of any anthocyanin, and the acetylated and coumaroylated forms of malvidin were also in high abundance in Cabernet Sauvignon and Merlot relative to the other four anthocyanins. Mild water deficit did not have any significant effects on anthocyanin abundance in any cultivar. Thus, all of the variation in metabolite composition could be attributed to the cultivar and not to water deficit.

2.3.5.2 Amino acid metabolism

The mature grape berry, via pressed must, provides a source of nitrogenous substances in the form of free amino acids and cleaved peptides, proteins and nucleic acid derivatives, and in mineral ammonium salts that collectively make up the fermentable nitrogen metabolized by yeast during alcoholic fermentation (Conde, Silva et al. 2007). Three glutamine synthetases (A5AP38, D7T6P4, and P51119) were identified in each cultivar; glutamine synthetase is an important enzyme for the condensation of glutamate and ammonia into glutamine. Glutamine synthetases (6.3.1.2) aid in berry nitrogen incorporation (Grimplet, Wheatley et al. 2009) and were the most abundant of the enzymes related to amino acid metabolism in each of the five cultivars, recording hundreds of spectra in each biological replicate. Of the mapped proteins quantified in each cultivar, all but ornithine aminotransferase (2.6.1.13) and ornithine carbamoyltransferase (2.1.3.3) were significantly different. Transcript

abundance differences between cultivars were muted, with the exception of an argininosuccinate lyase (4.3.2.1). Only the arginase (3.5.3.1) transcript (D7U7W7; VIT_15s0048g00420) in the microarray was significant for the cultivar x treatment term, but all transcripts were significant for cultivar. Chardonnay contained the highest amount of each mapped amino acid (arginine, glutamate, glutamine, ornithine, and proline), except for proline, which was highest in Cabernet Sauvignon (Figure 9 and Table S6). Proline was also the most abundant amino acid quantified by the GC-MS. Arginine abundance was not significantly different in any measured cultivar, but the abundances of glutamine, glutamate, ornithine and proline were significantly different between cultivars. The amino acids, glutamate and glutamine, are important sources of available nitrogen for yeast fermentation (Boulton, Singleton et al. 1996).

2.4 Discussion

The experimental design in this study allowed for a very powerful set of comparable analyses. First, all berry tissues were sampled from the same vineyard site, with vines exposed to the same environment, with nearly identical climate, water and soil (terroir). Second, studying five cultivars further allowed for phenotypic variation of berry metabolism at harvest to be assessed (Gilad, Oshlack et al. 2006). Third, the Omic analyses benefited from using aliquots of the same tissue, allowing us to better correlate changes between the proteome and transcriptome and observe variations in the intermediary and end products of metabolism. We also investigated the power of two transcriptomic methods, both closed and open platforms, that provided an opportunity to examine potential

cross-hybridization events of repeat elements, such as closely related gene family members.

2.4.1 Omic analyses

While previous proteomic analyses have investigated the proteome of grape berry skin (Deytieux, Geny et al. 2007, Negri, Prinsi et al. 2008, Grimplet, Wheatley et al. 2009, Wang, Bianchi et al. 2009), our approach estimated protein abundance changes by label-free quantification using spectral counting. A recognized challenge in quantitative proteomics stems from missing data values for a variety of reasons (e.g. peptides present in high abundance but not detected, peptide abundance below detection limits, and missing peptides), a challenge encountered while analyzing the proteomic data set presented here (Karpievitch, Dabney et al. 2012). For example, nearly 3,000 proteins were identified in five wine grape cultivars, but only 1,211 were selected for quantification and further analysis because they were detected in all samples. The removal of some proteins for quantitative analysis stemmed from missing spectral counts in one or more biological replicates, which qualified their removal under the NSAF method. Despite the high dynamic range for identifying large numbers of proteins, current label-free proteomic methods are disadvantaged for the detection and quantification of low abundant proteins (Bantscheff, Lemeer et al. 2012, Li, Adams et al. 2012). Thus, limited replication challenged our assessment of abundance differences due to treatment. Nevertheless, the proteomic results from this study did provide further insight into a large number of proteins residing within a mature berry at harvest, allowing the detection of

hundreds of differences in protein abundance in three red and two white cultivars.

Transcript profiling of grapevine can be used to assess specific interactions related to cultivar or treatment affects. Both whole and incomplete genome microarrays have been previously utilized in our research (Cramer, Ergul et al. 2007, Deluc, Grimplet et al. 2007, Grimplet, Deluc et al. 2007, Deluc, Quilici et al. 2009, Deluc, Decendit et al. 2011, Cramer, Ghan et al. 2014) to investigate berry development and the effects of water and salinity stress in both vegetative and berry tissues. For example, a recent investigation of berry pulp and skin revealed a dynamic and active ripening process occurring in the late stages of berry development, with ethylene signaling appearing to play a bigger role in non-climacteric fruit ripening than previously thought (Cramer, Ghan et al. 2014). Transcriptionally, the mature berry was very active, and this was evident with the number of transcripts significantly changed for each factor and interaction term. In our study, only transcription was sensitive enough to detect the treatment effect, likely due to the mild treatment level. In addition, the use of five biological replicates in the microarrays and the detection accuracy of the RNAseq may have increased the ability to detect significantly changing transcripts. In another study, water deficit was investigated in fruit from Chardonnay and Cabernet Sauvignon, revealing distinct expression patterns in the cultivars for ABA, isoprenoid and stilbene biosynthesis (Deluc, Quilici et al. 2009, Deluc, Decendit et al. 2011). The transcript data presented here offers a

rich data set of cultivar differences at harvest that can be used in future analyses by the grape research community.

In addition to our own research, other grape researchers have used high-throughput expression profiling technologies to globally characterize gene expression (Fasoli, Dal Santo et al. 2012, Sweetman, Wong et al. 2012, Vannozzi, Dry et al. 2012, Cavallini, Matus et al. 2015). Dal Santo *et al.* (Dal Santo, Tornielli et al. 2013) examined the phenotypic plasticity of Corvina berries from the three most important wine regions around Verona, Italy at various stages of development that revealed a number of non-plastic genes that display stage-specific expression increases or decreases irrespective of vineyard, such as PR and photosynthesis-related transcripts. The observation of non-plastic transcriptome programming partly explains the strong presence of the PR proteins detected in our analysis that accumulate as a disease-prevention strategy. Comparative Omic analysis can also been used to thoroughly investigate specific metabolic pathways, similar to the metabolic profiling done in this study. Profiling of Sauvignon Blanc with whole genome microarrays (Young, Lashbrooke et al. 2012) putatively identified forty-two carotenoid biosynthesis genes that updated our understanding of one pathway responsible for flavor and aroma production in grapes. More recently, the measurement of individual gene expression using RNAseq technologies have been used to further our understanding of the transcriptome and are greatly benefited by the higher dynamic range for detection of expression. With unprecedented sensitivity, Zenoni *et al.* (Zenoni, Ferrarini et al. 2010) were the first group to utilize RNAseq

to profile grape gene expression through berry development; with this approach they were able to identify differential splicing activity and single nucleotide polymorphisms. The observation of unique reads that did not directly map to the reference genome was particularly interesting, further highlighting the power of RNAseq. For example, *de novo* assembly of the Corvina transcriptome (Venturini, Ferrarini et al. 2013) revealed 180 new or unique genes (the authors referred to them as private genes) not annotated in the PN40024 reference genome (Jaillon, Aury et al. 2007). RNAseq has also been used to describe the expression of specific transcription factors over-expressed at single developmental stages, such as those belonging to the ERF, WRKY and UPBEAT transcription factor families (Sweetman, Wong et al. 2012). Knowledge of the timing of transcription factor activity can be used for generating new hypotheses for testing the regulation of berry developmental. Collectively, these studies have assisted in furthering our understanding of grapevines and improving the functional annotation of the genome (Grimplet, Van Hemert et al. 2012). These transcription studies are very powerful, often for the information not mentioned directly in the text but contained in their corresponding data sets.

The availability of the grape genome coupled with microarray and next generation sequencing technology allows global gene expression profiling. Platform concordance was informative of how well each of the technologies performed at measuring transcript abundance. Similar workflows were used beginning with identical tissue and methodology for total RNA extraction and quality assurance checks (Mantione, Kream et al. 2014). Samples also went

through similar cDNA syntheses prior to hybridization or library preparation. Closed platforms like microarrays are not readily adapted to improvements made to genomes as are gff3 annotation files and suffer from potential cross-hybridization events. Figure 7 illustrated the decrease of concordance between the platforms for annotated genes with the potential for one or more probe cross-hybridizations. Many of the lowly expressed transcripts in the RNAseq were not accurately modeled in the arrays with a wide range of expression values. The dynamic range of detection was not as high in the microarrays, evident by the right-tail in the pairwise plots. But, the expression profiles of the arrays did follow the relative abundance levels of transcripts seen with RNAseq.

Read numbers per gene are a complement of the expression level of the gene, what we're most interested in, the number of reads generated by the technology and the length of the transcript for those reads to align to. Of course, the transcript length of a given gene will not differ between samples, only differing between other genes, which are not directly compared with one another. Inefficiencies in measuring gene expression can be related to the degree of read mapping due to poor or incomplete annotations, RNA that is lost during extraction or during cDNA conversion and ligation to adaptors. Ultimately, measuring mRNA levels is only a proxy for protein level, when considering the importance of post-translational modifications affecting protein activity. While the two-transcriptomic platforms were highly correlated with each other, neither platform was an overall good predictor of protein abundance. The finding that the abundance of most transcripts is not correlated with the abundance of proteins

from the same gene is consistent with many other findings in plants (Chen, Gharib et al. 2002, Gallardo, Firnhaber et al. 2007, Fu, Fu et al. 2009, Haider and Pal 2013, Zhang, Egger et al. 2014).

2.4.2 Minor effects of water deficit

Water deficit treatment did not significantly alter the abundance of proteins or metabolites in the five cultivars. Berry physiology was also unaffected by water stress, which indicated that the stress was mild. Matthews *et al.* has shown that mild water deficit does not significantly affect levels of soluble sugars, titratable acidity or berry diameter (Matthews, Anderson et al. 1987, Matthews and Anderson 1988, Matthews and Anderson 1989), but does, however, produce wines with significantly different flavor and aroma profiles (Matthews, Ishii et al. 1990). In contrast, more severe water deficit causes significant reductions in berry diameter in Cabernet Sauvignon (Grimplet, Deluc et al. 2007) and Chardonnay (Deluc, Quilici et al. 2009) and significantly alters metabolite composition and abundance. The lack of significant differences observed in the present study was possibly related to the mild water deficit, thus inducing only small differences in metabolite abundance. With a higher number of replications, statistically significant changes in metabolite abundance in response to water deficit may have been detected.

Another explanation for little significant differences may partly be attributable to the single sampling time point at maturity. Dai *et al.* (Dai, Leon et al. 2013) surveyed a number of central metabolic signatures from whole berry samples displaying developmental specificity, with large abundance changes primarily occurring shortly before, through, and shortly after veraison. This

argument is further supported by a fruit development experiment comparing Cabernet Sauvignon and Shiraz berry skins (Degu, Hochberg et al. 2014), which showed similar developmental trends in both central and secondary metabolites where large metabolic changes occur early in development rather than at near-maturity. Additionally, the mild water deficit, very likely caused subtle Omics changes that made it difficult to detect common responses with this level of replication. Post-veraison, the berry undergoes rapid cellar expansion and increases in soluble sugars for a time, but as development continues, progressively towards senescence, the berry undergoes withering or dehydration. Perhaps, the poor detection of treatment related effects was simply due to both treatments having experienced a degree of water deficit-related stress, although no visible withering or shrivel was observed. The high abundance of peroxiredoxin proteins across cultivars is known to be elevated in water deficit in (Cramer, Van Sluyter et al. 2013), although other environmental stress factors such as high light or UV intensity could also influence protein abundance. In an extreme case, Corvina berries undergo a withering process to make the famous ripasso and amorone wines (Venturini, Ferrarini et al. 2013). As a result of the mild water deficit in our study, cultivar effects were the dominant differentiating factor in metabolic content.

2.4.3 Model assessment and correlation

The relationship of protein abundance by mRNA expression level was low as a whole (Pearson's correlation coefficient of 0.27 and r^2 of 0.07), when fit to linear regressions for the entire set of quantifiable proteins against either

transcriptional platform. The regulation of gene expression can be controlled at many different stages, which may partly explain the poor observed correlation (Venturini, Ferrarini et al. 2013, Degu, Hochberg et al. 2014). For example, transcriptional and post-transcriptional regulation related to the processing of RNA (e.g. alternative or differential splicing) and the stability of the RNA itself can determine the level of expression, where tissue specificity or stress response determines a specific isoform (Vitulo, Forcato et al. 2014). The general translation of mRNA into protein can also be affected by translational regulation from different regulatory elements (e.g. depletion of ternary complex or hormone signaling) (Melcher, Ng et al. 2009, Ishihama and Yoshioka 2012). Protein stability (often measured as a half-life) might also be influenced by the specific isoform or by the conditions that lead to its formation; to add to these examples the possibility of post-translation modifications of the protein (Mazzucotelli, Mastrangelo et al. 2008) only increases the complexity and reduces the probability for a high correlation of transcript abundance with protein abundance. Yet subsets of different transcript-protein pairs were strongly correlated, particularly pathogenesis-related proteins. At least in the mature berry, the regulation of these genes appears to be tightly controlled at levels upstream of translation.

Transcript-protein pair relationships that lack any correlation can also reveal insights into the biology shared amongst all the cultivars. For example, three of the top most abundant proteins quantified (D7SKR5/ VIT_06s0004g03550; F6HUD1/ VIT_02s0025g03600; D7TBK8/

VIT_11s0016g03630) assist in scavenging H_2O_2 and are involved in ascorbate-glutathione metabolism; they can offer protective qualities to a maturing berry, irrespective of cultivar, and benefit vine fitness (Matamoros, Loscos et al. 2010, Dietz 2011). Both the protein and transcript abundances of ascorbate peroxidase and a glutathione peroxidase were high in each of the cultivars. These data support the hypothesis that high protein abundance levels at this berry developmental stage are important for sustained H_2O_2 scavenging and antioxidant activities.

2.4.4 Effects on berry skin phenolics at harvest

Phenylpropanoids, derived from phenylalanine, are a diverse class of secondary metabolites and are important factors that influence both grape and wine quality. The biosynthesis of small molecular weight phenolics, such as caffeic acid and caftaric acid, peak around the onset of ripening (veraison) and then decrease in the weeks thereafter (Cavallini, Matus et al. 2015). As in Castellarin *et al.* (Castellarin, Pfeiffer et al. 2007), we wanted to link observable changes in our transcriptional and translational data sets with changes in metabolism following a seasonal water deficit treatment. In the present study, numerous protein-transcript pairs and metabolites involved in phenylpropanoid biosynthesis were mapped (Fig. 6), showing the phenotypic diversity of various organoleptic properties (e.g. color and astringency) and berry biochemistry. Enzymes related to anthocyanin biosynthesis were highly abundant relative to other enzymes mapped. Similarly, Deytieux *et al.* (Deytieux, Geny et al. 2007) observed high relative abundance of chalcone synthase, flavanone 3-

hydroxylase and UDP glucose:flavonoid 3-O-glucosyltransferase enzymes that initiate the gradual accumulation of these phenolic compounds.

Many of the phenylpropanoids were among the most abundant metabolites measured, with the genotype determining the abundance distributions. Metabolic profiling of anthocyanins in the three red cultivars revealed variation in the relative metabolic content of each selected metabolite (Supplemental File 9). Our results for high levels of malvidin were consistent with those reported previously for Cabernet Sauvignon (Degu, Hochberg et al. 2014), Malbec (Fanzone, Pena-Neira et al. 2010) and Yan73 (Muscat Hamburg x Alicante Bouschet) (He, Liu et al. 2010). The strong effect of cultivar was evident in protein and metabolite differences observed between the cultivars.

Stilbene abundance also varied between cultivars when compared at harvest. In Cabernet Sauvignon and Shiraz fruit, levels of *trans*-resveratrol accumulated at similar levels from veraison to maturity, whereas its glucoside, *trans*-piceid only increased in Shiraz (Degu, Hochberg et al. 2014). Similarly, our cultivars displayed divergent stilbene levels at harvest, with the highest levels observed in Pinot Noir. This is consistent with two comprehensive studies of cultivar comparisons of stilbene concentrations (Gatto, Vrhovsek et al. 2008, Lambert, Richard et al. 2013), in which Pinot Noir was the cultivar that had the highest stilbene concentrations. Under more severe water deficit, *trans*-piceid metabolite abundance increases 5-fold along with increasing steady state transcript abundance in Cabernet Sauvignon, but not in Chardonnay (Deluc, Decendit et al. 2011). These observations are further supported by a 3-year

survey of 78 Italian red, white and pink grape cultivars, where large variability in stilbene abundance was consistent with gene expression analysis in the healthy, developing grape berries (Gatto, Vrhovsek et al. 2008). The abundance of different stilbenes, like other phenylpropanoids, can distinguish one cultivar from another.

2.4.5 Importance of assimilable nitrogen in berry skins

Assimilable nitrogen within grape must (fermenting juice) can be a limiting factor to yeast growth during fermentation (Henschke and Jiranek 1993). The total nitrogen content is distributed primarily in skins and seeds of ripe berries, with the amino acid content ranging from 30 to 40% depending upon cultivar (Boulton, Singleton et al. 1996). Proline, arginine, glutamine, alanine, and glutamate are the major amino acids in fresh grape juice, but the specific composition and concentration of amino acids varies by cultivar, vineyard location and winemaking practices (Huang and Ough 1991, Henschke and Jiranek 1993, Negri, Prinsi et al. 2008). By sampling and processing berry tissues from the same experimental vineyards, we hoped to remove some of the bias introduced in our previous studies where the metabolisms of Cabernet Sauvignon and Chardonnay were compared from grapes grown in different geographic locations, root stock and trellis systems (Deluc, Quilici et al. 2009). Transcripts related to glutamine and glutamate metabolism were significantly different between cultivars. The metabolite abundance for these two amino acids in this study was low, and reflected different cultivar distributions (Fig. 7). Levels of glutamine and glutamate abundance decrease overtime from veraison to

maturity in studies located in Israel and Australia (Stines, Naylor et al. 1999, Degu, Hochberg et al. 2014). Proline is one of the major amino acid constituents in both juice and wine, and is formed from 1-pyrroline-5-carboxylate (Ough and Stashak 1974, Etiévant, Schlich et al. 1988, Huang and Ough 1991). In two studies, Chardonnay, Cabernet Sauvignon and Shiraz berry skins saw large increases in proline relatively late in the ripening process (post-veraison) peaking at maturity (Stines, Naylor et al. 1999, Degu, Hochberg et al. 2014). High proline abundance was observed in each of the cultivars in our study. Ornithine, derived from the urea cycle, can function as a substrate for further amino acid biosynthesis when converted to glutamate 5-semialdehyde by ornithine aminotransferase, which links proline and arginine metabolism (Negri, Prinsi et al. 2008). Non-protein amino acids like ornithine and γ-aminobutyric acid (GABA) also contribute to total available nitrogen content within grape must (Etiévant, Schlich et al. 1988). Bach *et al.* (Bach, Sauvage et al. 2009) observed varying GABA concentrations amongst 21 cultivars that changed with region, cultivar and year of harvest, observing the highest GABA levels in Chardonnay. We did not directly measure GABA in this study, but we can hypothesize that GABA levels like other nitrogen contributing compounds measured in this study varied with the cultivar.

2.5 Conclusions

In summary, the measured variance in each of the Omics analyses concordantly separated the five cultivars. The integration of multiple high-throughput Omic datasets revealed complex biochemical variation amongst five

cultivars of an ancient and economically important crop species. The phenotypic variation in the cultivars resulted in unique and large differences in abundance in many of the most common classes of proteins and metabolites measured in berry skins. Only transcript analyses were sensitive enough to detect significant induced changes from the moderate water deficit treatment. Overall, transcript abundance was poorly correlated with protein abundance. Omic analyses elucidated cultivar differences in phenylpropanoid biosynthesis and amino acid metabolism that influence winemaking, including color, astringency and yeast assimilable nitrogen levels. There were significant differences in the classes of pathogenesis proteins in the berry skins of each cultivar in the absence of pathogenic pressures. The information presented here exposes clear differences between the skins of mature berries of different cultivars, their molecular responses to water deficit and the diversity of molecules that can impact wine quality.

Table 1. *Mid-day stem water potentials at harvest time point. Measurements conducted on mature, fully expanded leaves. Values are mean* ± SE.

Vineyard[a]	Treatment[b]	ψ_w (MPa)[b]	n[c]
North	Water deficit	-0.84 (± 0.11)	6
	Well watered	-0.61 (± 0.03)	8
South	Water deficit	-0.95 (± 0.04)	15
	Well watered	-0.68 (± 0.04)	14

[a] North = Cabernet Sauvignon and Chardonnay; South = Merlot, Pinot Noir, and Semillon

[b] MPa = megapascal

[c] Inconsistencies between sample size were due to damaged leaves at time of sampling

Table 2. *Berry physiological measurements at the harvest time point. Values are mean ± SE, with n = 3 for berry diameter and n = 6 for ˚Brix and titratable acidity (TA) measurements. Differences between treatments were determined to be significant (p-value<0.01) by the Student's t-test.*

Varietal	Treatment[a]	Berry diameter (mm)[b]	˚Brix[c]	TA (g l^{-1})[cd]
Cabernet Sauvignon	WW	11.16 (± 0.07)	23.11 (± 0.20)	8.43 (± 0.25)
	WD	11.09 (± 0.07)	23.66 (± 0.27)	8.42 (± 0.29)
Merlot	WW	11.72 (± 0.09)	22.99 (± 0.23)	5.50 (± 0.38)
	WD	11.55 (± 0.08)	23.31 (± 0.30)	6.06 (± 0.43)
Pinot Noir	WW	12.09 (± 0.07)	22.85 (± 0.46)	5.80 (± 0.09)
	WD	11.51 (± 0.07)*	22.95 (± 0.46)	6.12 (± 0.24)
Chardonnay	WW	12.11 (± 0.06)	23.35 (± 0.39)	9.18 (± 0.26)
	WD	12.07 (± 0.07)	23.42 (± 0.25)	8.83 (± 0.44)
Semillon	WW	13.47 (± 0.09)	23.18 (± 0.40)	6.40 (± 0.32)
	WD	13.29 (± 0.09)	23.82 (± 0.33)	6.53 (± 0.28)

[a] WW = well watered; WD = water deficit

[b] Measurements conducted on individual berries

[c] Measurements conducted on whole clusters

[d] Expressed in g l^{-1} tartaric acid

Table 3. *Comparative Omic analyses.*

Data set	n
Proteins (nanoLC-MS/MS)	2,867
Transcripts (microarray)	29,549
Transcripts (RNAseq)	27,252
Metabolites measured by GC-MS	67
Metabolites measured by LC-MS	42

Table 4a *Top ten most abundant proteins quantified within each cultivar. The number within each cultivar column represents the abundance rank for that cultivar, with the number '1' being the highest.*

			Cultivar[b]				
UniProtKB	V1 ID	Annotation[a]	CS	ME	PN	CD	SM
D7TBK8	VIT_11s0016g03630	Peroxiredoxin-5	1	2	10	6	5
F6GY46	VIT_18s0072g00380	Abscisic stress ripening protein 2	2	–	–	5	9
A5BQN6	VIT_03s0038g01930	Peptidyl-prolyl cis-trans isomerase ROC5	3	3	–	–	–
Q9M4H4	VIT_06s0004g02560	Kiwellin Ripening-related protein grip22	4	–	–	–	–
Q9M4H7	VIT_12s0059g00590	Allergenic protein Pt2L4	5	–	–	10	–
F6HUD1	VIT_02s0025g03600	Phospholipid hydroperoxide glutathione peroxidase	6	–	–	–	
Q7XAU6	VIT_05s0094g00340	Chitinase class IV	7	7	5	–	6
D7SKR5	VIT_06s0004g03550	L-ascorbate peroxidase 1, cytosolic	8	10	–	–	7
F6HLL9	VIT_08s0007g06040	Beta-1, 3-glucanase	9	8	7	8	10
A5BAX1	VIT_01s0011g05110	Major latex protein 22	10	4	8	7	3
F6HUH1	VIT_02s0025g04330	Thaumatin VVTL1	–	1	1	1	2
D7TXF5	VIT_14s0081g00030	Pathogenesis-related protein-4 (Chitinase)	–	5	4	–	4
Q9FS43	VIT_05s0077g01580	Pathogenesis protein 10	–	6	–	–	–
A5C9F1	VIT_02s0025g04300	Thaumatin	–	9	3	4	8
F6HUG9	VIT_02s0025g04310	Thaumatin	–	–	2	2	–
F6HUG6	VIT_02s0025g04280	Osmotin	–	–	6	9	–
F6GXX3	VIT_08s0058g01230	Non-specific lipid-transfer protein	–	–	9	3	1

Table 4b *Top ten most abundant transcripts (microarray) within each cultivar.*

UniProtKB	V1 ID	Annotation[a]	Cultivars[b]				
			CS	ME	PN	CD	SM
F6H8W9	VIT_12s0034g01970	Cupin	1	1	4	–	–
F6H8M1	VIT_05s0049g00520	Putative uncharacterized protein	2	2	1	1	1
Q9M4H8	VIT_16s0022g00960	Invertase/pectin methylesterase inhibitor	3	3	3	7	6
Q9M4H7	VIT_12s0059g00590	Allergenic protein Pt2L4	4	–	9	9	8
Q7XAU6	VIT_05s0094g00340	Chitinase class IV	5	5	6	8	5
Q6VEQ6[c]	VIT_05s0049g00760	Putative ripening-induced protein 1	6	4	7	2	2
D7SLR0	VIT_15s0021g02700	Beta-expansin (EXPB4)	7	–	–	–	–
F6HFY8[c]	VIT_01s0010g01260	23S ribosomal RNA	8	–	–	–	7
A5B118	VIT_08s0007g03830	fructose-bisphosphate aldolase cytoplasmic isozyme	9	10	–	–	–
F6GXB0[c]	VIT_11s0052g01680	Photosystem II protein D1	10	9	8	5	4
F6HUG9	VIT_02s0025g04310	Thaumatin	–	6	2	3	–
F6HUH1	VIT_02s0025g04330	Thaumatin VVTL1 [Vitis vinifera]	–	7	5	4	10
F6GV13	VIT_06s0004g04650	Metallothionein	–	8	–	6	–
A5C670[c]	VIT_13s0064g01210	Zf A20 and AN1 domain-containing stress-associated protein 2	–	–	10	–	–
F6GXX3	VIT_08s0058g01230	Non-specific lipid-transfer protein	–	–	–	10	3
F6HPX1[c]	VIT_13s0101g00220	Ribosomal RNA 16S	–	–	–	–	9

Table 4c *Top ten most abundant transcripts (RNAseq) within each cultivar.*

			Cultivar[b]				
UniProtKB	V1_ID	Annotation[a]	CS	ME	PN	CD	SM
F6H8M1	VIT_05s0049g00520	Putative uncharacterized protein	1	1	1	1	1
Q6VEQ6[c]	VIT_05s0049g00760	Putative ripening-induced protein 1	2	2	2	2	2
F6HEL0	VIT_19s0090g01370	Putative uncharacterized protein	3	6	–	7	–
F6H8M0[c]	VIT_05s0049g00510	Ethylene response factor ERF1	4	7	7	–	5
Q7XAU6	VIT_05s0094g00340	Chitinase class IV	5	3	3	5	6
F6GY46	VIT_18s0072g00380	Abscisic stress ripening protein 2 (ASR2)	6	4	5	4	3
Q9M4H7	VIT_12s0059g00590	Allergenic protein Pt2L4	7	10	10	9	8
D7T852	VIT_19s0090g01340	Putative uncharacterized protein	8	–	9	–	–
D7T853	VIT_19s0090g01350	Aspartyl protease	9	–	–	–	–
F6GU22	VIT_06s0004g02560	Kiwellin Ripening-related protein grip22	10	–	–	–	–
F6HUH1	VIT_02s0025g04330	Thaumatin VVTL1 [Vitis vinifera]	–	5	4	3	4
Q9M4H8	VIT_16s0022g00960	Invertase/pectin methylesterase inhibitor	–	8	6	–	–
F6GV13	VIT_06s0004g04650	Metallothionein	–	9	–	–	–
F6HUG9	VIT_02s0025g04310	Thaumatin	–	–	8	–	–
F6GXX3	VIT_08s0058g01230	Non-specific lipid-transfer protein	–	–	–	6	10
D7TAI4	VIT_01s0010g02030	Gamma-thionin precursor	–	–	–	8	–
F6HMP0[c]	VIT_08s0056g01600	Putative uncharacterized protein	–	–	–	10	9
D7T2C8	VIT_05s0094g00350	Chitinase class IV	–	–	–	–	7

[a] Annotation by Grimplet et al. (2012)
[b] CS=Cabernet Sauvignon; ME=Merlot; PN=Pinot Noir; CD=Chardonnay; SM=Semillon
[c] Not identified in protein data set.

Table 5. *Statistically significant results from each Omics dataset adjusted for multiple testing using FDR (0.05).*

Dataset	Treatment	Cultivar	Cultivar x Treatment
Proteins	0	832	0
Transcripts			
Microarray	195	27,064	1,546
RNAseq	1	15,149	241
Metabolites			
GC-MS	0	63	0
LC-MS	0	40	0

Table 6. *Probesets (1 to 4) with potential for cross-hybridization. Pearson's correlation of transcripts annotated for cross-hybridization potential. Affected transcript counts for all transcripts and the subset paired with protein data.*

Probe count[a]	Coefficients[b]	Number of transcripts	Paired-to-protein
0	0.93	15,945	830
1	0.91	3,280	177
2	0.83	2,061	101
3	0.69	2,036	63
4	0.51	3,746	30

[a] Flagged transcripts from Cramer *et al.* 2014.
[b] Correlation between RNAseq & microarray.

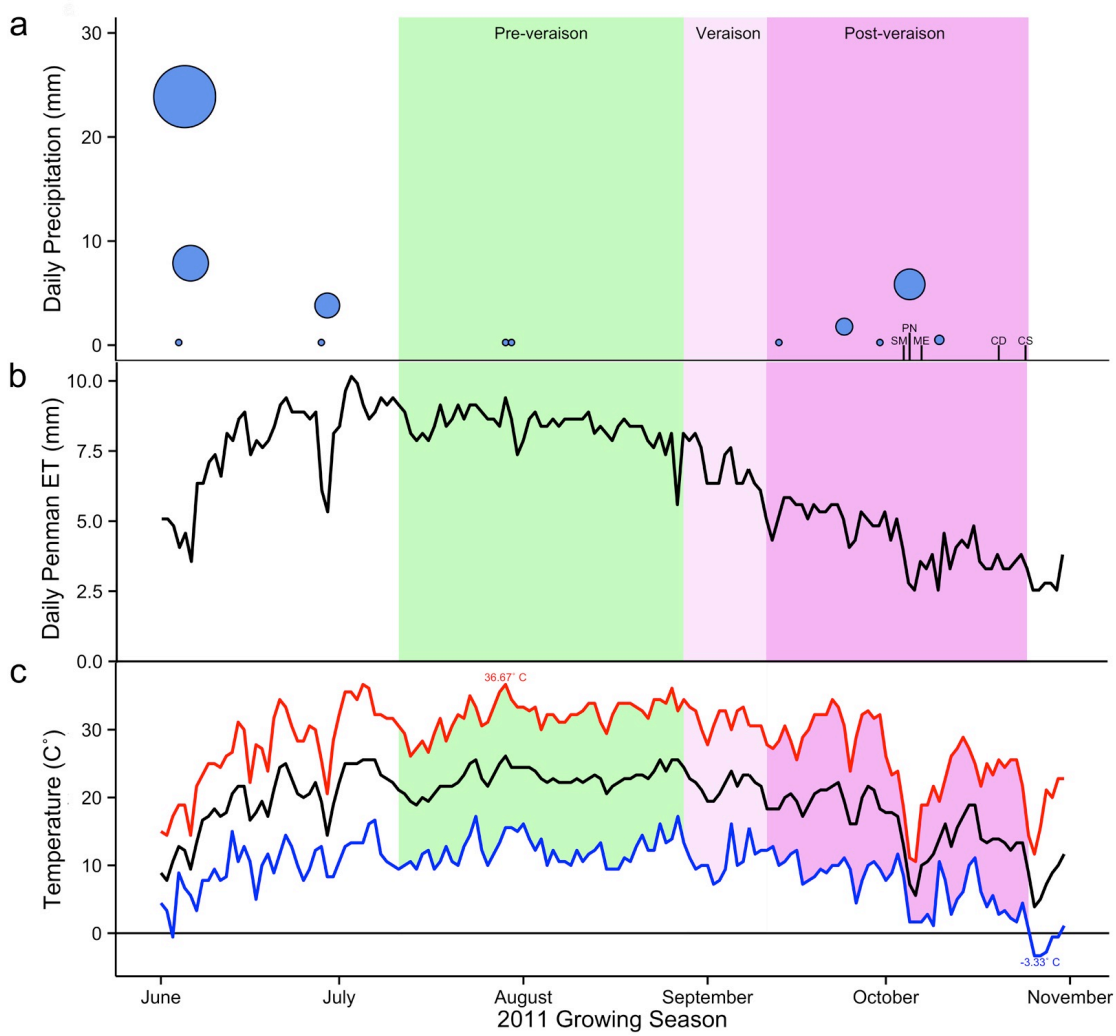

Figure 1. Seasonal precipitation and temperature at the Nevada Agricultural Experiment Station Valley Road Vineyard were collected from the Desert Research Institute's weather station. The double sigmoidal phases of berry development are highlighted: Pre-veraison in green refers to fruit set and enlargement before color change; Veraison in pink refers to the transition in color of berries; and Post-veraison in purple refers to full color change and heightened sugar and decreased organic acid levels until harvest. Harvest time points in October 2011 are denoted by cultivar abbreviations in their respective order of harvest: Semillon (SM), Pinot Noir (PN), Merlot (ME), Chardonnay (CD), and Cabernet Sauvignon (CS). (a) Daily precipitation (mm) values are illustrated by blue circles, scaled to the amount of precipitation on a given day. (b) The daily total Penman evapotranspiration (mm) values were based on the 82 Kimberly-Penman equation. (c) The daily high (red), low (blue) and mean (black) temperatures and the extreme high (36.7 ˚C) and low (-3.33 ˚C) are indicated.

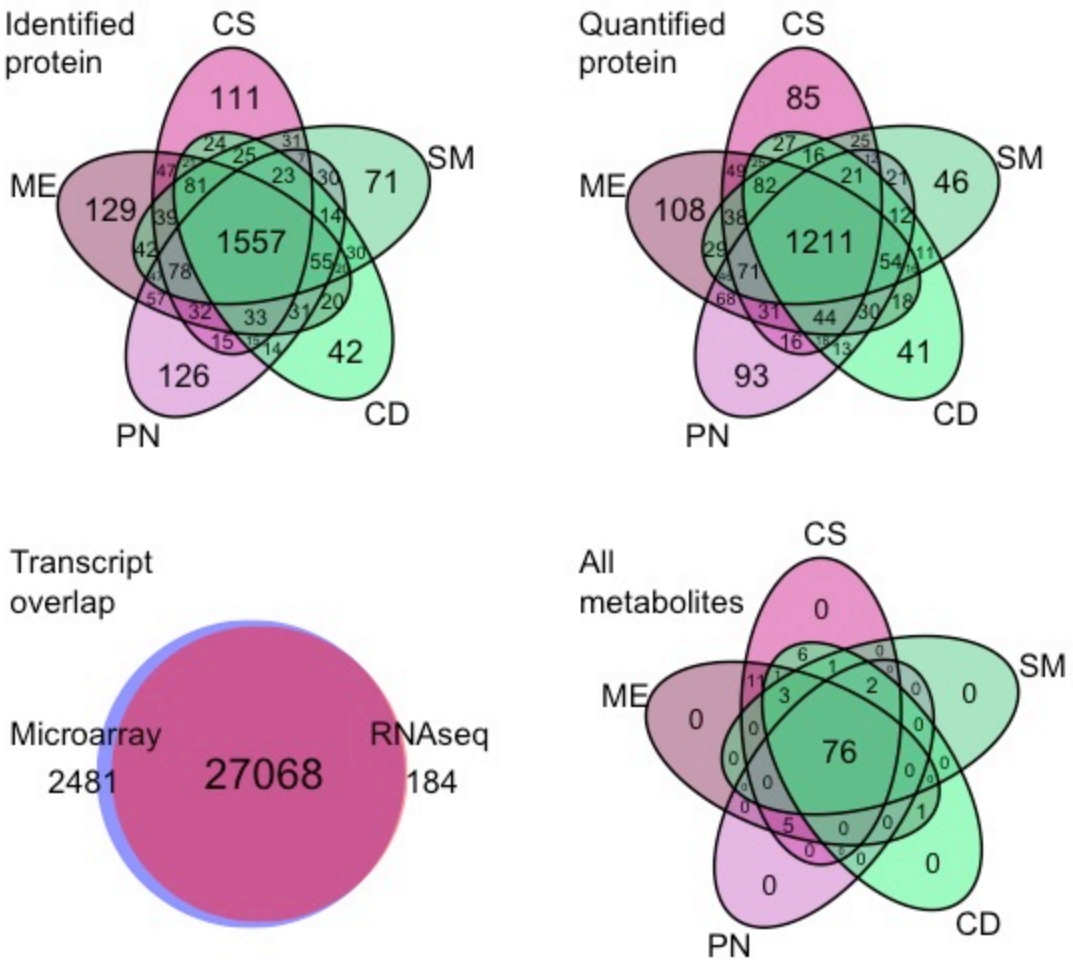

Figure 2. Venn diagrams of the (a) identified and (b) quantified proteins, the overlap of (c) transcripts assessed with either platform, and (d) all the metabolites measured in each cultivar, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

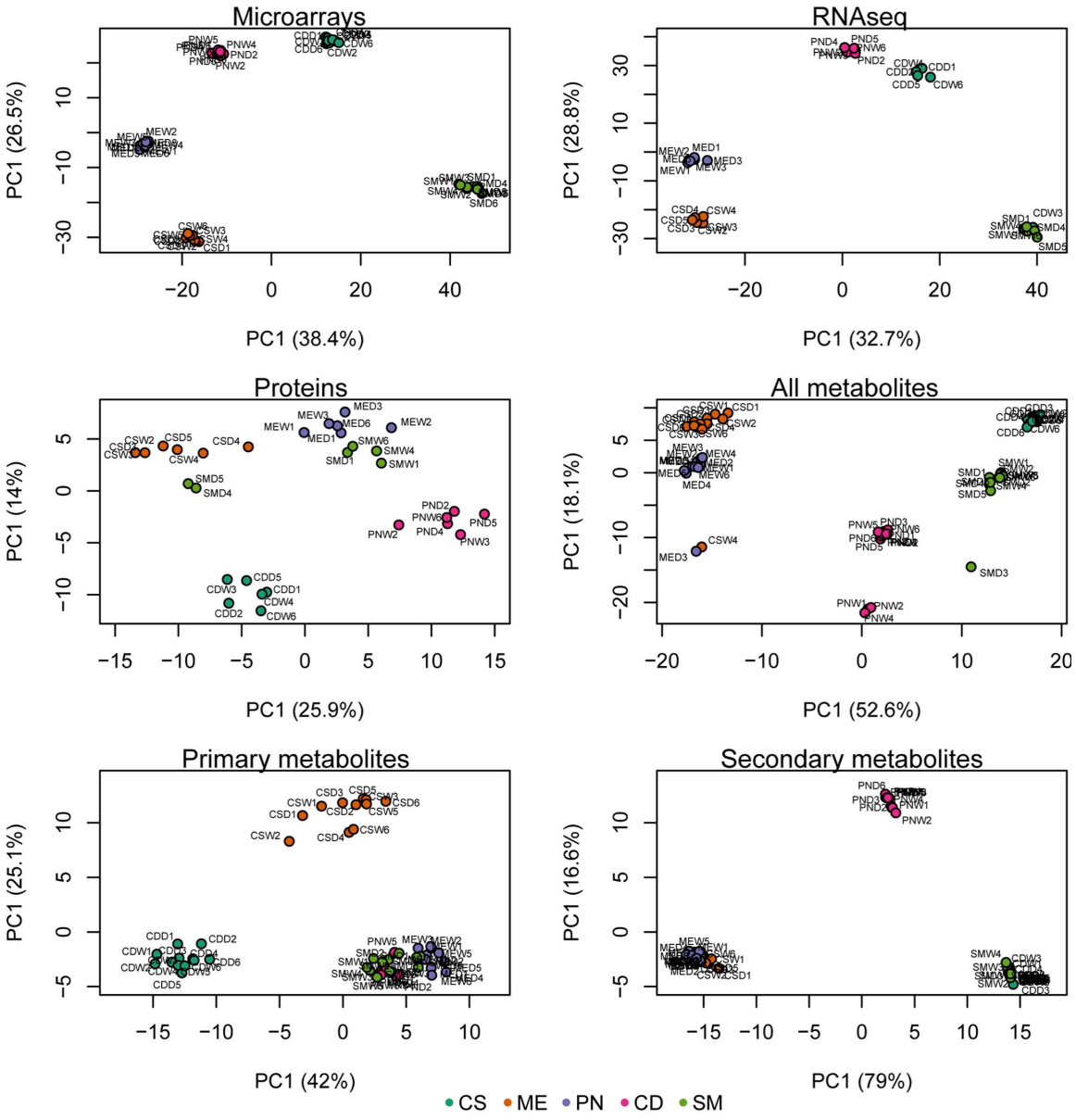

Figure 3. Principal components analysis of each Omic platform. Biological replicates are labeled and colored consistently in each platform, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

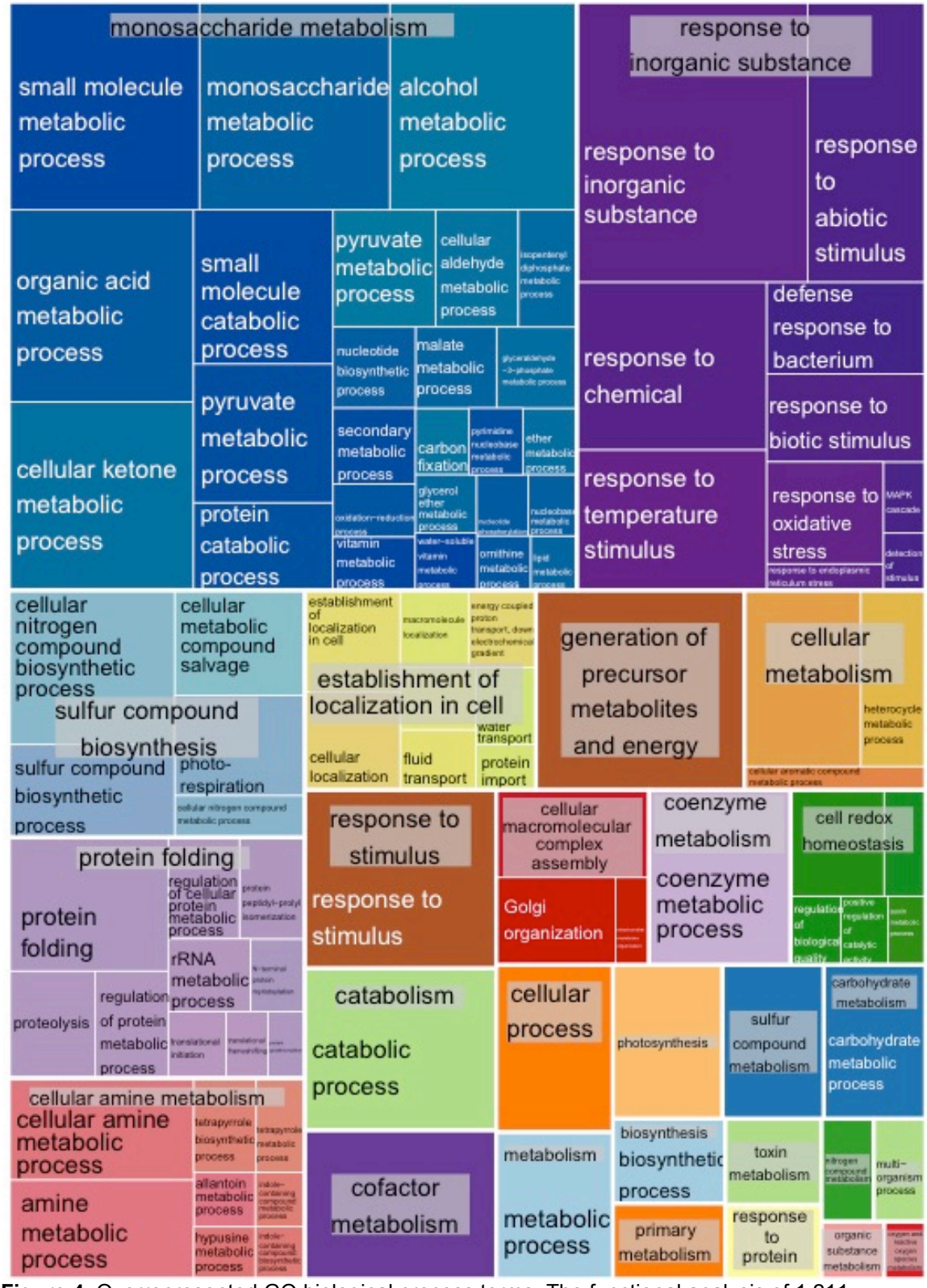

Figure 4. Overrepresented GO biological process terms. The functional analysis of 1,211 quantifiable proteins visualizes related terms by color, and rectangles were size adjusted to reflect their enriched p-value.

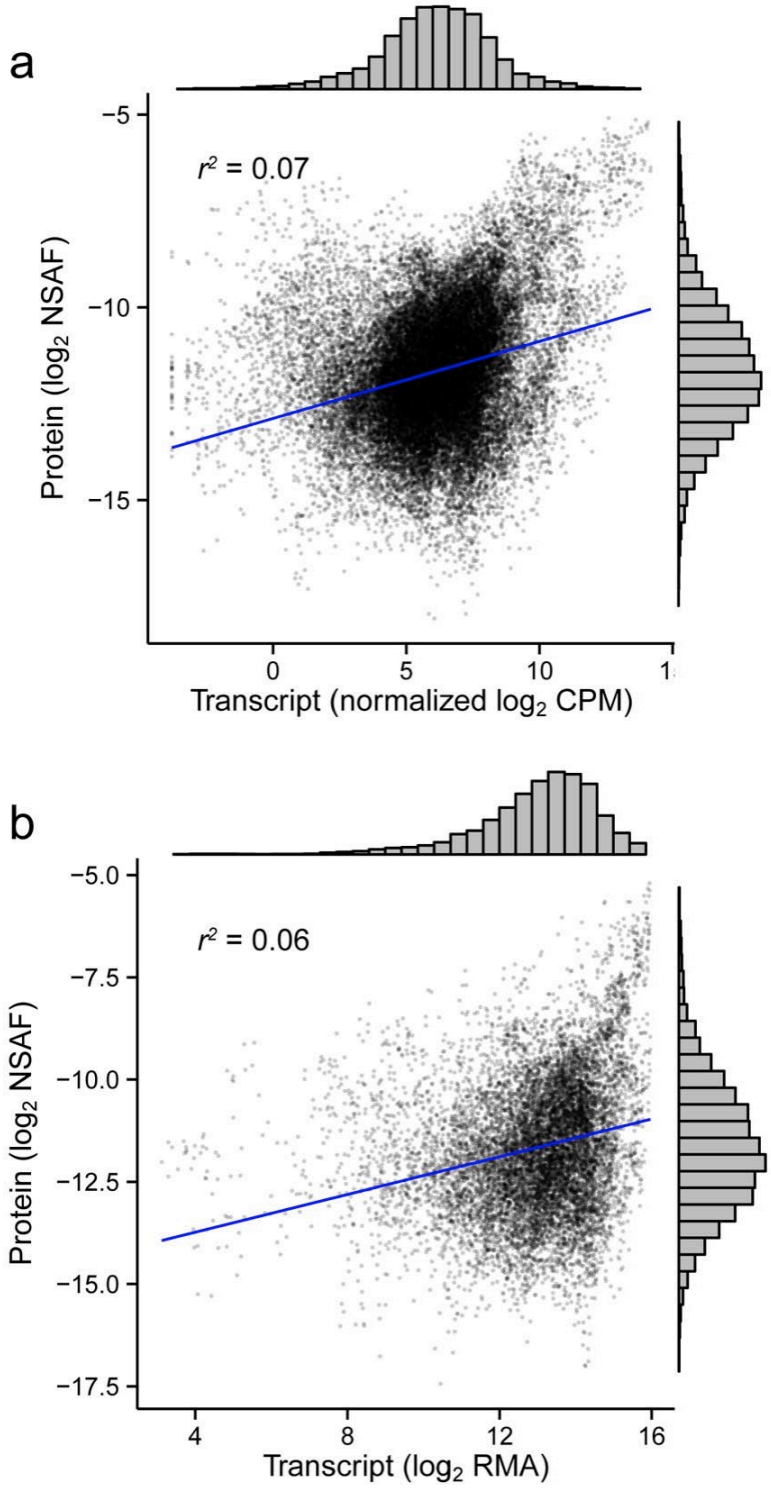

Figure 5. Correlations between log2 transform of the normalized protein and transcript abundance of five grapevine cultivars. The correlation between 1,201 transcript-protein pair abundance levels from either (a) RNAseq or (b) microarray analyses.

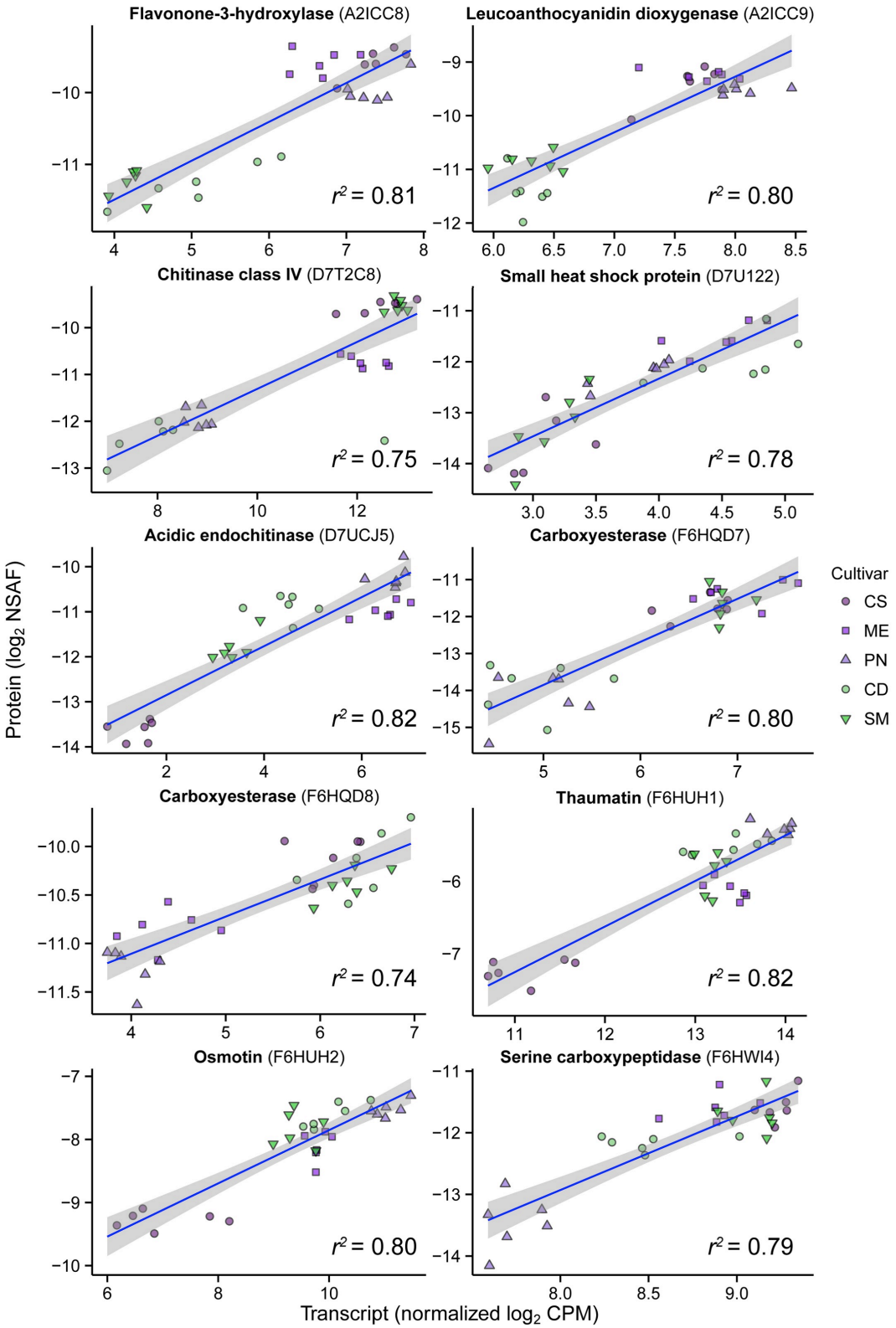

Figure 6. Individual correlations between ten of the highest correlated protein-transcript pairs. Linear regressions and Pearson's correlation of RNAseq and protein data sets were direct sample-to-sample comparisons.

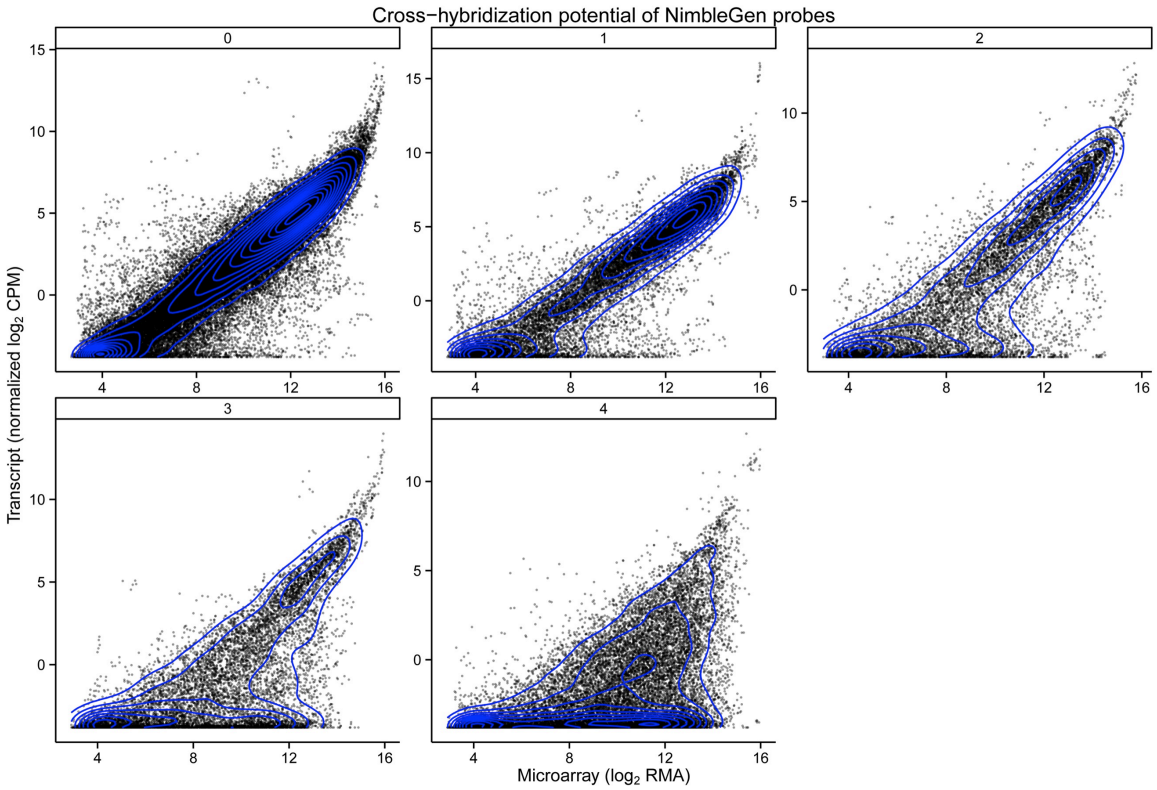

Figure 7. Pairwise platform comparisons of measured transcripts. Transcripts are subset by the number of probes (0, 1, 2, 3 or 4) with cross-hybridization potential on NimbleGen microarrays. Transcript expression values are the average treatment and cultivar expression level due to unequal biological replicates between platforms, n=3 for RNAseq and n=5 for microarrays.

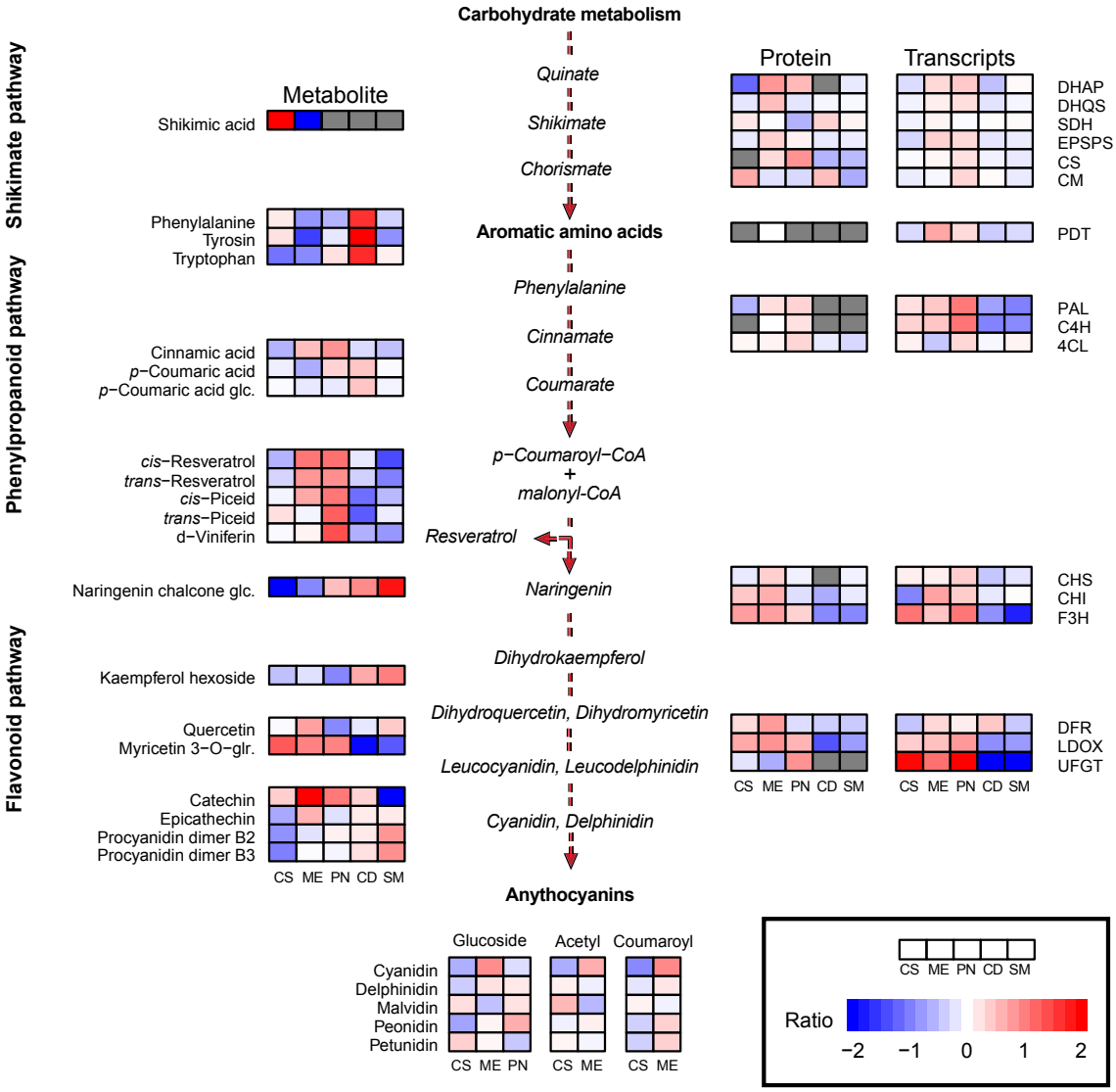

Figure 8. A simplified phenylpropanoid pathway from carbohydrates to anthocyanins in three Omic data sets. Enzymes and transcripts are given as EC numbers: 3-deoxy-7-phosphoheptulonate synthase (DHAP, 2.5.1.54), 3-dehydroquinate synthase (DHQS, 4.2.3.4), shikimate dehydrogenase (SDH, 1.1.1.25), 3-phosphoshikimate 1-carboxyvinyltransferase (EPSP, 2.5.1.19), chorismate synthase (CS, 4.2.3.5), chorismate mutase (CM, 5.4.99.5), prephenate dehydratase (PDT, 4.2.1.91), phenylalanine ammonia-lyase (PAL, 4.3.1.2.4), trans-cinnamate 4-monooxyygenase (C4H, 1.14.13.11), 4-coumarate-CoA ligase (4CL, 6.2.1.12), chalcone synthase (CHS, 2.3.1.74), chalcone isomerase (CHI, 5.5.1.6), flavanone 3-hydroxylase (F3H, 1.14.11.9), dihydroflavonol 4-reductase (DFR1.1.1.219), leucoanthocyanidin dioxygenase (LDOX, 1.14.11.19), and UDP glucose:flavonoid 3-O-glucosyltransferase (UFGT, 2.4.1.115). Cultivar order is from left to right: Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM). Abundance ratios are of the cultivar average relative to the average of all cultivars. Only transcripts (RNAseq) paired to proteins are shown. The five anthocyanidins measured are organized into rows (anthocyanidin) and columns (glycosylated, acetylated and coumaroylated moieties). Results were derived from biological replicates (n=3 for proteins, n=3 for transcripts, and n=6 for metabolites). Proteins and metabolites absent for a specific cultivar are colored grey.

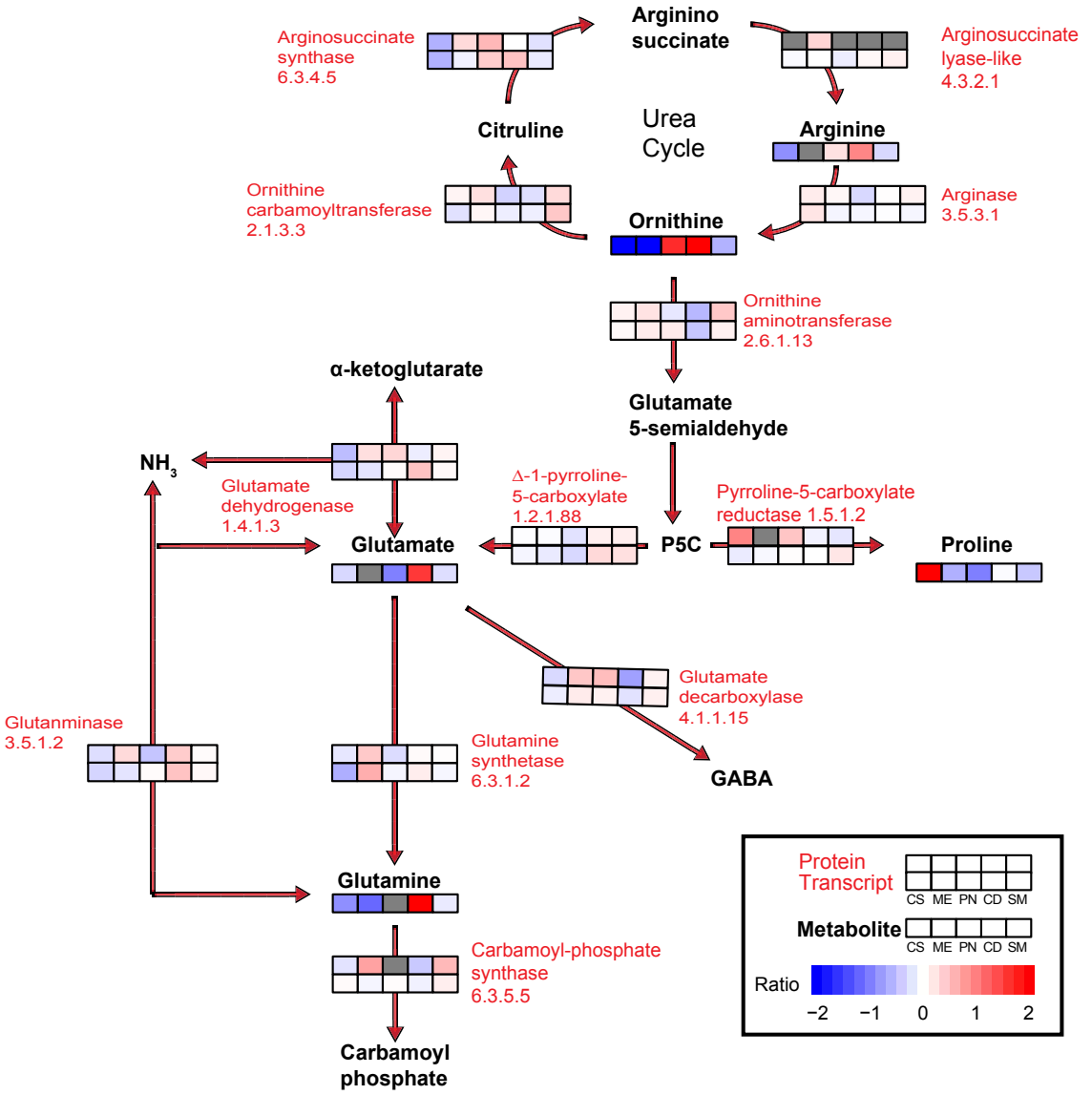

Figure 9. Comparative analysis of three Omic data sets related to amino acid metabolism. Enzymes and transcripts are given as EC numbers: ornithine carbamoyltransferase (2.1.3.3), argininosuccinate synthase (6.3.4.5), argininosuccinate lyase (4.3.2.1), arginase (3.5.3.1), ornithine aminotransferase (2.6.1.13), pyrroline-5-carboxylate reductase (1.5.1.2), L-glutamate gamma-semialdehyde dehydrogenase (1.2.1.88), glutamine synthetase (6.3.1.2), glutamate dehydrogenase (1.4.1.3), glutaminase (3.5.1.2), carbamoyl-phosphate synthase (glutamine-hydrolyzing) (6.3.5.5), and glutamate decarboxylase (4.1.1.15). Abbreviated products and intermediaries: γ-aminobutyic acid (GABA) and 1-pyrroline-5-carboxylate (P5C). Abundance ratios are of the cultivar average relative to the average of all cultivars. Only transcripts (RNAseq) paired to proteins are shown. Cultivar order is from left to right Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM). Results were derived from biological replicates (n=3 for proteins, n=3 for transcripts, and n=6 for metabolites). Proteins and metabolites absent for a specific cultivar are colored grey.

CHAPTER 3:

ELUCIDATION OF A CORE SET OF GRAPE (*VITIS VINIFERA* L.) GENES DIFFERENTIALLY EXPRESSED IN THE LATE STAGES OF BERRY RIPENING

This Chapter is based on a manuscript that is currently being prepared for submission to *Journal of Experimental Botany*

Ghan, R., Tillett, R., Schlauch, K.A., Fait, A., Cramer, G.R. Elucidation of a Core Set of Grape (*Vitis vinifera* L.) Genes Differentially Expressed in the Late Stages of Berry Ripening. *Journal of Experimental Botany,* In Preparation, (2015).

3.1 Introduction

The fruit of flowering angiosperms are specialized organs for seed dispersal. Fleshy fruits sufficiently ripened are financially important agricultural commodities, representing active areas for scientific research and discovery. Fruits are also beneficial for the many bioactive compounds, like polyphenols and carotenoids that signal via bright colors their health related benefits from ingestion (Jimenez-Garcia, Guevara-Gonzalez et al. 2013).

Ripening in fleshy fruits involves complex metabolic interactions that coordinate physical and molecular changes within plant tissues, including induction of color (Jaakola 2013, Jimenez-Garcia, Guevara-Gonzalez et al. 2013), softening of fruit tissues (Carreño, Cabezas et al. 2014, Moore, Fangel et al. 2014), evolution of volatile compounds (Kalua and Boss 2009, Nieuwenhuizen, Chen et al. 2015), and increases in soluble sugars. The culmination of these physiological and biochemical processes at maturity or peak ripeness produces attractive targets for human, avian and other vectors of seed dispersal. Thus, fruit ripening serves an evolutionary programmed effort for survival and palatability.

The translocation and accumulation of sucrose within fruit is an easily assessable metric for ripeness besides color change. Sugars can transcriptionally regulate gene activity (Bläsing, Gibon et al. 2005, Cordoba, Aceves-Zamudio et al. 2015), which can allow for fine-tuned regulation of metabolism with changing sugar levels (Conde, Silva et al. 2007). Furthermore,

carbohydrate-mediated control coupled with light exposure can effectively suppress the translation of mRNA (Rook, Gerrits et al. 1998).

Much has been written about climacteric and non-climacteric fruit and their hormonal responses during ripening. Climacteric fruit have classically been defined by a respiratory CO_2 burst that precedes a rise in ethylene and the onset of ripening (Gapper, McQuinn et al. 2013). Studies in mutant tomatoes have revealed numerous ripening related genes like *rin* (*ripening inhibitor*) (Lincoln and Fischer 1988) and *cnr* (*colorless nonripening*) (Martel, Vrebalov et al. 2011). More recently, a look at the tomato methylome showed epigenetic control over ripening that was tissue and developmental specific (Zhong, Fei et al. 2013). O

Other hormonal interactions control different aspects of ripening. At veraison (color change) in grape, levels of auxin, an inhibitor of ripening (Davies, Boss et al. 1997), have been reported as low prior to the accumulation of sugars (Coombe and Hale 1973). The application of synthetic auxins causes delays in ripening in grape that result in retarded accumulations of sugars, anthocyanins and altered gene expression of ripening associated transcripts (Davies, Boss et al. 1997, Bottcher, Boss et al. 2011, Böttcher, Boss et al. 2012).

In the present study, a transcriptional analysis investigated the commonalities between four red-skinned and three white-skinned cultivars: Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, Sauvignon Blanc and Semillon, respectively. Cabernet Sauvignon skin and pulp tissues were previously investigated over a range of ˚Brix levels (between 22 and 37 ˚Brix) revealing significant induction of genes associated with ethylene

signaling and flavor pathways in the skin (Cramer, Ghan et al. 2014). To focus in on specific markers or targets when transcriptional regulators are activated in grape a narrower set of °Brix levels were selected for observation. Near optimum °Brix levels of grape ripeness (Heymann, LiCalzi et al. 2013) were selected that both precede and follow optimum ripeness. Because they are the primary source of aroma, flavor and color in the fruit, we examined transcriptional changes in mature berry skins to identify potential markers that affect fruit and ultimately wine quality. We aimed to broadly assess the commonalities of transcription in grapes cultivars, and to describe novel observations. Sequencing of mature grape berry fruit at increasing concentrations of soluble sugars yielded various candidates for future exploration for ripening-related transcriptional markers.

3.2 Materials and Methods

3.2.1 Plant materials

Vitis vinifera L. cultivars Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, Sauvignon Blanc and Semillon were grown at the University of Nevada, Reno's Experimental Vineyard. Each cultivar was surveyed over the course of several weeks in September and October 2012, depending upon the berry maturity of each cultivar. Berry maturity was assessed by measuring soluble sugars (˚Brix) with a digital refractometer (HI 96811, Hanna Instruments, Woonsocket, RI, USA) zeroed with deionized water before each measurement. Berry clusters were collected between 11.00 h and 13.00 h to minimize the temporal response patterns related to circadian regulated transcription. Pretesting sugar (˚Brix) levels for determining the day of sampling

of berries was done on separate days. On the day of sampling, whole clusters were removed and individual berries from the entire cluster were squeezed to measure the individual berry °Brix level on a digital refractometer. Based on the reading, berry skins were separated and place into 50 mL centrifuge tubes in liquid nitrogen. The tubes were marked in 1 ± 0.5 ˚Brix level increments from 19 to 27 ˚Brix. Frozen skins were ground to a fine powder using a RETCH-mill (Retsch MM301, Newtown, PA, USA) with pre-chilled steel holders and grinding beads. Sometimes berries varied as much °8 Brix on a single cluster. In this way berries were collected over many days from multiple clusters from multiple vines from 3 different individually irrigated blocks in the vineyard. Each block was considered an experimental replicate.

3.2.2 RNA extraction

Three experimental replicates from each cultivar at 20, 22, 24 and 26 ˚Brix were used for sequencing. Total RNA was extracted from ~250 mg of finely ground skin tissue using a modified CTAB extraction protocol based on (Chang, Puryear et al. 1993, Jaakola, Pirttila et al. 2001, Tattersall, Ergul et al. 2005, Gambino, Perrone et al. 2008) followed by an additional on column DNase digestion using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA quality and quantity were assessed with a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA LabChip assays (Agilent Technologies, Santa Clara, CA, USA).

3.2.3 RNAseq library preparation and sequencing

Eighty-four 50 bp single-end, bar-coded libraries were constructed and sequenced by the Neuroscience Genomics Core at the University of California, Los Angeles (Los Angeles, CA, USA) using Illumina TruSeq RNA library prep kits (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The libraries were pooled and multiplexed, using Illumina TruSeq chemistry (version 3.0) and a HiSeq2000 sequencer (Illumina Inc., San Diego, CA, USA). Due to multiplexing, individual experimental replicates were thus sequenced on each of the four flow-cells to reduce technical variation.

3.2.4 Gene expression analysis

Reads were quality filtered with the NGS QC Toolkit (Patel and Jain 2012), and demultiplexed. The TopHat2 (version 2.0.10) splice alignment software package (Kim, Pertea et al. 2013) was used with data from the PN40024 *Vitis vinifera* reference genome and annotation obtained at plants.ensembl.org to align the quality filtered reads, with the "--b2-very-sensitive" and "--transcriptome-index" options. Approximately 93% of reads from all libraries were mapped. Samtools (Li, Handsaker et al. 2009) and HTSeq (Anders, Pyl et al. 2015) were used to generate a feature count from BAM alignment files. Using the "union" mode, HTSeq was run using the "union" mode, with the "-i gene_id -t exon -s no" options. Count filtering, normalization and differential expression analysis were performed with edgeR (3.8.6) (Robinson, McCarthy et al. 2010). Genes with zero counts for all sample libraries were removed, and genes with less than one count per million in three experimental replicates of the sample set were likewise filtered before normalization. Counts

for each gene were fit to negative binomial generalized log-linear models. A design model that defined each ˚Brix-cultivar combination as an element of a group (~ 0 + Group) was used to test for differential expression using simple contrasts between subgroups of interest. Statistically significant transcript abundance changes were found below the adjusted p-value (false discovery rate) of 0.05 (herein referred to as “significant” throughout this paper). Benjamini and Hochberg’s procedure was used to control the false discovery rate (Benjamini and Hochberg 1995). Adjusted log-counts-per-million were computed with the cpm() function in edgeR for data visualization and clustering. RNAseq data were deposited with the Sequence Read Archive database at NCBI with BioProject identifier PRJNA260535 (Leinonen, Sugawara et al. 2011).

3.2.5 Gene and transcription factor family annotation

Transcription factors annotated in this study were individually identified by Cramer and Grimplet (Cramer, unpublished results; (Grimplet, Van Hemert et al. 2012) by BLAST against the gene models currently annotated in the V1 version of the *Vitis vinifera* reference genome using known transcription factor domains from three plant transcription factor databases: PlnTFDB v3.0 (Perez-Rodriguez, Riano-Pachon et al. 2010), PlantTFDB 3.0 (Jin, Zhang et al. 2014) and iTAK *(bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi*). The individual annotations from Cramer and Grimplet were compared and combined for this study. The annotated gene names were updated in June of 2015 to follow the International Grape Genome Program guidelines (Grimplet et al 2014 reference). If a *Vitis* gene had an *Arabidopsis* ortholog that was identified in Gramene.org, the *Vitis*

name was given the ortholog name or symbol used for Arabidopsis. This facilitated functional and comparative analyses of the genes.

3.3.6 Functional enrichment of GO (Gene Ontology) categories

Using GO categories that were assigned to the *Vitis vinifera* V1 genes from plants.ensemble.org in June, 2015, functional category enrichment of biological processes was determined with the BinGO plugin application (version 3.0.2) (Maere, Heymans et al. 2005) in Cytoscape (version 3.2.1) (Shannon, Markiel et al. 2003). Gene ontology membership classifies function hierarchically from broad to specific. Multiple testing correction adjusted p-values were determined using the Benjamini & Hochberg False Discovery Rate at a 0.05 threshold.

3.3.7 Soft clustering of transcription factors

Clusters were formed from standardized significant transcription factor expression results using fuzzy c-means with the Mfuzz package (2.28.0) (Futschik and Carlisle 2005, Gillespie, Lei et al. 2010) using the R (3.2.1) statistical and graphic software (R Core Team 2015). The $\log_2$-transformed CPM values were standardized across each transcription factor so that the standardized values of each transcription factor had mean equal to zero and standard deviation equal to one. The fuzzy c-means algorithm can potentially cluster the same gene into multiple clusters, with similar profiles. A membership threshold of 0.2 was applied so each transcription factor remained in only one cluster.

3.3 Results

Over several weeks in September and October of 2012, whole berry clusters were harvested from seven grape cultivars: Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, Sauvignon Blanc and Semillon. Individual berries were measured with a digital refractometer to separate berry skins by their sugar (˚Brix) levels (e.g. 20, 22, 24 and 26 ˚Brix) from both seed and pulp. Total RNA was then extracted with a modified CTAB protocol and checked for RNA integrity (see methods). The construction and sequencing of single-end, 50 bp reads was conducted on an Illumina HiSeq2000. RNAseq based profiling of major changes in the abundance of berry skin transcripts during the late stages of development was then conducted using the PN40024 reference genome for read alignment (Jaillon, Aury et al. 2007). Genes with zero counts for all sample libraries were removed, and genes with less than one count per million in three experimental replicates of the sample set were likewise filtered before normalization. There were 27,926 expressed genes out of 29,971 annoted genes in the grape 12x V1 genome (Jaillon, Aury et al. 2007). Filtering of lowly expressed genes by minimum counts per million resulted in 19,056 genes for analysis. Our investigation focused upon common changes in transcript abundance amongst the seven grape cultivars in response to different °Brix levels.

3.3.1 Sugar content explains variance in PCA

A principal components analysis was performed (Fig. 1) to validate sample uniformity and investigate the degree of separation between cultivar and ˚Brix effects. Cultivars were distinctly separated on the 1st principal component

explaining 28.2 % of the variance, with red and white cultivars separating together and away from one another. One exception, Pinot Noir was separated more closely with the white cultivars. ˚Brix levels were separated along the 2nd principal component explaining 22.1% of the variance, in some cases distinctly from one another (e.g. Merlot at 20 ˚Brix and Semillon & Chardonnay both at 26 ˚Brix). The degree of separation between ˚Brix levels in the PCA may reflect the degree of transcriptional differences between time points. Perhaps transcriptional changes are discretely different after 20 ˚Brix in the case of Merlot.

3.3.2 Differential expression of increasing ˚Brix levels

Differential expression analysis was performed with edgeR (Robinson, McCarthy et al. 2010). A design model that defined each ˚Brix-cultivar combination as an element of a group was used to test for differential expression. Simple contrasts were used between groups to compute significantly changing transcripts below the adjusted p-value (false discovery rate) of 0.05 (herein referred to as “significant” throughout this paper) (Tables 2 and S3). The most differentially expressed genes observed were related to the 26 vs. 24 ˚Brix comparison (5,801), followed by 22 vs. 20 ˚Brix (3,008) and 24 vs. 22 ˚Brix (2,643). Significantly changing transcripts between ˚Brix levels accounted for 8,238 overlapping transcripts between the seven cultivars.

3.3.3 Gene set enrichment analysis

A functional analysis (Table S4) was performed to identify overrepresented (enriched) GO categories for the significant transcripts during late berry ripening. This analysis focused on common transcriptional changes

with °Brix for all cultivars. Over 500 GO categories were overrepresented after correcting for FDR (adjusted p-value of 0.05). This result indicates that grape berry ripening is complex. Some of the many overrepresented GO categories included chromosome & histone modification, transcriptional regulation, postembryonic development, fruit development, and light. Categories that influence flavor development were also prevalent in the overrepresentation. Isoprenoid metabolism was one example of a volatile and sensory-related GO term, with 130 associated genes. The remaining results attempt to highlight some of the important transcriptional relationships in late berry ripening.

3.3.4 Transcription factors changing with ˚Brix

Transcription factors (TF) that were significantly changing with increasing ˚Brix were further investigated for potential developmental regulators. There were 809 TFs significantly changing with increasing ˚Brix or ~4% of all expressed genes (Table 3). TFs changing with ˚Brix also represented ~32% of all annotated TFs in the reference genome, from 81 different families/domains. The C3H family contained the most differentially expressed TFs (106), followed by MYB (64), bHLH (49), AP2/ERF (43), and PHD (30) families.

To identify gene expression profiles, the TFs were clustered using a fuzzy c-means approach (Futschik and Carlisle 2005) and a membership threshold was applied so each TF remained in only one cluster. The intent of clustering was to identify groups of co-expressed and possibly co-regulated genes under increasing levels of sugar. Clustering resulted in eight main clusters. A subset

(26) of transcripts did not follow the general profiles and were further clustered with the fuzzy c-means algorithm into three subclusters.

The eight TF clusters (Fig. 2A) mostly followed two general profiles of expression with increasing ˚Brix, upward or downward. The upward trending transcripts included clusters 1, 3, 4, 7, and 8, and were predominantly associated with C3H, MYB, bHLH, PHD and AP2/ERF families. Downward trending transcripts included 2, 5, and 6, and were associated with CH3, MYB, AP2/ERF, bHLH and WRKY families. The three subclusters (Fig. 2B) had less defined profiles but were comprised of similar families of TFs: AP2/ERF, bHLH, C2H2 and MYB. The diversity of TF profiles, with diverse function as positive and negative regulators indicated that berries were very active transcriptionally during the late stages of berry development.

3.3.5 Post-embryonic development

Post-embryonic development (GO:0009791) was a surprisingly enriched category. Why would embryonic genes be changing in ripe berry skins? One hypothesis is that seed-to-skin signaling might indicate seed ripeness and its readiness for distribution. For example, a DUF642 class protein associated with cell wall proteomes has been classified as a marker for viable seeds (Jamet, Canut et al. 2006, Garza-Caligaris, Avendano-Vazquez et al. 2012). However, the grape ortholog (VIT_13s0064g00460) was markedly decreasing in all cultivars with increasing ˚Brix.

In *Arabidopsis,* the *AtBRIZ1* gene (AT2G42160) forms a hetero-oligomer with *AtBRIZ2* that helps form an ubiquitin E3 ligase complex required for normal

seed germination and the initiation of post-germination development (Hsia and Callis 2010). The grape ortholog of *AtBRIZ1*, A zinc finger/BRCA1-associated protein, *VviBRIZ1* (VIT_11s0016g05600) significantly increased with ˚Brix. *VviBRIZ1* and two other zinc finger TFs (VIT_12s0028g03300 and VIT_00s0125g00250 the VviBRIZ2 ortholog) were also associated with seed germination (GO: 0010029).

Down-regulated transcripts involved with seed embryogenesis or the activity of repressors could be one clue. For example, the major regulators of seed embryogenesis and maturation are ABA INSENSITIVE 3 (*ABI3*), LEAFY COTYLEDON 1 & 2, and FUSCA3, which contain B3 DNA binding domains (Kroj, Savino et al. 2003, To, Valon et al. 2006). These transcriptional regulators had zero-counts in all or most of the cultivar replicates that made comparisons unclear. In grape, *VviABI3* peaks in expression prior to the onset of ripening (veraison) (Deluc, Grimplet et al. 2007). Interestingly, a splicing factor, SUPRESSOR OF ABI3-5 (*SUA*), induces an alternative spliced *ABI3-β* only during late seed maturation (Sugliani, Brambilla et al. 2010, Roscoe, Guilleminot et al. 2015). *VviSUA* (VIT_02s0012g00870) had increasing expression profiles with increasing sugar, although none significantly different in the three contrasts.

3.3.6 Light: response, radiation & photosynthesis

The transcript abundance of most of the core components of the circadian clock characterized in *Arabidopsis* (Harmer 2009), highly conserved in plant and animal species (Panda, Hogenesch et al. 2002), and presumed to be functional in grape, had common responses to °Brix in all cultivars (Figs. 3 & S1). These

included orthologs of *AtLHY, AtPRR5, AtELF3, AtELF4, AtRVE1* and others that repress or alleviate clock components in a complex interplay with the 24-hour photoperiod (Hsu and Harmer 2014). The plant clock effectively self-regulates in a transcriptional feedback loop in *Arabidopsis*. Interestingly, *VviTOC1*, normally repressed by a complex of *CCA1*, *LHY*, *DET1*, *COP1* and *DDB1* during the day (Lau, Huang et al. 2011, Johansson and Staiger 2015), and *VviLHY* are not under oscillatory control in grape (Carbonell-Bejerano, Rodriguez et al. 2014). .

Berry composition is inextricably linked with sunlight and day length, being the source for energy and sugar production as well as affecting circadian clock regulation. Day length decreased with advanced berry ripening and the onset of autumn. Many blue light responsive genes, including *ZTL* and *XAP5* (XCT), had common responses to °Brix level. These genes are known to measure day length and adjust the circadian clock. Could some interaction between sugar concentration and photoperiod-related genes affect transcription in ripening berry? A constans-like 4 gene (*VviCOL4*, VIT_04s0008g07340), very similar to *VviCOL3*, was significantly upregulated with ˚Brix x Cultivar. Constans-like genes were first identified in flowering and are important sensors of day-length and light-driven redox signaling (Valverde 2011). CONSTANS-like 13 (VIT_07s0104g01360) belongs to Group III of CO-like TF (Griffiths, Dunford et al. 2003, Almada, Cabrera et al. 2009). Almada et al. (2009) reported both spatial and temporal expression patterns for the *VviCOL1*, with a reduction of expression in maturing berries, a pattern seen in all cultivars.

Photosynthesis related transcripts were also highly affected in late ripening fruit. The ripening berry is a sink organ for photosynthate, losing its photosynthetic capacity with time and changing color as chloroplasts are degraded or converted to other plastids with changing carotenoid production (Fanciullino, Bidel et al. 2014). In tomato, chloroplasts are converted to chromoplasts, which are the source of the red pigments. In grapes, it is not clear what the chloroplasts become as it is not well studied. The red-purple-blue pigments, anthocyanins, are generally stored in the vacuoles of the skin cells. Nearly all-photosynthetic transcripts were constitutively decreasing with ˚Brix, such as Cytochrome c6 (VIT_01s0011g01850) or light harvesting complex II type I CAB-1 (VIT_10s0003g02890). Some of these photosystem genes appear to be completely shutting down. Only two transcripts associated with photosynthesis were increasing in expression, such as a pentatricopeptide (PPR) repeat-containing protein (VIT_03s0063g00900) and ferritin (VIT_08s0058g00440). This indicates that chloroplasts were becoming non-functional for photosynthesis or possibly even degraded. Chloroplasts are also the location for isoprenoid, carotenoid and terpenoid metabolism, and thus a source of important volatiles and aromas.

3.3.7 Hormone & signaling response

Hormones & signaling responses tightly regulate developmental stages in grape and other fruit during ripening. Indeed, the initial formation of individual berries is itself a response from auxin-stimulated gibberellic acid (GA) synthesis within the skin and pulp (Sundberg and Ostergaard 2009). Specificity for genes

encoding GA oxidases is tissue-specific and likely regulated by hormone abundance (Giacomelli, Rota-Stabelli et al. 2013). Examination of GA oxidases were therefore unsurprisingly either decreasing with ˚Brix (VIT_16s0098g00860), low (VIT_05s0020g01310) or not expressed (VIT_19s0177g00020).

Auxin response factors (AFRs) specifically bind to auxin response elements found within the promoter region of auxin-inducible genes (Liu, Wu et al. 2014) and shown to be upregulated after veraison by Deluc *et al.* (2007). *VviIAA16*, an AUX/IAA-induced protein (VIT_14s0081g00010), was significantly decreasing and contains a bHLH-binding site for *VviCEB1* that was described in Nicolas *et al.* (2013). *VviIAA* genes are components in auxin signaling but the endogenous role of auxin during berry development remains fully unexplained (Davies and Böttcher 2009). However, it is known that higher concentrations of auxin delays veraison and sugar accumulation in Riesling and Shiraz (Bottcher, Boss et al. 2011, Böttcher, Boss et al. 2012), as well as altering the expression of ripening-related genes (Davies, Boss et al. 1997). Cellular expansion related to fruit ripening and the regulation of genes affecting expansion were shown to be affected by the overexpression of the fruit-specific bHLH TF, (VIT_01s0244g00010), mitigated hormonally only by auxin (Nicolas, Lecourieux et al. 2013). Under increasing concentrations of sugar, the bHLH, *VviCEB1*, was significantly decreased at 26 vs. 24 ˚Brix.

Transcripts related to ethylene included ethylene response factors, receptors, and regulators (Fig. 4). ACC synthase (VIT_15s0046g02220) limits the production of ethylene and was essentially not expressed in any cultivar but Pinot

Noir, with the other cultivars having low or zero counts. Synthesis of ethylene from 1-aminocyclopropane-1-caryboxylate by *ACO* (VIT_11s0016g02380) was less clear with differing profiles in the cultivars. The MADS-box TF RIN, in tomato, regulates *ACS* and *ACO* that lead to ethylene biosynthesis (Martel, Vrebalov et al. 2011). The RIN ortholog (VIT_01s0011g00110) belonged to cluster 5 of TFs and was progressively decreasing. *CTR1* (VIT_08s0007g03910), a serine/threonine protein kinase, is activated in the absence of ethylene by ethylene receptors (Kieber, Rothenberg et al. 1993). *CTR1* was elevated, peaking at 24 ˚Brix. The ethylene receptors, *VviETR1*, *VviETR2*, *VviEIN4*, were less clear. Ethylene-insensitive 3 (*EIN3*) TF (VIT_06s0009g01380) is another important promoter of ethylene signaling in *Arabidopsis*. *AtEIN3* activates ethylene target genes in the presence of ethylene (Merchante, Alonso et al. 2013). The transcript abundance of EIN3 was decreased with increasing °Brix. Furthermore, the transcript abundance of *XAP5* (VIT_03s0038g01810) was increased with increasing °Brix level. *XAP5* acts as an inhibitor of ethylene signaling downstream of *EIN3* (Ellison, Vandenbussche et al. 2011). A large number of ethylene responsive element binding factors (ERFs), were decreased with °Brix (e.g. *VviERF017*, VIT_11s0016g00660; *VviERF037*, VIT_11s0016g03350; and *VviERF021*, VIT_18s0001g05890). The overall response of most of these ethylene signaling genes indicate that the berry skins may becoming less sensitive to ethylene during late berry maturation.

Sugar, particularly sucrose, participates in a feedback loop with ABA, whereby each stimulates the production of the other (Jia, Chai et al. 2011). Three

isoforms of NCED, the rate-limiting enzyme of abscisic acid (ABA) biosynthesis, were persistently expressed in all cultivars and sugar levels (Fig. 5). Only *VviNCED2* expression had a similar profile in all cultivars, while *VviNCED1* and *VviNCED4* had varying patterns. Investigation of genes involved in ABA signaling and perception were not entirely clear (Fig. 6). ABA receptor *VviPYL1/RCAR12* appeared to be decreasing with sugar, whereas *VviPYL8/RCAR3* seemed to peak between 22 – 24 ˚Brix before decreasing. Two type-2 protein phosphatases (*VviPP2Cs*) (VIT_06s0004g05460, VIT_13s0019g02200), the key negative regulator of ABA signaling (Ma, Szostkiewicz et al. 2009, Umezawa, Sugiyama et al. 2009), were constitutively expressed. Though not significantly different, the *PP2Cs* did appear to have divergent profiles after 24 ˚Brix. *PP2Cs* inactivate further ABA signaling by dephosphorylating sucrose non-fermenting 1-related protein kinase 2 (*SnRK2*) whose action is inhibited in the presence of ABA (Park, Fung et al. 2009). *VviSnRK2.3* and *2.4* (VIT_12s0035g00310, VIT_07s0031g03210) both were significantly decreasing. Likewise, orthologs of several transcriptional regulators known to regulate ABA production decreased with increasing sugar accumulation. One, a homeobox-leucine zipper TF (VIT_15s0048g02870) is a target of ABA signaling, acting as positive (*PP2C* genes) and negative (*PYR/PYL* receptors) regulators that effectively down-regulate the ABA-sensitivity in Arabidopsis (Valdes, Overnas et al. 2012). The other is an ortholog of a tomato zinc finger protein 2 (*ZFP2*), which affects the regulation of ABA biosynthesis in fruit ripening by targeting the binding motifs of promoters, as well as accelerating ripening when down-regulated (Weng, Zhao et

al. 2015). *VviZFP2* (VIT_11s0052g01120) also was decreasing. It appears that ABA signaling was undergoing a downregulation by 26˚Brix level, indicated by the increasing *PP2Cs* and decreasing regulators and *SnRKs*.

3.3.8 ROS

A consequence of continued respiration & UV exposure upon the berry is the formation of highly reactive and damaging oxygen species (ROS) that accumulate at color change and softening (Pilati, Brazzale et al. 2014). The transcript abundance of many ROS-related genes was affected by ˚Brix level (Fig. 7). These included three catalases; two similar to the *Arabidopsis* (AT1G20630) CATALASE 1 (VIT_04s0044g00020, VIT_18s0122g01320) were significantly decreasing with ˚Brix, and another transcript (VIT_00s0698g00010) related to CATALASE 3 (AT1G20620) that was significantly upregulated. Both *AtCAT1* & *AtCAT3* peak at midday in WT *Arabidopsis* (Lai, Doherty et al. 2012). Four ascorbate peroxidases from at least eight that are annotated in grape were also significantly differentially expressed between ˚Brix levels. A thioredoxin reductase 2 (VIT_04s0044g01750) was among the oxidative stress and reactive oxygen species associated genes. mRNA abundance for this thioredoxin was progressively decreasing with ˚Brix. Thioredoxin has recently been characterized as a master regulator of the tricarboxylic acid cycle in mitochondria, chloroplasts and the associated citrate shunt pathway (Daloso, Muller et al. 2015), where interorganelle communication between the two are facilitated by regulatory mechanisms controlled at the level of the gene (Balmer, Vensel et al. 2004, Leister, Wang et al. 2011).

3.3.9 Chromatin organization and regulation of transcription

The transcript abundance of many genes related to chromatin silencing or chromosome organization that negatively regulate gene transcription was observed with increasing ˚Brix (Fig. 8**)**. These included histone methyltransferases and a number of sucrose non-fermentable 2 transcripts. SNF2 domain-containing proteins participate in epigenetic regulation of gene transcription to control development in plants and other organisms (Hu, Zhu et al. 2013). For example, ALTERED SEED GERMINATION 3 (AtASG3), a DNA helicase SNF2 domain-containing protein (VIT_15s0021g02180), significantly increased with ˚Brix, and Photoperiod Independent Early Flowering1 (*PIE1*) (VIT_08s0007g06370) also containing a SNF2 domain, was increasing. Similarly, a *VviDDM1* (decrease in DNA methylation) TF (VIT_04s0023g01610) also peaked at 26 ˚Brix. *DDM1* also belongs to the Lsh subfamily of SNF2 proteins (Knizewski, Ginalski et al. 2008, Hu, Zhu et al. 2013). *SUVH4* (SUPPRESSOR OF VARIEGATION 3-9 homolog 4) (VIT_14s0068g01090) a H3K9 methyltransferase that, like *DDM1*, is involved in DNA methylation and histone modification (Pikaard and Scheid 2013) was significantly decreasing with ˚Brix. Cytosines methylated at CHG motifs function as binding sites for *SUVH4* to modify H3K9. Another TF, methyl-CPG-binding Domain 9-like (*MBD9*) (VIT_14s0066g01450) was also significantly increasing.

With the above observations surrounding chromosomal rearrangement and chromatin modification, transcripts involved in DNA methylation of cytosines were also investigated and observed as active in late ripening berries. These

included domains rearranged methylases, *VviDRM2* (VIT_14s0066g01040) and *VviDRM3* (VIT_05s0020g00450), which are responsible for *de novo* methylation of cytosine residues (Cao and Jacobsen 2002, Cao, Aufsatz et al. 2003). Only *VviDRM3* was significantly changed with ˚Brix. Neither of the two *VviMET1*-like transcripts (VIT_07s0130g00390 and VIT_07s0130g00380), which primarily perform symmetric CG cytosine methylation (Kishimoto, Sakai et al. 2001), were significantly changing with ˚Brix. Likewise, the plant specific chromomethylase 3, *VviCMT3* (VIT_06s0004g01080) that maintains the CHH asymmetric methylation sites (Bartee, Malagnac et al. 2001) did not significantly change. The activity of transcripts overtime may suggest some maintenance of cytosine methylation in berries. The overall response indicates that chromatin is being remodeled, DNA is being methylated, and many genes are being silenced during late berry maturation.

3.4 Discussion

3.4.1 High-throughput profiling of the mature berry transcriptome

This study investigated grape berries sampled at four concentrations of total soluble sugars, 20, 22, 24 and 26 ˚Brix, during late ripening. Transcripts from the mature skins were profiled to examine subtle transcriptional changes that may influence different aspects of grape quality. In this way, 84 sequencing libraries from seven cultivars were investigated for common transcriptional responses to increasing concentrations of ˚Brix. Each ˚Brix level, serving as a developmental marker, was compared with the immediately previous time point. The presented results summarize some of the important transcriptional

relationships observed in late berry ripening that was either unique to this study or less well described in grape.

By sampling individual berries in a ‘time course’ of different soluble sugar levels, an alternative approach to capturing gene expression profiles was used to investigate sugar related transcripts. Besides confirming the uniformity between samples, PCA showed separation by sugar level. This observation was similar to other studies where developmental stages were separated by ˚Brix (Pilati, Perazzolli et al. 2007, Lijavetzky, Carbonell-Bejerano et al. 2012, Dai, Leon et al. 2013, Gouthu, O'Neil et al. 2014). Grape berries ripen throughout the cluster in an asynchronous manner, with a range of soluble sugar levels (Coombe 1992). The lack of uniformity in sugar concentration can range from 5 – 7 ˚Brix within a grape cluster (Pagay and Cheng 2010). Indeed, our own observations of ˚Brix lacked uniformity but followed a normal distribution within a cluster while separating individual berries for this study. Ripening related asynchronicity within a cluster has been shown to be overcome by maturity with fruit of different classes synchronizing (Gouthu, O'Neil et al. 2014), but this process was not complete in our berries, where we observed differences in berries on a single cluster of approximately 4°Brix (sometimes as much as 8°Brix was observed). The late stages of ripening thus underwent continued and extensive transcriptional changes.

Grape berry sugar concentrations increase dramatically after veraison, where soluble sugars are actively transported via the phloem while vines are photosynthetically active (Keller, Smith et al. 2006, Conde, Silva et al. 2007,

Choat, Gambetta et al. 2009). In this study, many transcription factors were significantly different between ˚Brix levels, mostly displaying upward or downward expression profiles. Sugar can induce the transcription of some genes in grape berry, such as increasing the expression of a glucose-6-phosphate transporter that facilitates sucrose transport for starch conversion in plastids (Noronha, Conde et al. 2015). Some bZIP TFs also contain a sucrose-controlled upstream open reading frame that exhibits repressed expression under increasing molarities of sugar (Wiese, Elzinga et al. 2005, Thalor, Berberich et al. 2012). Also in grape, the promoter sequence of a dihydroflavonol reductase gene (VIT_18s0001g12800) contains a G-box binding domain, MYB and sucrose box domains that can be induced by sucrose, glucose and fructose, constituents of a ripening berry (Gollop, Even et al. 2002).

Many ripening related process were observed in our data, far too many to report. As many other groups have found, softening genes like polygalacturonases and expansins were expressed in the mature berry. Cell wall degradation and other processes related to fruit softening with advanced maturity are among the late stages of ripening prior to senescence. This included the continued downregulation of most photosynthetic transcripts, which might indicate the deactivation or degradation of chloroplasts, such as Cytochrome c6 (VIT_01s0011g01850) and light harvesting complex II type I CAB-1 (VIT_10s0003g02890). Additional ripening processes included the activity of repressors and downregulation of seed embryogenesis genes. For example, Vvi*ABI3* was perhaps unsurprisingly inactive in the skin, whereas a known

splicing factor and suppressor of *ABI3*, *VviSUA* remained active. *AtSUA* splices a cryptic intron producing a truncated protein upon seed maturation (Sugliani, Brambilla et al. 2010), which may explain the absence of expression.

Not all genes appear to be directly related to the sampled ˚Brix levels. Over a thousand genes in two *Vitis vinifera* cultivars were recently observed expressing distinctive circadian rhythms throughout the light-dark cycle (Carbonell-Bejerano, Rodriguez et al. 2014). For example, *VviLHY* and *VviTOC1* did not oscillate, whereas *VviRVE1* and *VviELF3* of the core clock genes did show circadian rhythm in grape (Carbonell-Bejerano, Rodriguez et al. 2014). The authors attribute the differences in clock gene expression to grape maintaining a simplified clock in ripening fruit. Furthermore, secondary processes seemed more responsive to circadian oscillation in late ripening stages than primary metabolism, such as stilbene synthases and phenylalanine ammonia lyase (Lai, Doherty et al. 2012, Carbonell-Bejerano, Rodriguez et al. 2014).

Circadian clock genes like *CCA1, ELF3*, *LUX*, and *TOC1* are also partly involved in regulating transcription of ROS genes, including catalase and peroxidase activity in the early morning (Lai, Doherty et al. 2012). H_2O_2 (in cytosol) and also $^1O_2$ (in plastids) ROS species accumulate predominately in grape berry skins, peaking 1-2 weeks post-veraison before slowly decreasing into harvest (Pilati, Perazzolli et al. 2007, Pilati, Brazzale et al. 2014). We observed a downward trend in many ROS genes (Fig. 7), such as catalase and peroxidase, consistent with a reduction of ROS consumption and enzyme activity in the mature fruit (Pilati, Brazzale et al. 2014). Carbonell-Bejerano *et al.* (2014)

did not show circadian oscillation of these ROS genes occurring in Verdejo or Tempranillo cultivars; instead, constant expression of ROS genes was observed over the light-dark cycle. ROS accumulation and not downregulation of scavengers at the onset of ripening may participate in signaling of ripening genes (Pilati, Brazzale et al. 2014).

Day length decreased with advanced berry ripening and the progression of fall. Increasing ˚Brix was therefore a de facto separation of both berry development and of time. Many of the core clock genes displayed similar patterns of expression (Figs. 3 & S1). Genes that normally peaked after dawn would begin doing so later in the day as day length lessened towards the end of the season, shortening each successive day. This might be an explanation for the increasing profiles of many clock genes by 26 ˚Brix, despite sampling each cultivar at a similar time each day. Subtle to large differences in expression of cultivars that ripen at different times might then be expected. For example, Cabernet Sauvignon is the latest cultivar to ripen in our vineyard, normally in mid-to-late October, while Merlot and Semillon are two of the earlier cultivars to ripen.

3.4.2 Epigenetic control of ripening

DNA methylation plays an indispensible role in regulating endogenous gene transcription (Zilberman, Gehring et al. 2007). A link between DNA methylation and the regulation of fruit ripening was supported by gradual decreases in methylation of the promoter region of the *RIN* MADS-box TF in tomato (Zhong, Fei et al. 2013). Differential expressed methyltransferases, like *CMT*, *DRM* and *MET*, have been recorded during plant development in pear

(Huang, Li et al. 2014) and legume (Garg, Kumari et al. 2014). Our study also profiled cytosine methyltransferases. While the profiles of *VviCMT3*, *VviDRM2*, *VviDRM3*, *VviMET1a* and *VviMET1b* were divergent lacking a common grape profile, they each displayed persistent transcript abundance through late development. These findings strongly suggest an important role for normal fruit ripening through the regulation of DNA methylation, particularly in class of genes highly conserved in eukaryotic species (Feng, Cokus et al. 2010).

MBD9 in *Arabidopsis* can modulate DNA methylation and histone acetylation to regulate both flowering time and shoot branching by specifically binding methylated CpG dinucleotides (Zemach and Grafi 2003, Peng, Cui et al. 2006, Yaish, Peng et al. 2009). *Atmbd9* mutants flower earlier and show abnormal axillary bud outgrowth (Peng, Cui et al. 2006), displaying significantly methylated promoter and intronic regions of the FLOWERING LOCUS C (*FLC*) gene (Yaish, Peng et al. 2009). A common upregulation of *VviMBD9* in all grape cultivars was observed that raises the possibility for methylation of DNA and histones. Likewise, *DDM1* proteins have been observed co-localizing with *MBD* proteins forming protein complexes (Zemach, Li et al. 2005). *DDM1* in *Arabidopsis* (Vongs, Kakutani et al. 1993, Gendrel, Lippman et al. 2002) and rice (Higo, Tahir et al. 2012) have been shown to be necessary for genomic DNA methylation and chromatin remodeling, through preferential methylation of histone H3 lysine 9 (H3-K9) over transposable elements.

Genome-wide reduction of DNA methylation results in severe developmental and morphological defects in *ddm1* mutants (Pikaard and Scheid

2013). In *Arabidopsis*, *PIE1* forms part of the Swr1-like complex which deposits a histone variant, H2A.Z, onto chromatin around both the transcriptional start and stop sites on genes responsible for flowering repression (*FLC*, *MAF4* and *MAF5*) enabling their competence for activation by other factors (Deal, Topp et al. 2007). Thus, we can hypothesize a role for epigenetic regulation during the later stages of berry development.

3.4.3 Hormone and gene response in late ripening

Classically, a direct link between ripening and ethylene in non-climacteric fruit have been inconclusive, but we cannot deny the growing evidence that some ethylene signaling occurs in fruits like strawberry (Merchante, Vallarino et al. 2013), hot pepper (Kim, Park et al. 2014) and grape (El-Kereamy, Chervin et al. 2003, Chervin, El-Kereamy et al. 2004, Chervin, Tira-Umphon et al. 2008, Cramer, Ghan et al. 2014). Low levels of ethylene have been recorded before and during ripening of grapes (Coombe and Hale 1973). The MADS-box TF *RIN* regulates *ACS* and *ACO* that lead to ethylene biosynthesis (Martel, Vrebalov et al. 2011). *VviRIN* and ACC synthase were decreasing, while negative regulation of ethylene signaling factors such as *CTR1* and to a lesser extent *ETR2* were increasing. Its possible that ethylene production was decreasing as ethylene biosynthesis participates in a self-feedback loop where the presence of ethylene has the affect of self regulation (Hua and Meyerowitz 1998). Other grape transcriptomic studies also report the over and under expression of ethylene responsive factors, receptors and regulators (Sweetman, Wong et al. 2012, Cramer, Ghan et al. 2014). An abundance of ethylene responsive genes were

affected by varying ˚Brix levels in berry skin, including *VviEIN2*, which decreased in expression from 25 to 36.7 ˚Brix (Cramer, Ghan et al. 2014), however, in that study many ERFs were upregulated that are down-regulated in this study. There are clearly changes in ethylene signaling, although it is not clear if this is developmentally or environmentally (abiotic and biotic) regulated. Ethylene response can occur from both types of effects. More research is needed to untangle such possible interactions.

Ethylene abscission and maturing berry are interrelated. Indeed, “ripening” fruit is just one step on a program of senescence. Additional evidence for the action of ethylene, in the form of an ethephon treatment, upon shoots and leafs increased the rate of abscission relative to the control in mature grape leafs (Hedberg and Goodwin 1980). More recently, ethylene production in berry was shown to be greatly increased in fruit exposed to a combination of 1-aminocyclopropane-1-carboxylate and methyl jasmonate treatment, enhancing abscission in ripe fruit (Uzquiza, Martin et al. 2014). Ethylene responses possibly precede the eventual abscission of fruit.

In relation with its accumulation profile, numerous reports suggested that ABA may play a major role in controlling several ripening-associated processes of grape berry at the beginning of ripening at the veraison stage, including coloration, sugar accumulation, and softening (Jia *et al.*, 2011). It appears that ABA signaling may have been down-regulated by 26˚Brix level, indicated by the increasing *VviPP2Cs* and decreasing regulators and *VviSnRKs*. However, *VviNCEDs* were expressed indicating ABA biosynthesis continued through late

ripening. ABA has been hypothesized as regulating ripening-associated processes in grape (Coombe 1992, Davies and Böttcher 2009). An ABA-responsive element-binding protein 2 (*AREB2*, VIT_18s0001g10450) that localizes in the nucleus was recently characterized as belonging to the bZIP family and is responsive to ABA (Nicolas, Lecourieux et al. 2014). Expression levels of *VviAREB2* did not significantly change but maintained elevated expression over all ˚Brix levels. *AREB2* targets include LATE EMBRYOGENESIS ABUNDANT (*VviLEA*; VIT_08s0007g04240), NO APICAL MERISTEM [NAM], ARABIDOPSIS TRAN- SCRIPTION ACTIVATION FACTOR [ATAF], CUP-SHAPED COTYLEDON [CUC] (*VviNAC*; VIT_19s0014g03290), and Benzodiazepine Receptor (*VviBenzoR*; VIT_07s0005g00140). These findings further support the hypothesis that ABA may play a role in continued ripening of the grape berry.

Auxin response factors (AFRs) bind auxin response elements within the promoters of auxin-inducible genes (Liu, Wu et al. 2014). Auxin related genes are also expressed (e.g. *IAA16)* during the ripening of hot pepper, like grape a non-climacteric fruit (Lee, Chung et al. 2010). Auxin response factors (ARFs) were split into increasing and decreasing profiles (clusters 2, 4, & 5 of Fig. 2) For example, *VviCEB1* has a role in controlling cellular expansion in skin, and responds negatively to increasing auxin levels (Pires and Dolan 2010, Nicolas, Lecourieux et al. 2013). Nicolas *et al*. (2013) showed the fruit preferentially expressed *VviCEB1* peaking at 60 dpa and then beginning to decline around 100 dpa. Our data showed stable expression of *VviCEB1* that significantly decreased

from 24 to 26 ˚Brix in all cultivars. *VviCEB1* also specifically binds these auxin related genes, such as pectate lyase (VIT_05s0051g00590) that was progressively down-regulated beginning at 20 ˚Brix. Cellular expansion is rapid post-veraison but slows towards maturity and the activity of *VviCEB1* maybe a marker for maturity.

3.5 Conclusions

In conclusion, our data suggest a continued role for the transcriptional regulation of fruit ripening that involves several families of transcription factors, including C3H, MYB, AP2/ERF and bHLH. Data also support continued hormonal control through late ripening that involve interplay between ABA, auxin, and ethylene. Curiously, a circadian clock signature for key clock components was observed that warrants further study. In addition, genes related to DNA methylation suggest that epigenetic programming may be involved in berry ripening at maturity. A key signal from the seed indicating seed maturity may play a role affecting berry ripening and senescence. Our results provide practical information for the grape and fruit communities at large for further research into late ripening processes.

Table 1 *Sequencing, read mapping and feature count statistics.*

Cultivars investigated	7
Library type	Single-end
Number of libraries	84
Read length (bp)	50
Total number of reads	2,901,803,214.00
Average total reads	34,545,276.36
Total number of filtered reads[1]	2,877,839,522.00
Average filtered reads[1]	34,259,994.31
Percentage of filtered reads[1]	99.18%
Alignment not unique[2]	3,410,081.85
Ambiguous[2]	219,495.24
No feature[2]	2,692,823.24
Not aligned[2]	0.00
Too low a quality[2]	0.00

[1] High quality (Phred score = 20)
[2] HTSeq, Anders *et al.* (2014)

Table 2 *Summary of significant transcript results for each ˚Brix contrast tested with edgeR.*

	22 vs. 20	24 vs. 22	26 vs. 24
Overall	3,008	2,643	5,801
Chardonnay	62	109	238
Cabernet Franc	1	2,125	0
Cabernet Sauvignon	144	0	4,872
Merlot	1,669	6	802
Pinot Noir	68	9	66
Sauvignon Blanc	1,273	133	511
Semillon	154	566	597

Table 3 *Cluster membership of transcription factors significantly changing with ˚Brix*

Cluster	Top TF families	n	Profile trend
1	C3H (11), MYB (11), PHD (8)	115	Up
2	C3H (12), MYB (11), AP2/ERF (7)	109	Down
3	C3H (11), MYB (9), PHD (8)	112	Up
4	C3H (16), AP2/ERF (7), HB (6), MYB (6)	82	Up
5	C3H (22), MYB (16), WRKY (10)	113	Down
6	C3H (19), AP2/ERF (11), bHLH (10)	112	Down
7	C3H (14), bHLH (11), AP2/ERF (6)	100	Up
8	bHLH (8), C2H2 (3), WRKY (3)	40	Up
S1	ARF, bHLH, C2H2, C3H, MYB, RWP-RK, TIFY	8	Mixed
S2	SWI/SNF-BAF60b (2), AP2/ERF, bHLH, DBP, LBD, MYB-related	8	Mixed
S3	AP2/ERF, C2H2, FAR1, GRAS, GRF, HSF, MADS-box, MYB, WRKY	10	Mixed

Plant transcription factor databases sourced: PlnTFDB v3.0 (Perez-Rodriguez *et al.*, 2010), PlantTFDB 3.0 (Jin *et al.*, 2014) and iTAK *(*bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi)

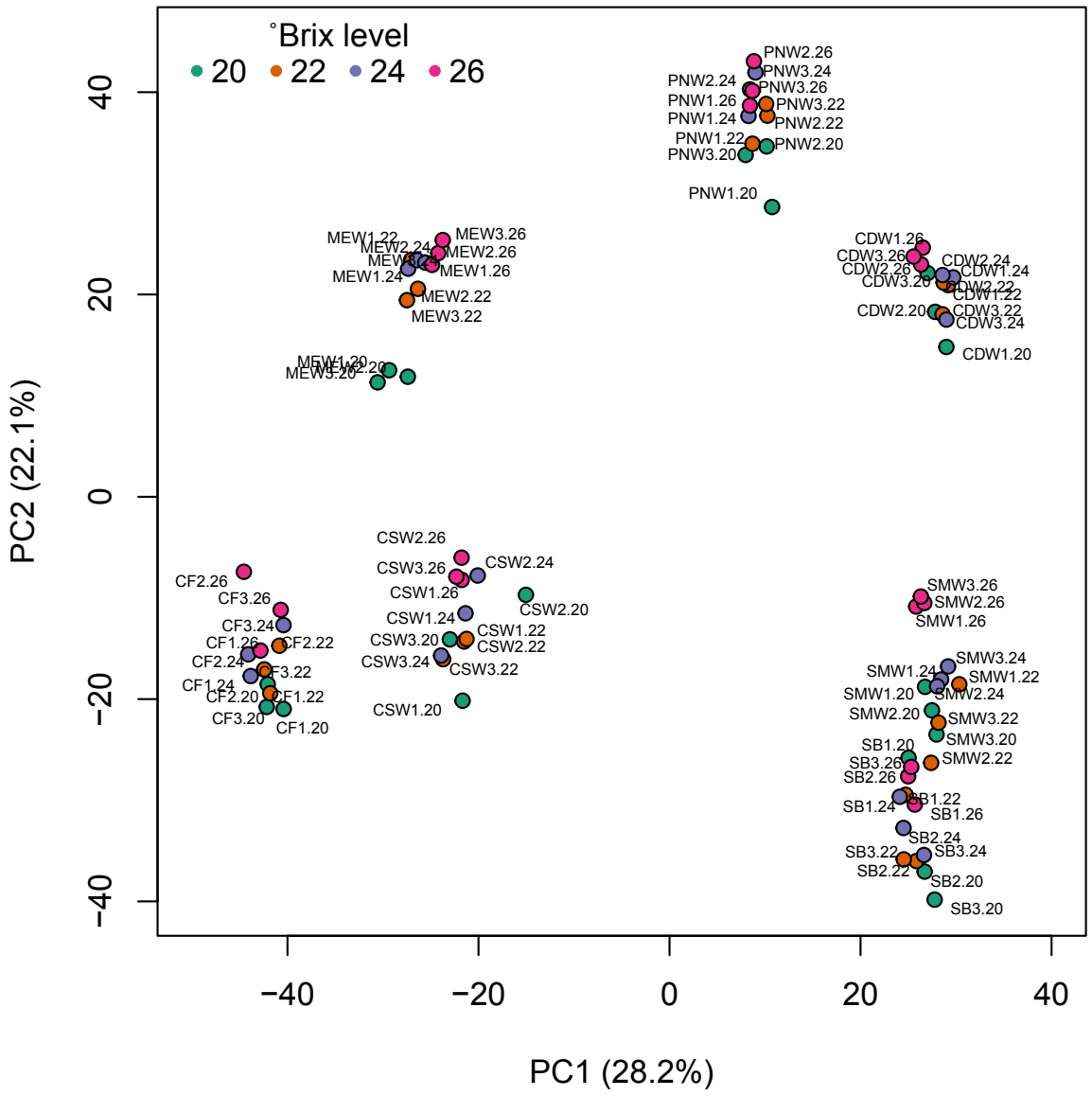

Figure 1. PCA plot of skin ripening samples according to their normalized counts per million. The first (PC1) and second (PC2) components are represented. Samples corresponding to three biological replicates from four ˚Brix levels were analyzed. ˚Brix levels are colored across cultivars. Sample abbreviation and number indicate cultivars and replicates: Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

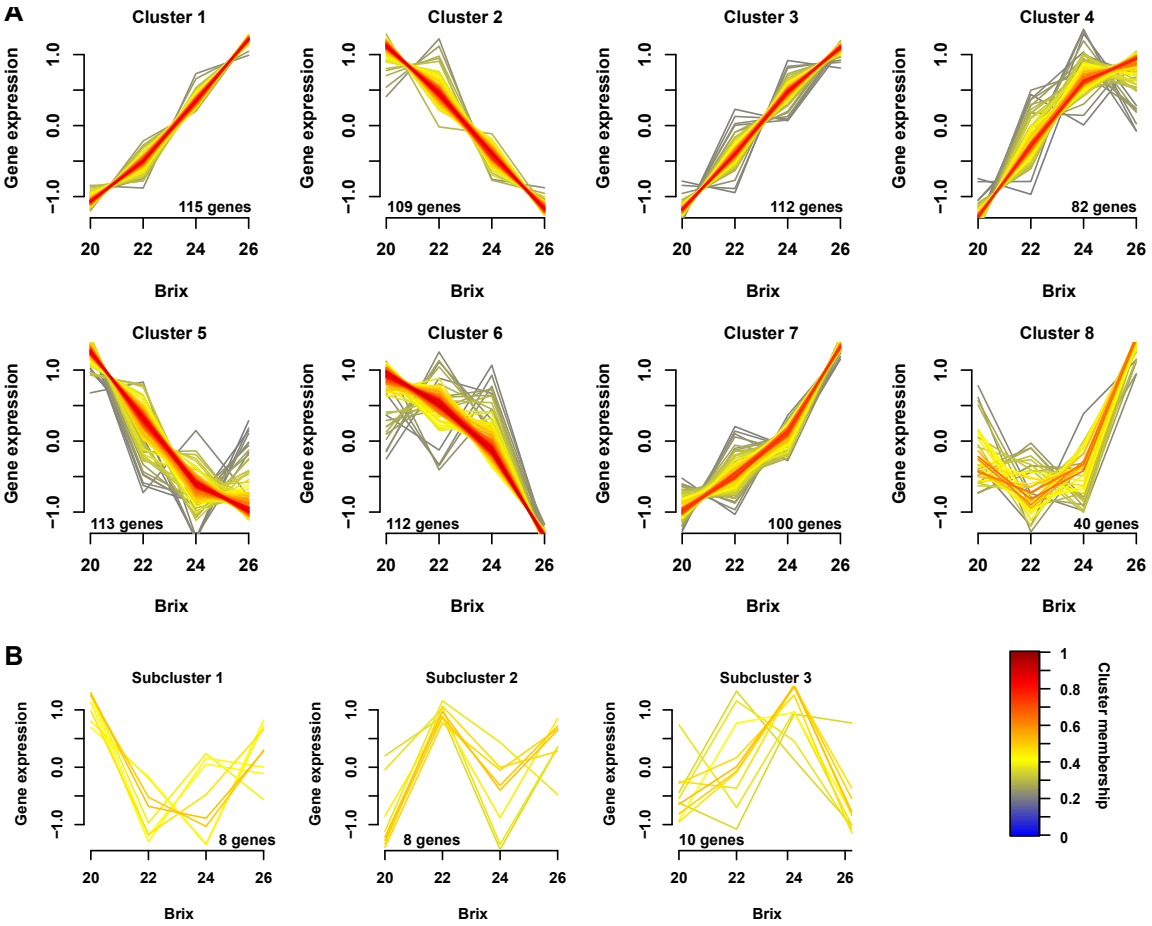

Figure 2. Profiles of significant differentially expressed transcription factors clustered with fuzzy c-means soft clustering. (A) Eight main clusters were formed at a minimum membership threshold of 0.2. (B) The remaining 26 transcripts were then clustered into three subclusters.

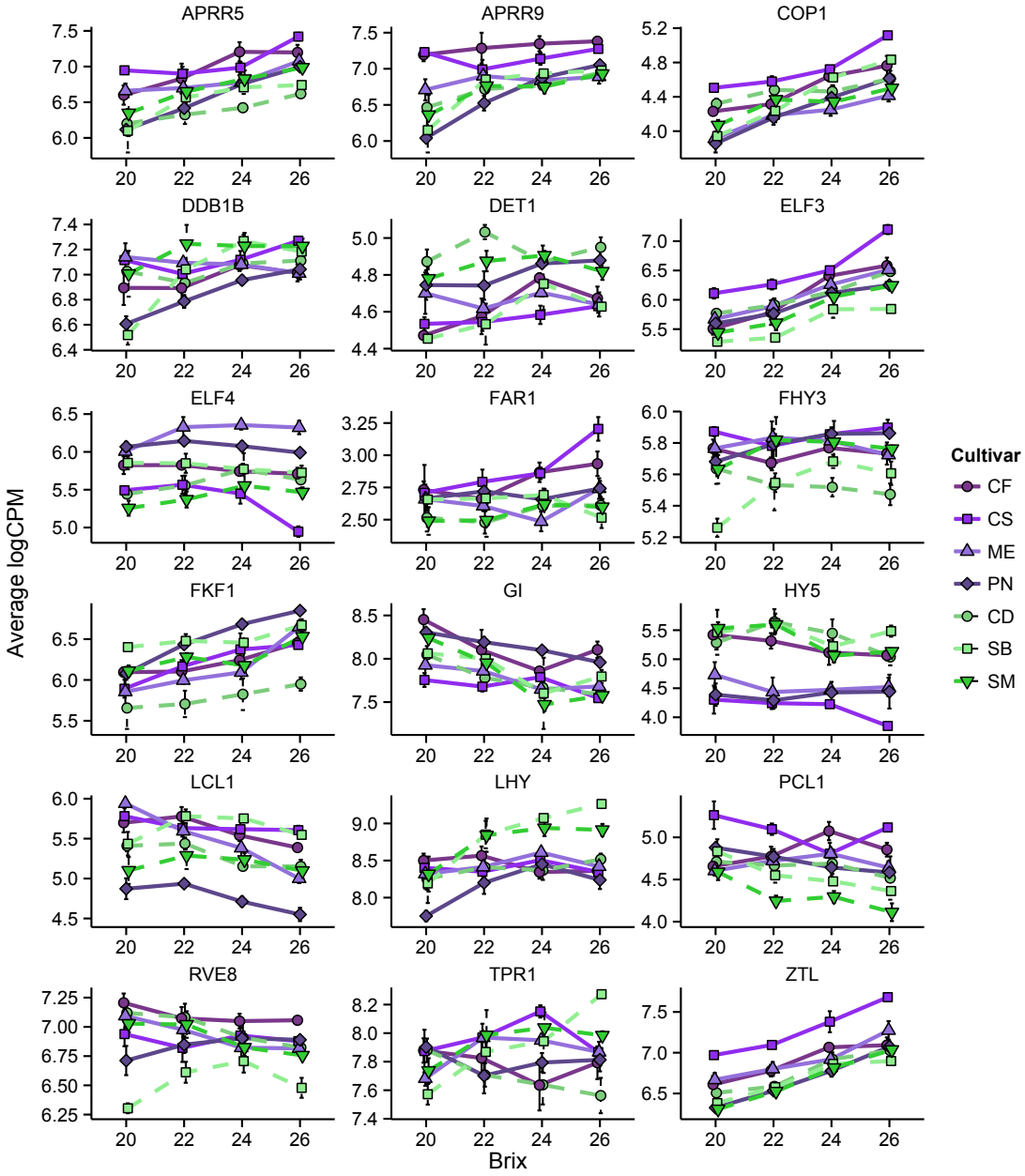

Figure 3. The transcript abundance of key components of the circadian clock. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

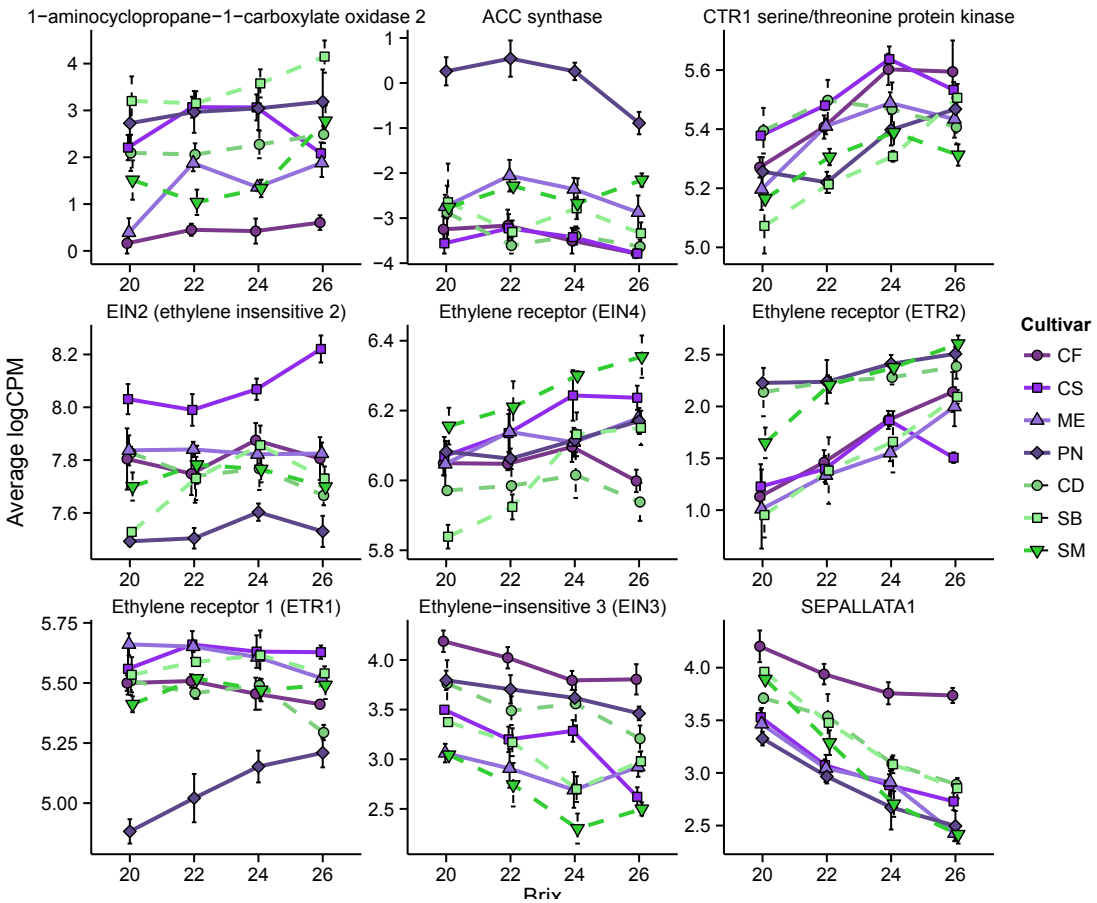

Figure 4. The transcript abundance of key components of the ethylene-signaling pathway. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

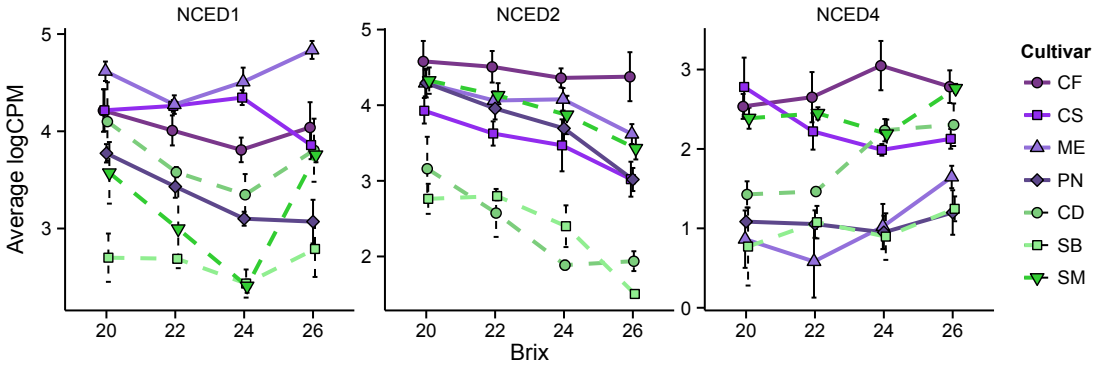

Figure 5. Expression profiles of rate limiting step of ABA biosynthesis by 9-cis-epoxycarotenoid dioxygenases. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

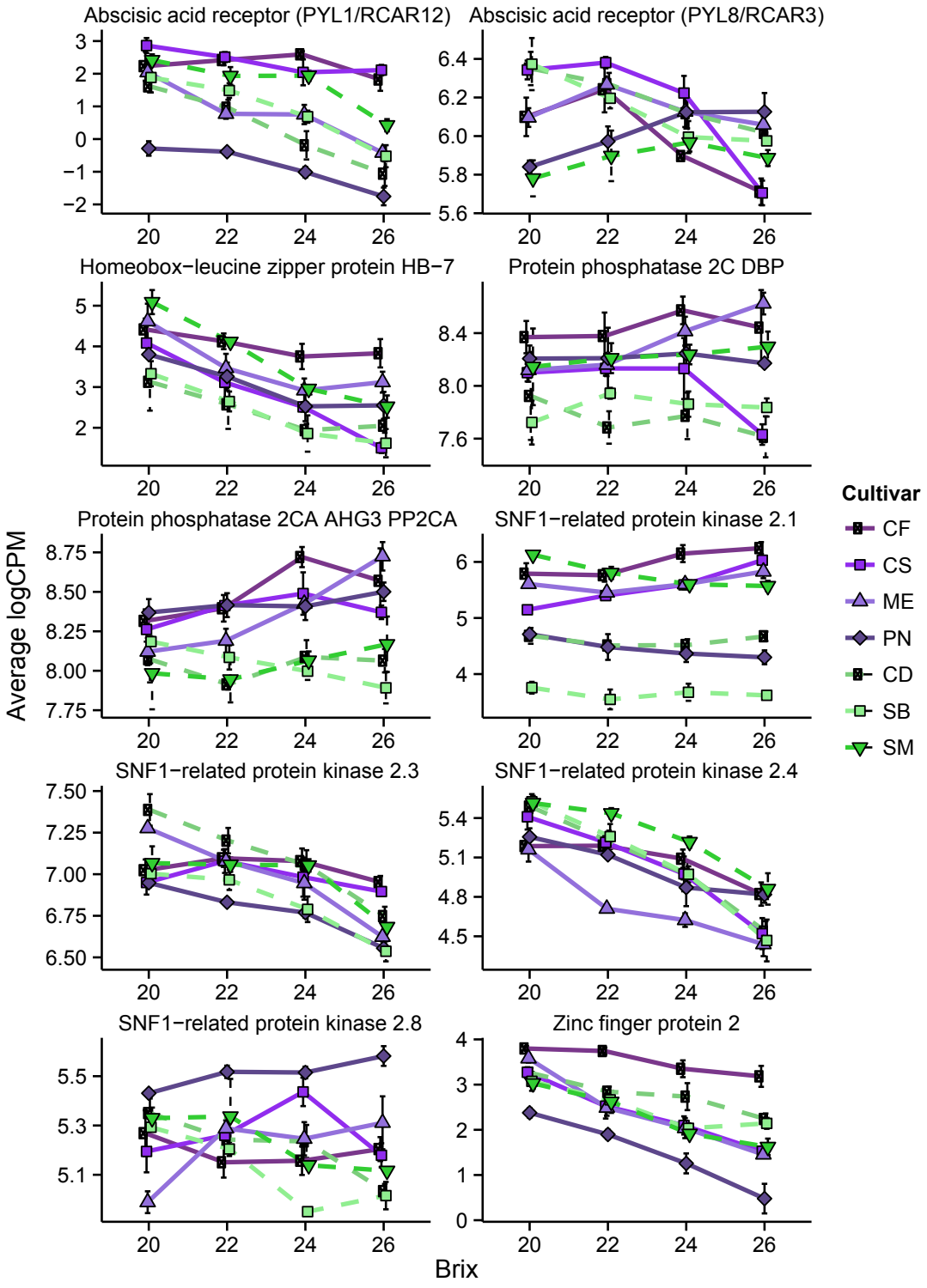

Figure 6. Transcript abundances essential to the perception and signaling of ABA. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

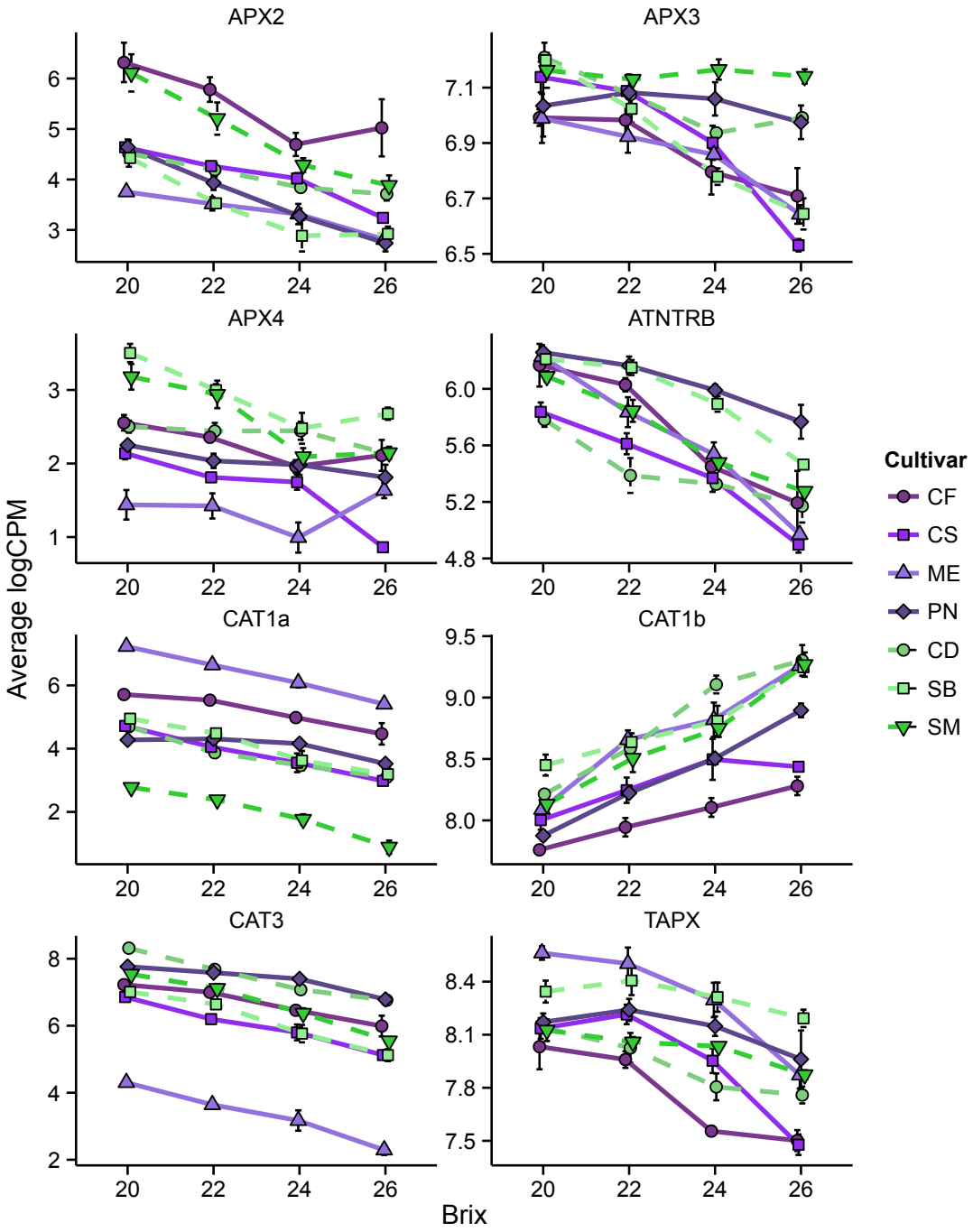

Figure 7. Expression profiles of ROS signaling and scavenging transcripts. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

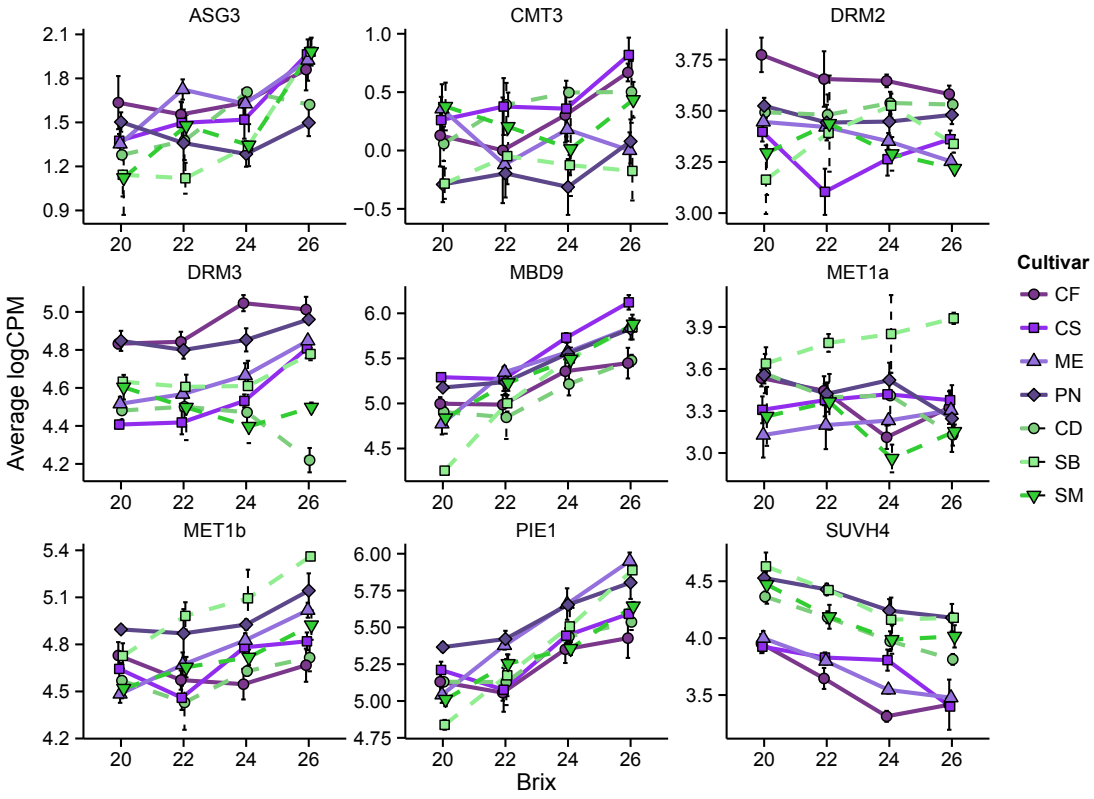

Figure 8. The transcript abundance of transcripts that perform chromosomal rearrangement, chromatin modification and the methylation of DNA. Symbols represent mean ± SE n=3, Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

CHAPTER 4:

CHARACTERIZATION OF PATHOGENESIS-RELATED PROTEIN FAMILIES IN GRAPE BERRY SKINS AT HARVEST

This Chapter is based on a manuscript that is currently being prepared for submission to *Journal of Agricultural Food Chemistry*

Ghan, R., Van Sluyter, S.C., Hopper, D.W., Tillett, R., Schlauch, K.A., Fait, A., Cramer, G.R. Characterization of Pathogenesis-related Protein Families in Grape Berry Skins at Harvest. *Journal of Agricultural Food Chemistry,* In Preparation, (2015).

4.1 Introduction

Coinciding with the buildup of soluble sugars and the expansion and softening of the berry at veraison, pathogenesis-related (PR) proteins begin accumulating in grape through the latter half of maturation until harvest (Tattersall 1997, Ferreira, Piçarra-Pereira et al. 2001, Monteiro, Picarra-Pereira et al. 2007). PR-proteins are defined as plant proteins induced in pathological or related situations, but also include stage specific proteins on the basis of their sequence homology and enzymatic or biological activity (Antoniw and White 1980, van Loon, Pierpoint et al. 1994). Proteins remaining after the wine fermentation process are considered 'nuisance' proteins in the wine industry and contribute to wine haze are largely made up of PR-proteins (Waters, Shirley et al. 1996, Ferreira, Piçarra-Pereira et al. 2001). These small-sized proteins remain stable at wine pH (2-4), display resistance to proteolysis, and can withstand fermentation, which collectively contribute to the presence of soluble haze-forming proteins in white wines, primarily from thaumatin/osmotin-like and chitinase-like proteins (Pocock, Hayasaka et al. 2000, Ferreira, Monteiro et al. 2004). Interestingly, reduction of solar UV radiation by UV exclusion effectively reduces the total content of phenolics, thaumatins and chitinases in Sauvignon Blanc skins (Tian, Harrison et al. 2015). Anecdotally, white wines produced from drought-stressed vines in our high elevation (high UV) experimental vineyard have routinely displayed the classical haze characteristic of high concentrations of PR-proteins over the past ten years of winemaking (G.R. Cramer, unpublished data).

The PR-proteins consist of 17 unrelated protein families and are highly conserved across plant species (Van Loon and Van Strien 1999, Sels, Mathys et al. 2008, Sinha, Singh et al. 2014). In *Vitis*, PR-protein accumulation has previously been reported in response to abiotic stress, fungal pathogen infection (Fung, Gonzalo et al. 2008), and phytoplasma infection (Margaria, Abba et al. 2013). Additionally, glucan endo-1,3-beta-glucosidase-like (PR-2), chitinase-like (PR-3, 4, & 8), and thaumatin-like (PR-5) protein classes have been reported as some of the dominant proteins found either at harvest or within wine (Cilindre, Castro et al. 2007, Deytieux, Geny et al. 2007, Cilindre, Jegou et al. 2008, Wigand, Tenzer et al. 2009). Both class IV chitinases (PR-4) and lipid transfer proteins (PR-14), from either the fruit or wine have been identified as allergens to humans (Pastorello, Farioli et al. 2003, Schad, Trcka et al. 2010). The PR-10 family characterized in grape contains 14 complete PR10 related sequences (Lebel, Schellenbaum et al. 2010). The maturing berry expresses a suite of pathogenesis-related proteins developmentally regulated in normal conditions and induced under stressful conditions (Deytieux, Geny et al. 2007, Negri, Prinsi et al. 2008, Negri, Robotti et al. 2011). Grape composition at harvest can therefore impact the quality of the juice and finished wine.

In the present study, pathogenesis-related proteins which accumulated in skins of three red-skinned and two white-skinned cultivars: Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay and Semillon, were characterized *in silico*. Better characterizations of the PR-proteins that potentially impact overall quality of the wine berry were of particular interest. Pathogenesis-related proteins represented

an additional class of highly abundant skin proteins, representing ~4% of all proteins identified.

4.2 Materials and Methods

4.2.1 Classification of pathogenesis-related protein families

Protein and RNAseq data from Chapters 2.2 & 3.2 were further investigated. The protein database used for identification was compiled in (Chapter 2.2) three sources: 1) all reviewed *V. vinifera* protein entries in UniProt, "Taxonomy:29760 AND reviewed:yes" (164 sequences); 2) *V. vinifera* proteins predicted by the International Grape Genome Program, "Taxonomy:29760 AND author:vitulo AND reviewed:no" (29803 sequences); 3) mitochondrial proteins associated in UniProt (81 non-redundant sequences) (Van Sluyter, Marangon et al. 2009). The protein database used for spectrum-peptide matching (GPM Cyclone XE and X!Tandem Cyclone version 2011.12.01.1) contained protein annotations from manually curated annotations, including 580 known and putative pathogenesis-related protein sequence annotations (Table S1).

4.2.2 Data analysis

Non-redundant *Vitis vinifera* proteins identified by nanoflow liquid chromatography-mass spectrometry (Chapter 2.2.2) were further queried for domain structure and gene ontology from plants.ensemble.org, using the R (3.2.1) bioconductor (3.1) package, biomaRt (2.24.0) (Durinck, Moreau et al. 2005, Durinck, Spellman et al. 2009, R Core Team 2015). Queried databases from Ensemble included Interpro, PFAM, and PANTHER. The protein abundance and transcript expression profiles (Chapters 2.2 & 3.2) of transcripts matching

identified proteins were scaled and then used to create annotated heat maps with the R package ComplexHeatmap (1.0.0) (Gu 2015). Transcripts were grouped into pathogenesis-related families, and the experimental replicates were hierarchically clustered by Spearman correlation. The linear relationship of transcript level with protein abundance was computed in R that fit linear regression models to the 118 matching transcript-protein pairs and computed Pearson's correlation. A direct experimental sample-to-sample comparison was performed, grouping each protein into different pathogenesis-related families.

4.3 Results

This study explored the pathogenesis-related (PR) proteins briefly discussed in Chapter 2, which represented an additional class of highly abundant skin proteins. The skins from cultivars Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, and Semillon, were the focus of this investigation. Within the 2,867 non-redundant *Vitis vinifera* proteins identified by nanoflow liquid chromatography-mass spectrometry (Chapter 2.3.2), over 100 proteins were classified as pathogenesis-related. Despite representing only ~4% of all proteins identified, many of the most abundant proteins and the transcripts from the berry skins of each of the five cultivars were PR-proteins.

4.3.1 Pathogenesis-related proteins in mature berry skins

Spectrum-peptide matching was performed with X!Tandem and the GPM Cyclone (www.thegpm.org) against a custom grape database. Sequences for over 500 putative PR-proteins were amongst the annotated proteins in the grape database. There were 123 unique pathogenesis-related proteins identified across

the five cultivars that encompassed 15 of the unrelated PR-families (Table 2). Each putative PR-protein was further queried for domain and gene ontology classification from plants.ensemble.org.

Domain descriptions of identified proteins contained overlapping identifiers that grouped each protein into their respective protein family (Tables 2 & S2). PR-proteins were found in 15 of the 17 known families of PR-proteins. Almost half of the PR-families were annotated as 'Defense Response' (PR-2, 4, 6, 8, 10, 12, 15/16). Multiple families contained (PR-2, 3, 4, 8) glycol-hydrolase and chitin binding domains. Haze-forming proteins in wines, primarily the thaumatin (PR-05) and chitinases families (PR-3, 4, 8), were well represented in each cultivar (Pocock, Hayasaka et al. 2000, Robinson and Davies 2000, Monteiro, Picarra-Pereira et al. 2007), but, while well represented, did not accumulate with more specificity in the white cultivars over red (Figure S1). A single gamma thionin or plant defensin (PR-12; D7TAI4) was identified. This class of PR-proteins has functionally been characterized as inhibiting pathogen growth *in vitro*, showing developmental (veraison through ripening) and organ (berry) specificity (de Beer and Vivier 2008, Carvalho Ade and Gomes 2009). PR-17 is a less well-characterized family. Database searches describe the only identified domain as a class of basic secretory proteins, likely participating in plant defense response (Christensen, Cho et al. 2002). Protein D7SXW6 (VIT_03s0091g00160) was the only protein identified that matched PR-17.

Over 100 PR-proteins were identified at harvest in each cultivar (Table 1). Many of these PR-proteins had large spectral counts into the hundreds of

spectral counts indicative of their large abundance relative to other classes of proteins (Table S2, Figure S1). The PR-proteins were predominately glucan endo-1,3-beta-glucosidases (PR-02), thaumatins (PR-05), plant peroxidases (PR-09), and oxalate oxidases (PR-15/16) (Sels, Mathys et al. 2008, Sinha, Singh et al. 2014). These classes of PR-protein have also been reported as some of the dominant proteins found either at harvest or within wine (Cilindre, Castro et al. 2007, Deytieux, Geny et al. 2007, Cilindre, Jegou et al. 2008, Wigand, Tenzer et al. 2009). Proteins from PR-11 & 13 families were not detected. Not all plant species contain each PR-family (Van Loon and Van Strien 1999). For convenience, we classify families 15 & 16 together due to their domain similarity, classified as oxalate oxidase and oxalate oxidase-like.

The majority of protein abundance differences in Chapter 2.3 were from the cultivar-effect, which included 47 significant PR-proteins (Chapter 2.3). Profiles for proteomic and transcriptomic abundance levels in each PR-family are presented as a heat map in Figure 1 for easy visualization and comparison at harvest. Experimental samples were clustered by Spearman correlation. Obvious cultivar differences were observed in both protein and transcript data sets. For example, Cabernet Sauvignon had much lower protein abundance for most PR-families, and this observation was magnified in the transcript levels. The low abundance in Cabernet Sauvignon was particularly apparent in PR-05 for both protein and transcript (Figs. 1, S1). Both Pinot Noir and Merlot had higher abundance levels of proteins and transcripts, such as in PR-families -15/16, in Merlot, and PR-05, in Pinot Noir. The white cultivars, Chardonnay and Semillon,

were more similarly clustered by transcript abundance than protein, with Chardonnay clustering closely with Pinot Noir in PR-05.

The expression profiles (Chapter 3.3) from skin tissue separated by ˚Brix levels (20, 22, 24, 26) in 2012 were also briefly investigated for expression differences, as these samples were obtained from the same vineyard and under similar growing conditions as the 2011 (cluster average ~23.3˚Brix, Chapter 2.3.1). A heatmap of matching transcripts-to-identified proteins was made to globally observe patterns in expression data under increasing levels of ˚Brix (Fig. 2). Overall, each cultivar had relatively constant expression levels with higher sugar concentration, but differences between the cultivars themselves were similar to (Fig. 1). For example, relatively constant expression was observed in Pinot Noir for most PR-families, such as the chitinases, PRs -03, -04, and -08. Overall expression in Merlot and Pinot Noir was noticeable higher in most PR-families, similar to the transcript levels from 2011 for both cultivars. Cabernet Sauvignon PR transcript expression was again clustered with the white cultivars, and many of its transcripts were much lower relative to the other cultivars. Expression levels decreased noticeably at 26˚Brix in Chardonnay, Cabernet Sauvignon, and Merlot, and to a lesser extent, Pinot Noir. Though, this was most apparent in Cabernet Sauvignon, the cultivars displayed decreased levels for peroxidases of PR-09 and the lipid transfer proteins of PR-14 at 26˚Brix.

4.3.2 Correlations between proteomic and transcriptomic data

Several matching transcript-protein pairs of PR-proteins were fit to linear regression models (Chapter 2.3.3) showing strong positive relationships. To

further investigate the correlative relationship of transcript level with protein abundance, we fit linear regression models to the transcript-protein pairs for all PR-families. Thus, 118 proteins were matched to a corresponding transcript and modeled by family (Fig. 4, Table S4). Interestingly, correlations of the PR-families displayed a range of coefficients of determination from high, in PRs -10 & -17 (r^2 = 0.63 & 0.66, to low, in PRs -01 & -12 (r^2 = 0.07 & 0.02). Despite being the two of the predominant families of PR-proteins based upon total number of enzymes quantified, protein abundance and transcript abundance correlated poorly for some families, such as in PR-02 (r^2 = 0.17) and PR-05 (r^2 =0.11) (Table S4).

We further examined the abundance of individual proteins from several PR-families, as well as making correlations of their transcript-protein pairs (Fig. 4, Table S4) to observe how well the abundance of particular transcripts could approximate protein abundance in these families. Nearly half of the individual protein-transcript pairs had high Pearson correlation coefficients (> 0.5), with the thaumatins, ribonuclease-like and β-1,3-Glucanses being better correlated overall (Fig. 1, Table S4) (Ning, Fermin et al. 2012). Whereas, the protein-transcript pairs for the L-ascorbate peroxidases of PR-09 were not correlated well. The correlations for individual protein-transcript pairs within each family also contained examples with higher correlations than their entire families, such as PR-03 protein D7T2C8 (r^2 = 0.75 vs. the family average of 0.me48), PR-05 protein F6HUH1 (r^2 = 0.82 vs. the family average of 0.11), and PR-10 protein F6HFH0 (r^2 =0.8 vs. the family average of 0.63). However, many individual matching protein-transcript pairs also displayed low correlation despite their

families on a whole being correlated, such as D7SY83 (r^2 =0.004, PR-10 vs. the family average of 0.63) and F6HVL6 (r^2 =0.002, PR-04 vs. the family average of 0.61) (Table S4). These results indicate that there may be different rates of transcript and protein turnover in grape skins by harvest for different families and different proteins within families. However, transcript abundance for some families, like the thaumatins, may be good indicators for protein abundance.

4.4 Discussion

Pathogenesis-related protein families were further classified in five grape cultivars, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, and Semillon, grown at our experimental vineyards. In this way, grapevines were grown under nearly identical environmental conditions, free from disease and pathogen pressures. As a group, the 123 pathogenesis-related proteins made up a large number (approximately 4 %) of identifiable (2,867) proteins in the five cultivars. Many of these PR-proteins recorded hundreds of spectral counts in each experimental replicate (Table S2) indicative of their high abundance in mature berry skins relative to other less abundant proteins.

PR-proteins can be divided into 17 novel families of peptides (Table 1), each with different plant defense related properties (Tables 2 & S3) (Van Loon and Van Strien 1999, Sels, Mathys et al. 2008, Sinha, Singh et al. 2014). Numerous PR-family proteins were observed in the berry skins despite the absence of pathogens in our dry climate desert vineyard. There were 52 individual protein-transcript pairs, from 13 PR-families, with Pearson's correlation coefficient > 0.5, which suggests a possible relationship between transcription

and protein degradation for selected PR-proteins (Fig. 1). The thaumatins, ribonuclease-like and β-1,3-Glucanses had the most correlated protein-transcript pairs (Table S4). Although some of the families did not correlate well as a group, such as the PRs -09 and -14 (Figs. 1 & 3), a subset of individual matching protein-transcripts that included several thaumatins and β-1,3-Glucanses were correlated well with one another (Fig. 4). T

With the exception of PRs -12 & -17, each family had multiple proteins assigned to it (Table 1). These included families less investigated in grape, such as L-ascorbate peroxidases of PR-09 (16 proteins) and oxalate oxidases of PR-15/16 (12 proteins). Interestingly, shoot tips from Cabernet Sauvignon had different distributions of PR-proteins, being less abundant in terms of spectral counts and total number of PR-families (ten total for shoot tips vs. 15 for berry skins), with L-ascorbate peroxidases (PR-09) as the most abundant class of PR-protein observed (Cramer, Van Sluyter et al. 2013). However, not all plant species contain each PR-family (Van Loon and Van Strien 1999). For example, proteins from PR-11 & 13 families were not detected in the skin samples. Additionally, studies of chitinase in stems, roots, leaves, and berries of grape showed that not all inducible or constitutive isoforms from one tissue (berries or roots) could be induced, either by infection or salicylic acid treatment in another tissue class (leaves) (Derckel, Legendre et al. 1996).

Significant attention of PR-proteins (the chitinases and thaumatins, PRs-3 & -5, respectively) occurring in grapes has primarily been focused on wine proteins or nuisance proteins (Waters, Shirley et al. 1996, Robinson, Jacobs et

al. 1997, Tattersall 1997, Marangon, Van Sluyter et al. 2011). Haze accumulation can affect aesthetic qualities in white wines. Even recent-related PR studies in grapes quantified and identified these haze-forming classes (Tian, Harrison et al. 2015, Tian, Harrison et al. 2015) to include elucidation of thaumatin crystal structures (Marangon, Van Sluyter et al. 2014). Significant cultivar differences were observed in the protein and transcript data sets (Chapter 2.3**)**, and this was seen also for cultivar PR-family profiles. Neither of the white cultivars, known for haze formation, seemed to accumulate a larger amount of these haze-forming protein families over the other cultivars. If anything, Pinot Noir had the higher abundance levels (Fig. 1 & 2).

Pathogen infection is also sufficient to transcriptionally reprogram the expression of PR-04 & -10 in the red cultivars, Cabernet Sauvignon (Fung, Gonzalo et al. 2008) and Trincadeira (Agudelo-Romero, Erban et al. 2015). PR-encoding transcripts under increasing ˚Brix levels displayed decreased expression profiles for peroxidases and lipid transfer proteins noticeably at 26˚Brix (Chardonnay, Cabernet Sauvignon, Merlot, and Pinot Noir). Cabernet Sauvignon had much lower protein and transcript abundance in 2011 than did the other cultivars (Fig. 1). In 2012, a similar pattern was observed, but the separation of ˚Brix allowed for finer resolution of expression (Fig. 2). However, many of the PR-encoding transcripts had relatively constant levels of expression in each ˚Brix level, which may Cabernet Sauvignon ripened later and was harvested last of the cultivars in both seasons, which may partially explain their lower abundance relative to other cultivars. Overall, the PR-proteins represented

a class of proteins found in highest abundance in berry skins regardless of cultivar (Chapter 2.3.2).

To a lesser extent, β-1,3,glucanases represent a smaller class of haze-forming proteins (Esteruelas, Poinsaut et al. 2009, Sauvage, Bach et al. 2010) that are ripening-induced and more abundant in skins than grape pulp (Wang, Bianchi et al. 2009). The β-1,3,glucanase of PR-02 in this study included 14 different proteins; this class was one of the largest classes observed. The enzymatic activity of chitinase and β-1,3,glucanase isoforms has been demonstrated previously as increasing from color change to maturity (Deytieux, Geny et al. 2007), functioning in a synergistic manner in plant defense through the hydrolysis of fungal hyphae and induction of resistance (Minic 2008).

The large accumulation of PR-proteins has been demonstrated, particularly from veraison until maturation and harvest (Tattersall 1997, Ferreira, Piçarra-Pereira et al. 2001, Monteiro, Picarra-Pereira et al. 2007). The early and lasting PR presence likely offers a long-term defense strategy for post-veraison berries against the increasing appeal to birds, insects and microorganisms of berries that are softening and increasing in soluble sugars. In Semillon and Sauvignon Blanc model wines, chitinase and thaumatin proteins begin to unfold at 55 and 62 ˚C respectively, but have long lasting half-lives at temperatures below 20 ˚C (Falconer, Marangon et al. 2010, Van Sluyter, McRae et al. 2015). Thaumatin-like proteins being resistant to degradation likely proved an evolutionarily advantageous trait. Though, the constitutive presence of PR-proteins does not, however, preclude pathogen infections like gray mold in

grapes caused by *Botrytis cinerea* (Deytieux, Geny et al. 2007, Williamson, Tudzynski et al. 2007).

4.5 Conclusions

In conclusion, many identified proteins were classified as pathogenesis-related in berry skins, more so than were previously observed in shoot tips. Several PR-families had numerous protein members in skins, which maybe a tissue specific occurrence. The transcript abundance was well correlated to the protein abundance in thaumatins of PR-05, but not so in the L-ascorbate peroxidases of PR-09. Haze-forming proteins, while well represented, did not accumulate with more specificity in the white cultivars and were mostly higher in the red cultivar, Pinot Noir. Large accumulations of PR-proteins in skins at harvest provide support for a prolonged and possibly constitutive defense mechanism that protects a maturing seed within the berry.

Tables

Table 1. *Classification of pathogenesis-related (PR) protein families identified in the skins of five grape cultivars at harvest.*

		Cultivars[a]				
Family	Properties	CS	ME	PN	CD	SM
PR-01	Antifungal	2	3	4	3	4
PR-02	β-1,3-Glucanse	12	14	14	13	12
PR-03	Endochitinase	8	7	8	7	7
PR-04	Antifungal and chitinase	5	5	5	5	5
PR-05	Thaumatin/Osmotin-like	13	15	15	15	15
PR-06	Proteinase-inhibitor	6	7	6	6	6
PR-07	Endoproteinase	6	6	6	6	6
PR-08	Chitinase III	3	4	4	3	4
PR-09	Peroxidase	16	14	16	16	16
PR-10	Ribonuclease-like	13	14	15	11	14
PR-11	Chitinase	-	-	-	-	-
PR-12	Defensin	1	1	1	1	1
PR-13	Thionin	-	-	-	-	-
PR-14	Lipid-transfer protein	10	10	10	10	10
PR-15/16[b]	Germin-like, Oxalate oxidase, Oxalate oxidase-like'	12	11	12	12	12
PR-17	Unknown	1	1	1	1	1
	Cultivar summary	108	112	117	109	113

Table was adapted from J.Sels *et al.* (2008) and Sinha *et al.* (2014)

[a] CS = Cabernet Sauvignon; ME = Merlot; PN = Pinot Noir; CD = Chardonnay; SM = Semillon

[b] Family differentiation was unclear

Table 2. *Domain and gene ontology annotation was derived from pathogenesis-related proteins identified in five grape cultivars.*

Family	Interpro domain, short description	Go accession name
PR-01	Allrgn V5/Tpx1, V5 allergen, CAP domain, Allrgn V5/Tpx1 CS	extracellular region
PR-02	Glyco hydro 17, X8, Glyco hydro catalytic dom, Glycoside hydrolase SF	hydrolase activity, acting on glycosyl bonds, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process, metabolic process, catalytic activity, anchored component of membrane, anchored component of plasma membrane, cation binding, asymmetric cell division, root morphogenesis, regulation of cell size, pattern specification process, growth, cation binding, auxin polar transport, regulation of meristem growth, glucan endo-1, 3-beta-D-glucosidase activity, response to chitin, cell wall, plasmodesma, defense response to nematode, defense response to fungus, incompatible interaction, vacuolar membrane, response to biotic stimulus
PR-03	Glyco hydro 19 cat, Chitin-bd 1, Glyco hydro 19, Chitin-binding 1 CS, Lysozyme-like dom, , UBN2 3	cell wall macromolecule catabolic process, chitin binding, chitin catabolic process, carbohydrate metabolic process, chitinase activity, hydrolase activity, acting on glycosyl bonds, metabolic process, polysaccharide catabolic process, cell wall macromolecule catabolic process, chitin binding
PR-04	Barwin, Barwin-related endoglucanase, Barwin-like endoglucanase, Barwin CS, Chitin-bd 1, Chitin-binding 1 CS, Expansin/allergen DPBB dom,	defense response to fungus, defense response to bacterium, chitin binding, ribonuclease activity, defense response to fungus, incompatible interaction, response to nitrate, nitrate transport, extracellular region
PR-05	Thaumatin, Thaumatin CS	response to other organism, plant-type cell wall, plant-type cell wall organization, cell wall modification, plasmodesma, cytokinesis by cell plate formation
PR-06	Prot inh cystat, Prot inh cystat CS, Prot inh cystat cons-reg, Cystatinat cons-reg, Cystatin, Prot inh Kunz-lg, Kunitz inhibitor ST1-like	serine-type endopeptidase inhibitor activity, response to wounding, peptidase inhibitor activity, negative regulation of peptidase activity, cysteine-type endopeptidase inhibitor activity, hyperosmotic response, peptidase inhibitor activity, negative regulation of peptidase activity, cysteine-type endopeptidase inhibitor activity, cobalt ion binding, response to water deprivation, endoplasmic reticulum, response to cold, cytosol, response to oxidative stress, negative regulation of endopeptidase activity, endopeptidase inhibitor activity, programmed cell death, defense response to bacterium, mitochondrion, response to nitrate, nitrate transport
PR-07	Protease-assoc domain, Inhibitor I9, Peptidase S8 subtilisin-rel, Peptidase S8/S53 dom, Peptidase S8 subtilisin-rel, Peptidase S8A TPPII, Peptidase S8 His-AS, Peptidase S8 Ser-AS	serine-type peptidase activity, peptidase activity, negative regulation of catalytic activity, identical protein binding, serine-type endopeptidase activity, extracellular region, proteolysis, hydrolase activity
PR-08	Glyco hydro18cat, Glyco hydro 18 chit AS, PIN dom, DUF652, Glyco hydro catalytic dom, Glycoside hydrolase SF, PIN domain-like	hydrolase activity, acting on glycosyl bonds, hydrolase activity, hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process, metabolic process, catalytic activity, polysaccharide catabolic process, defense response to fungus, chitin catabolic process, chitinase activity, metabolic process, catalytic activity, small-subunit processome

Table 2 continued. *Domain and gene ontology annotation was derived from pathogenesis-related proteins identified in five grape cultivars.*

Family	Interpro domain, short description	Go accession name
PR-09	Peroxidase pln, Haem peroxidase pln/fun/bac, Haem peroxidase, Peroxidases heam-ligand BS, Peroxidases AS	hydrogen peroxide catabolic process, extracellular region, metal ion binding, heme binding, response to oxidative stress, peroxidase activity, oxidation-reduction process, oxidoreductase activity, anther development, plant-type cell wall, cell wall, regulation of meristem growth, vacuole, plasmodesma, extracellular region, polarity specification of adaxial/abaxial axis, meristem initiation, determination of bilateral symmetry, L-ascorbate peroxidase activity, peroxisomal membrane, vacuolar membrane, mitochondrion, chloroplast envelope, vacuole, chloroplast, response to hydrogen peroxide, response to high light intensity, protein folding, response to heat, glucosinolate metabolic process, pentose-phosphate shunt, stomatal complex morphogenesis, starch biosynthetic process, thylakoid lumen, photosynthesis, light reaction, chlorophyll biosynthetic process, response to far red light, response to red light, response to blue light, thylakoid, chloroplast thylakoid lumen, chloroplast thylakoid membrane, chloroplast thylakoid, rRNA processing, isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway, photosynthesis, nucleus, protein binding
PR-10	Bet v I dom, START-like dom, MLP dom, Bet v I allergen	response to biotic stimulus, mRNA modification, defense response, membrane
PR-12	Knot1, Gamma-thionin, G Purothionin	defense response
PR-14	Plant LTP, Bifunc inhib/LTP/seed store, Hydrophob seed	lipid binding, lipid transport, positive regulation of transcription, DNA-templated, plasmodesma
PR-15/16	Germin, Cupin 1, DUF594, RmlC Cupin, RmlC-like jellyroll, DUF4220n 1, DUF594, DUF4220, 11S seedstore pln, Germin Mn-BS	nutrient reservoir activity, manganese ion binding, extracellular region, metal ion binding, extracellular matrix, photosynthesis, light reaction, stomatal complex morphogenesis, cellular cation homeostasis, divalent metal ion transport, defense response to bacterium, nucleus, cell wall, plant-type cell wall, 5-formyltetrahydrofolate cyclo-ligase activity, folic acid-containing compound biosynthetic process, ATP binding
PR-17	Uncharacterised_BSP	

Figures

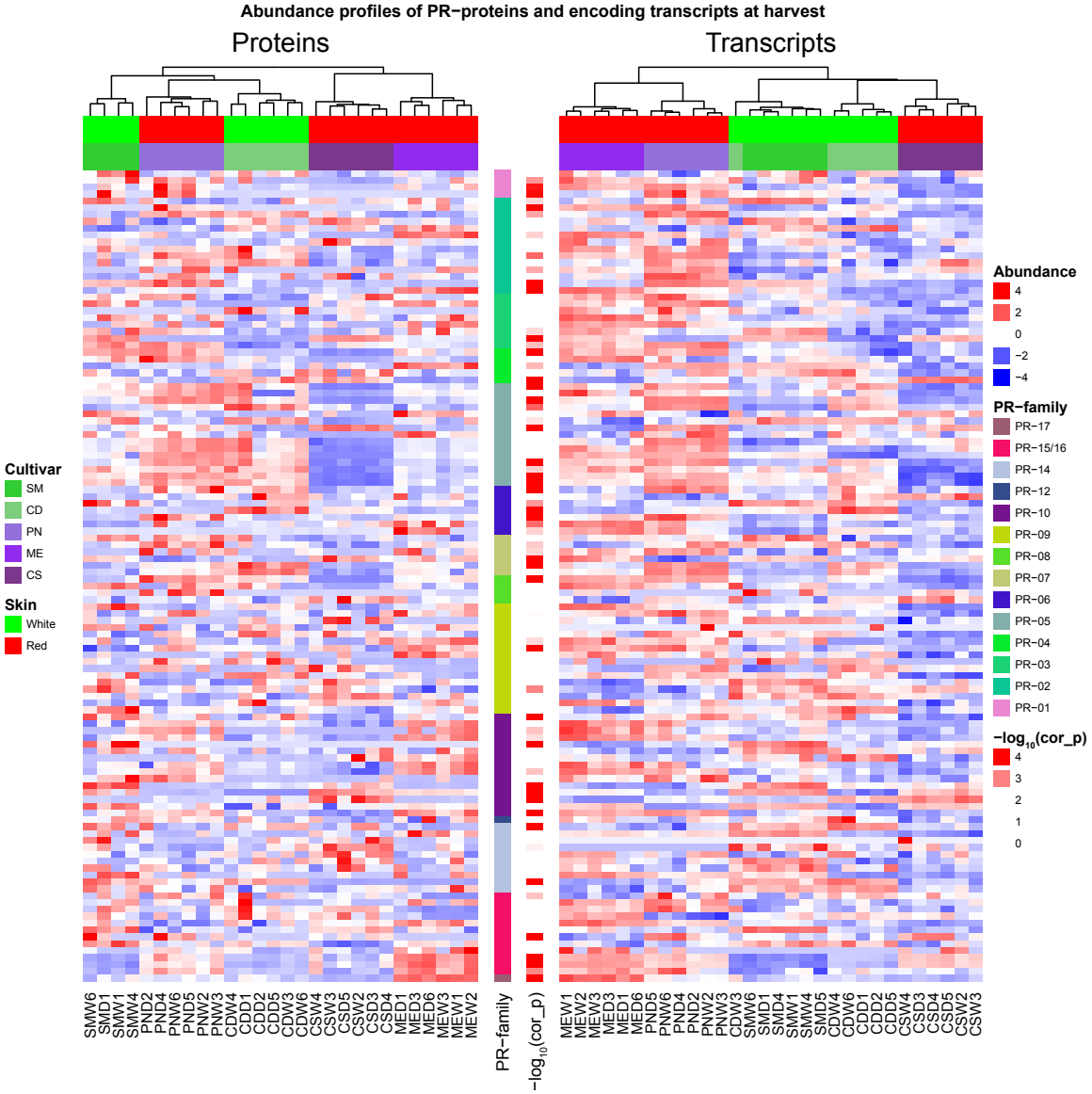

Figure 1. Cultivar abundance profiles of protein and their encoding transcripts were grouped by pathogenesis-related (PR) family membership from harvested berry skins. Experimental replicates were hierarchically clustered by Spearman correlation. The degree of correlation between protein and transcript was calculated from the negative logarithm of the Pearson's correlation p-value, under -$\log_{10}$(cor_p). Legend abbreviations: Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

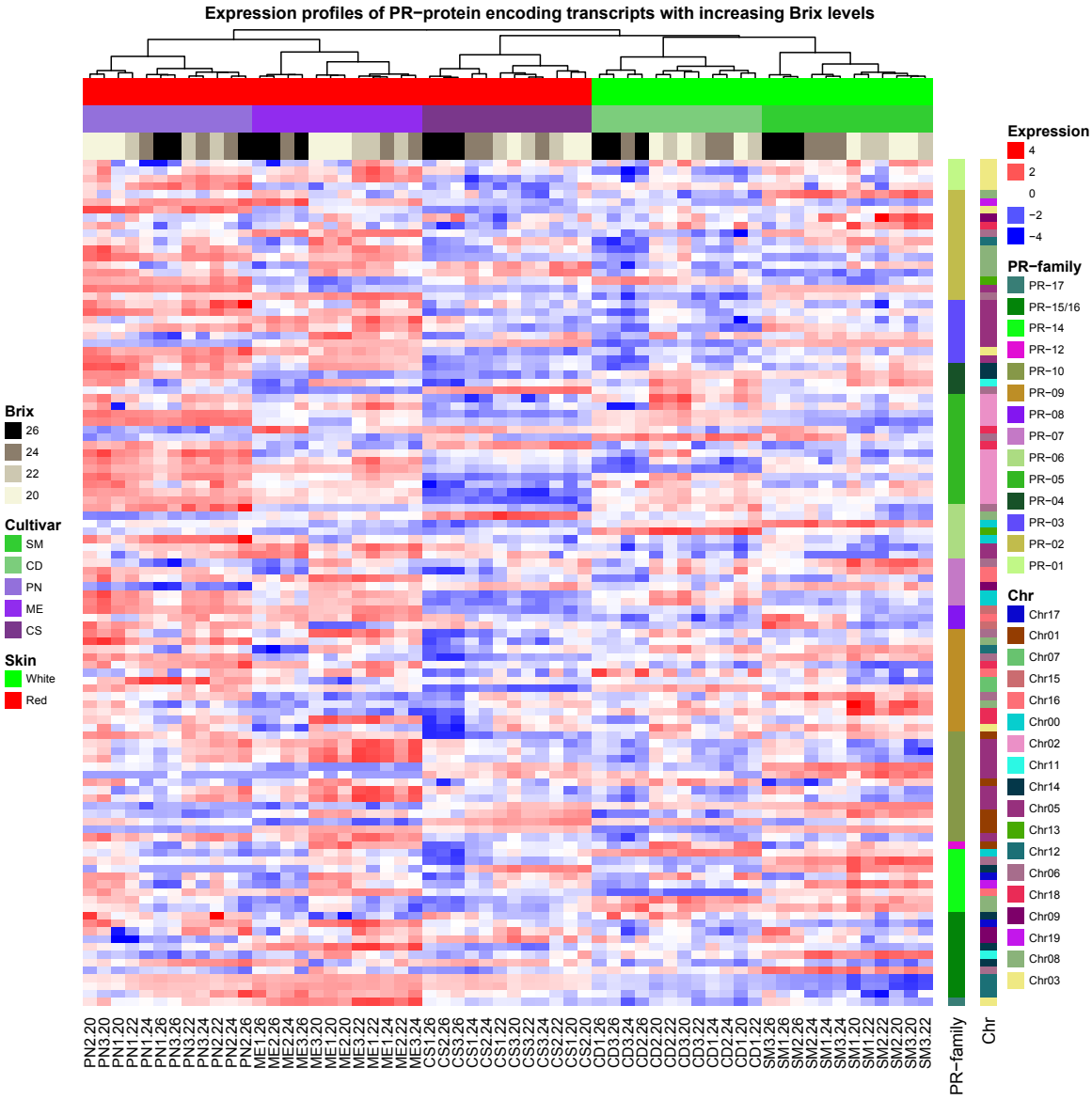

Figure 2. Transcript expression profiles from five grape cultivars were sampled under increasing ˚Brix levels (20 – 26). Each transcript was grouped by pathogenesis-related family membership, and experimental replicates were hierarchically clustered by Spearman correlation. ˚Brix level, cultivar and skin color annotations are visible along the top of the heat map. Legend abbreviations: chromosome membership (Chr), Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

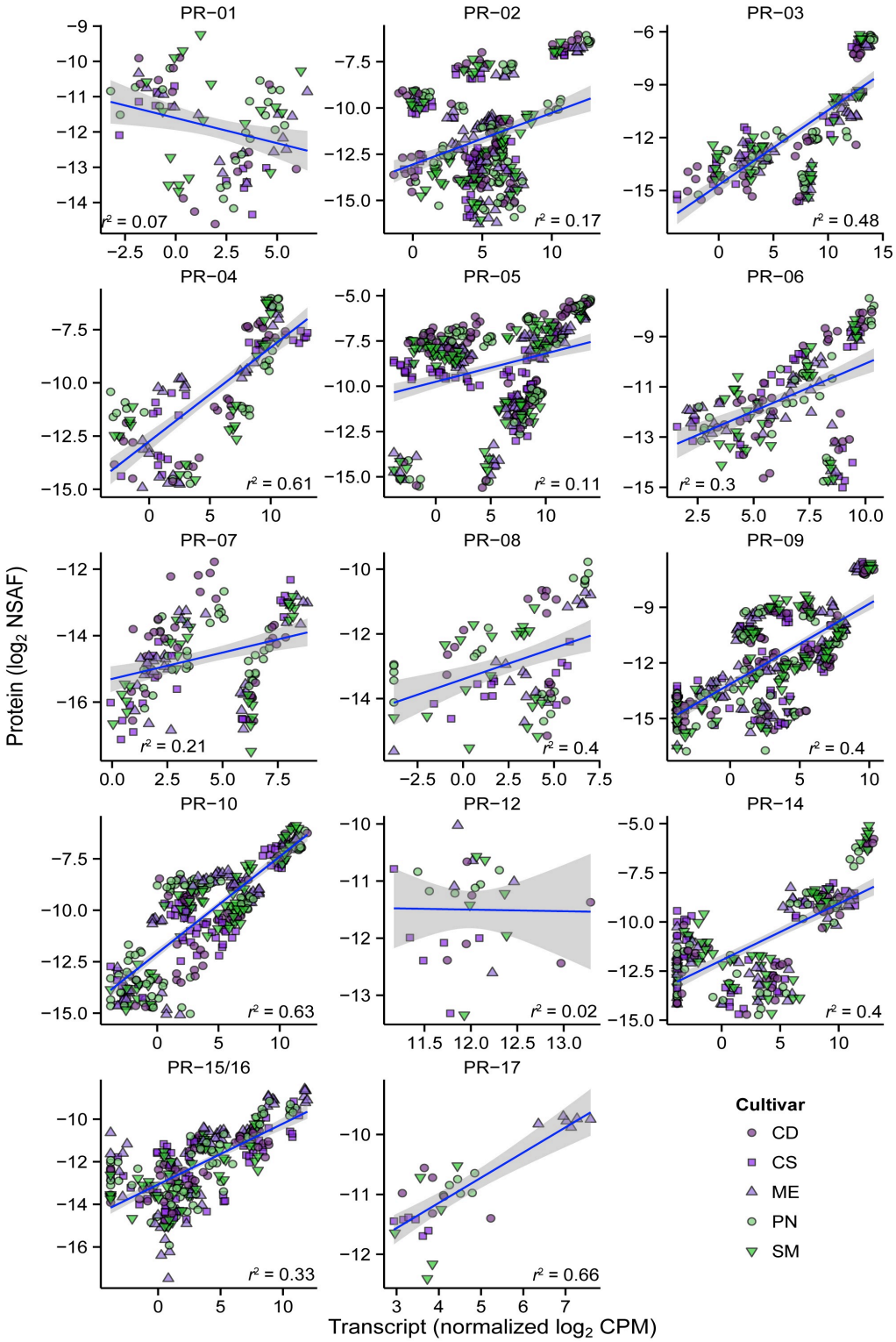

Figure 3. Correlations of proteins and transcript abundance for each family of pathogenesis related proteins. Coefficient of determination = r^2. Legend abbreviations: chromosome membership (Chr), Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

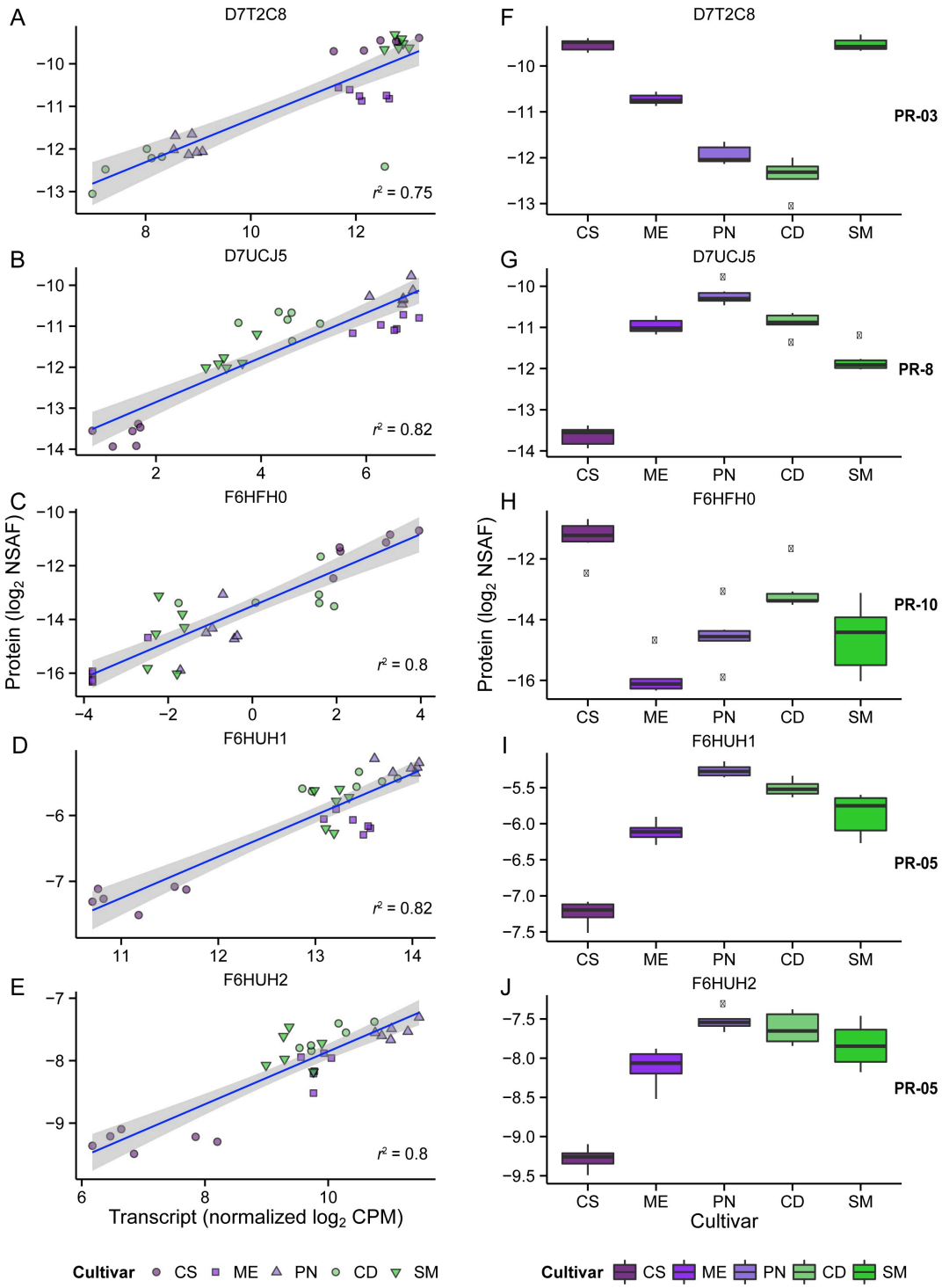

Figure 4. Individual correlations between (**A-E**) five of the highest correlated protein-transcript pairs, with corresponding (**F-J**) protein abundance distributions by cultivar, n=6. UniProt IDs D7T2C8 = endochitinase; D7UCJ5 = chitinase III; F6HFH0 = ribonuclease-like; F6HUH1 & F6HUH2 = thaumatins. Legend abbreviations: chromosome membership (Chr), Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD) and Semillon (SM).

CHAPTER 5

CONCLUSION

5.1 Summary of presented research and developed methods

Grapes (*Vitis vinifera* L.) are an ancient and economically important crop species (Martínez-Esteso, Sellés-Marchart et al. 2011). Several thousand cultivars are grown for a variety of commercial purposes, each defined by traits selected and maintained overtime. Our investigation of grape berry skins focused upon the late stages of ripening and at harvest. A series of assays were undertaken to better characterize this fruit species.

First, a systems biology approach integrating multiple high-throughput Omic datasets revealed complex biochemical variation amongst five cultivars. The phenotypic variation in the cultivars resulted in unique and dramatic differences in abundance in many of the most common classes of proteins and metabolites measured in berry skins. Only transcripts were sensitive enough to detect significant induced changes from the moderate water deficit treatment, although overall transcript abundance was poorly correlated with protein abundance. Omic analyses emphasized cultivar differences in phenylpropanoid biosynthesis and amino acid metabolism that influence winemaking, including color, astringency and yeast assimilable nitrogen levels. The information presented here exposes clear differences between the skins of mature berries of different cultivars, their responses to water deficit and the diversity of molecules that can impact wine quality.

Second, our data suggest a continued role for the transcriptional regulation of fruit ripening that involves several families of transcription factors, including C3H, MYB, AP2/ERF and bHLH. Data also support continued hormonal

control through late ripening that involve interplay between ABA, auxin, and ethylene. Curiously, a circadian clock signature for key clock components was observed that warrants further study. In addition, genes related to DNA methylation suggest that epigenetic programming may be involved in berry ripening at maturity. A key signal from the seed indicating seed maturity may play a role affecting berry ripening and senescence. Our results provide practical information for the grape and fruit communities at large for further research into late ripening processes.

Finally, data from our earlier studies were combined and leveraged to better characterize the highly abundant classes of pathogenesis-related proteins in the berry skins of each cultivar in the absence of pathogenic pressures. Many of identified proteins were classified as pathogenesis-related in berry skins, more so than what were previously observed in shoot tips. Several PR-families had numerous protein members in skins, which maybe a tissue specific occurrence. The transcript abundance was well correlated to the protein abundance in thaumatins of PR-05, but not so in the L-ascorbate peroxidases of PR-09. Haze-forming proteins, while well represented, did not accumulate with more specificity in the white cultivars and were mostly higher in the red cultivar, Pinot Noir. Large accumulations of PR-proteins in skins at harvest provide support for a prolonged and possibly constitutive defense mechanism that protects a maturing seed within the berry.

5.2 Future Research Directions

5.2.1 The problem of cross-hybridization of highly similar probes

Numerous *Vitis* gene families (e.g. stilbene and terpene synthases) contain multiple members with high sequence similarity (Vannozzi, Dry et al. 2012, Matarese, Cuzzola et al. 2014). During our analysis of several grape genome arrays, we discovered a peculiar bi-modal distribution of expression values likely caused by cross-hybridization of similar (and identical) probes on the array. The NimbleGen whole genome grape arrays (090918_Vitis_exp_HX12) are based on 29,971 gene annotations from the 12x V1 assembly of the grape genome. Probe selection was based on a scoring algorithm developed by NimbleGen to identify highly repetitive regions and then exclude them from probe selection. Thus, the -3' of each transcript was targeted with four unique oligo probes (60 nt in length). Our initial investigation aligned probes onto the genome as well as blasting them against the NCBI non-redundant database that identified approximately 13,000 genes with cross-hybridization potential. For example, a stilbene synthase (VIT_10s0042g00910) (Fig. 1a) had six probes with the potential to hybridize, making accurate quantification of this gene ambiguous. The grape arrays also contain duplicated probe set-sequences on multiple probes targeting different genes, such as VIT_16s0013g00950 (Fig. 1b & c). The probe cross-hybridization problem was briefly discussed in Chapter 2 and (Cramer, Ghan et al. 2014), but a more thorough analysis is required to adequately characterize the potential for similar genes to cross-hybridize on the commonly used array platform.

5.2.3 Future directions for data analysis

Weighted gene coexpression network analysis (WGCNA) is a way to globally model the systems' network based on the input data (e.g. gene expression or protein abundance data). WGCNA will extract subsets of the network that are connected with respect to the weighted correlation (Langfelder and Horvath 2008). The clustered outcomes can then be used to screen for functional similarity, generate new hypotheses, and screen for functional hubs, like the highly correlated pollen-specific modules in petunia (Broderick, Wijeratne et al. 2014). Gene networks have been used to successfully associate genes to biological processes and they demonstrate great potential to gain further insights into the functionality of genes (Broderick, Wijeratne et al. 2014, Korber, Bus et al. 2015). Each of the data sets produced during this project are suitable for further analysis by WGCNA (Langfelder and Horvath 2008, Zhao, Langfelder et al. 2010), further complementing our understanding of grape berries at harvest, and no additional costs would be incurred other than time for investigation. Even with the continuous advancements in biological models, it is still a challenge to assign recognized functions to specific genes.

5.3 Concluding Remarks

With the completion of the presented projects, new techniques were introduced into the Cramer lab. These include methodologies for analyzing high-throughput protein and sequencing data. The data analysis was able to leverage multiple data sets to examine snap shots of biochemical activity in mature harvested berry fruit. These analyses bring new insights into the similarities and

cultivar-specific responses of many of the most popular cultivars enjoyed by consumers, such as Cabernet Sauvignon, Chardonnay and Pinot Noir.

My time at the University of Nevada, Reno, has been incredible. I have learned an assortment of new skills, visited interesting countries, and collaborated with many excellent researchers in multiple fields. I feel extremely lucky to have attended university through the next-generation-sequencing era that has exploded in terms of research being conducted, offering a promising future of career possibilities.

Figures

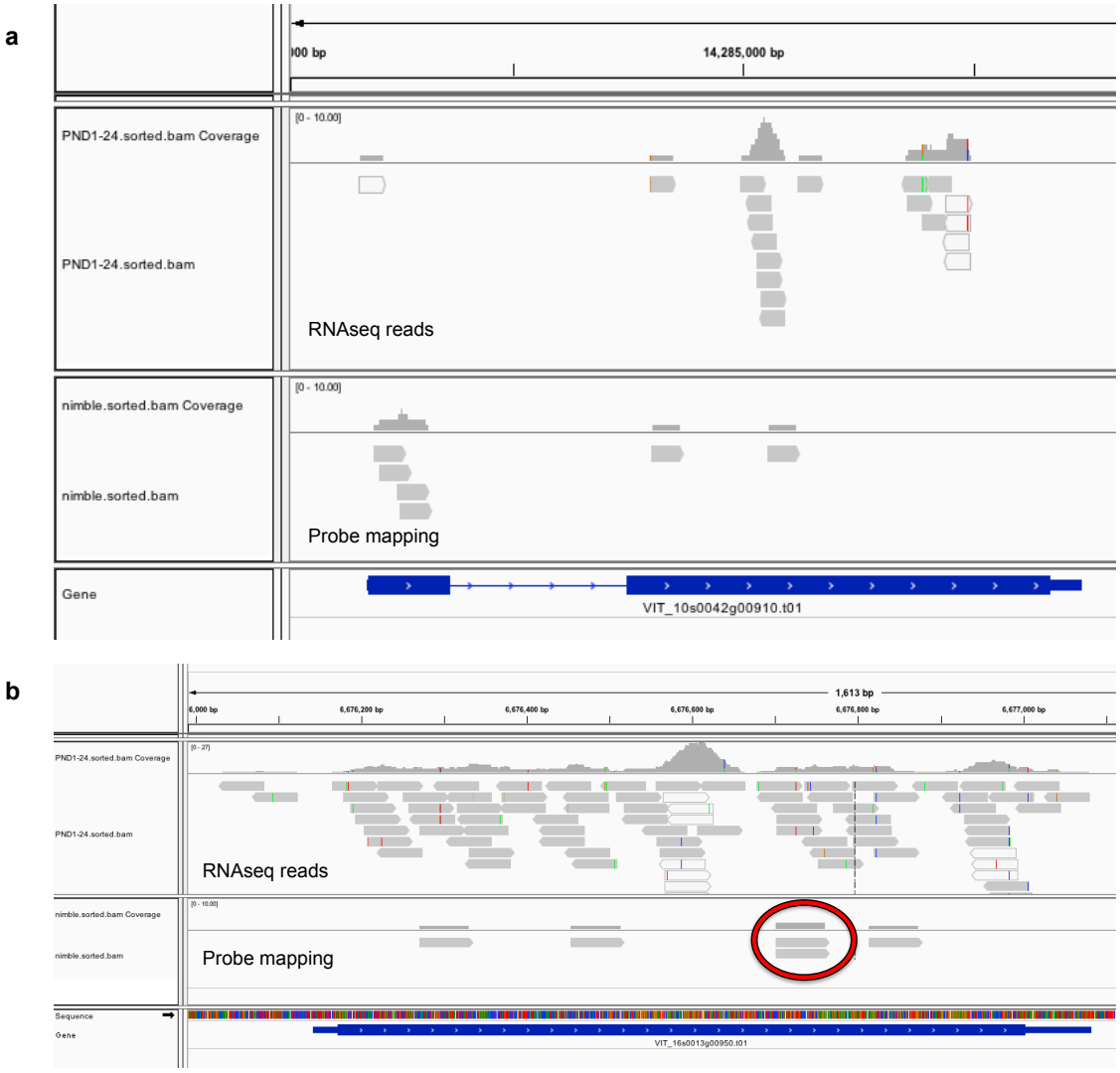

Figure 1. Examples of NimbleGen probe sequences mapped to (**a**) stilbene synthase (VIT_10s0042g00910) and an ethylene response factor (VIT_16s0013g00950). RNAseq 50 bp reads are also shown at the top of each panel. (**a**) Six probes were mapped to VIT_10s0042g00910. (**b**) Two probes mapped to VIT_16s0013g00950 (**c**) with identical oligo sequences, in VitusP00084947 (CHR16_JGVV13_72_T01) and VitusP00084956 (CHR16_JGVV13_74_T01).

APPENDICES

CHAPTER 2: CONCORDANT COMPARISONS OF FIVE GRAPEVINE (*VITIS VINIFERA* L.) CULTIVARS UNDER SEASONAL WATER DEFICIT USING FIVE OMIC ANALYSES

Supplemental File 1: Stem water potential measurements (MPa) for the North and South vineyards. Water potential measurements were averaged across cultivars, Cabernet Sauvignon and Chardonnay in the North and Merlot, Pinot Noir and Semillon in the South. Symbols represent mean ± SE; n = 6 (North) and 9 (South). WW = well watered, WD = water deficit.

Supplemental File 2: Annotation, protein spectral counts, Normalized Spectral Abundance Factor (NSAF) values and log2 transformed NSAF values for each replicate and protein identified, with '.count', '.NSAF', and '.NSAF.log2' suffixes respectively. Cultivar and treatment abbreviations for biological replicates: Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM) grown under well-watered (W) and water deficit (D) conditions, n=3.

Supplemental File 3: ANOVA results for the quantifiable (1,211) proteins (log2 NSAF) in five grape cultivars. Cultivar and treatment abbreviations for biological replicates: Cabernet Sauvignon (CD), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM) grown under well-watered (W) and water deficit (D) conditions, n=3.

Supplemental File 4: Annotation, transcript abundance values, and ANOVA results of all genes on the NimbleGen Whole-Genome microarray measured in five grape cultivars. Red highlighted rows identify the possibility of cross-hybridization of probes with other genes from Cramer *et al.* 2014. Cultivar and treatment abbreviations for biological replicates: Cabernet Sauvignon (CD), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM) grown under well-watered (W) and water deficit (D) conditions, n=5.

Supplemental File 5: Annotation, read counts, transcript normalized log2 counts per million (CPM) values, and edgeR statistical results of all genes with unique counts assigned from Illumina RNAseq, with '.count' and '.log2CPM' suffixes respectively. Cultivar and treatment abbreviations for biological replicates: Cabernet Sauvignon (CD), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM) grown under well-watered (W) and water deficit (D) conditions, n=3.

Supplemental File 6: Mean relative abundance values, M/Z, and results from the ANOVA for all primary and secondary metabolomic details for all metabolites (67) analyzed by GC-MS and (42) analyzed by LC-MS in five grape cultivars. Cultivar

and treatment abbreviations for biological replicates: Cabernet Sauvignon (CD), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM) grown under well-watered (W) and water deficit (D) conditions, n=6.

Supplemental File 7: BinGO results for overrepresented GO biological process functional categories for all quantifiable proteins (1,211).

Supplemental File 8: Correlations of protein and transcript abundance. Protein data are log2 NSAF values, n=3, RNAseq data are log2 normalized counts per million (CPM), n=3, and microarray data are log2 RMA values, n=5. Relationships of proteins with either RNAseq (CPM) or microarray (RMA) are indicated.

Supplemental File 9: The effect of water deficit upon the relative metabolic content of five anthocyanidins and their glycosylated, acetylated and coumaroylated moieties within the red cultivars. All metabolites were significant at the Cultivar level except malvidin 3-O-(6-p-coumaroyl)glucoside and petunidin 3-O-(6-acetyl)glucoside. Error bars represent mean ± SD n=6.

CHAPTER 3: ELUCIDATION OF A CORE SET OF GRAPE (*VITIS VINIFERA* L.) GENES DIFFERENTIALLY EXPRESSED IN THE LATE STAGES OF BERRY RIPENING

Supplemental File 1: Read counts uniquely mapped to the PN40024 grape genome.

Supplemental File 2: Log_2 counts per million of filtered and normalized read counts.

Supplemental File 3: Differential expression results from edgeR.

Supplemental File 4: A functional gene enrichment using BinGO. Specific enrichments are accessible under each excel tab.

Supplemental File 5: Membership of transcription factors significantly changed with ˚Brix.

CHAPTER 4: CHARACTERIZATION OF PATHOGENESIS-RELATED PROTEIN FAMILIES IN GRAPE BERRY SKINS AT HARVEST

Supplemental Figure 1: Protein abundances (log_2 normalized spectral abundance factor, NSAF) of each pathogenesis-related protein, separated into 14 different protein families, n=6. Cultivar abbreviations: Cabernet Sauvignon (CD), Merlot (ME), Pinot Noir (PN), Chardonnay (CD), and Semillon (SM).

Supplemental File 1: FASTA containing amino acid sequences queried within the GPM for peptide-to-spectrum matching.

Supplemental File 2: Count and NSAF data for identified pathogenesis-related protein.

Supplemental File 3: Classification of pathogenesis-related protein family domains, gene ontology (GO) and closest Arabidopsis ortholog(s).

Supplemental File 4: Correlations of individual protein and transcript pairs.

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