Identification of transcripts associated with postharvest withering in *Vitis vinifera* L. 'Cabernet Franc' grape berry

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Abstract

The withering of grape berries following harvest is known in Italian as *appassimento*, and it used as a tool to alter the composition of the berries prior to winemaking. Specific gene expression patterns were determined by RNA Sequencing in the red-skinned berries of *Vitis vinifera* L. 'Cabernet Franc' for three time points during withering, at both slow and fast rates, up to 40.7 and 31.2 percent cluster weight loss respectively, with a target of 29 $\pm$ 1 ^{o}Brix. Slow withering was associated with a higher number of differentially expressed genes, and those commonly up-regulated across all time points were associated with a higher number of gene ontology categories, including phenylpropanoid and cellular aromatic compound metabolism and biosynthesis. Commonly up-regulated genes across fast drying were only related to response to abiotic and biotic stimuli terms. Both slow and fast withering were associated with an up-regulation of numerous WRKY and MYB transcription factors, as well as stilbene synthase, phenylalanine ammonia lyase and trehalose-phosphate phosphatase. With respect to anthocyanin biosynthesis, there was no change in expression for *MYBA* genes, and UDP-glucose:flavonoid 3-O-glucosyltransferase was down-regulated transiently in fast withering and showed no change in expression in slow. Slow withering was also associated with an up-regulation of a dehydrin and the *CBF3* gene. The results of the present study suggest that fast withering may be associated with primarily an intense stress response, while slow withering may be associated with a senescence or over-ripening, in combination with a gradual adaptation to a less intense dehydration stress. As such, the influence of rate during withering will likely have a strong impact on the final berry composition, and thus will influence the characteristics of the resulting wines.

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4CL – 4-coumarate-CoA ligase

ABA – Abscisic acid

ACC oxidase – 1-aminocyclopropane-1-carboxylate oxidase

AFLP-TP – Amplified fragment length polymorphism-transcriptional profiling

ADH – Alcohol dehydrogenase

ANOVA – Analysis of variance

AP2/ERF – APETALA2 ethylene response factor

Borkh. – Moritz Balthasar Borkhausen

bp – Base pairs

CBF – C-repeat binding factor

cDNA – Complimentary deoxyribonucleic acid

CH4 – Cinnamate 4-hydroxylase

CHS – Chalcone synthase

COR – Cold regulated

CRT – C-repeat

DE – Differentially expressed

DNA – Deoxyribonucleic acid

DRE – Dehydration response element

DREB – Dehydration response element binding factor

E-L – Eichhorn-Lorenz

ERF – Ethylene response factor

FDR – False discovery rate

FLS – Flavonol synthase

FW – Fast withering

GO – Gene ontology

H – Harvest

Heynh. – Gustav Heynhold

L. – Carl Linneaus

L.H. Bailey – Liberty Hyde Bailey

LOX – Lipoxygenase

Mig. – Emil Friedrich August Walter Migula

Michx. – André Michaux

mRNA – Messenger RNA

NCBI – National Centre for Biotechnology Institute

NGS – Next-generation sequencing

ORF – Open reading frame

PAL – Phenylalanine ammonia lyase

PC1 – Principal component 1

PC2 – Principal component 2

PEAR – Paired-end read merger

Pers. – Christiaan Hendrik Persoon

qPCR – Quantitative polymerase chain reaction

RH – Relative humidity

RNA – Ribonucleic acid

RNA-Seq – RNA Sequencing

rRNA – Ribosomal RNA

ROS – Reactive oxygen species

RPC – Reusable plastic container

RT-PCR – Reverse transcription polymerase chain reaction

STAR – Spliced transcripts alignment to a reference

STS – Stilbene synthase

SW – Slow withering

S. Watson – Sereno Watson

TF – Transcription factor

Torr. – John Torrey

TSS – Total soluble solids

UFGT – UDP-glucose:flavonoid-3-O-glucosyltransferase

Chapter 1. Introduction and Literature Review

1.1 Appassimento wine

Wine produced from grapes which have undergone postharvest withering is referred to in Italian as *appassimento*. *Appassimento*-style wine production is based on a traditional technique of drying grapes to produce a rich premium wine or a fine sweet wine, which are commonly known as Amarone or Recioto respectively. Amarone wine is a high-quality, distinctive red wine with special sensory attributes, which is produced in the region of Valpolicella in north-eastern Italy, whereby slow drying traditionally occurs in barns under natural environmental conditions (Paronetto and Dellaglio, 2011). The withering process can last from 90 to 150 days (Accordini, 2013) and grapes are usually crushed when they have lost 35 to 40% of their original weight (Paronetto and Dellaglio, 2011; Tonutti *et al*., 2004). This moisture loss produces changes in the composition and the chemical-physical properties of the berries, which concentrates and develops aromatic compounds, sugars and polyphenols, and in turn affects the flavour and aroma of the resulting wine (Bellincontro *et al*., 2004; Bonghi *et al*., 2012; Paronetto and Dellaglio, 2011). Several cultivars are used in the production of Amarone, and these are required to include 'Corvina' and 'Rondinella' in specific proportions (Paronetto and Dellaglio, 2011).

Beyond Italy, the *appassimento* technique is also a wine-making tool of interest in cool climate regions such as Ontario, Canada, which experiences a relatively short growing season, especially in terms of red grape production. The climate in Ontario in the months after harvest make drying grapes in uncontrolled environments quite challenging, due to berry damage and loss from pests, animals and weather, as well as the development of fungal infection and disease. In recent years, various drying technologies have been

developed to assist with controlling postharvest withering through modulation of the environmental parameters, which in turn affects the rate of dehydration (Tonutti *et al*., 2004). Controlled withering creates the ability to maintain high quality grapes over a postharvest period of 100 days or more in regions such as Ontario, where it would otherwise be extremely difficult, and in Italy it reduces the risk associated with dependence solely on the climate. The use of drying technologies in *appassimento*-style wine production has also allowed for the ability to reduce the traditional 100-day withering period and to dry grapes rapidly over a matter of weeks.

Postharvest withering for *appassimento*-style wine production induces changes in the berry which are the result of water loss and concentration of metabolites, but are also due to the modulation of metabolism in response to dehydration stress (Pedreschi and Lurie, 2015; Tonutti and Bonghi, 2013). Typical characteristics of partially withered berries include increased sugar concentration, reduced titratable acidity, modified volatile and phenolic profiles, as well as an increased tartaric-to-malic acid ratio and decreased glucose-to-fructose ratio (Accordini, 2013; Bellincontro *et al*., 2004; Chkaiban *et al*., 2007; Rizzini *et al*., 2010; Tonutti and Bonghi, 2013).

1.2 Postharvest withering of grape berries

1.2.1 Ripening and senescence in grape

The process of senescence is the final phase of fruit ontogeny and consists of the initiation of a series of normally irreversible events beginning at the inception of ripening, which eventually lead to cellular breakdown and death (Sacher, 1973). Ripening is recognized as a senescent phase, and in fleshy fruit, ripening senescence is the dominant phase of ontogeny (Blackman and Parija, 1928). The development of the common grape

berry, *Vitis vinifera* L., follows a double sigmoid pattern of growth (**Figure 1.1**), with ripening initiated at the beginning of the second development phase (Coombe, 1995). The onset of ripening in grape is known as *véraison*, which is characterized by a reduction in titratable acidity, an increase in sugar accumulation, softening and enlargement of berries, accumulation of flavour compounds and the initiation of anthocyanin production (Chervin *et al*., 2004, Coombe, 1995; Robinson and Davies, 2000).

Ripening in fruit is viewed as a dynamic process involving structural, biochemical and physiological changes, which eventually lead to the production of an edible fruit and then continue beyond this point to end in cell death (Tonutti and Bonghi, 2013). There is an abundance of information available on the physiological, molecular and biochemical changes associated with grape berry ripening up to the point of optimal edible composition. However, less biochemical and molecular information exists regarding those phases of over-ripening and senescence (Tonutti and Bonghi, 2013). These phases in grapes can be studied both on-the-vine, or investigated in clusters following harvest.

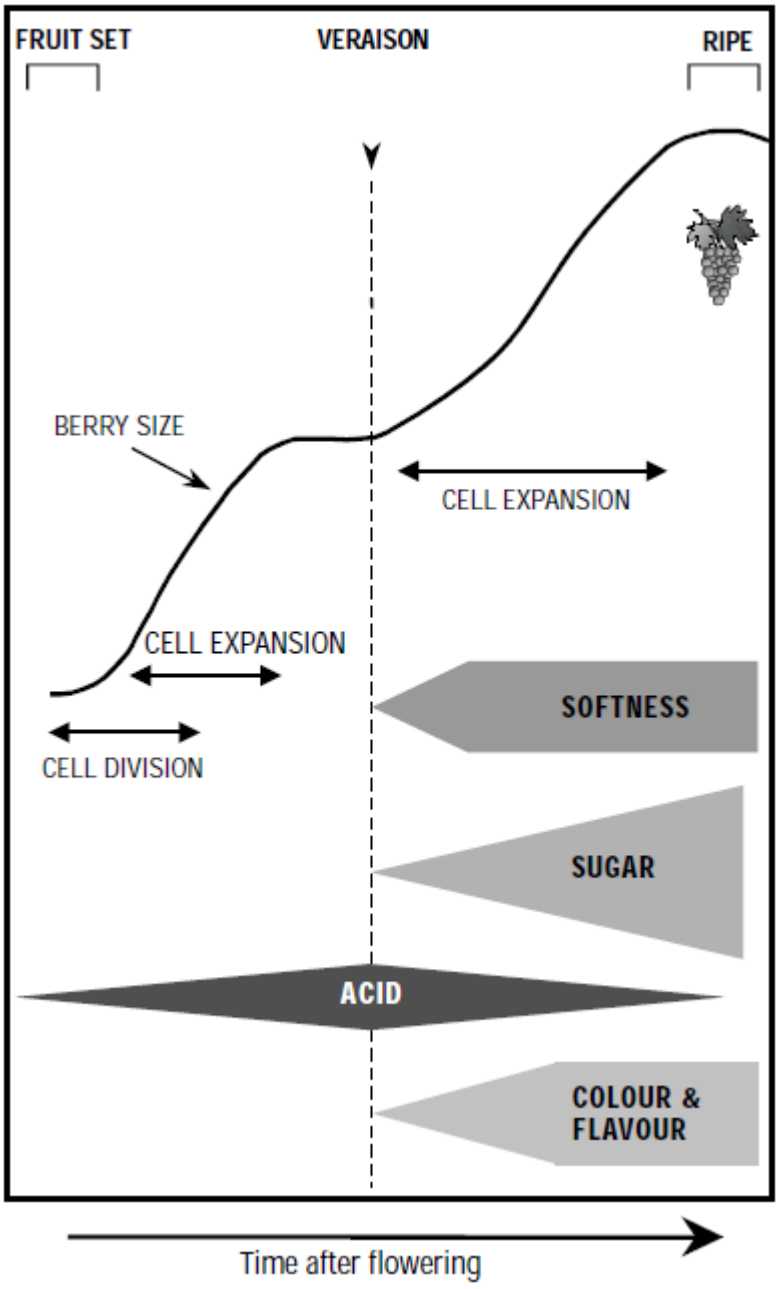

Figure 1.1 Schematic representation of grape berry development. The curve indicates changes in berry size. The inception of ripening (*véraison*) is shown by the vertical dashed line. Softening, sugar accumulation, metabolism of organic acids and synthesis of colour and flavour compounds all occur after *véraison* (Robinson and Davies, 2000).

After crops are severed from the plant at harvest they are still alive and respiring, and grapes, like all perishable crops, continue to metabolize actively until cell death occurs, reacting to environmental stimuli and stresses, which in turn affects their composition (Grierson, 2002; Pedreschi and Lurie, 2015; Rizzini et al., 2009; Tonutti and Bonghi, 2013). The metabolism of detached grapes will be different from the metabolism of berries on-the-vine, which is an important factor in *appassimento* withering. This difference in metabolism is due to both the lack of attachment to a supply of water, minerals and energy from the vine's vasculature, and also as a result of the specific environmental conditions and stresses to which the berries are exposed during the postharvest period (Pedreschi and Lurie, 2015; Tonutti and Bonghi, 2013).

1.2.2 Postharvest dehydration stress

Once grape clusters are detached from the mother plant they can be exposed to a range of stresses including dehydration (Pedreschi and Lurie, 2015). Postharvest water stress is greater than water stress experienced on-plant, as the harvested crop is dependent solely on its internal water supply, and cells cannot overcome this stress through vascular uptake (Cirilli *et al*., 2012; Pedreschi and Lurie, 2015; Zamboni *et al*., 2008). Postharvest grapes are also affected by the rate and intensity of water loss, which increases ethylene production and respiration, induces cell wall enzyme activity, and impacts polyphenol and volatile levels (Bellincontro *et al*., 2004; Costantini *et al*., 2006). The extent and rate of dehydration in the grapes during withering is influenced by the specific postharvest conditions (Chkaiban *et al*., 2007).

As water stress increases in perishable fruits, changes in metabolism and respiration occur, as well as loss of cellular turgor pressure and accumulation of solutes (Hsaio, 1973).

In order to cope with abiotic stresses such as drought, plants and their harvested products react by the induction or repression of specific stress-responsive genes, which in turn affect metabolic processes (Pedreschi and Lurie, 2015; Xiao *et al*., 2006). Abiotic stress in plants generates a number of common responses including the accumulation of "compatible" metabolites such as trehalose, sorbitol, mannitol and the amino acid proline, which protect them against water loss. In addition, the biosynthesis of secondary metabolites (polyphenols) that function as stress protectants occurs and scavenging enzymes for reactive oxygen species (ROS) are accumulated (Pedreschi and Lurie, 2015). Furthermore, drought, heat and cold stress in plants also share similar response features, in that they all have a common dehydration element. The dehydration response specifically includes: accumulation of compatible osmoprotectants such as proline, sugars and sugar-alcohols; changes in carbon metabolism and glycolysis; accumulation of anthocyanin and phenylpropanoid metabolites; induction of C-repeat binding factor (CBF) transcription factors which are involved in regulation of cold acclimation gene expression; and accumulation of ROS scavenging enzymes (Pedreschi and Lurie, 2015).

Postharvest water stress is often exploited in grapes in order to alter their physical or biochemical structure for the purposes of generating completely dehydrated berries (raisin production), or to create partially withered berries used in the production of various wines, such as the previously described *appassimento*-style wines (Bellincontro *et al*., 2004; Rizzini *et al*., 2009). There is limited information with respect to the effect of water stress on the metabolism, biochemistry and physiology of grape berries after harvest (Rizzini *et al*., 2009), and very little is known in any fruit about the effects of the postharvest stage and associated stresses specifically at the molecular level (Zamboni *et*

al., 2008). Postharvest studies in withering grapes have shown that there is an active modulation of metabolic pathways, including stress-related responses and secondary metabolism, and have also demonstrated that berries are still metabolically and transcriptionally active at advanced stages of dehydration (Di Carli *et al*., 2011; Versari *et al*., 2001).

1.2.3 The grape berry withering response and associated factors

The response of grapes to postharvest withering is determined by both exogenous and endogenous factors; that is, the drying conditions, as well as the physical and biochemical properties of the berries themselves (Mencarelli and Bellincontro, 2013). The main environmental conditions which are considered during drying are the air flow rate, the temperature and the relative humidity (RH), which have been shown to affect berry metabolism (Bellincontro *et al*., 2004; Mencarelli and Bellincontro, 2013). The behaviour of different grape varieties during drying depends on the berry's specific characteristics, including their surface to volume ratio, skin thickness, micropore abundance, presence of cracks and cuticle thickness (Mencarelli and Bellincontro, 2013).

An initial stress response during postharvest withering was associated with a shift from aerobic to anaerobic metabolism when grapes sustained a 10 to 15% cluster weight loss (Bellincontro *et al*., 2004). This initial response was confirmed by Costantini *et al*. (2006), who additionally observed increases in lipoxygenase (LOX) activity, as well as in concentrations of abscisic acid (ABA) and proline. ABA is a plant hormone involved in numerous processes including modulation of water stress (Ferrandino and Lovisolo, 2014), and proline can function as a osmoprotectant during dehydration (Penna *et al*., 2006). LOX is an oxidative enzyme involved in cell protection, and during dehydration its activation

may be due to the presence of ABA and/or the modification of cell structure as a result of turgor loss (Costantini *et al*., 2006). Costantini *et al*. (2006) also observed a second metabolic stress response at greater than 19.5% weight loss, which was associated with an increase in total protein and proline content. In addition, the researchers observed an increase in alcohol dehydrogenase (ADH) activity and ethyl acetate content. The first 5 to 10% cluster weight loss does not apparently affect the main metabolism of the berry, as it is primarily due to water loss from the rachis (stems and cluster branches), which creates a pressure differential and draws a limited amount of water from the berry, a process which continues until the rachis is completely dry (Centioni *et al*., 2014; Cirilli *et al*., 2012; Mencarelli and Bellincontro, 2013). The rachis is highly permeable due to the presence of numerous stomata and lenticels, while the berry does not readily lose moisture, as it has no stomata and is covered in a thick waxy cuticle layer. Once the rachis is dry, cluster water loss is due solely to transpiration from the berries which begin to experience dehydration stress in response to the environmental conditions (Cirilli *et al*., 2012).

The rate and intensity of the dehydration process both play an important role in the postharvest stress response of perishables (Kays, 1997), as well as the resulting properties of the grape berries, with consequences to the quality of the wines (Tonutti *et al*., 2004). Since grapes are alive following harvest, their metabolism during withering will impact the compounds they produce, which in turn contributes to the wine quality. As a result, if the drying process is too fast, there is no opportunity for further development of compounds and aromas (Mencarelli and Bellincontro, 2013). Such is the case for raisins, where the goal is only a concentration of sugars, and achieving an aromatic product is not a concern, thus a very rapid and high temperature drying process is employed. However, if the goal is

to produce a grape with a composition that is suitable for the production of a high-quality wine, it is imperative to carefully manage the rate and intensity of the drying parameters, as both have an effect on the withering process and subsequently impact the metabolism and composition of the berry (Barbanti *et al*., 2008; Mencarelli and Bellincontro, 2013; Rolle *et al*., 2013).

Temperature is a key factor modulating both the speed of grape dehydration as well as their metabolism, with higher respiration rates observed in berries withered at 20 ^{o}C as compared to 10 ^{o}C (Bellincontro *et al*., 2009). The rate of dehydration affected the range of volatiles produced by the berries (Bellincontro *et al*., 2004), as well as the total phenol and anthocyanin content (Bellincontro *et al*., 2004; Bellincontro *et al*., 2009). Lower temperatures (10 ^{o}C or under) required more drying time while generating less oxidation, maintaining varietal volatiles (Cirilli *et al*., 2012; Mencarelli and Bellincontro, 2013) and resulted in improved berry structure during the withering process (Bellincontro *et al*., 2009). Maintenance of the cellular structure allows for water to move slowly from cell to cell, decreasing water stress and allowing for a slower production of important secondary metabolites such as volatiles and polyphenols, along with slower anaerobic fermentation, which delays the production of metabolites such as ethanol, acetaldehyde and acetic acid (Crilli *et al*., 2012; Mencarelli and Bellincontro, 2013).

Air speed also affects berries during dehydration, with higher ventilation (2.5 m/sec) at 10 ^{o}C associated with an increase in respiration and drying rate, as compared to lower ventilation (1.5 m/sec) at the same temperature (Bellincontro *et al*., 2009). In addition, RH is an important factor to consider in the drying process. As expected,

decreasing the RH during postharvest withering of grapes has been demonstrated by Barbanti *et al*. (2008) to increase the rate of dehydration.

1.3 Molecular changes during postharvest withering

Only a small number of studies have investigated the response of grape berries to postharvest withering at the gene expression level. Changes in metabolism during postharvest withering in grape were initially demonstrated to be controlled at the gene level by Versari *et al*. (2001). Stilbene synthase (STS) catalyzes the biosynthesis of resveratrol (*trans*-3,5,4'-trihydroxystilbene) in grape, and Versari *et al*. (2001) investigated the accumulation of STS messenger RNA (mRNA) in 'Corvina' berries. The researchers employed a Northern blot analysis to demonstrate an up-regulation of the *STS* gene, accompanied by an accumulation of resveratrol during the dehydration process. A similar observation was made by Cirilli *et al*. (2012), who demonstrated that sensitivity to dehydration stress occurred at the gene level for ADH, before evidence of other physiological effects became apparent. The researchers investigated ADH activity over time, along with its gene expression through the use of quantitative polymerase chain reaction (qPCR) analysis. They demonstrated an up-regulation of the *VvAdh2* gene prior to a corresponding increase in ADH activity.

The use of transcriptome analysis allows for the study of the expression behaviour and transcript accumulation of a large number of genes, and over the past decade, five separate transcriptome analysis studies have been reported during postharvest withering of grape berries. These analyses have employed both a microarray (Fasoli *et al*., 2012; Rizzini *et al*., 2009; Zamboni *et al*., 2010; Zoccatelli *et al*., 2013) and amplified fragment length polymorphism-transcriptional profiling (AFLP-TP) approach (Zamboni *et al*., 2008). The

studies were conducted using ‘Corvina’, ‘Sangiovese’ and ‘Oseleta’ berries (Zoccatelli *et al*., 2013) and de-seeded ‘Corvina’ grape berries which were withered in uncontrolled natural environmental conditions (Fasoli *et al*., 2012; Zamboni *et al*., 2010; Zamboni *et al*., 2008), as well as using the skins of ‘Raboso Piave’ berries in a comparison of both controlled fast withering and uncontrolled slow natural withering conditions (Rizzini *et al*., 2009).

Transcriptome analysis of berries during postharvest dehydration has revealed a unique withering response, involving the modulation of specific genes during the process that are not modulated during on-plant drying (Zamboni *et al*., 2008). In addition, the rate and intensity of postharvest withering has been shown to affect the distribution of expressed genes within functional categories. Furthermore, an increased intensity of water loss from 10 to 30% cluster weight has demonstrated a greater number of differentially expressed probes, indicating that grape berries are still transcriptionally active, even at high levels of dehydration (Rizzini *et al*., 2009). Common genes characterizing postharvest withering in grapes include those involved in abiotic and biotic stress responses, transport, carbohydrate and secondary (phenolic) metabolism, regulation of transcription (transcription factors) and ethylene metabolism (Bonghi *et al*., 2012; Cirilli *et al*., 2012; Mencarelli *et al*., 2010; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Tonutti and Bonghi, 2013; Versari *et al*., 2001; Zamboni *et al*., 2008).

1.4 Functional and regulatory withering stress responses

Plants react to abiotic stresses such as drought and cold temperature by the induction or repression of specific stress-responsive genes, which in turn affect various developmental, physiological and biochemical processes (Xiao *et al*., 2006). Stress-

responsive genes produce proteins which can have a functional role, such as antifreeze and water channel proteins, or those that alter the levels of various metabolites such as proline, sugars and sugar alcohols. Stress-responsive genes can also produce proteins with a regulatory role (Akhtar *et al*., 2012), which includes a network of transcription factors (TFs) that control a cascade of genes involved with stress modulation in plants (Mizoi *et al*., 2012). Transcription factors are able to bind to specific elements in promoter regions of target genes and in doing so they regulate their expression.

1.4.1 CBF transcription factors

Although grape has been considered a non-climacteric fruit, meaning that ripening is independent of the plant hormone ethylene, it has been demonstrated in recent years that a weak increase in the production of ethylene is experienced by berries at the onset of ripening (Chervin *et al*., 2004). This peak in ethylene production is coupled with an increase in the activity and transcript accumulation of the enzyme responsible for the last step of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (Chervin *et al*., 2004). In addition to its role in ripening, ethylene is associated with stress responses, and an increase in the amount of mRNA from genes encoding ethylene biosynthesis and response functions was seen in the berries of vines subjected to water-deficit conditions, including ACC oxidase (Grimplet *et al*., 2007). An up-regulation of ACC oxidase, independent of the rate and intensity of dehydration (Rizzini *et al*., 2009), and also an up-regulation of *S*-adenosyl methionine synthase, which is involved in ethylene biosynthesis (Zamboni *et al*., 2008), has been shown during withering in grape berries, indicating that ethylene may be involved in the postharvest water stress response.

The role of ethylene in response to stress is likely regulated by transcription factors, such as those in the APETALA2 ethylene response factor (AP2/ERF) family, which share a conserved DNA-binding domain (Yin *et al*., 2012; Zhuang *et al*., 2009). There are five subfamilies of AP2/ERF, including the dehydration response element binding factor (DREB) subfamily and the ethylene response factor (ERF) subfamily. *ERF* genes have only been studied in a few species of plants and almost all reported stress-related investigations have been performed on vegetative tissues. A recent study was conducted using harvested kiwifruit that were subjected to low and high temperature, high CO_2 and high water loss conditions, and it was found that *ERF* genes were important for modulating the postharvest stress response (Yin *et al*., 2012). C-repeat binding factor/DREB1 (CBF/DREB1) proteins are members of the DREB subfamily and are collectively referred to as CBF proteins; these transcription factors bind to C-repeat (CRT) or dehydration-responsive elements (DRE) to activate cold regulated (*COR*) genes in the CBF pathway, whose expression convey tolerance to low temperatures and drought (Siddiqua and Nassuth, 2011; Xiao *et al*., 2006). A CBF encoding gene was originally discovered in *Arabidopsis thaliana* (L.) Heynh., with a total of six now identified in this plant; CBF proteins have since been found in a large number of different plants (Akhtar *et al*., 2012; Siddiqua and Nassuth, 2011).

Four *CBF* genes, *CBF 1-4* have been identified in the temperature-sensitive *Vitis vinifera*, as well as in the wild, freeze-tolerant *Vitis riparia* Michx. (Xiao *et al*., 2006; Xiao *et al*., 2008). Transcripts of *VvCBF1*, *2* and *3* were shown to accumulate to relatively high levels in detached young *V. vinifera* 'Chardonnay' leaves under drought stress within 15 minutes after exposure, and these genes were also induced after exposure to low

temperature (4 ^{o}C) treatment in both *V. vinifera* and *V. riparia* young leaves (Xiao *et al*., 2006). Although no *CBF* genes have been reported thus far to be modulated in grape berries during postharvest withering, the up-regulation of two ethylene biosynthesis genes during drying, along with the accumulation of *CBF* gene transcripts in response to drought in grape leaves suggests that this could be an area worthy of investigation.

1.4.2 WRKY transcription factors

Members of the WRKY transcription factor family are part of a large group of regulatory proteins which show high binding affinity to the W-box DNA promotor sequence, and are primarily involved in biotic and abiotic stress tolerance in plants (Pandey and Somssich, 2009; Ülker and Somssich, 2004). These transcription factors all share the WRKY DNA-binding domain, comprising a superfamily with 74 members in *Arabidopsis* and 59 in *V. vinifera* (Wang *et al*., 2015).

Postharvest withering in grape berries has induced the up-regulation of a transcript which shows homology to the WRKY6 transcription factor from *Nicotiana attenuata* Torr. ex S.Watson (Zamboni *et al*., 2008). In numerous plant species, *WRKY* genes show rapid and strong up-regulation in response to wounding, drought, hot and cold temperature, as well as to biotic stress (Ülker and Somssich, 2004). The accumulation of transcripts for a putative WRKY transcription factor during appassimento drying indicates that these proteins may have a role in the postharvest withering stress response in grape berry (Zamboni *et al*., 2008).

1.4.3 MYB transcription factors

MYB transcription factors share the MYB binding domain and have been found to accumulate in plants in response to environmental stimuli, such as light and salt stress

(Martin and Paz-Ares, 1997). There are estimated to be over 100 MYB transcription factors in *Arabidopsis* and some of these proteins are also known to be involved in the control of phenylpropanoid metabolism (Martin and Paz-Ares, 1997).

Many phenylpropanoid compounds play a role in abiotic and biotic stress tolerance, such as the flavonoids and stilbenes. In both tobacco and *Antirrhinum majus* L., commonly known as snapdragon, the MYB305 protein activated the gene for phenylalanine ammonia lyase (PAL), which encodes for the enzyme responsible for the first step of the phenylpropanoid biosynthetic pathway (Martin and Paz-Ares, 1997). Transcriptome profiling of grape berries during postharvest withering identified homologues to apple MYBR2 (Zamboni *et al*., 2008) and to *Vitis labruscana* L.H. Bailey MYBB (Bonghi *et al*., 2012) transcription factors, which were both up-regulated and might be linked to changes in phenylpropanoid metabolism during drying. In contrast, the down-regulation of a *MybA* transcription factor during postharvest withering has also been observed (Bonghi *et al*., 2012; Rizzini *et al*., 2009). MYBA is responsible for the control of anthocyanin biosynthesis and its repression during drying is opposite to what has been observed in berries from vines that have experienced water-deficit conditions (Castellarin *et al*., 2007; Grimplet *et al*., 2007), thus suggesting that anthocyanin biosynthesis is affected in different ways by each process.

1.4.4 Phenylpropanoid metabolism

Phenylpropanoid metabolism is a type of secondary metabolism in plants involving changes to compounds which are initially derived from phenylalanine, including simple phenols, stilbenes and flavonoids (**Figure 1.2**). Anthocyanins and flavonols belong to the group of phenolic compounds called the flavonoids. The concentration and ratios of

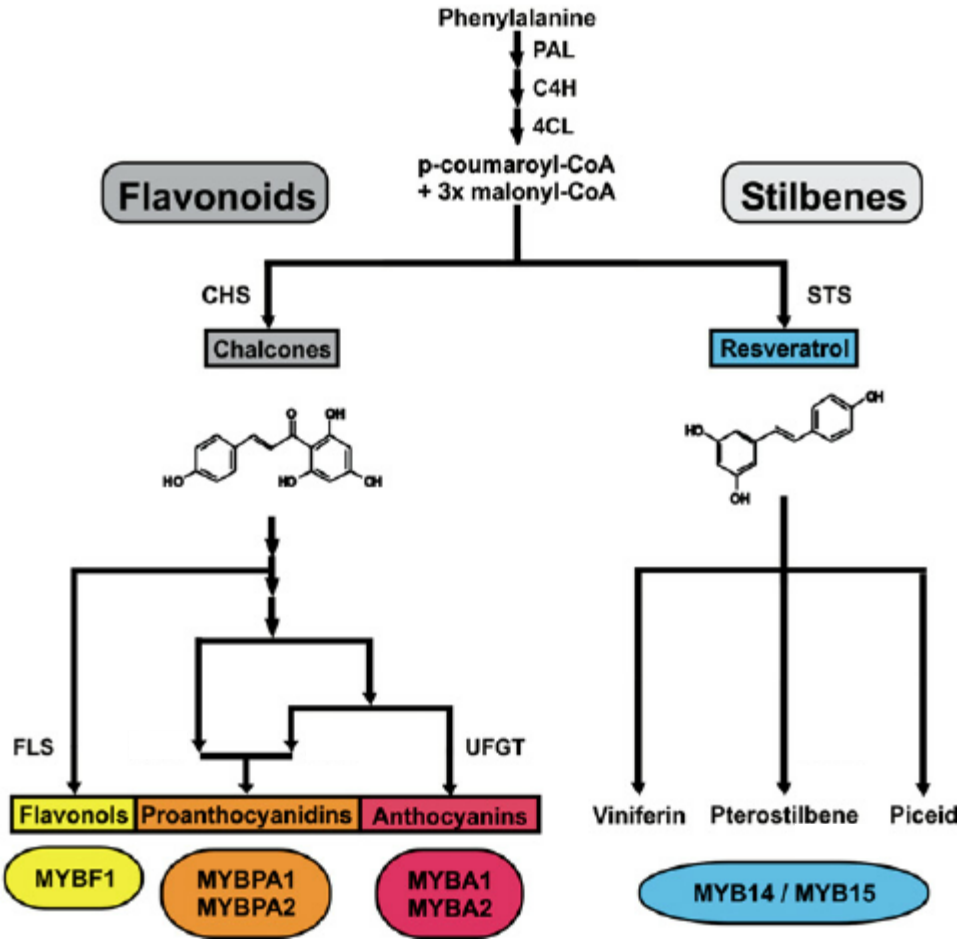

Figure 1.2 Simplified representation of the grapevine phenylpropanoid biosynthetic pathway leading to the production of flavonoids and stilbenes. Transcriptional regulation of the general enzymes is conducted by MYB transcription factors including MYBF1, MYBPA1, MYBPA2, MYBA1, MYBA2, MYB14 and MYB15. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; FLS, flavonol synthase; UFGT, UDP-glucose:flavonoid-3-O-glucosyltransferase; STS, stilbene synthase (Höll *et al*., 2013).

different phenolic compounds are important in determining the quality of wine, with the colour of red wines due to the presence of anthocyanin pigments, and astringent and bitter properties mainly a result of flavan-3-ols and flavonols (Corradini and Nicoletti, 2013; Moreno *et al*., 2008). Beyond their role in wine quality, phenolics are also involved in plant defense. Anthocyanins and flavonols have antioxidant capabilities, and it is suggested that they may also be able to absorb ultraviolet light, in addition to various other protective roles (Chalker-Scott, 1999; Martin and Paz-Ares, 1997). Numerous flavonoids, along with other phenylpropanoids such as stilbenes, are involved in abiotic and biotic stress responses (Martin and Paz-Ares, 1997).

The literature contains conflicting views regarding changes in concentrations of various phenolic compounds during postharvest withering, which is likely due to the variability in drying conditions, rate and intensity of dehydration, as well as type of tissue and grape variety used (Tonutti and Bonghi, 2013). With respect to molecular changes in phenylpropanoid metabolism during drying, an increase in transcripts coding for phenylalanine ammonia lyase (PAL) has been shown in both fast and slow drying withering treatments (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Zamboni *et al*., 2008). PAL represents the first step in the phenylpropanoid pathway (**Figure 1.2**) and this protein is encoded by a multigene family. In addition to an up-regulation of some *PAL* gene members during withering, Bonghi *et al*. (2012) also observed a selective down-regulation of two other *PAL* genes across different drying conditions. The increase in transcripts of *PAL* observed during postharvest drying (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Zamboni *et al*., 2008) is similar to what has been observed in the field following water-deficit (Grimplet *et al*., 2007). The rate of drying has a notable effect on

the expression pattern of *PAL*, which was demonstrated by Tonutti *et al*. (2004) in 'Corvina' grape berries, where an increase in transcripts in fast dry samples was only detected at the end of the withering period at 42% cluster water loss, while slow dry samples showed accumulation beginning at 14% water loss.

The postharvest withering process appears to induce a general activation of the phenylpropanoid pathway due to an increase in *PAL* transcripts. However, gene expression for pathways involved in the biosynthesis of various classes of phenylpropanoids appears to be affected differently. Using Northern Blot analysis, transcripts encoding for chalcone synthase (CHS), the first committed step in flavonoid biosynthesis, were reported to remain at levels similar to harvest in 'Corvina' berries throughout withering in both fast and slow drying conditions (Tonutti *et al*., 2004). However, two *CHS* genes were down-regulated in skins of 'Raboso Piave' berries during both slow and rapid rates of postharvest withering at 30% cluster weight loss (Bonghi *et al*., 2012). The down-regulation of *CHS* genes suggests a general suppression of the flavonoid pathway, however one category of flavonoids, the flavonols, appears to be positively affected by the withering process (Tonutti and Bonghi, 2013). In skins of 'Raboso Piave' berries dehydrated up to 30% cluster weight loss, flavonol synthase (*FLS*) was induced, coupled with an increase in the concentration of the flavonol quercetin (Bonghi *et al*., 2012). Flavonols have the ability to reduce ROS in oxidative stress conditions including desiccation, which may explain their increase and role during postharvest withering (Rothschild and Mancinelli, 2001).

Structural genes involved in the anthocyanin biosynthetic pathway were also investigated during postharvest withering by Bonghi *et al*. (2012), and they were found to either undergo no changes in expression or to be down-regulated. Such was the case for

UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) which regulates anthocyanin biosynthesis, as no change in the relative level of gene expression (Tonutti *et al*., 2004) or in transcript accumulation were observed (Bonghi *et al*., 2012). These observations are in contrast with the up-regulation of genes involved in anthocyanin production which has been observed in field cultivated grapes exposed to water-deficit (Castellarin *et al*., 2007; Grimplet *et al*., 2007). The literature offers conflicting information regarding the changes in concentration of anthocyanins in withered berries, with an increase found in 'Corvina' (Toffali *et al*., 2011) and in 'Sangiovese' (Bellincontro *et al*., 2004), a decrease in 'Cesanese' (Bellincontro *et al*., 2009), along with no significant changes reported in 'Pinot Noir' (Moreno *et al*., 2008) and 'Raboso Piave' (Bonghi *et al*., 2012) grapes. These discrepancies are likely to be a result of differences in experimental conditions and the intensity of dehydration, as well as genotypic variability between cultivars (Antelmi *et al*., 2010; Tonutti and Bonghi, 2013).

Stilbenes, such as resveratrol, have been shown to inhibit ROS production (Gresele *et al*., 2008) and represent another group of grape phenolpropanoids induced by biotic and abiotic stresses (Soleas *et al*., 1997). As previously discussed, an up-regulation in *STS* gene expression during withering has been demonstrated by Versari *et al*. (2001), and this observation has since been confirmed by Tonutti *et al*. (2004), Zamboni *et al*. (2008), Mencarelli *et al*. (2010) and Bonghi *et al*. (2012). However, this response appears to be modulated by drying temperature, with a down-regulation of *STS* observed by Mencarelli *et al*. (2010) at 30 ^{o}C conditions. Accumulation has been observed to begin at 7% cluster weight loss in slow dried samples, however no change in *STS* transcript abundance was detected until 17% water loss in fast dry conditions (Tonutti *et al*., 2004). The up-regulation

of *STS* which has been observed during drying, coupled with an increase in resveratrol concentration, suggests that resveratrol may play a role in modulating the postharvest stress response.

1.4.5 Dehydrins

The most common proteins that accumulate in plants in response to water or cold temperature stress are dehydrins (Close, 1997). Dehydrins comprise a large group of hydrophilic proteins found in a number of organisms including all higher plant species examined (Artlip and Wisnieski, 2002; Rorat, 2006). In *Arabidopsis,* six genes have been characterized (Puhakainen *et al*., 2004) and two similar dehydrin genes have been identified in both *Vitis vinifera* and *Vitis riparia* (Xiao and Nassuth, 2006). Dehydrins are believed to play a key role in promoting dehydration stress tolerance, although their exact mode of action is not understood. They are thought to prevent structural damage by stabilizing membranes and macromolecules, by maintaining essential activities of enzymes and by acting as scavengers of ROS (Close, 1997; Rorat, 2006; Xiao and Nassuth, 2006).

The induction of *DHN1a*, encoding dehydrin 1a, in vegetative tissues of *V. vinifera* and *V. riparia* has been observed in response to drought, cold temperature or ABA treatments (Xiao and Nassuth, 2006). An increase in *DHN1a* transcripts in *V. vinifera* has also been observed in shoot tips under water deficit and salinity stress (Cramer *et al*., 2007). Transcriptional profiling in 'Corvina' berries during postharvest withering has also demonstrated the up-regulation of *DHN1a* in both uncontrolled off-plant and on-plant withering, with a higher level observed in off-plant berries (Zamboni *et al*., 2008), which is consistent with its expected involvement in modulating the dehydration stress response.

1.4.6 Alcohol dehydrogenase

As berries begin to lose water during withering they experience shrinkage, which is an important consequence of the fruit drying process (Ramos *et al*., 2004). Shrinkage causes a modification of the size and often the shape of the fruit, and it is directly related to the water loss experienced (Ramos *et al*., 2004). A gradual shrinkage of the overall cell parameters was observed by Ramos *et al*. (2004) during the first stage of air drying in grape berry quarters. The change in cell structure experienced by berries during withering results in reduced intracellular spaces and the squeezing of cells, which alters membrane functionality (Ramos *et al*., 2004). This process is accompanied by an activation of LOX which has a role in altering membrane permeability, and finally leads to the formation of a barrier to gaseous diffusion (Chkaiban *et al*., 2007; Costantini *et al*., 2006; Kays, 1997). The restriction of gas diffusion generates a change in the gas concentration within cells and intracellular spaces, contributing to the creation of a partially modified atmosphere within the berries (Cirilli *et al*., 2012). This concentration change occurs at a time when oxygen demand is higher in the cells due to increased respiration associated with water stress, and ultimately contributes to the shift from aerobic to anaerobic metabolism which has been observed during withering, as well as the subsequent activation of ADH (Costantini *et al*., 2006; Cirilli *et al*., 2012; Franco *et al*., 2004).

ADH is an enzyme responsible for catalyzing the inter-conversion of acetaldehyde to ethanol, which under anaerobic conditions leads to fermentative metabolism (Tesnière and Abbal, 2009). It is known that ADH is induced in response to various types of stress in plants and it can be activated in *Arabidopsis* at a certain level of water loss (Dolferus *et al*., 1994). Its activity has also been shown to increase in potato during dehydration (Matton

et al., 1990), and a strong ADH activation has been demonstrated during intracellular anaerobic fermentation in grape (Flanzy *et al*., 1974). Six *ADH* genes have been discovered in *Vitis vinifera* (Tesnière and Abbal, 2009), with *VvAdh2* transcripts found to be the most abundant in berries among the isogenes (Tesnière and Verriès, 2000). As previously discussed, during postharvest withering in grapes, an increase in ADH activity associated with greater than 19.5% cluster weight loss in 'Malvasia' grapes has been demonstrated (Costantini *et al*., 2006), and similar results were found in 'Aleatico' berries at comparable drying parameters and weight loss levels (Cirilli *et al*., 2012).

The relative gene expression of *VvAdh1* and *VvAdh2* during postharvest withering in 'Aleatico' grape berries was investigated by Cirilli *et al*. (2012) under different drying conditions. No transcripts of *VvAdh1* were detected, however there was an increase in *VvAdh2* transcripts by 10% cluster weight loss in all temperatures, although the relative transcript amount and subsequent effects were different for each of the treatments. The highest relative gene expression was observed at 10 ^{o}C and 10% weight loss. In contrast to what has been observed for *VvAdh2*, in both slow and rapid drying conditions at 30% cluster weight loss Rizzini *et al*. (2009) found a decrease in accumulation in a putative *Adh7* transcript in 'Raboso Piave' berries. Taken together, the research conducted thus far supports the involvement of *VvAdh2* in the postharvest withering stress response in grape berries.

1.4.7 Osmoprotectants

A common response to osmotic stress and dehydration in plants is the accumulation of osmoprotectants or non-toxic, compatible osmolytes (Hare *et al*., 1998; Penna *et al*., 2006; Rontein *et al*., 2002). Although their actual physiological roles during water stress

remain a subject of some controversy, osmoprotectants are believed to function to stabilize molecules (Garg *et al*., 2002), and are also accumulated for the purposes of free radical scavenging, osmotic adjustment and water retention (Hussain, *et al*., 2012; Penna *et al*., 2006; Smirnoff, 1998). These compounds aid in osmotic adjustment through an influx of water into cells and/or a reduction in the amount of water lost from cells, which accompanies increases in their concentration (Hare *et al*., 1998). Compatible osmolytes include certain amino acids like proline, sugars such as trehalose and fructan, as well as sugar alcohols such as mannitol and sorbital (Garg *et al*., 2002; Hare *et al*., 1998; Penna *et al*., 2006).

There is some evidence for the involvement of osmoprotectants in the postharvest withering response in grape. As previously discussed, in 'Malvasia' berries, the dehydration stress response involves an accumulation of proline until a 11.7% weight loss, followed by a second significant rise in proline at 19% weight loss (Costantini *et al*., 2006). In addition, transcriptional profiling in 'Corvina' berries during postharvest drying has shown the up-regulation of a trehalose-phosphate phosphatase transcript during late withering (Zamboni *et al*., 2008). This enzyme is responsible for the last step in trehalose biosynthesis (Crowe *et al*., 2001). Trehalose is a disaccharide of glucose, which is accumulated in high amounts in many organisms capable of surviving complete dehydration, such as yeast (Crowe *et al*., 2001), and it has been shown to protect against dehydration stress at increased levels in *Escherichia coli* (Mig. 1895) Castellani and Chalmers 1919 (Garg *et al*., 2002). Trehalose has a unique reversible water-absorption feature (Hussain *et al*., 2012) and can stabilize proteins and membranes in the dry state (Crowe *et al*., 2001), forming a glass-like, high viscosity liquid structure (Goddijn *et al*.,

1999), which is thought to function to prevent the denaturation of biomolecules, thus allowing them to retain functionality upon rehydration (Fernandez *et al*., 2010). Trehalose does not seem to be accumulated in most plants to high levels, with the exception of a group of extremely desiccation-tolerant 'resurrection' plants that can recover from extreme dehydration (Goddijn *et al*., 1999; Penna *et al*., 2006). Besides resurrection plants, drought resistance has been associated with increased levels of trehalose in common bean (*Phaseolus vulgaris* L.) (Farias-Rodriguez *et al*., 1998), transgenic rice (Garg *et al*., 2002), tobacco (Zhao *et al*., 2000), tomato (Cortina and Culiáñez-Macia, 2005) and potato (Yeo *et al*., 2000).

Another group of osmoprotectants are sugar alcohols, which are believed to function by stabilizing macromolecules through maintaining an artificial sphere of hydration, due to their ability to imitate the structure of water (Penna *et al*., 2006). They may also have a role in the scavenging of ROS, which helps to protect against cell damage (Smirnoff, 1998). Sorbitol in particular is found most commonly in woody members of the Rosaceae, including all members of the genera *Malus* (apples), *Rosa* (Roses), *Prunus* (stone fruits) and *Pyrus* (pears), where it is the primary photosynthetic product (Loescher, 1987; Penna *et al*., 2006). Sorbitol is believed to function as an osmoprotectant, but its method of action and physiological role are not known (Ohta et al., 2005; Penna *et al*., 2006). Water stress has been associated with the accumulation of sorbitol in leaves of micropropagated apple (*Malus domestica* Borkh.) plants (Li and Li, 2005). In addition, transgenic *Nicotiana tabacum* L. plants which accumulate sorbitol also display increased drought tolerance (Sheveleva *et al*., 1998; Tao *et al*., 1995).

The up-regulation of a sorbitol-related enzyme during earlier stages of postharvest withering in 'Corvina' grape berries has been observed by Zamboni *et al*. (2008), and the up-regulation of a sorbitol transporter and dehydrogenase has also been reported in berries in the field after experiencing water-deficit (Grimplet *et al*., 2007). The up-regulation of both a sorbitol-related enzyme and of a trehalose-phosphate phosphatase during postharvest withering could positively affect the synthesis of sorbitol and trehalose respectively, and contribute to the water stress response in drying grape berries (Zamboni *et al*., 2008).

1.5 Conclusion and objectives

Recent research in transcriptional profiling of the postharvest withering of grape berries has been conducted primarily under uncontrolled, natural environmental conditions in Italy, using microarray and AFLP-TP approaches (Fasoli *et al*., 2012; Rizzini *et al*., 2009; Zamboni *et al*., 2010; Zamboni *et al*., 2008). Transcriptional profiling has revealed that the intensity and rate of postharvest dehydration affects the abundance of numerous gene transcripts in grape berry skins, with a higher number of differentially expressed genes associated with both a slower rate, as well as a more intense dehydration level (Rizzini *et al*., 2009).

The use of different rates of postharvest withering in grapes will each produce berries with their own unique compositions, which as a result will contribute to the production of diverse *appassimento* wine styles. However, a transcriptional profiling study comparing gene expression responses between controlled fast and slow withering has yet to be preformed, and previous gene expression studies have not employed the use of next generation sequencing technologies. The objective of this research was to use a completely

controlled, patented grape drying technology, in combination with RNA Sequencing (RNA-Seq), in attempts to characterize differential gene expression trends in both fast and slow *appassimento* methods, and to offer further insight into the postharvest withering stress response in grapes. The Vineland novel controlled drying technology can be used as a tool to create both fast and slow drying *appassimento* conditions, and gene expression information can provide a foundation for further research to investigate how similarities and differences between withering rates may be related to berry composition, and ultimately to the different *appassimento* wines produced.

Chapter 2. Materials and Methods

Contribution: The maintenance and monitoring of the postharvest withering process, as well as set-up of the grapes in the system was carried out by Kimberley Cathline. The design and construction of the experimental drying system, as well as arrangement of environmental conditions, was performed by Bernard Goyette (Vineland Research and Innovation Centre). All aspects of the sampling, berry compositional analysis, weight loss monitoring and RNA extraction, including sample preparation for RNA-Seq and RT-PCR, were carried out by Kimberley Cathline. RNA-Seq service for this research was carried out by the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada). Bioinformatics was led by Travis Banks (Vineland Research and Innovation Centre). Processing of sequencing data was performed by Joseph O'Neill (Vineland Research and Innovation Centre) with direction and oversight from Travis Banks. Analysis of sequencing data was carried out by Travis Banks, Joseph O'Neill and Kimberley Cathline.

2.1 Plant material

Healthy red skinned grape clusters (*Vitis vinifera* L. 'Cabernet Franc') were carefully harvested by hand on October 30, 2014 from a commercial vineyard located in the Niagara Region appellation, Beamsville Bench Sub-Appellation, of Ontario, Canada. Harvest occurred when berries had reached the "Harvest-Ripe E-L 38" grapevine growth stage, according to the Modified Eichhorn-Lorenz (E-L) system for identifying grapevine growth stages (Coombe, 1995). At harvest, berries had an average total soluble solids (TSS) content of 21.7 ^{o}Brix.

2.2 Postharvest withering of 'Cabernet Franc' grapes

2.2.1 Withering treatments

High quality clusters with sound and healthy berries were harvested and gently placed into reusable plastic containers (RPC), containing openings in the sides and bottom (SmartCrate™, model IPL 6411, IPL Inc., Saint-Damien, Québec, Canada). The RPCs measured 60 cm long x 40 cm wide x 12 cm high (**Figure 2.1**) and are designed and optimised to allow air to circulate through and around a product (Vigneault and Goyette, 2002) when placed inside the proprietary ventilation technology described in section **2.2.2**. Each RPC contained an average of 8.6 kg of grape clusters. Grapes were stored under two different environmental conditions using a novel controlled drying technology designed at Vineland Research and Innovation Centre (Vineland, Ontario, Canada), in order to allow for two different withering rates:

a) Fast Withering, FW – Clusters were stored for 20 days at an average temperature and RH of 25 ^{o}C and 65% respectively, and an air speed of 0.2 m/s.

b) Slow Withering, SW – Clusters were stored for 132 days at an average temperature and RH of 6 ^{o}C and 77% respectively, and an air speed of 0.2 m/s.

Drying systems were monitored continuously using temperature and RH data loggers (HOBO® RX3000 and HOBO® U23 Pro v2, Onset Computer Corp., Bourne, Massachusetts, USA). The drying process was followed by monitoring TSS values almost daily in the fast withering treatment, and approximately on a weekly basis in the slow withering treatment. The drying process was considered complete once a target total soluble solids of 29 $\pm$ 1 ^{o}Brix was achieved.

Figure 2.1 Reusable plastic container used for harvest and postharvest withering of grapes, (SmartCrate™, model IPL 6411, IPL Inc., Saint-Damien, Québec, Canada).

2.2.2 Vineland's proprietary ventilation technology

A proprietary ventilation technology designed at Vineland Research and Innovation Centre (United States Patent Application Number - 14/032,619; Canadian Patent Application Number - 2,828,028) was used as an *appassimento* grape drying system in this experiment (**Figure 2.2**). This system consists of a plenum box equipped with a fan to create vertical air flow through the grapes, together with cooling and heating units used for environmental control of the temperature and RH. The fan component of the system creates suction in the plenum, which is connected to specially designed pallets. The pallet acts as a ducting system which transports the air that passes vertically through the grapes from the top to the bottom of the stacked RPCs wrapped in polyurethane film. Wrapped pallets are placed in a row on the side of the plenum, in such a way that the openings on the plenum fit the opening of the row of the pallets. Under these controlled conditions, air passes through the grapes to absorb humidity. The system is completely controllable and allows for tight modulation of the drying parameters.

A small-scale version of Vineland Research and Innovation Centre's (Vineland) drying system was used to conduct both fast and slow withering experiments described in this thesis. This system used the same proprietary ventilation technology as the commercial system and consisted of 10 individual RPCs stacked together on a plenum box, which was equipped with a fan (**Figure 2.3**). Cluster weight loss was monitored by weighing five separate RPCs periodically, from harvest through to completion of withering. Weights were measured using a scale accurate to 0.0005 kg with a 30 kg capacity (Ranger® RC30LS, Ohaus® Corp., Parsippany, New Jersey, USA), and weight loss was expressed as a percentage of original weight.

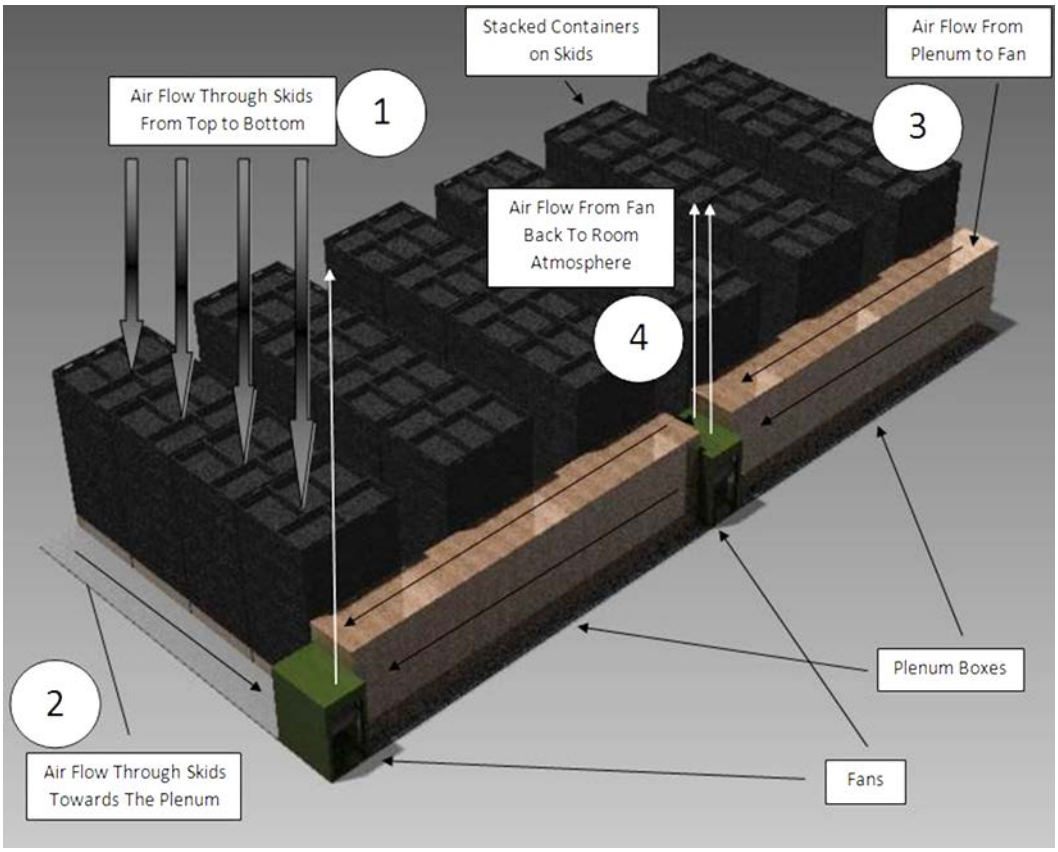

Figure 2.2 Schematic representation of Vineland Research and Innovation Centre's novel drying system (United States Patent Application Number - 14/032,619; Canadian Patent Application Number - 2,828,028).

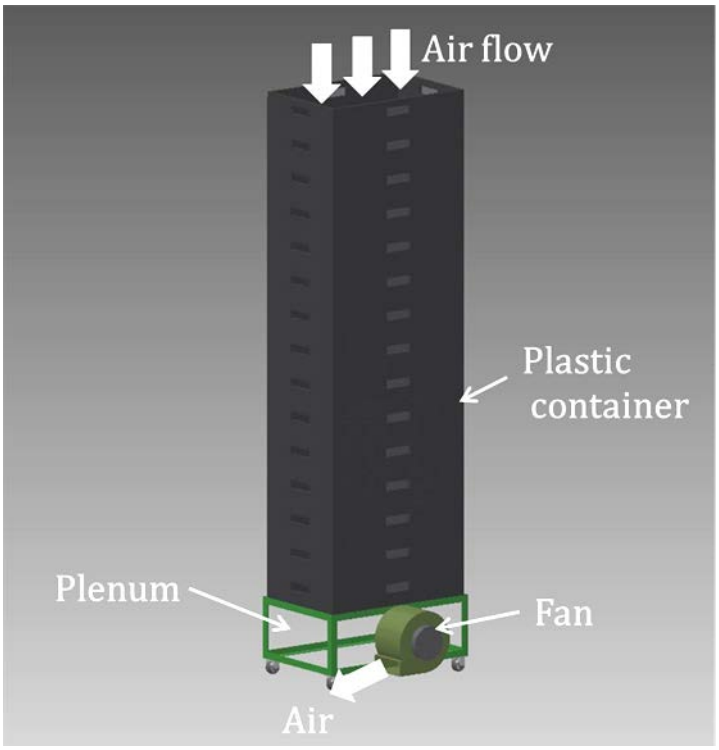

Figure 2.3 Schematic representation of a small-scale version of Vineland's novel drying system which was used to conduct both fast and slow withering in this experiment.

2.3 Sample collection and compositional analysis

Samples were collected at harvest (H), and at three time points (I, II, III) during both fast withering (FW) and slow withering (SW), with the last sampling point occurring on the final day of drying (**Table 2.1**). A total of 240 berries from the top, middle and bottom portions of 80 randomly selected clusters were collected for each sampling time point and withering treatment. Of these 240 berries, 180 berries, (taken from 60 clusters as described above), were collected by clipping the pedicel while leaving a portion attached to the berry, for immediate transfer and storage at –80 ^{o}C. The remaining 60 berries, (taken from 20 clusters as described above), were collected without pedicels to be homogenized in a blender, and the juice was filtered through two layers of cheesecloth for measuring the TSS (^{o}Brix) using a hand-held, temperature-compensating, digital refractometer (ATAGO® PAL-1, Atago Co., Ltd., Tokyo, Japan). Two independent measurements were made for each sample and averaged to obtain a final TSS (^{o}Brix) value. The pH and titratable acidity (g/L tartaric acid) of two ml of the filtered juice, diluted in 50 ml Milli-Q® water, was determined with an automatic titrator (Titrino 848, Metrohm AG, Switzerland), and titration was accomplished with 0.1 N sodium hydroxide to a pH 8.2 endpoint. Two independent titrations were conducted for each sample and averaged to obtain a final titratable acidity (g/L tartaric acid) value.

Table 2.1 Sampling time points during fast and slow postharvest withering of *Vitis vinifera* 'Cabernet Franc' grape berries.

Sampling time point	Days after harvest
Harvest: H	0
Fast withering: FW	
I	5
II	16
III	20
Slow withering: SW	
I	53
II	103
III	132

2.4 Statistical analysis of berry composition and percent weight loss

Berry compositional analysis values are expressed as the mean of two technical replications, comprised of 60 berries taken from the top, middle and bottom portions of 20 randomly selected clusters. Percent weight loss values are expressed as the mean of five replicates, each comprised of one separate RPC filled with clusters. Berry compositional analysis values, as well as percent weight loss measurements were subjected to analysis of variance (ANOVA) using Minitab 17 (Minitab, Inc., State College, Pennsylvania, USA). Differences were accepted as statistically significant when $P \leq 0.05$. Mean values were compared by Tukey's test ($\alpha = 0.05$) and significant difference attributed by letters.

2.5 Extraction of total RNA

Total RNA was extracted from grape berry tissue (skin and flesh) using PureLink® Plant RNA Reagent (Abion™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) according to the manufacturer's protocol (Publication

Number MAN0000243), with some modifications as follows. Success with this extraction protocol was achieved with careful optimization of the technique, including minimizing exposure to ribonucleases, which rapidly degrade RNA, by working on ice, carrying out steps rapidly, autoclaving reagents and glassware, baking mortars and pestles, and the use of clean gloves (Pilcher *et al*., 2007). This protocol was optimized after numerous repetitions to isolate total RNA from approximately 200 mg of tissue. Reagent volumes should be scaled appropriately if higher amounts of tissue are used. All microcentrifuge tubes and pipette tips were autoclaved and cooled completely prior to use. Mortars and pestles, along with tools such as tweezers and spatulas, were wrapped in tin foil and baked at 250^{o}C for 24 hours and cooled completely prior to use. All surfaces, pipettes and additional non-disposable items were sprayed and treated with RNaseZAP™ (Sigma-Aldrich Co., St. Louis, Missouri, USA) prior to commencing the extraction, and care was taken to periodically re-treat surfaces as needed throughout the process. Proper aseptic techniques were used throughout the extraction procedure, including the use of disposable gloves which were changed frequently. All solutions were prepared with sterile Milli-Q® water (EMD Millipore Corp., Billerica, Massachusetts, USA), autoclaved and cooled completely prior to use. All reagents and microcentrifuge tubes were cooled on ice prior to use and centrifugation was performed at 4^{o}C (Centrifuge 5418 R, Eppendorf AG, Hamburg, Germany). Each step was performed on ice where possible and unless specified otherwise.

For each time point, ten randomly selected berries from those stored at –80 ^{o}C were ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. Seeds and pedicles were carefully removed from frozen berries and discarded prior to grinding.

Approximately 200 mg of ground, frozen berry tissue was added to a pre-weighed 2.0 mL microcentrifuge tube. Remaining berry tissue was stored at –80 ^{o}C. To the microcentrifuge tube containing the frozen tissue, 1 mL of PureLink® Plant RNA Reagent was added, followed by briefly vortexing until the sample was fully suspended. The tube was then incubated at room temperature for 5 minutes by laying it horizontally to maximize surface area, during which time it was periodically shaken gently. After incubation, the solution was clarified by centrifugation for 5 minutes at 15,000 rpm and supernatant was transferred to a clean 2.0 mL microcentrifuge tube. The clarified extract was treated with 200 µL of 5 M sodium chloride and mixed by gently tapping the tube for 10 seconds. The sample was then treated with 600 µL of chloroform and mixed by gently inverting the tube repetitively for 10 seconds. The sample was then centrifuged for 30 minutes at 15,000 rpm to separate the mixture into two phases. The upper, aqueous phase was transferred to a clean 1.5 mL microcentrifuge tube, to which an equal volume of isopropyl alcohol was added, followed by gentle repetitive inversion for 10 seconds. The sample was then let stand on ice for 20 minutes to precipitate the RNA, followed by centrifugation at 15,000 rpm for 30 minutes. Supernatant was decanted gently and 1.5 mL of 75% ethanol was added to the pellet, followed by gentle repetitive inversion for 10 seconds. Sample was then pelleted again by centrifugation at 15,000 rpm for 5 minutes and supernatant gently decanted. The ethanol wash, subsequent centrifugation and decanting step were repeated three additional times. After the fourth time, the tube was then briefly centrifuged to collect the residual liquid, which was removed carefully with a pipette. RNA was resuspended by adding 20 µL of sterile Milli-Q® water to the tube and by gently tapping, in combination with carefully pipetting the liquid up and down over the pellet. Extractions were repeated three times for

each of the seven sampling time points, using 10 randomly selected berries each time, to generate three biological replicates and thus a total of 21 samples.

RNA quantity and quality were determined by spectrophotometer analysis through measuring the absorbance (NanoPhotometer® Classic, Implen U.S.A., Inc., Westlake Village, California, USA) at 230, 260 and 280 nm. The absorbance at 260 nm provides a measurement of quantity, the A_{260}/A_{280} (absorbance at 260 nm divided by the absorbance at 280 nm) and A_{260}/A_{230} ratios were calculated to determine the purity of the RNA sample. In addition, the integrity of RNA was verified by visualization of intact ribosomal bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis using 5 µL of RNA sample. Gels were visualized and photographed using a Molecular Imager® Gel Doc™ XR+ system with Image Lab™ Software (Bio-Rad Laboratories, Inc., Hercules, California, USA).

In order to look for the presence of full-length gene transcripts, RNA extraction samples from all seven time points underwent reverse transcription polymerase chain reaction (RT-PCR) using primers designed to amplify the open reading frame (ORF) of three different genes. Total RNA was used as a template for first-strand cDNA (complimentary deoxyribonucleic acid) synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions. The presence of full-length transcripts of a phenylalanine ammonia lyase and a trehalose-phosphate phosphatase which have both shown up-regulation during withering of grape berries (Zamboni *et al*., 2008), as well as a lipid transfer protein that has been associated with water deficit in shoot tissue of grapes in the field (Cramer *et al*., 2007), were chosen to

investigate using PCR. Oligonucleotide primers were designed (Integrated DNA Technologies Inc., Coralville, Iowa, USA) to amplify the full-length ORF of the phenylalanine ammonia lyase (DFCI Grape Gene Index accession number TC150103), the trehalose-phosphate phosphatase (DFCI Grape Gene Index accession number TC67690) and the lipid transfer protein (NCBI gene accession number LOC100258473) from cDNA. The sequences of the primers were: phenylalanine ammonia lyase 5'-ATGGATGCAACGAACTGCCA -3' (forward) and 5'-CTAGCAGATTGGGAGAGGAGC -3' (reverse); trehalose-phosphate phosphatase 5'-ATGGATCTGAAGTCCAATCATTCT -3' (forward) and 5'-TTATAGTGCACTTGACTTCTTCCAC -3' (reverse); and lipid transfer protein 5'-ATGAATCCAGTGCCTTTCCCG -3' (forward) and 5'-TCATTTAGGGTGGACCAGGT -3' (reverse). The PCR reaction for all three genes was carried out using Taq DNA Polymerase (2X Taq FroggaMix, FroggaBio, Inc., Toronto, Ontario, Canada) under the following cycling conditions: initial denaturation at 94 ^{o}C for 3 minutes, then 34 cycles of 94 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, followed by a final extension period of 72 ^{o}C for 10 minutes (C 1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, California, USA). The presence of full-length gene transcripts was verified by visualization of bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis. A majority of samples showed clear bands corresponding to the expected ORF sizes of 2133 base pairs (bp) (phenylalanine ammonia lyase), 1158 bp (trehalose-phosphate phosphatase) and 1203 bp (lipid transfer protein). The same PCR reaction was also run using RNA in place of cDNA from all seven sampling time points to check for product amplification as an indication of

DNA contamination. No bands were present upon visualization on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis.

2.6 RNA sequencing

RNA-Seq service for this research was carried out by the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada) using an Illumina® HiSeq 2500 ultra-high-throughput sequencing system using v4 chemistry (Illumina, Inc., San Diego, California, USA). The 21 total RNA samples (three biological replications of seven time points, **Table 2.1**) were prepared for shipping following McGill University and Génome Québec Innovation Centre's (Génome Québec) instructions (User Guide: Illumina® sequencing technologies, version 5.4). Once samples were received at Génome Québec, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Due to an unfamiliarity with *V. vinifera* and an absence of the required quality control metrics, Génome Québec was not able to provide confirmation that the RNA was intact and of good quality using their bioanalyzer method of analysis. Génome Québec also indicated a concern that the A_{260}/A_{230} ratios associated with the samples may indicate the presence of contamination that could possibly interfere with sequencing. The initial information provided by the spectrophotometer analysis, specifically the A_{260}/A_{280} ratios, along with the visualization of intact ribosomal bands after agarose gel electrophoresis (**2.5, Materials and Methods**) indicated that the RNA was intact and of good quality for all samples. However, without the ability of Génome Québec to confirm this information, and due to their concern surrounding the A_{260}/A_{230} ratios, it was important to attempt to further verify the positive quality observations made prior to

shipping the samples, and to address the concern regarding the A_{260}/A_{230} ratios, before proceeding with authorizing the RNA-Seq.

Modifications to the original RNA extraction protocol (**2.5, Materials and Methods**) were attempted, along with several additional techniques and tools, however all efforts resulted in much lower yields, as well as reduced A_{260}/A_{280} and A_{260}/A_{230} ratios. Following these unsuccessful attempts, a consultation was arranged with Norgen Biotek Corporation (Thorold, Ontario, Canada). Norgen Biotek Corporation employed the use of their Plant/Fungi Total RNA Purification Kit to extract RNA from ripe *V. vinifera* 'Cabernet Franc' berries that had not undergone postharvest withering. Unfortunately, the kit resulted in extremely low yields of RNA, making it an unacceptable choice for extraction for the purposes of this research. The kit was also unable to generate an increase in the A_{260}/A_{280} and A_{260}/A_{230} ratios and showed results that were comparable to what was achieved with the original RNA extraction protocol used in this experiment.

A final test was conducted before deciding whether or not to proceed with authorizing the RNA-Seq. In order to verify the presence of full-length gene transcripts in these specific samples, four sub-samples covering a range of RNA quantities and A_{260}/A_{280} and A_{260}/A_{230} ratios were chosen to undergo RT-PCR. These sub-samples were taken from the exact same RNA extractions of which samples were sent to Génome Québec. Total RNA was used as a template for first-strand cDNA synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions, with a uniform 1.422 µg of RNA reverse-transcribed for each sample in a 20 µL reaction volume. The presence of full-length transcripts of the previously described lipid transfer protein (Cramer

et al., 2007) were chosen to investigate using PCR. The same oligonucleotide primers as previously described were used to amplify the ORF of the lipid transfer protein gene from cDNA (NCBI gene accession number LOC100258473). The PCR reaction was carried out using Taq DNA Polymerase (2X Taq FroggaMix, FroggaBio, Inc., Toronto, Ontario, Canada) under the following cycling conditions: initial denaturation at 94 ^{o}C for 3 minutes, then 34 cycles of 94 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, followed by a final extension period of 72 ^{o}C for 10 minutes (C 1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, California, USA). The presence of full-length gene transcripts was verified by visualization of bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis. All four samples showed clear bands corresponding to the expected ORF size of 1203 bp.

The total RNA quality and quantity based on the A_{260}/A_{280} ratios, the detection of intact ribosomal bands after agarose gel electrophoresis and the ability to amplify full-length gene products following RT-PCR triggered an authorization to Génome Québec to proceed with the RNA-Seq of the provided samples. At Génome Québec, cDNA libraries were created from 250 ng of total RNA using the Illumina® TruSeq® Stranded mRNA Sample Preparation Kit (Illumina, Inc., San Diego, California, USA), as per the manufacturer's recommendations, which included mRNA enrichment using poly-T oligo attached magnetic beads using two rounds of purification, mRNA fragmentation and priming for cDNA synthesis using random hexamers. Second strand cDNA synthesis involved the incorporation of dUPT, in order to quench the second strand during amplification. Following double stranded cDNA synthesis a single 'A' nucleotide was added to the 3' ends of the fragments to allow for ligation of the sequencing primers, which

had a corresponding 'T' nucleotide overhang on their 3' end. Fragments were then enriched using PCR, followed by hybridization to flow cells for sequencing. Libraries were sequenced with an Illumina® HiSeq 2500 ultra-high-throughput sequencing system using v4 chemistry (Illumina, Inc., San Diego, California, USA), following the manufacturer's instructions. Prior to sequencing, solid-phase bridge amplification was used to generate clusters of up to 1,000 copies of each single fragment in close proximity to each other. An Illumina® PhiX library spike-in was used as a control and mixed with the sample libraries at a level of one percent (Illumina, Inc., San Diego, California, USA). Four fluorescently-labelled reversible terminator nucleotides were used to sequence clusters in parallel and 125-bp paired-end sequences were generated. Illumina® Real-Time Analysis (RTA) software, Version 1.18.64, (Illumina, Inc., San Diego, California, USA) calculated the number of reads per sample and the average *phred* quality score (*Q*-score) for each sample (Ewing and Green, 1998).

2.7 Sequencing data processing and analysis

For pre-analysis and quality control, the sequence reads from all 21 samples were analyzed using FASTQC software, Version 0.11.5, (Babraham Bioinformatics, Cambridge, UK) as an initial step. For each sample, forward and reverse paired-end reads were merged using PEAR (Paired-End reAd mergeR) software, Version 0.9.6 (Zhang *et al.*, 2014). Both merged reads and reads that could not be merged were kept for downstream analysis, but they were retained separately. Illumina® adapter sequences were clipped and low-quality bases/reads were removed from sequence reads in each sample (minimum *Q*-score 13, minimum read length 25 bp) using FaQCs software, Version 1.34 (Lo and Chain, 2014). For overall alignment rates, raw sequence reads were mapped to the current National

Centre for Biotechnology Institute (NCBI) *Vitis vinifera* Annotation Release 101 genome (http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Vitis_vinifera/101/) using Bowtie software, Version 1.1.2 (Langmead *et al*., 2009). For differential gene analysis, sequence reads were aligned to protein-coding regions against the *V. vinifera* reference genome using STAR (spliced transcripts alignment to a reference) software, Version 2.5 (Dobin *et al*., 2013). For each sample, the STAR aligner outputs a reads-per-gene file. Using a custom script, these multiple reads-per-gene files were merged into a master reads-per-gene matrix, where columns are samples and rows are genes. This matrix was imported into the R software environment, Version 3.3.1 (The R Foundation, www.r-project.org). Analysis of differential gene expression was conducted by comparing each sampling time point to the harvest time point in R with the *limma* package, Version 3.28.21 (Ritchie *et al*., 2015). Based on the distribution of P-values for unfiltered reads-per-gene data, a cut-off was calculated which allowed genes with very low reads to be removed from each differential gene expression analysis. The threshold for a differentially expressed gene was set as: false discovery rate (FDR) $\leq$ 0.01, $\log_2$ fold change (lfc) $\geq$ 1. Principal component analysis (PCA) was performed using the R software environment. Comparison of expression patterns for genes of interest over time was performed in the Perl software environment, Version 5.20 (www.perl.org). The numerical identifiers for the genes of interest were first identified in the reference genome (NCBI *Vitis vinifera* Annotation Release 101) and then these identifiers were used to locate the same genes within withering samples to follow general expression over time.

Gene ontology (GO) identifiers were assigned to each of the genes in the NCBI *Vitis vinifera* Annotation Release 101 genome by the use of the Basic Local Alignment

Search Tool (BLAST) method, in combination with the *Vitis vinifera* Genoscope database (http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ML/data/12X/annotation/Vitis_vinifera_peptide.fa.gz). The top hit from the Genoscope database was used to identify the GO identifier for the associated gene in the NCBI genome, as long as the top hit had a P-value < 0.0001. In cases where the top hit had a P-value greater than the established threshold, no GO identifier was assigned. Distribution of DE genes by GO term was analyzed using agriGO (Du *et al*., 2010). Using this web-based tool, significantly enriched GO terms represented within lists of Genoscope GO identifiers were determined by means of Fisher statistical test with a significance level of $P \leq 0.05$ using the following settings: singular enrichment as the analysis tool, *Vitis vinifera* as the species and the suggested background as the reference. Significantly overrepresented GO terms associated with $\geq$ 5 percent of the total DE expressed genes analyzed were included in the results.

Botrytis cinerea Pers. (1794) alignment rates were determined by aligning raw reads to the latest version of the *Botrytis cinerea* B05.10 reference genome in NCBI's Genbank (Amselem *et al*., 2011; Staats and van Kan, 2012). Alignment and statistical analysis was performed using Bowtie software, Version 1.1.2 (Langmead *et al*., 2009). Alignment rates were subjected to ANOVA ($P \leq 0.05$). Mean values were also compared by Tukey's test ($\alpha = 0.05$).