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MOLECULAR ANALYSIS OF EPIGENETIC MEMORY OF STRESS ESTABLISHMENT AND LONG-TERM MAINTENANCE IN A PERENNIAL WOODY PLANT

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MOLECULAR ANALYSIS OF EPIGENETIC MEMORY OF STRESS ESTABLISHMENT AND LONG-TERM MAINTENANCE IN A PERENNIAL WOODY PLANT

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

MOLECULAR ANALYSIS OF EPIGENETIC MEMORY OF STRESS ESTABLISHMENT AND LONG-TERM MAINTENANCE IN A PERENNIAL WOODY PLANT

Plants adapt to extreme environmental conditions through physiological adaptations, which are usually transient. Recent research has suggested that environmental conditions can activate a memory of stress that can result in a primed response to subsequent stress events. While the effect of priming has been observed in many plants, the underlying mechanisms are puzzling and seldom studied. A large body of research has been developed in the last decade linking response to stress, stress priming, and memory of stress with epigenetic mechanisms. This understanding of plant epigenetics has opened the door to the application of epigenetics to crop improvement, such as the use of epigenetic breeding for the generation of more resilient crops. Although well-studied in annual and model species, research on epigenetic memory of stress in perennials is still minimal. Viticulture, a perennial form of agriculture, is highly dependent on climatic conditions, not only for yield but also for fruit quality, which is the most important factor affecting produce value at the farm gate and would benefit from more in-depth knowledge on epigenetic memory of stress.

Here we present the results of an experiment conducted over two growing seasons, which constitute the first comprehensive study providing insights into the memory of stress establishment and temporal maintenance, and its potential effect on priming in a perennial crop. Gene expression and DNA methylation data were obtained from 222 plants exposed to the most common forms of abiotic stress faced by vineyards (drought, heat, and combined drought and heat). Our results indicate that the effect of the combined stress on physiology and gene expression is more severe than that of individual stresses, but not simply additive. Common genes expressed under both individual and combined treatments included heat-shock proteins, mitogen-activated kinases, and sugar-metabolizing enzymes, while phenylpropanoid biosynthesis and histone-modifying genes were unique to the combined stress treatment. We also found evidence of the establishment of memory of stress after the heat and combined stress, but not after drought, and that epigenetic chromatin modifications may play an important role during this process. Additionally, we identified genes that are differentially expressed in primed plants one year after their initial exposure to environmental insult and in the absence of recurrent stress. Moreover, primed plants showed a stronger response in gene expression to recurrent stress than plants exposed for the first time to that same stress.

Finally, we explored the effect that two types of vegetative propagation may have on the maintenance of epigenetic memory of stress in primed grapevines. Briefly, although primed propagules generated using callused cuttings presented more differentially expressed genes in response to a second stress than those propagated using layering, only

primed layered propagules showed differentially expressed genes in the absence of a recurrent stress, suggesting that the established stress memory is, at least partially, lost during cutting propagation.

Collectively, our results constitute the first molecular evidence of long-term stress memory in grapevine and lay the foundation for the development of a comprehensive model integrating plant response to stress, the establishment of epigenetic memory of stress, and its maintenance, over time and during vegetative propagation in perennial plants.

KEYWORDS: Stress priming, Stress response, Grapevine, Transcriptome, Epigenome, Perennial crops

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03/31/2023

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DEDICATION

In memory of *my father*.

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(This chapter has been submitted to Frontiers in Horticulture as a review with coauthor Dr. Carlos M. Rodríguez López, and it is currently under review.)

1.1 Abstract

Climate change is expected to increase the occurrence of extreme environmental conditions. Viticulture, as agriculture in general, is highly dependent on climatic conditions, not only for yield but also for fruit quality, which is the most important factor affecting produce value at the farm gate. This demands the development of novel plant breeding techniques that will lead to the accelerated production of more resilient grape varieties, as conventional breeding programs for perennials are often prolonged. Recent research has suggested that environmental conditions can activate a memory of stress that could result in a primed response to subsequent stress events. This is a process capable of increasing plant's resilience to abiotic stimuli, allowing plants to better adapt to extreme environmental conditions. While the effect of priming has been observed in many plants, the underlying mechanisms are puzzling and seldom studied in perennial crops. A large body of research has been developed in the last decade linking response to stress, stress priming, and memory of stress with epigenetic mechanisms. This understanding of plant epigenetics has opened the door to the application of epigenetics to crop improvement, such as the use of epigenetic breeding for the generation of more resilient crops. Perennial

crop agriculture in general, and viticulture in particular, would benefit from more in-depth knowledge on epigenetic memory of stress.

Keywords: Review(article), grapevine, perennial crops, epigenomics, epi-breeding, stress memory, stress priming

1.2 Introduction

Wine grapes are considered the most important fruit crop in the world in terms of production and economic importance (Alston and Sambucci, 2019). It has been reported that there are nearly 8 million hectares of vineyards worldwide and the global annual production have reached approximately 90 million tons (http://faostat.fao.org). In the United States alone, which ranks fourth in the volume of wine production behind Italy, France, and Spain (Stevenson, 2005), wine, grapes, and grape products contribute $276 billion to the economy in 2022 (https://wineamerica.org/economic-impact-study/2022-american-wine-industry-methodology/). The importance of grape cultivation for wine production, however, goes beyond its bare contribution to the economy. Wine consumption has moved from a source of nutrition to a cultural phenomenon with a large tourist industry associated with it. For this reason, the wine industry has helped fix local populations in rural areas by diversifying the job markets in such regions (https://wineamerica.org/economic-impact-study/2022-american-wine-industry-methodology/). The majority of cultivated grapes belong to Vitis vinifera subsp. vinifera; but the cultivation of other Vitis *sensu stricto* species, including hybrids, and the related

subgenus *Muscadinia* are also common in regions where the climate and/or disease pressure are not suitable for V. vinifera (Hickey et al., 2019).

Climate change is expected to severely affect the major viticultural regions of the world by reducing the areas where most grapevine cultivars can be cultivated economically, due to an increase in abiotic stress pressure (Diffenbaugh et al., 2011), and in the incidence of pests and diseases (Gullino et al., 2018). Although the long domestication and breeding history of *V. vinifera* in particular, for wine and fresh and dried fruit consumption has led to desirable traits such as berry color, sugar content, and berry size (Aradhya et al., 2003; Myles et al., 2011; Zhou et al., 2017), the attempts to utilize more disease/environmental tolerant wild non-*vinifera* species to cross with *V. vinifera* has been compromised by negatively perceived flavors, prominently in wine production (Liu et al., 2015; Yang et al., 2016). Therefore, the future success of the wine industry will require the development of novel varieties better suited to the climatic conditions predicted under the scenario of climate change.

Plants have acquired many adaptation strategies, activated and controlled by changes in gene expression and nuclear organization (Budak et al., 2015) to cope with ever-changing environmental conditions. Progress in plant molecular biology has enable the identification of major stress response pathways, leading into a deeper understanding of the plant responses that constitute such strategies (Hirayama and Shinozaki, 2010). The availability of the complete grapevine genome sequence has allowed the identification and characterization of various stress-inducible genes, cis-regulatory elements and transcription factors (Jaillon, 2007). More recent studies have shown that epigenetic mechanisms, some with the potential to be inherited, play an important role in plant

response to environmental stress (Miryeganeh, 2021). Although the current knowledge on the role of epigenetic regulation in response to the environment in the grapevine is still limited, the demonstration of the involvement of epigenetic mechanisms in model plants has led to an increased interest in their role in crop resilience to environmental stress (Varotto et al., 2020).

Here we summarize the current knowledge on, environmental factors that affect grape and wine qualities, transcriptomic approaches that have been utilized to study the effect of environmental factors on grapevine, and finally recent studies focusing on epigenetic mechanisms, particularly those involved in plant response to environmental changes, which have led to proposing epigenetic breeding as a new tool for the generation of climate resilient grapevines.

1.3 Factors affecting grape and wine quality

Fruit and wine quality are determined by the interaction between the cultivar(s) planted (including the interaction between rootstock and scion), the local environmental conditions (climate, topography, soil, etc.), and the viticultural and enological practices implemented to grow the grapes and produce the wine (Van Leeuwen et al., 2004). Such interaction has been traditionally termed *terroir* (Seguin, 1986) (Figure 1.1).

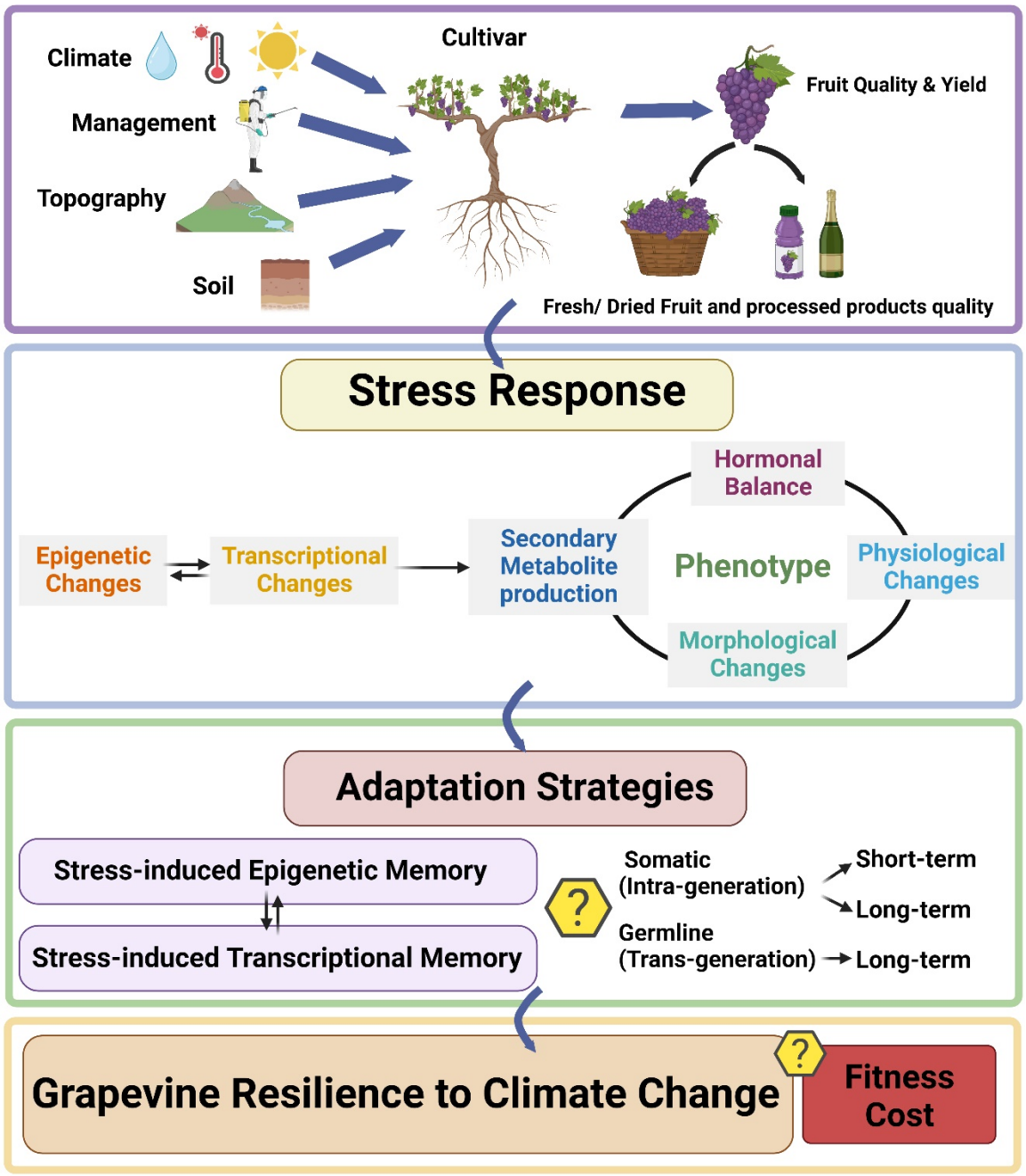


Figure 1.1 Grapevine response to the environment.

The top panel represents the different factors (climate, soil, topography, management, and planted rootstock/scion genotype) contributing to grapevine growth and development, berry composition, fruit quality and yield. Middle panel represents the stress response triggered at a molecular level leading to a change in phenotype. Panel 3 represents the adaptation strategies employed by grapevine to establish different types of memory, leading to grapevine resilience to environmental stress. Question marks in panels 3 and 4 denote the current limited knowledge about the establishment and maintenance of epigenetic memory in grapevine, and of the potential deleterious fitness cost of epigenetic priming.

1.3.1 Climate

Among those factors, climate conditions determine the suitability to grow a particular variety, as the most desirable composition of grapes requires certain climatic conditions (Gladstones, 1992). Common climate factors that are important for grape and wine quality are temperature, radiation, and rainfall (Romero et al., 2016) (Figure 1.1).

1.3.2 Temperature

Temperature is widely accepted to affect grapevine phenology, vegetative cycles, grape quality, and the timing of grape harvest (Cook and Wolkovich, 2016; Jones and Alves, 2012; Winkler, 1974). Photosynthesis is among the first physiological functions to be directly affected by temperature variations, as it is reduced before other symptoms appear when the temperature rises above an optimum limit, which differs among species (Luo et al., 2011; Sharma et al., 2020; Xiao et al., 2017). Most of the physiological processes decline at below 10°C and heat acclimation mechanisms are activated at temperatures over 35°C (Bernardo et al., 2018). At extreme high temperature, i.e., above 40°C, the photosynthetic apparatus is disrupted (Venios et al., 2020). Elevated temperature during berry growth and maturation largely impacts size and composition (Carbonell-Bejerano et al., 2013). More specifically, higher temperatures lower the acidity and increase the sugar content of berries, resulting in unbalanced wines with higher alcohol content and deprived of freshness and aromatic complexity (Martínez-Lüscher et al., 2016). More sugar and less organic acids in berries, and altered secondary metabolites composition, mainly in aroma precursors, have been observed with increased temperature

(Van Leeuwen and Destrac-Irvine, 2017). It has been reported that berry size and weight are reduced at temperatures above 30°C (Hale and Buttrose, 1974), while metabolic processes and sugar accumulation may completely stop (Downey et al., 2006). In addition, despite tartaric acid being relatively stable with regards to temperature, malic acid levels are tightly dependent on maturity and temperature, as higher temperature leads to lower malic acid content (Santos et al., 2020). In general, elevated temperature is associated with increased potassium levels and decreased total acidity, and thus is associated with increased pH levels (De Orduna, 2010). Higher temperatures also modify the biosynthesis and accumulation of flavonoids in berries. Temperatures above 30°C led to lower anthocyanin synthesis (Spayd et al., 2002; Tarara et al., 2008), which can be completely and irreversibly inhibited at 37°C (Yang et al., 2018). This suggests that in warm climates, grapevine berries can suffer from the inhibition of anthocyanin formation and hence reduce grape color (Downey et al., 2006). Conversely, low temperature leads to an increase in anthocyanin accumulation and total soluble solids (Mori et al., 2005). It is important to consider, however, the degree to which high temperature affects the anthocyanin to sugar ratio is believed to be cultivar dependent, due to different sensitivity of berry anthocyanin to critical ranges of temperature (Fernandes de Oliveira et al., 2015).

1.3.3 Radiation

Solar radiation, along with temperature and thermal amplitude are highly influential for grape phenological stages (Zapata et al., 2017). In general, higher levels of radiation are likely accompanied by higher temperatures, which leads to a higher photosynthetic rate and increased metabolic activity (Arias et al., 2022; Jackson and Lombard, 1993).

Additionally, photosynthesis can be inhibited when the radiation intensity is too high and accompanied by elevated temperatures (Iacono and Sommer, 1996). The natural intensity of ultraviolet (UV) radiation can alter grapevine physiology (Núñez-Olivera et al., 2006), and change grape production and composition (Berli et al., 2011; Kolb et al., 2003). In general, Ultraviolet B (UV-B) radiation at high-altitude can reduce shoot length, leaf expansion, photosynthesis and stomatal conductance; and augmented leaf thickness, photoprotective pigments, proline accumulation and the antioxidant capacity of leaves (Berli et al., 2013; Martínez-Lüscher et al., 2016). Moreover, UV-B is associated with flavonols accumulation in berries (Gregan et al., 2012; Marfil et al., 2019). However, increased levels of UV-B can have a potentially damaging effect on grapevine leaves and berries (Kolb et al., 2003), e.g., total amino acid concentration and total carotenoid pigment content both reduced by exposure to ambient level UV-B (Schultz, 2000). Conversely, UV-C radiation induces the synthesis of stilbene, via the phenylpropanoid pathway (Bais et al., 2000). Stilbenes are important for their defensive roles in plants, pharmacological value and beneficial effects on human health (Kiselev et al., 2019; Vannozzi et al., 2012).

1.3.4 Water

Rainfall or water available for grape production is a crucial factor that affects grapevine characteristics. Water management can be used to manipulate vine and berry attributes (Smart and Coombe, 1983), as changes in water status at critical phenological stages have a direct effect on grape composition and quality attributes by influencing vegetative growth, yield, canopy microclimate, and fruit metabolism (Ezzahouani et al., 2007; Pellegrino et al., 2005; Van Leeuwen and Seguin, 2006). Vine water stress is thought

to enhance fruit quality for wine production (Jackson and Lombard, 1993), but it can at the same time reduce berry size and therefore lower yield (Salón et al., 2005). This has been exemplified by studies where water stress reduced the berry size but increased the phenolic compounds, soluble solids, and the berry anthocyanin concentration at harvest (e.g., Deluc et al., 2009; Savoi et al., 2017). However, significant changes in anthocyanin levels under water stress have not been observed in some of the studies, indicating that this response is common but not universal (Bonada et al., 2015; Brillante et al., 2018). Contrasting results have also been reported among studies on the impact of water stress on tannins (e.g., Casassa et al., 2015; Castellarin et al., 2007; Deluc et al., 2009; Savoi et al., 2017). Similar inconsistencies have been observed for stilbene accumulation (e.g., Deluc et al., 2011; Hochberg et al., 2015; Vezzulli et al., 2007). Still, a strong relationship has been observed between improved grape quality and water stress before veraison (Van Leeuwen et al., 2004). Other studies found that in addition to reduced berry size, sugar content and total acidity were also lowered with water stress. Under mild water stress, grape aroma potential was highest in vines, while severe water stress limits such potential (Des Gachons et al., 2005). A recent meta-analysis indicated that sugars and organic acids negatively and positively correlated, respectively, with grapevine stem water potential (Mirás-Avalos and Intrigliolo, 2017). To conclude, it should be important to note that different varieties respond differently to water deficit and that season conditions affect their responses (Gambetta et al., 2020; Herrera et al., 2017).

1.3.5 Cultivar

The cultivar has a significant impact on berry composition at maturity. A study conducted by Van Leeuwen et al. (2004), found that fruit composition (e.g., malate, sugar, and Potassium content) is especially dependent on the cultivar. Although the same study also showed that the impact of climate and soil was greater than that of cultivars on vine development and berry composition, the impact of cultivars is still a crucial factor to consider. Ripening speed is another crucial factor contributing to fruit composition that varies among cultivars (Costantini et al., 2008). As previously observed, different cultivars also respond to different environmental factors differently. For example,, different cultivars respond differently to water stress, where the impact of water stress on anthocyanin accumulation was greater in Shiraz and Cabernet Sauvignon berries (Hochberg et al., 2015), subsequently influencing the yield and the quality of the berry (Dal Santo et al., 2016).

1.3.6 Soil

Grapevines can be grown on a large variety of soils, and one type of soil might be ideal for vine growth but not ideal for winemaking. For example, vines are vigorous and highly productive in deep, and rich soils, but better wines are generally produced when the vines are cultivated on poor soils (Van Leeuwen and Seguin, 2006). The complex effect of soil on vine and berry composition is due to factors such as vine mineral nutrition, water uptake, rooting depth, and the temperature in the root zone. Among the minerals found within the soil, nitrogen is believed to be one of the most influential regarding vine vigor,

yield, and grape maturation (van Leeuwen and Seguin, 2006). Soil structure and chemistry are believed to influence grapevine composition and wine quality (Mackenzie and Christy, 2005). Analysis of the effects of vine water and nitrogen status, linked to soil type for grafted Cabernet Sauvignon suggested that limited nitrogen uptake is associated with decreased vine vigor, berry weight, and yield, and also with increased sugar, anthocyanin, and tannin accumulation, which consequently increased quality in red wine production (Chone et al., 2001). In addition, Van Leeuwen et al. (2004) found that berry weight is mainly influenced by the soil type, and that grape quality is higher under moderate water stress, especially on clayey soils where water stress occurs early in the season.

Finally, soils contain the richer and more functionally active of all the plant's microbiota (Rodriguez et al., 2019). It is now well stablished that soil microbial communities provide multiple benefits to plants, including better access to nutrients, enhanced growth, and improved tolerance to stress (Corbin et al., 2020). Moreover, soil microbial communities have also been linked to *terroir* at a local (Zhou et al., 2021) and global scale (Gobbi et al., 2022).

1.3.7 Topography

Topography variation is one of the main causes of vineyard variability, such variability can affect the yield (Bramley and Hamilton, 2004), vegetative development (Johnson et al., 2003; Acevedo-Opazo, 2008), and grape composition (Bramley, 2005). Different terrain attributes are factors causing topography variations, such as slope, elevation, and aspect/exposure (Yau et al., 2013). Those factors are then impacting soil depth, water holding capacity, air and soil temperature, radiation exposure, and others (Victorino et al., 2017). The elevation is a good example of how multiple agronomically

important environmental conditions can be impacted by a single topographic factor. Vineyard elevation has been linked to vigor, as low elevation induced higher vigor vineyards due to higher temperatures (Fraga et al., 2014), while at the same time affect berry metabolomic profiles (Tarr et al., 2013) as elevation can have a profound effect on the UV levels experienced by vines (approximately 1% increase every 70 m gain in altitude) (Xie et al., 2017).

1.3.8 Management practices

Management practices refer to the idea of human factors at the vineyard level affecting fruit quality, as suggested by Van Leeuwen and Seguin (2006). Different management practices such as canopy management (Dry, 2000), floor management (Guerra and Steenwerth, 2012; Tesic et al., 2007), which includes practices such as soil management (Likar et al., 2015; Muganu et al., 2013), and weed management (Sanguankeo et al., 2009), have been shown to affect grapevine growth, yield, and berry quality traits. For example, the use of cover crops can increase juice soluble solids, anthocyanins, and other phenolic components and decrease acidity and pH (Guerra and Steenwerth, 2012). In addition, mineral composition varies significantly between differently managed vineyards, e.g., increased bioaccumulation of potassium and phosphorus is associated with sustainably managed vineyards (which utilizes biodynamic or organic farming practices to minimize environmental impacts and ensure economic viability), while increased zinc bioaccumulation is associated with conventional vineyards (Likar et al., 2015). This is significant since the soil concentrations of potassium, iron, and copper, organic matter content, and vesicular colonization, strongly affect the mineral composition of the grapes.

Moreover, differences in soil management have also been associated with vine growth, bud break time, and total soluble solids and anthocyanin contents (Muganu et al., 2013). The goal of canopy management practices is to optimize sunlight interception, photosynthetic capacity, and fruit microclimate. The combination of these factors has been shown to affect the berry composition of red and white grape cultivars, where the combination of leaf removal and either shoot thinning or cluster thinning resulted in higher total soluble solids and anthocyanin content, and lower malic acid and potassium content (Satisha et al., 2013).

To conclude, the concept of *terroirs* is dynamic, and will most likely be affected by climate change (Brillante et al., 2020), similar to other agronomical important crops. Environmental variability can be managed by deeper understandings of the vine/environment interactions, and through the application of innovative agriculture techniques designed to make grapevines more resilient to environmental challenges (Brillante et al., 2020).

1.4 Consequences of climate change related stress on grape quality, yield, and wine production

Stress can be classified into biotic and abiotic. Biotic stresses are caused by biological agents such as fungi, bacteria, viruses, and insects, whereas abiotic stresses are caused by physical environmental factors. Common abiotic factors unfavorable for plant growth and crop yield include drought, saline soils and irrigation, heat, and cold. Worldwide, extensive agricultural losses result from heat stress, often in combination with drought (Vogel et al., 2019). It is expected that the effects of combined drought and heat stress will become more severe as the climate continues to warm (Raza et al., 2019; Zhao

et al., 2017), as it is predicted that an increase in global temperature of 1.5°C will cause more extremely hot days on land, and an increase in the intensity and frequency of drought and precipitation deficits (Masson-Delmotte et al., 2018).

Agriculture is highly dependent on climatic conditions during the growing season. Climate determines the ability to successfully grow a particular variety and can greatly affect the value of the fruit produced (Bai et al., 2022; Jones, 2006; Jones and Davis, 2000). Grape production in general is particularly vulnerable to environmental stress as the environmental conditions occurring during one growing season contribute to the quality and yield of the next vintage (Edwards and Clingeleffer, 2013; Martínez-Lüscher and Kurtural, 2021; Mullins et al., 1992). Viticulture is commonly practiced in regions with a Mediterranean climate (Cs climate according to the updated Koppen-Geiger climate classification (Peel et al., 2007)), where the growing season is characterized by low rainfall, the majority occurring in winter, and by high air temperature and evaporative demand (Fraga et al., 2012). In addition to the coastal regions of the Mediterranean Sea, this includes, the West coast of the Iberia Peninsula, the Pacific coast of Chile and the United States, Cape Town region in South Africa, and portions of the West and South Coast of Australia (Peel et al., 2007). Recent studies have shown that temperature rise is highly correlated with an earlier onset of many growth stages in the grapevine (Alikadic et al., 2019). It has been proposed that an increase in ambient temperatures will constitute the primary cause of water shortages for viticulture due to increased evaporative demand (Schultz, 2010), and may eliminate production in many areas (Diffenbaugh et al., 2011; White et al., 2006). Similarly, climate change is also expected to affect plant-pathogen interactions causing severe damage to grapevine and leading to extensive yield and quality

losses (Gullino et al., 2018; Yu et al., 2012). The maintenance of stable and high-quality supplies of grapes and derived products will demand the implementation of measures such as relocation of vineyards to northern zones or higher altitude areas with lower average temperature (White et al., 2006) or the development of novel and faster breeding programs.

1.5 Transcriptomic approaches to understand the responses of grapevine to stress

Studying the regulation of gene expression can provide a deeper understanding of the molecular regulation of the physiological mechanisms used by grapevine to respond to various stresses such as elevated temperatures (heat) or drought. Earlier efforts included the use of Expressed Sequence Tags (ESTs), which resulted in the development of a microarray containing a set of 3,200 Unigenes from *V. vinifera* to study grape development (Terrier et al., 2001; Terrier et al., 2005). The number of unigenes present on the microarray rapidly increased with newer technologies such as the Operon (Camps et al., 2010) or Affymetrix (Deluc et al., 2009) grape arrays. The complete sequence of the grapevine genome became available after the sequencing and assembly of the PN40024 line (Jaillon, 2007). With that being available, NimbleGen microarrays were utilized to study grape transcriptome (Pastore et al., 2017). With the advance of technology, full coverage of the grapevine transcriptome was made possible by next-generation sequencing, namely RNA-sequencing (Zenoni et al., 2010). Since then, both genome wide-microarrays and RNA-sequencing have been widely used to characterize the response of grapevine to various stress. Some examples include heat (i.e., Rienth et al., 2016), drought (i.e., Berdeja et al., 2015), and UV-B stress (Du Plessis et al., 2017). The high-throughput sequencing technology has been proven useful in revealing potential key stress response genes, which

could be highly beneficial for breeding new grape cultivars that can better adapt to the changing environment. Examples of the key genes that have been characterized as playing a role in grapevine stress response, include *leafy cotyledon1-like* (*LEC1*) and *somatic embryogenesis receptor kinase* (*SERK*) (*VvL1L* and *VvSERK*, respectively in grapevine), which are key regulators of grapevine development and stress response (Maillot et al., 2009). *Abscisic acid-insensitive 3* (*ABI3*), a gene that is involved in abscisic acid (ABA) signaling and drought response (Mittal et al., 2014; Rattanakon et al., 2016). Various calcium-dependent protein kinases (CDPKs), such as *VaCPK20* and *VaCPK29* identified from *V. amurensis* have been shown to be involved in drought and cold tolerance, and to heat and osmotic stresses, respectively, when being overexpressed in transgenic grape cell cultures and in *Arabidopsis thaliana* (Dubrovina et al., 2015, 2017). Several dehydration responsive proteins associated genes and transcription factors regulated by ABA, including *dehydration responsive element-binding protein1a* (*DREB1A*), have been identified as regulators of stress-responsive genes against drought tolerance (Cardone et al., 2019), while apoptosis related-proteins associated genes were shown to be involved in the regulation of programmed cell death and defense against biotic stress (Repka, 2006). The exact role and mechanism of action of these genes can vary depending on the type of stress and the grapevine genotypes being studied and they are often a part of a much more complex stress signaling pathways. Additionally, Zha et al. (2020) used transcriptomic analysis to study grapevine response to heat stress and identified two important genes central to grapevine's response to heat stress, *heat shock factor a2 and a7* (*VvHSFA2* and *VvHSFA7*, respectively). Cochetel et al. (2020) showed that more drought tolerant grapevine genotypes are more responsive transcriptionally in terms of ABA signaling and

biosynthesis than less drought tolerant ones. The authors also identified core genes to drought stress as well as gene clusters and sub-networks that are associated with drought tolerance in grapevine.

Transcriptomic analyses are not without limitations. Rienth et al. (2014) showed that the transcriptome of grapevine plants under heat stress can vary drastically depending on the time of the day the stress is being applied. The results from this study suggested that future grapevine transcriptomic analyses should rely on standardized experimental designs. Additionally, the quantitation of the applied stress factor and the physiological impact on the plant should be measured carefully (Berdeja et al., 2015). Moreover, a large body of research has suggested the need to go beyond classical differentially expressed gene (DEG) analysis, and use more detailed tools and analyses such as weighted gene co-expression network (WGCNA) and cluster analysis. Those will provide more in-depth knowledge on stress response by revealing co-regulated gene modules and potential master switch/hub genes that might be key for abiotic stress responses in plants (Cochetel et al., 2017; Hopper et al., 2016; Palumbo et al., 2014). Moreover, although stress conditions in the natural environment often occur in combination (e.g., heat and drought stress tend to occur simultaneously in grapevine cultivating regions), a majority of grapevine transcriptomic studies deal with only one abiotic stress factor, where such a factor is often applied in controlled or semi-controlled conditions. Therefore, it has been suggested that transcriptomic studies should integrate stress combinations in their experimental design (Gomès et al., 2021). We integrated these recommendations in our most recent global transcriptomic and gene co-expression network analysis to reveal core genes central to grapevine response to combined heat and drought stress (Tan et al., 2023). Interestingly,

this work also found that epigenetic chromatin modifications may play an important role in grapevine responses to combined drought and heat stress through the establishment of an epigenetic memory of stress.

1.6 Epigenetic mechanisms in the context of plant adaptation to stress

Plants have developed various mechanisms to adapt to daily environmental conditions, and the regulation of gene expression through both transcriptional and post-transcriptional regulation is particularly important for their survival. Among those strategies are a suit of molecular mechanisms studied under the umbrella term of epigenetics. Waddington (1942) first proposed the term *epigenetics*, referring to the study of the interactions between genes and the environment. The current definition of the term refers to heritable changes in gene function without changes to their underlying DNA sequence (Wu and Morris, 2001) that are usually mediated by three main types of changes: DNA methylation, histone post-translational modifications (PTMs), and the expression of small RNAs (sRNAs) (Agarwal et al., 2020).

1.6.1 DNA Methylation

DNA methylation generally refers to the addition of a methyl group to carbon 5 of cytosine bases, thus forming 5-methylcytosine or 5mC. Although other forms of DNA methylation has been detected in plants, including N^6-methyladenine (6-mA), and 5-hydroxymethylcytosine (5-hmC) (Kumar and Mohapatra, 2021), we will focused on 5mC. The establishment and maintenance of plant cytosine methylation depends on the cytosine sequence context (i.e., CG, CHG, or CHH, *H* = a nucleotide other than G), and is catalyzed

by DNA methyltransferases. CG and CHG methylation is regulated by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively (Zhang et al., 2018a), while CHH methylation is maintained by either DOMAINS REARRANGED METHYLASE 2 (DRM2) or CHROMOMETHYLASE 2 (CMT2) (Zemach et al., 2013) depending on the genomic region. In general, cytosine methylation impacts genome stability and influences chromatin structure, thus also controlling the accessibility of genetic information (Bouyer et al., 2017; Jin et al., 2011; Zhang et al., 2018a). The effect of cytosine-methylation on gene expression is proposed to be determined by its genic context, i.e., cytosine methylation occurring within the promoter usually act to repress transcription, although in some cases it promotes gene transcription (Zhang et al., 2018a). On the other hand, gene-body methylation and transcription has been observed to be positively associated at some level (Yang et al., 2014), however, its function remains unknown (Bewick and Schmitz, 2017).

Numerous studies have examined the potential roles of cytosine methylation in plant response to various biotic and abiotic stress factors, including but not limited to heat, cold, drought, salinity, and pathogen infections (e.g., Eichten and Springer, 2015; Konate et al., 2018; Liu et al., 2017). Many early studies have shown that stress not only induces genome-wide cytosine methylation and/or demethylation patterns but also loci specific changes, and that these changes in cytosine methylation may be associated with the transcriptional regulation of genes involved in plant stress response (Khan et al., 2013; Yong-Villalobos et al., 2015; Zhang et al., 2018b).

1.6.2 Histone post-translational modification (PTMs)

Histone PTMs, such as phosphorylation, lysine acetylation, arginine and lysine methylation, ubiquitylation, proline isomerization, ADP ribosylation, arginine citrullination, SUMOylation, carbonylation, and, with some controversy, biotinylation, are essential elements of the chromatin signaling pathway (Arnaudo and Garcia, 2013; Seet et al., 2006). Among those, histone acetylation/deacetylation and histone methylation/demethylation are well characterized. Their effect depends both on the type of modification and on the histone residues being modified, for example, di-methylation and tri-methylation on lysines 9 and 27 of Histone 3 (H3K9 and H3K27 respectively) result in gene expression repression, compared to the gene transcription activating mono-methylated forms, while acetylation of those same residues is associated with transcription activation. Moreover, the repressive transcriptional state of both transposable elements and repetitive sequence-enriched heterochromatic regions are maintained by H3K9 monomethylation and dimethylation (H3K9me1 and H3K9me2, respectively) in plants. Heterochromatin regions are also associated with H3K27me1, while the repression found in euchromatin regions is associated with H3K27 trimethylation (H3K27me3) (Liu et al., 2010). The involvement of histone modification in regulating plant responses to stresses by mediating gene expression has been extensively studied. Some examples include the involvement of histone acetyltransferase (HATs), deacetylases (HDACs), and demethylases (HDMs), which play important roles in the response to various stress in a variety of plants (e.g., Ueda and Seki, 2020).

1.6.3 Non-coding RNA-mediated regulation

The third main epigenetic mechanism involves two species of RNA molecules, i.e., small-interfering RNAs (siRNAs) and microRNAs (miRNAs), which have been shown to regulate gene expression at the transcriptional and post-transcriptional levels (Wei et al., 2017). In general, miRNAs are processed from single-stranded RNA (ssRNA) stem-loop precursors by DICER-LIKE 1 (DCL1) ribonucleases (Axtell, 2013) and when loaded into Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC), they regulate gene expression post-transcriptionally, by directing mRNA degradation and translational repression (Rogers and Chen, 2013). On the other hand, siRNAs are processed from double-stranded RNA (dsRNA) precursors and can be further classified into multiple subclasses depending on their size (i.e., 21, 22, or 24 nucleotides (nt) long). The 21-nt and 22-nt siRNAs are associated with mRNA cleavage, while 24-nt siRNAs regulate DNA methylation, with those participating in the RNA-directed DNA methylation (RdDM) pathway being the most abundant (Matzke and Mosher, 2014). Based on the number of nucleotides, these siRNAs either participate in canonical RdDM pathway (24-nt siRNAs) that target transposable elements (TEs) and other repeats to induce DNA methylation and reinforce their transcriptional silencing (Du et al., 2015; Matzke and Mosher, 2014) or participate in noncanonical RdDM pathway (a small fraction of 21-22nt siRNAs) to establish the silencing of novel TEs at new target loci, both transcriptionally and post-transcriptionally (Nuthikattu et al., 2013). The functional outcome of a specific 21-22nt siRNA depends on the associating AGO protein. The association with AGO4, AGO6, or AGO9 will result in a noncanonical RdDM pathway and DNA methylation, while the association with other AGOs will result in post-transcription gene silencing

(PTGS) through the cleavage of mRNAs (Cuerda-Gil and Slotkin, 2016; Matzke and Mosher, 2014).

The involvement of miRNA and siRNA in plant stress response by regulating gene expression has been studied extensively. A large number of miRNAs and putative siRNAs such as miRNA156 have been shown to play important roles in stress response in plants (e.g., Ito et al., 2011; Sunkar and Zhu, 2004).

In conclusion, these epigenetic mechanisms are thought to be closely related, acting together to coordinate gene activity at the transcriptional level and regulate different cellular processes and responses to environmental stimuli (Bartels et al., 2018) despite having their own regulatory mechanisms.

1.7 Epigenetics in grapevine

Fortes and Gallusci (2017) proposed grapevine as a model to study epigenomics in perennial woody plants of agricultural importance due to its characteristics. Which include a genome and methylome more like those of other crops than those of the most widely used model plant, *Arabidopsis thaliana* (Lee and Kim, 2014). In addition to a set of important agronomic characteristics, which have been previously associated with epigenetic mechanisms, the grapevine is considered to be one of the models for non-climacteric fruit development (Fortes et al., 2015), (1) due to the usage of grafting and vegetative propagation (Lewsey et al., 2016); (2) vine age and vineyard location (Grigg et al., 2018; Grigg, 2017; Xie et al., 2017) have been traditionally associated with fruit production and quality; and (3) grapevine flower development has been shown to be programmed and affected by the environmental conditions one year in advance (Guilpart et al., 2014),

indicating the establishment and maintenance of long-term memory of the environmental conditions (López et al., 2022).

Although multiple studies have shown that the main driver of DNA methylation variability in grapevine is the genotype (Dal Santo et al., 2018; Varela et al., 2021), recent studies have suggested that the growing environment can have a significant effect on the methylome of the vine and that such environmentally induced epigenetic changes could be the molecular basis of *terroir* in grapevine. In 2017 (Xie et al.) showed that the main contributor to differences in DNA between 22 *V. vinifera* cv. Shiraz vineyards in six sub-regions of South Australia was geographic distance (with 9% of the identified differentially methylated genes being associated with response to environmental stimulus), followed by vineyard management and altitude. A later study comparing DNA methylation patterns in two *V. vinifera* cultivars (i.e., Merlot and Pinot Noir) planted in contrasting climatic regions showed that a significant amount of DNA methylation variability (roughly 80% and 71% of Merlot and Pinot Noir, respectively) was associated to geographical location (Baránková et al., 2021).

The regulation of the biosynthesis of metabolites and accumulation of phenolic compounds in grapevine also are found to be associated with epigenetic mechanisms. In *V. amurensis* cell cultures treated with 5-Azacytidine, a demethylating agent, the methylation level of a stilbene synthase gene was significantly reduced, while expression of the same gene and synthesis of resveratrol were significantly increased, which led to a high level of resveratrol compared to the control cell culture, suggesting that the DNA methylation may be involved in the control of resveratrol biosynthesis during *in vitro* culture (Kiselev et al., 2013). DNA methylation also has been reported to have a role in the regulation of stilbene

synthase genes (Kiselev et al., 2013) and anthocyanin accumulation during berry maturation (Jia et al., 2020) in grapevine. In addition, UV-B was associated with flavonol accumulation in *V. vinifera* cv. Malbec berries and hydroxycinnamic acids in early fruit shoots, and these changes might be DNA methylation-dependent (Marfil et al., 2019). Interestingly, in a study that analyzed ten grape varieties, a negative correlation between gene body methylation and gene expression variation between grapevine varieties was observed. The authors proposed that a higher number of transposable elements (TEs) within the grapevine genes may be responsible for this negative association between gene body methylation and expression (Magris et al., 2019). Pereira et al. (2022) were able to characterize nine grapevine DNA methyltransferase genes and suggested that changes in grapevine genome methylation are associated with the establishment of compatible and incompatible interactions with *Plasmopara viticola*. A following study by Azevedo et al. (2022) observed that DNA methylation is affected by *P. viticola* inoculation and that differences in the DNA methylation levels might be related to the different susceptibility to *P. viticola*. These studies provided useful insights into the role of epigenetic mechanisms in grapevine defense against downy mildew and their potential implications for future breeding programs such as improving tolerance to powdery mildew in grapevine and reducing the massive current and recurring use of chemicals. Additionally, the use of DNA methyltransferases blockers (including but not limited to 5-azacytidine, 5-aza-2'-deoxycytidine, 1-beta-D-arabinofuranosyl-5-azacytosine and dihydro-5-azacytidine) has been proposed as an approach to generate epigenetic variation for crop improvement (Amoah et al., 2012).

1.7.1 Stress memory, priming, and epi-breeding

Similar to other crop breeding, classical grapevine breeding relies on the transfer of desirable traits by crossing and recurrent selection of genetic variants. However, the reliance on limited germplasm has resulted in an irreversible loss of genetic diversity, known as genetic erosion (Gallusci et al., 2017), making grapevine genetic improvement difficult (Esquinas-Alcázar, 2005). This has been exposed by the vulnerability of current varieties to rapid climate changes (Esquinas-Alcázar, 2005). Epigenetic mechanisms, on the other hand, play an essential role in the interactions between genes and the environment (Baulcombe and Dean, 2014; Bräutigam et al., 2013). As the study of epigenetics has advanced, it has provided novel directions to drive plant-breeding strategies by exploiting epigenetic variation and/or manipulating the epigenome to improve adaptation to various environmental stresses and ensure yield and quality (Gupta and Salgotra, 2022; Pecinka et al., 2020; Rodríguez López and Wilkinson, 2015; Tirnaz and Batley, 2019) (Figure 1.2). Indeed, studies have revealed the relevance of epigenetic regulation of stress response in many crop and model species such as arabidopsis (e.g., Tricker et al., 2012), barley (e.g., Konate et al., 2018), maize (e.g., Steward et al., 2002), rice (e.g., Zheng et al., 2017), soybean (e.g., Song et al., 2012), tomato (e.g., González et al., 2013), and wheat (e.g., Wang et al., 2016). Moreover, the study on both natural and artificial epigenetic diversity could contribute to improvement of current breeding programs, via multiple strategies, including the identification of epigenetic biomarkers capable of predicting plant performance in a given environment (Kakoulidou et al., 2021) and the selection epigenetic variability in genomic regions that modulate gene expression of traits of interest, after the validation of the functional association between a given epiallele and a given trait. The

origin of such variability can be genotype dependent (Rodríguez López and Wilkinson, 2015), or exogenously generated through the application of chemicals capable of randomly altering the epigenetic profile of the target genome (Amoah et al., 2012) and/or via targeted gene editing approaches (Volta et al., 2016). Moreover, the plastic and potentially heritable dual nature of environmentally induced epigenetic variability provides the potential of generating epigenetically-controlled adaptive traits to accelerate crop breeding (Rodríguez López and Wilkinson, 2015) (Figure 1.2).

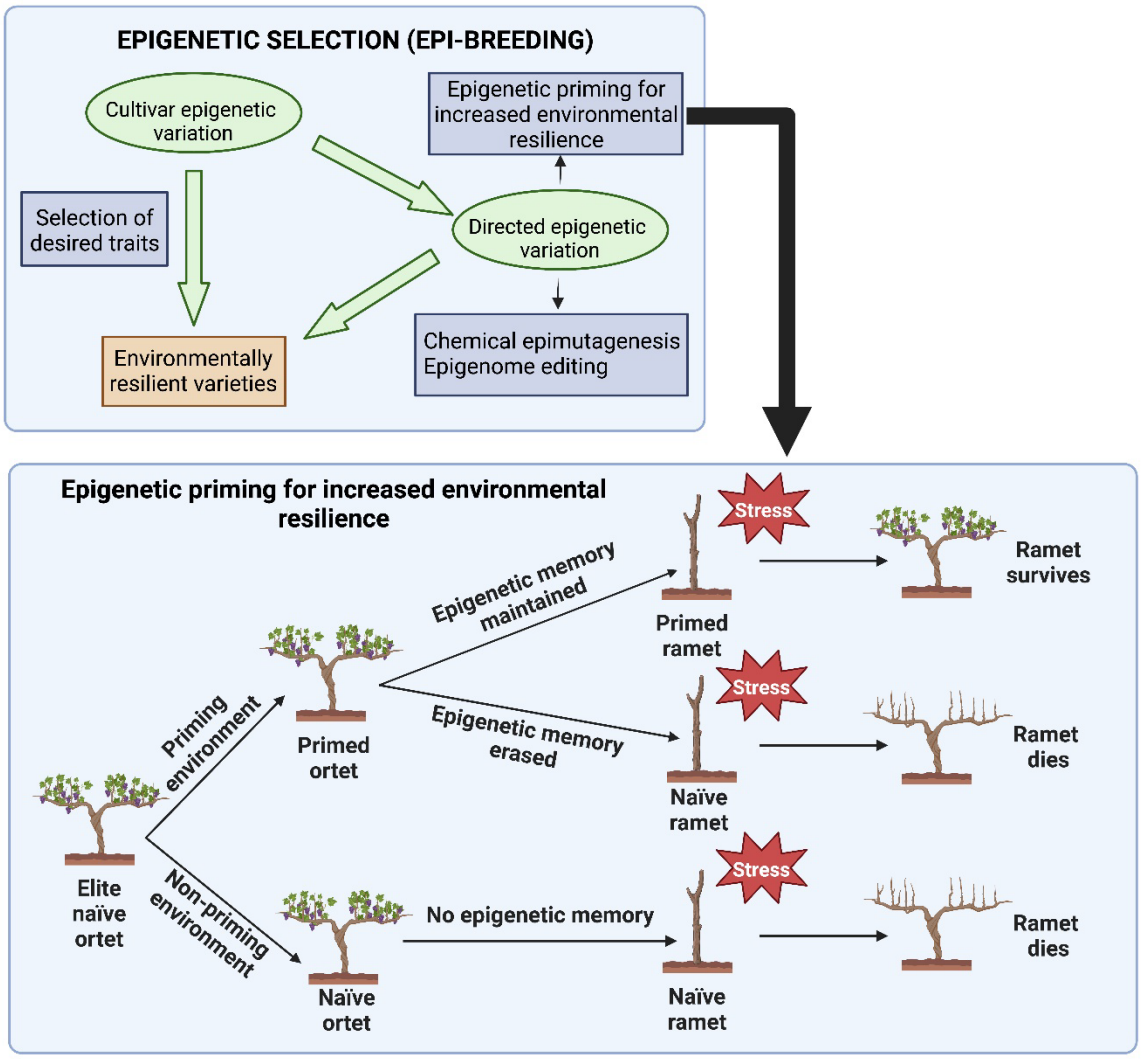


Figure 1.2 Epigenetic priming for the production of environmentally resilient grapevine cultivars.

The top box shows two epi-breeding approaches for the production of environmentally resilient grapevine varieties via the selection of epigenetic variant of agronomic interest (adapted from Rodriguez Lopez and Wilkinson 2015). The bottom box shows the proposed method to enhance stress tolerance through epigenetic priming maintenance in perennial crops (modified from Rodriguez Lopez, 2019).

Stress and environmental stimuli can indeed induce epigenetic variation in the genome, leading to phenotypic plasticity, where different phenotypes can arise from the same genome due to alterations in the epigenetic marks (Asensi-Fabado et al., 2017; Fortes and Gallusci, 2017). The acclimation and response process are thought to be related to the development of stress memory in plants (Figure 1.1). Stress memory is often associated with a phenomenon called stress priming, which is trigged by extreme conditions that inhibit normal growth and development. Priming has occurred when a plant shows a modified response to stress, after an initial exposure to a stimulus, as compared to a plant in the naïve (unprimed) state (Aranega-Bou et al., 2014). Priming is evidenced by positive effects like stronger or faster response to stress (Bruce et al., 2007; Conrath, 2009; Crisp et al., 2016). Studies have shown that plants have a memory of the first (priming) stress and are able to retrieve the remembered information upon encounter with the later stress when there is a period of no stress between the two stress events (Hilker and Schmülling, 2019). Additionally, studies have shown that priming is effective at various stages of the plant life cycle, starting from seed (i.e., seed priming) to seedlings and subsequent adult stages (Mozgova et al., 2019). While this priming and subsequent stress memory has provided valuable information on breeding more vigorous crops via various products and techniques (e.g., Brzezinka et al., 2016), the underlying molecular mechanisms that establish, regulate, and even erase such memory has been puzzling (Iwasaki, 2015; Roberts and López Sánchez, 2019; Varotto et al., 2020).

Studies have, however, identified several mechanisms of storage and retrieval of this stress memory, which include epigenetic regulation, transcriptional priming, the primed conformation of proteins, or specific hormonal or metabolic signatures (Crisp et

al., 2016; Ding et al., 2012; Hake and Romeis, 2019; He and Li, 2018; Heil and Karban, 2010). It is important to consider that a transcriptional response is usually triggered when plants are exposed to stress. After physiological recovery, the previously stressed plant enters the primed state, during which the transcription of the majority of stress-responsive genes will return to their original expression levels. The degree and time of recovery depends on the intensity of the environmental cue (Avramova, 2015). The encounter of a second stress will trigger a different response to that shown by unprimed plants. The triggered response can be faster, stronger, more sensitive, and/or different (altered) than the first one (Lämke and Bäurle, 2017).

Some stress-inducible genes are linked to establishing a memory of stress, and they do not necessarily revert to their non-stress transcriptional state and are therefore termed stress memory genes (Charng et al., 2007; Charng et al., 2006; Ding et al., 2012; Lämke et al., 2016; Liu et al., 2018). Currently, memory genes are classified into two groups based on their transcriptional profile: Type I – those whose change in expression pattern persists through the recovery phase, and Type II – those whose response is modified during a second exposure compared to the initial stress response (reviewed in Bäurle (2018), and Bäurle and Trindade (2020)) and it is usually stronger and faster than the first response (Mozgova et al., 2019; Roberts and López Sánchez, 2019) (Figure 1.3). Built upon this knowledge, more evidence suggests that stress memory and the modified transcriptional response are heavily epigenetic-based and involve mechanisms such as chromatin remodeling, DNA methylation, nucleosome position, histone modification, and noncoding RNA-mediated regulation (Liu et al., 2022).

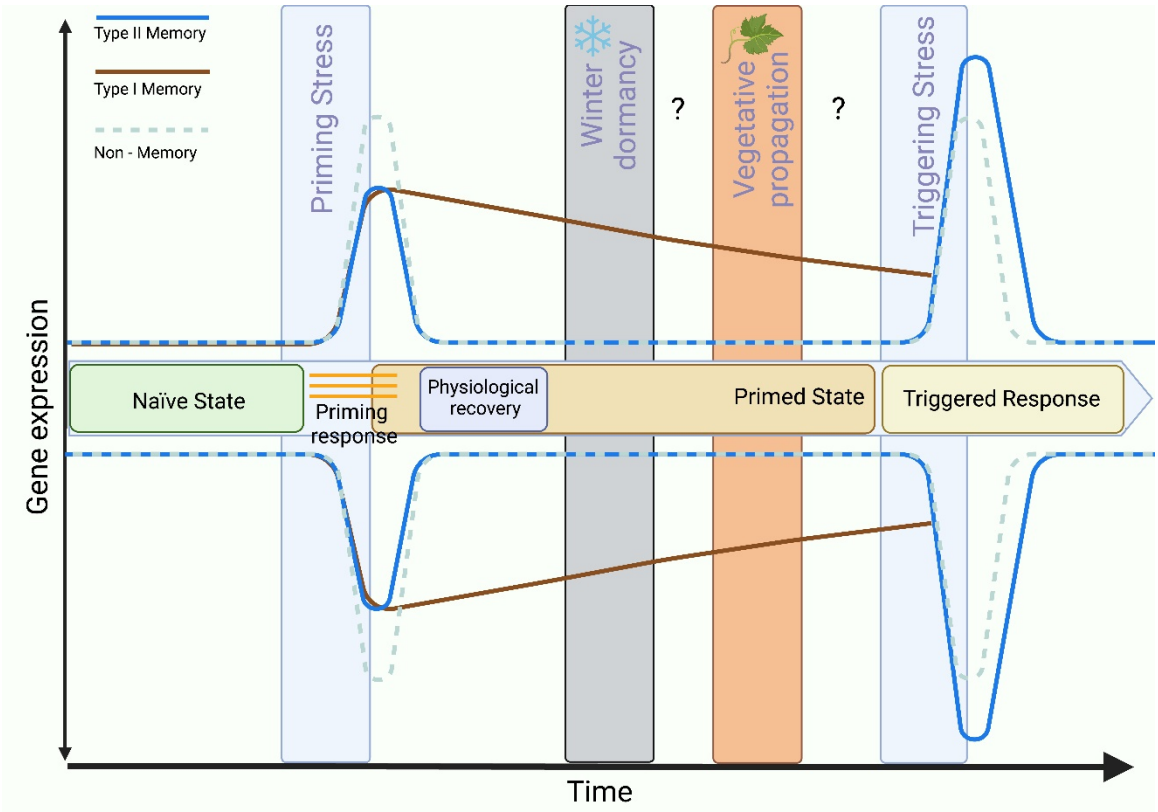


Figure 1.3 Effect of somatic memory of environmental stress on plant gene transcription in the context of perennial vegetatively propagated plants.

Solid and dashed lines represent the transcriptional changes of stress-responsive genes triggered by the first stress encountered by naïve plants (priming stress), and by subsequent stress (triggering stress) encountered by primed plants. Stress-responsive genes can be classified into three categories based on their transcriptional profiles during priming and triggering stress events: (1) Non-memory genes, the stress-induced transcriptional changes are identical in naïve and primed plants; (2) Type I memory genes, the stress-induced transcriptional changes are sustained after stress removal and through physiological recovery; (3) Type II memory genes, the magnitude of the stress-induced transcriptional changes is larger in primed than in naïve plants (Bäurle, 2017). Current research in annual plants suggests that the primed state is maintained for a finite period within the same generation (somatic memory) and that it can also be inherited by the offspring of primed plants (inter-/transgenerational memory, not shown here), however, the effect of dormancy cycle and vegetative propagation on the maintenance of priming has not been sufficiently studied in perennial plants.

Although the mechanisms underlying the stable status of epigenetic traits are not fully understood, stress induced epigenetic traits can be stable and therefore be inherited by the next generation as part of an adaptive form of memory (Johnson and Tricker, 2010). The effect of this stress memory can be observed through the physiological, transcriptional, and biochemical modifications occurring in the plant when re-exposed to the stress, resulting in the plant becoming more resilient (or sensitive) to the same stress (de Freitas Guedes et al., 2018; Perrone and Martinelli, 2020; Tricker et al., 2013a) or a different stress (Tricker et al., 2013b). The duration of this memory varies from days to weeks or months for somatic memory (intergenerational), but it can be stable and inherited by offspring to one or more stress-free generations (transgenerational) (Bäurle, 2018; Lämke and Bäurle, 2017; Tricker et al., 2013a; Blödner et al., 2007). In annual plants, the key to keeping the transcriptional state associated with the primed response across generations is the repeating stress in the progeny (Boyko et al., 2010; Wibowo et al., 2016) and a stress recovery phase of the mother plants (López Sánchez et al, 2021).

The potential importance of persistent stress for establishing DNA methylation-dependent stress memory through priming in plants has been highlighted and studied in annual plants, such as arabidopsis (e.g., Ding et al., 2012; Tricker et al., 2013a; Tricker et al., 2013b), maize (e.g., Forestan et al., 2020), and rice (e.g., Cong et al., 2019). How this translates to perennial plant species, which can be exposed many times during their life span, has not been studied to the same level. Studies on the effect of priming and establishment of stress memory on perennial species have been limited, a majority of the studies that have been conducted, have been focused on forest trees such as poplar (reviewed in Amaral et al., 2020; Le Gac et al., 2018; Sow et al., 2018). Other recent studies

have addressed the effect of stress on the epigenome of different perennial plant species, including coffee (de Freitas Guedes et al., 2018), the perennial grass species tall fescue (Bi et al., 2021), and wild strawberry (López et al., 2022). Taken collectively, these studies show that the plant epigenome is versatile and plastic in response to environmental stress and that the resulting change could potentially prime the plants against future stress (López et al., 2022). Viticulture could benefit from a deeper understanding of how this memory of stress is established, maintained, and even reset, potentially leading to the production of more resilient grape varieties.

1.8 Future prospects, potential challenge, and gaps in knowledge

As described in this review, there is growing evidence that epigenetic mechanisms play an important role in increasing crop resilience to stresses and therefore may be an important tool in the development of more resilient grapevine cultivars. Some additional examples include: Lämke et al. (2016) have described the methylation of histone H3 lysine 4 (H3K4) is involved in the heat stress-induced genes in arabidopsis. Moreover, Surdonja et al. (2017) showed that DNA methylation and target gene repression by small non-coding RNAs were involved in the drought stress response in barley. Similarly, the presence of possible epi-marks that are drought inducible and inheritable across generation were observed in rice and that multigenerational drought exposure improved the adaptability of rice plants to drought conditions (Zheng et al., 2017). Taken collectively, these showed that epigenetic modifications play important roles in stress response and the long-term adaptation to changing environmental conditions (Zheng et al., 2017).

There are some challenges to the utilization of epigenomics to design environment resilient grapevine, such as the stability and heritability of the epigenetic variation, which are important for the potential transmission to the progeny (Eichten et al., 2014; Iwasaki and Paszkowski, 2014; Vriet et al., 2015). Most of the stress-induced epigenetic modifications have been observed to return to basal levels when the stress is removed, but some of the modifications can be inherited mitotically and meiotically in plants (Sudan et al., 2018). Such epigenetically-mediated stress memory can lead to long-term adaptation and is a good indication of the possibility of using epigenetics as a tool to combat environmental stress. It is important to note, however, that further study is needed to understand various factors that might affect epiallele stability to avoid inducing epialleles that might be unstable during the breeding process (Hofmeister et al., 2017). Moreover, epigenetic variation also can be maladaptive and become an epigenetic trap (Consuegra and Rodríguez López, 2016), e.g., if the changes they induce do not match the environment experienced by the offspring. Also, the energetic cost associated to the maintenance of the acquired epigenetic state, that could negatively impact plant growth and development, and ultimately affect crop yield (Chinnusamy and Zhu, 2009) (Figure 1.1).

Another major challenge in creating epigenetic populations in crops is the uncertainty of whether epigenetic changes (i.e., alteration of DNA methylation patterns) induced by approaches developed in model species such as arabidopsis can be transferable to crops, since so few to none viable equivalent mutants have been produced in crop species (Hu et al., 2014; Kawakatsu and Ecker, 2019; Li et al., 2014). An alternative approach such as epimutagenesis and targeted epigenome editing can be utilized, as demonstrated in arabidopsis (Johnson et al., 2014; Springer and Schmitz, 2017). However, it will require

advancement and innovation in both technical and biological disciplines to develope the full potential of epigenomic variants and use them efficiently in the breeding of better stress-adapted crops (Varotto et al., 2020). Similarly, for the integration of epigenetics and epigenomics in crops, or more specifically grapevine breeding, more knowledge needs to be acquired on stress induced epigenetic memory in perennials. Acquisition of such knowledge should move beyond describing the correlation between epigenetic variation and the desired trait to demonstrating the functional association between acquired epialleles and enhanced tolerance to stress.

Among the plethora of epigenetic memory of stress and priming studies done in plants, only a small amount of them is perennial focused – even less on grapevine specifically. Contrary to the limited studies on epigenetic memory of stress and priming, there is no lack of observations of stress priming in grapevine. Some of the more recent studies that observed physiological, transcriptional, and biochemical modifications, which potentially indicative of established stress memory in grapevine, include Babajamali et al. (2022), these authors showed that drought stress priming improved freezing tolerance in shoot and root tissues of both drought-tolerant and sensitive grapevine cultivars. In addition, a study performed on dry-grown Cabernet Sauvignon suggested the more drought-resilient grapevines with superior vine water status, leaf gas exchange and berry size are likely due to long-term drought stress adaptation via stress priming (Pagay et al., 2022). Spray-induced gene silencing (SIGS) that targets a putative grape glutathione S-transferase (GST) gene (VvGST40) has been shown to prime vines resulting in increased resilience to severe drought (Nerva et al., 2022). In the response to salinity stress, it has been shown that 6-Benzylaminopurine (BAP) primes salt tolerance in *V. vinifera,* with

BAP-primed plants exhibiting higher intrinsic water use efficiency, photosystem-II efficiency, and growth than control plants (Montanaro et al., 2022). Moreover, grapevines infected with Grapevine fanleaf virus (GFLV) are more resilient to mild water stress than healthy vines, suggesting that biotic stress (GFLV) can potentially induce priming in grapevine (Jež-Krebelj et al., 2022). Many more studies, including biotic stress priming (e.g., Perazzolli et al., 2011; Trouvelot et al., 2008; Verhagen et al., 2010) and abiotic stress priming (e.g., Tombesi et al., 2018) provide evidence of priming effects in grapevine. Even with the ever-growing research on epigenetic regulations in the grapevine, to date, a limited amount of research is available on how this memory of stress and its underlying epigenetic mechanisms are established, maintained, and even reset. The long lifespan of woody perennials could be used to address some of the prevailing concerns in studies with annual plants, e.g., was the period of vegetative growth between the priming treatment and the second stress treatment long enough to test whether the stress was phenotypically effective and whether the changes in the epigenome were induced by the priming treatment (Sani et al., 2013). Therefore, studies in woody perennials can provide valuable insights into how long-term somatic memory is established and if it can be maintained past dormancy cycle. Similarly, the connection between vegetative propagation and epigenetic memory of stress establishment and maintenance also should be considered. The use of vegetative propagation (i.e., propagated through cutting or layering) in woody perennials could reveal novel and useful information on how permanent or transient long-term somatic memory is after vegetative propagation (Perrone and Martinelli, 2020). Viticulture could benefit greatly from an understanding of transient or stable modification to the epigenome of stress memory, as it may contribute to development of novel molecular approaches such as

targeted, gene-specific modifications to the epigenome for stress adaptation through plant breeding, leading to the production of more resilient grape varieties. However, when using epigenetic and epigenomics to develop stress resilient crop, the negative effects of stress memory on breeding in general should be considered, since the obtained stress memory could inhibit normal plant growth (Chinnusamy and Zhu, 2009). The prediction and assessment of the impact of stable epigenetic variation on plant phenotype and performance should be explored further, via machine learning and model training as demonstrated in several studies (Colicchio et al., 2015; Hu et al., 2015; N'Diaye et al., 2020).

Despite the gap in knowledge of stress memory establishment and maintenance, the advancement in technology and the employment of multi-omics approaches have allowed epigenetic breeding (epi-breeding) to be successful in various aspects (Rajnović et al., 2020), including generation of mutant lines (e.g., Yang et al., 2015), recurrent epi-selection (e.g., Greaves et al., 2014; Hauben et al., 2009), and epigenome editing (e.g., Park et al., 2016), as well as the usage of priming/stress memory (e.g., Lämke and Bäurle, 2017). One of the successful examples is the suppression of the nuclear-encoded *MutS HOMOLOGUE 1* (*MSH1*). The success of the *MSH1* system has been reported in arabidopsis and tomato, where the phenotypic changes that led to improved growth vigor and yield were linked to DNA methylation. These improvement can be repressed by 5-AzaC, while METHYLTRANSFERASE 1 (MET1) and HISTONE DEACETYLASE 6 (HDA6) played an important role in the phenotypic changes (Kundariya et al., 2020; Yang et al., 2015; Yang et al., 2020). In soybean, epigenetic selection has led to yield improvement for at least three generations (Raju et al., 2018). Moreover, after crossing the *msh1* mutant to the wild type, the created epi-population was shown to possess multiple yield-related traits

both in the greenhouse and in the field (Raju et al., 2018). Many other examples showing the potential of epi-breeding for plant adaptation to various stresses, including the usage of eustressors have been reviewed by Kakoulidou et al. (2021), and Villagómez-Aranda et al. (2022). For these successful examples to serve as future grapevine improvement strategies, their inherent characteristics (long-living perennial, highly heterozygous, high inbreeding depression) must be considered.

If the grapevine industry, and by extension other perennial crop industries, want to benefit from the potential use of epi-breeding approaches to produce climate resilient varieties, future multi-omics studies should be custom designed to (1) unravel how environmentally-induced epigenetic mechanisms interact with gene expression to affect the vine's phenotype, and (2) determine if environmental stress is followed by the establishment and maintenance of a memory of stress in grapevine. Such studies will lay the foundation for the development of a comprehensive model integrating plant response to stress, the establishment of transcriptional and epigenetic memory of stress, and their maintenance, over time and during vegetative propagation in perennial plants.

1.9 Outline of the dissertation

The major objective of this dissertation is to develop a comprehensive model for woody perennial plants that will cover:

1. Plant (grapevine) response to individual and multiple stresses.
2. The establishment of transcriptional and epigenetic memory of stress, and their maintenance, over time and,

3. During vegetative propagation.

Chapter two focuses on the stress response in grapevine to individual stress such as heat and drought, as well as the two stresses in combination. In this study, I studied the physiological and molecular response of grapevines under stress and identified 5 hub genes for the combined stress co-expression network. I observed differences in transcriptional response to the individual and combined stress and identified histone modifying genes to be involved in combined stress response. Overall, we observed that the effect of combined stress on physiology and gene expression is more severe than that of individual stresses and suggested that epigenetic chromatin modifications may play an important role in grapevine responses to combined drought and heat stress.

The majority of the content in this chapter has been published:

Tan, J.W., Shinde, H., Tesfamicael, K., Hu, Y., Fruzangohar, M., Tricker, P., Baumann, U., Edwards, E., & Rodriguez-Lopez, C.M. (2023). Global Transcriptome and Gene Co-Expression Network Analyses Reveal Regulatory and Non-Additive Effects of Drought and Heat Stress in Grapevine. *Frontiers in Plant Science, 14,* 10.3389/fpls.2023.1096225

Chapter three is mainly to study the maintenance of acquired long-term somatic memory of stress in grapevine. In this study, I observed that plants that have been stress-primed are more transcriptionally active compared to the plants that have not been exposed to stress before. Methylome analyses suggest that stress-induced expression changes are, at least partially, independent of DNA methylation. Overall, this study showed that a long-term somatic memory of stress can be maintained in grapevine even after a long period of time.

The majority of this chapter will be submitted:

Tan, J.W., Tesfamicael, K., Shinde, H., Hu, Y., Tricker, P., Edwards, E., & Rodriguez-Lopez, C.M. Transcriptome Analysis Reveals Long-Term Somatic Memory of Stress in the Woody Perennial Crop Grapevine.

Chapter four aimed to study the effect of the two commercially used vegetative propagation techniques on the maintenance of acquired long-term somatic memory of stress in grapevine. I observed a variation in the transcription level of plants propagated using the two techniques. Our results suggested that stress-induced DNA methylation changes cannot be stably inherited through hardwood cutting compared to layered plants. And that both transcriptional and epigenetic memory of stress established in the ortets, is at least partially, lost during callused cutting propagation.

The majority of chapter will be submitted:

Tan, J.W., Tesfamicael, K., Shinde, H., Hu, Y., Tricker, P., Edwards, E., & Rodriguez-Lopez, C.M. Maintenance of Long-Term Somatic Memory of Stress in Grapevine is Dependent on the Vegetative Propagation System.

Chapter five summarizes the overall findings of this dissertation and possible future directions.

All sections regarding DNA methylation will be submitted separately.

CHAPTER 2. GLOBAL TRANSCRIPTOME AND GENE CO-EXPRESSION NETWORK ANALYSES REVEAL REGULATORY AND NON-ADDITIVE EFFECTS OF DROUGHT AND HEAT STRESS IN GRAPEVINE

(The majority of chapter have been published in Frontiers in Plant Science, the section on DNA methylation will be submitted separately.)

2.1 Abstract

Despite frequent co-occurrence of drought and heat stress, the molecular mechanisms governing plant responses to these stresses in combination have not often been studied. This is particularly evident in non-model, perennial plants. We conducted large scale physiological and transcriptome analyses to identify genes and pathways associated with grapevine response to drought and/or heat stress during stress progression and recovery. We identified gene clusters with expression correlated to leaf temperature and water stress and five hub genes for the combined stress co-expression network. Several differentially expressed genes were common to the individual and combined stresses, but the majority were unique to the individual or combined stress treatments. These included heatshock proteins, mitogen-activated kinases, sugar metabolizing enzymes, and transcription factors, while phenylpropanoid biosynthesis and histone modifying genes were unique to the combined stress treatment. Following physiological recovery, differentially expressed genes were found only in plants under heat stress, both alone and combined with drought. Taken collectively, our results suggest that the effect of the combined stress on physiology and gene expression is more severe than that of individual stresses, but not simply additive,

and that epigenetic chromatin modifications may play an important role in grapevine responses to combined drought and heat stress.

Keywords: Vitis vinifera, transcriptome, heat, drought, stress, co-expression network, pathways

2.2 Introduction

Abiotic stress is a major limiting factor for plant growth and crop production in many regions of the world. Common abiotic factors unfavorable for plant growth and crop yields include drought, saline soils, heat, and cold. Worldwide, extensive agricultural losses result from heat stress, often in combination with drought (Vogel et al., 2019). It is expected that the effects of combined drought and heat stress will become more severe as the climate continues to warm (Zhao et al., 2017; Raza et al., 2019), as it is predicted that an increase in global temperature of 1.5°C will cause more extremely hot days on land, and an increase in the intensity and frequency of drought and precipitation deficits (IPCC, 2018).

Viticulture is highly dependent on climatic conditions during the growing season. Climate determines the ability to successfully grow a particular variety and can greatly affect the value of the fruit produced (Gladstones, 1992; Jones and Davis, 2000; Jones, 2006; Bai et al., 2022). Grape production for winemaking is particularly vulnerable to environmental stress as the environmental conditions occurring during one growing season contribute to the quality of the next vintage (Mullins et al., 1992; Edwards and Clingeleffer, 2013; Martınez-Lüscher and´ Kurtural, 2021). Viticulture is commonly practiced in regions with a Mediterranean climate, where the growing season is characterized by low rainfall,

the majority occurring in winter, and by high air temperature and evaporative demand, temperatures above 40°C are not uncommon. It has been proposed that an increase in ambient temperatures will constitute the primary cause of water shortages for viticulture due to increased evaporative demand (Schultz, 2010), and may eliminate production in many areas (White et al., 2006; Diffenbaugh et al., 2011). It is important to consider the effect of combined stress on grapevines since plants growing in vineyards will be affected by both these interacting factors (Mittler, 2006).

Long-lived perennials, including grapevine, have acquired a myriad of adaptions to cope with stress conditions such as heat and drought (Estravis-Barcala et al., 2020). The importance of identifying protection mechanisms of grapevine against abiotic stresses has motivated research both in the field and in controlled environments (reviewed in Carvalho and Amâncio, 2019). Physiological changes including limiting stomatal opening and a reduction in vegetative growth are common responses to drought, protecting the plant from extensive water loss (Chaves et al., 2002). Similarly, altered leaf structure and increased leaf rolling are also observed in grapevines under stress in relation to water use and status (Patakas et al., 2005; Kulkarni et al., 2007). In contrast, for example, under heat stress, leaf transpiration may increase because of high stomatal conductance, maintaining a cooler canopy temperature (Moore et al., 2021). The dissection of physiological traits to understand which might be synergistic or antagonistic during combined drought and heat stress may lead to the identification of more tolerant varieties. Common protective mechanisms against damage from various abiotic stresses include increases in concentrations of scavengers of free radicals and hormones involved in systemic stress signaling (Raja et al., 2017; Sachdev et al., 2021). RNA-sequencing analysis has revealed

important gene regulation patterns and potential stress tolerance genes under drought (Salman-Haider et al., 2017) and heat (Carvalho et al., 2015).

Plant responses to a combination of stresses can be hard to differentiate from the response to each of the individual stresses (Mittler, 2006) and the timing and persistence of stress and recovery also influence physiology and metabolism in a genotype by environment-dependent manner (Carvalho et al., 2015). Here we focused on the differential responses of *V. vinifera* L. cv. Cabernet Sauvignon (a relatively tolerant genotype) to drought, heat, and combined drought and heat stress to identify key gene co-expression networks and clusters associated with physiological changes, and the differentially expressed genes between different stress treatments to gain insight into the differences between grapevine responses to individual or combined stresses.

2.3 Materials and methods

2.3.1 Plant materials and experimental design

120 hardwood cuttings propagated from 6 donor grapevine (V. vinifera L. cv. Cabernet Sauvignon) plants were planted in UC potting mix and maintained in a plant propagator under high humidity until root establishment. Each cutting was individually labelled using a unique ID number, to allow the linkage of physiological and gene expression data to conduct downstream analyses. Plants were then transferred to 24 cm pots and randomly allocated into four different groups, each designated to a future treatment (i.e., Control (T0), drought (T1), heat (T2), and combined drought and heat (T3)). These were then randomly allocated into five blocks, such that there were six vines of each

treatment per block. The plant positions within a block were also randomized and each block was placed on a separate bench in a glasshouse (CSIRO, Waite Campus, Adelaide, South Australia, Australia) maintained at an air temperature of 27°C Day/20°C Night, until stress treatments were applied. Humidity and light were uncontrolled. Air temperature and humidity were continuously recorded using a TinyTag Plus 2 logger in a small Stephenson shield (Hastings Data Loggers, Port Macquarie, NSW, Australia).

The experimental method was adapted from Edwards et al. (2011) and incorporated drought and high temperature stresses in a factorial design. Utilizing this design had the advantages of providing greater statistical power to the main effects (drought stress, heat stress), whilst allowing a potential interaction between these two stresses to be specifically addressed. Capacity limits referred to only two levels (presence/absence) of each stress could be used. Heat stress was generated by allowing natural insolation to heat the glasshouse (i.e., cooling was not initiated until a higher set temperature was reached than the control). Drought stress was generated by reducing the volume of daily irrigation applied. Once the vines were established, irrigation was removed from the selected plants (T1 and T3) until they were under moderate to severe drought stress. Vine response was monitored by measuring stomatal conductance to water vapor (*gs*) using a Delta-T AP4 Porometer (MEA, Magill, SA, Australia). Vines were deemed to be under drought stress when *gs* was measured between 75 and 100 mmol/m^2/s. Once plants reached this stage, each pot was weighed and subsequently hand-watered to this weight daily for the duration of the treatment. Once the drought condition had been maintained for ten days, heat stress was applied to selected plants (T2 and T3) for 48 hours, by setting the thermostatically controlled evaporative air-conditioning system in the greenhouse to 45°C and allowing

insolation to heat the chamber. Nighttime temperatures were maintained at a minimum of 30°C using a gas heating system. Plants that were not selected for heat stress treatment (i.e., T0 and T1) were moved to an adjacent glasshouse with the same layout but with temperatures maintained at 27/20°C as previously. T0 and T1 plants were transferred back to the initial glasshouse after heat treatment, watering was reinitiated for drought-treated plants and temperature reduced to control conditions for heat-treated plants on the midnight of the 12th day of reduced irrigation. Plants exposed to one of the stress treatments were considered physiologically recovered when their *gs* showed no significant difference from that of the control plants (See Supplemental Figure S2.1 for a schematic representation of the experimental design).

2.3.2 Physiological measurements

A standardized set of measurements was established and undertaken before drought treatment initiation (ST1), immediately before heat stress initiation (ST2), during heat stress (ST3 and ST4), immediately following initiation of normal irrigation and the removal of heat stress (ST5) and after physiological recovery (ST6) (Supplemental Table S2.1). These measurements were combined with tissue sampling (see below). To avoid any impact of tissue sampling or leaf removal for stem water potential measurements on subsequent measurements, each plant was only sampled once (i.e., nsampling time= 20; 5 plants x 4 treatments). At sampling times ST1 and ST2, only plants from the control treatment, and the control and drought treatments, respectively, were sampled for stem water potential and molecular analyses.

Stomatal conductance to water vapor (*gs*): First fully expanded leaves were used for measuring gs using an AP4 Leaf Porometer (as above). Measurements were made at approximately 11 AM to avoid any potential impact of midday depression of gs, except for ST3 and ST4, which were measured at approximately 4 PM to assess the maximum stress.

Stem water potential (stemΨ): Grapevine water status during the experiment was determined by measuring the stemΨ of the second fully expanded leaf. A Scholander-type pressure chamber (model 3000, Soil Moisture Equipment Corp, Goleta, CA, USA) was employed to measure the second fully expanded leaf of plants selected at each sampling time (Supplemental Figure S2.1). Leaves were bagged with silvered plastic zip lock bags for a minimum of 20 minutes to ensure equilibration between leaf and stem.

Leaf temperature (LT): The effect of the applied stresses on leaf temperature was studied by measuring the surface LT of the third leaf counting from the plant main stem apex (non-fully expanded leaves), and the first fully expanded leaf of selected plants at each sampling time (Supplemental Figure S2.1) using a non-contact infrared thermometer (Fluke, USA).

The statistical significance of treatment effects on vine physiology was assessed using univariate ANOVAs fitted with a GLM (IBM SPSS Statistics version 27, New York, USA). The dataset was split into four time periods, pre-treatment, drought-only, combined stress period and recovery. If a time period included more than one measurement date, repeated measures ANOVA was used, with time as the within-subjects effect. For the combined stress and recovery periods a factorial model was used. For the pre-treatment and drought-only periods, a single factor (drought) ANOVA was used. Significance was assumed when an effect probability was below 0.05.

2.3.3 RNA extraction, library preparation, and sequencing

Sample collection: The second and third leaves counting from the plant's main stem apex were collected for nucleic acid extraction at each sampling time (Supplemental Table S2.1). Leaves were frozen immediately after collection using liquid nitrogen and stored at -80°C.

RNA was extracted from 100 mg of frozen and ground powder from the collected leaves using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's Protocol A. RNA quality and quantity were determined by spectrophotometric analysis (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington, DE, USA) and Experion™ RNA StdSens Chips (BIO-RAD, USA). Extractions presenting 260/280 and 260/230 absorbance ratios between 1.8-2.2 and an RNA quality indicator (RQI) above 7 were used in library preparations (i.e., 94/95 RNA extraction).

4 µg of total RNA per sample was used for ribosomal RNA depletion using Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. 5 ml of ribosomal depleted RNA was used to prepare 94 individually barcoded RNA-seq libraries using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, USA) following the manufacturer's instructions. The Illumina NextSeq 500 HighOutPut platform was used to produce 75 bp single end runs at the Australian Genome Research Facility (AGRF) in Adelaide, Australia. RNA-seq libraries not yielding >18,000,000 reads were re-sequenced, and results merged.

2.3.4 Whole Methylome Sequencing (WMS)

WMS was performed on genomic Library preparations were done following the manufacturer instructions of the NEBNext Enzymatic Methyl-seq Kit (New England BioLabs). Each individual sample of genomic DNA was spiked with internal controls to determine the enzymatic conversion efficiencies and the abundance of false positives and negatives (i.e., 0% methylated Lambda DNA, and 100% CpG methylated pUC19 DNA). Spiked DNA samples were then fragmented to 200 – 300 bp using the Covaris S220 ultrasonicator. The resulting individually barcoded libraries were sequenced using Nova Seq 6000, and PE150 with a paired-end sequencing approach.

2.3.5 Bioinformatic analyses

RNA-seq data analysis: Raw sequencing datasets were processed on the University of Adelaide High-Performance Computing Phoenix platform. AdapterRemoval (Lindgreen, 2012) was used to remove adaptors of the raw reads. Sequence quality control was performed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/(2015). Demultiplexed reads were mapped to the 12X grapevine reference genome (NCBI assembly ID: GCF_000003745.3) with the alignment tool (HISAT2) with default setting (Kim et al., 2015; Khalil-Ur-Rehman et al., 2017). The GTF reference of the Vitis vinifera genome was downloaded from the *Ensembl Plants* website (http://plants.ensembl.org/Vitis_vinifera/Info/Index). Samtools (Li et al., 2009) was used to generate Binary Alignment Map (BAM) files after mapping the reads to the genome.

2.3.5.1 Identification of gene expression associated to physiological measurements using weighted co-

expression network and co-expressed gene cluster analysis

Transcripts Per Million (TPM) of each plant sample were calculated from the BAM files using the TPMcalculator (Vera Alvarez et al., 2019). Normalized data (calculated TPMs) was used for the identification of gene expression clusters based on physiological measurements using *clust* v1.8.4 (Abu-Jamous and Kelly, 2018).

Gene co-expression networks and gene modules were identified using R package WGCNA (Langfelder and Horvath, 2008). Hierarchical clustering analysis was used to identify sample outliers using FlashClust (Langfelder and Horvath, 2012). The correlations amongst genes across samples were calculated using the WGCNA algorithm. The standard scale-free network was established after choosing the appropriate soft threshold power. Subsequently, module identification was performed with the dynamic tree cut method by hierarchically clustering the genes using the topological overlap matrix (TOM) as the distance measure with a deep split value of 2 and minimum module size (minClusterSize) of 50 for the resulting dendrogram. Modules showing high similarity were clustered and merged with a height cutoff of 0.25. Co-expression modules and gene information were extracted from each module using the WGCNA algorithm. The correlations between clustered modules and physiological variables (i.e., leaf temperature, stomatal conductance and stem water potential) were estimated by module eigengenes (MEs). The association of the individual module and each physiological variable was determined by Spearman's correlation. Modules were considered significantly associated with a given physiological

variable and retained for further analysis when their absolute correlation value was higher than 0.6 and their p-value < 0.05 (Wang et al., 2020).

2.3.5.2 Differentially expressed genes analysis

Gene expression was estimated using the edgeR package (Robinson et al., 2010) on Rstudio. The raw mapped data of each sample was normalized by edgeR's trimmed mean of M values (TMM). This normalization method estimates scale factors between samples to determine DEGs. Between controls and each treatment, a log2fold change(log2FC) of 2 and a false discovery rate adjusted P-value <0.05 using Benjamini and Hochberg's algorithm was adopted to indicate significant genes. The 'pheatmap' package (Kolde, 2012) was used to generate heat maps of gene expression patterns under drought, heat, and combined drought and heat stress treatments.

2.3.5.3 Gene ontology, KEGG pathway and network analysis

To interpret and classify the DEGs associated with drought, heat, and combined drought and heat stress, GO analysis was performed with agriGO v2.0 (Tian et al., 2017), along with WGCNA modules and clusters assembled by clust. DEGs of each treatment were used to attain the significant GO terms with agriGO v2.0 with the following criteria: Fisher's Exact test method, Yekutieli (FDR under dependency) multi-test adjustment method, significance level <0.05, and selecting complete GO as the gene ontology type. DEGs of each treatment, WCGNA modules, and clusters assembled by *clust* were used to attain the significant molecular pathways with Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) (Moriya et al., 2007). Visualization of

KEGG functional enrichment pathways of DEGs was generated using the "*clusterProfiler*" package (Yu et al., 2012). A Web tool "REVIGO" was used to summarize the long lists of GO terms (Supek et al., 2011); subsequently, the lists generated by REVIGO were visualized with CirGO (Kuznetsova et al., 2019). The visualization of GO terms identified and enriched for WGCNA modules and clusters were done through Cytoscape, only genes that has gene module membership > 0.5 are considered hub genes (Shannon et al., 2003).

2.3.5.4 Identification of differentially methylated cytosines and regions (DMCs and DMRs)

Adaptor sequences, low-quality reads, and contaminants were removed from WMS reads using Adapter Removal V2 software. The enzymatic conversion efficiency of unmethylated and methylated cytosines was calculated using pipelines (https://github.com/nebiolabs/EM-seq/blob/master/em-seq.nf) and the methylation control sequences (https://github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa) provided by the NEBNext Enzymatic Methyl-seq Kit manufacturer.

Genome indexing was performed with Bismark using '--bismark_genome_preparation' option (Krueger and Andrews, 2011) using the C-to-T and G-to-A versions of the reference grapevine genome (PN40024 v.4) created with Bowtie2 (Langmead and Salzberg, 2012). Sequencing coverage and depth were estimated using Samtools coverage and depth toolkits (Li et al., 2009). Methylation calling was performed with Bismark extractor (https://www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf) by calling '--comprehensive' and '--cytosine_report' option after the conversion to bedGraph. Both Differentially methylated cytosines and regions (DMCs and DMRs

respectively) were determined using the 'Methylkit' package (Akalin et al., 2012) with default parameters (minimum coverage threshold of 10 and 5 for DMCs and DMRs, respectively; q-value ≤ 0.05; minimum differential methylation level of 10%); sliding window for DMRs was 1000 bp). Genes were deemed differentially methylated when a DMR overlapped with their promoter (defined here as 1000 bp upstream of the transcription starting site (TSS)), or with the body of the given gene.

2.4 Results

2.4.1 Environmental conditions

Temperature control in the glasshouse consisted of evaporative cooling and gas heating, both thermostatically controlled. The evaporative cooler was unable to fully cool the glasshouse in the extreme heat that can occur during summer in Adelaide, Australia and was of limited effectiveness at night due to the relatively high humidity often seen in greenhouses. Consequently, the efficacy of the temperature control was variable, as can be seen in Supplemental Figure S2.1. Excluding the heat stress period, the mean daily maximum air temperature was 30.9°C, the mean daily minimum was 22.7°C and the overall mean was 25.9°C throughout the experiment. The mean daily maximum VPD was 1.81 kPa.

The heat stress treatment achieved a maximum air temperature of 38.5°C on the first day and 42.6°C on the second day. VPD increased to 4.2 and 5.3 kPa on days one and two of heat stress respectively. Following the removal of the heat stress, and during the

recovery period, glasshouse conditions (mean daily max/min air temperature) were within 0.5°C of the pre-stress conditions.

2.4.2 Physiological analysis

Stomatal conductance (g_s): No difference in g_s between the plants to be subjected to stress treatments and the controls was observed before the initiation of drought treatment (ST1), consequently, it was assumed that there was no pre-existing bias between the future stress treatments (Figure 2.1A). The desired level of drought stress was reached after three days of drought treatment initiation and maintained for six days before the initiation of heat stress treatment. At ST2 (immediately before the application of heat stress) g_s was measured at 362±77 mmol/m^{-2}/s^{-1} in the control plants and 55±13 mmol/m^{-2}/s^{-1} in the droughted plants, slightly lower than the aimed for 75-100 mmol/m^{-2}/s^{-1} (Figure 2.1A). The difference between control and drought treated plants was statistically significant ($p=0.016$), demonstrating that the intended drought stress was successfully applied to the relevant plants.

Whilst the progress of water deficit treatments are best, and traditionally, monitored using mid-morning g_s, to ensure the peak period of stress (late afternoon) was observed, the primary physiological measurements during the heat stress period were taken later in the day. The space, number of individual plants, and resources available prevented more sets of measurements being taken on a single day, so the direct effects of the stress treatments were compared during the ST3 and ST4. The g_s of control plants at ST3 and ST4 was lower than the mid-morning values observed during the rest of the experiment, reaching only half of the maximal (mid-morning) g_s values recorded during the experiment

(See Figure 2.1A), although such 'midday depression' of g_s is commonly observed in C_3 plants. Nevertheless, as with the mid-morning measurements prior g_s under drought stress (T1) measured during the afternoon at ST3 and ST4 remained significantly lower than control ($p<0.001$).

There was no significant (main) effect of heat stress (T2 and T3) on g_s. Additionally, the heat and drought interaction term was non-significant over the two days of the applied high-temperature event (ST3-4) (Figure 2.1A). Consequently, heat stress did not have an effect on g_s regardless of the plant's drought status.

Despite the lack of a heat stress effect on g_s being observed during the high-temperature event itself, there was a difference immediately after the removal of that stress (ST5), with g_s significantly higher in the previously heat-stressed plants (T2 & T3) than those not exposed to heat (T0 and T1) ($P < 0.001$). However, there was also a significant interaction between heat and drought treatments ($P=0.023$) due to a much larger absolute increase in g_s with heat treatment in the absence of drought (T2 vs T0) than where drought was present (T3 vs T1). The relative increase was similar in each case, approximately double. It cannot be ruled out that an impact of heat stress would have been observed if mid-morning measurements of g_s were available as the ST3 and ST4 measurements were made in the afternoon. The g_s of drought-treated plants remained significantly lower than controls ($P < 0.001$) at this time as the plants had not yet been re-watered.

Sixteen days after all plants removed from stress treatment (ST6), there were no significant differences in g_s between any of the treatments, indicating physiological recovery (Figure 2.1A).

Stem water potential (stemΨ): The stemΨ of control plants was consistent at all sampling times (~-0.4 MPa) and did not vary between morning and afternoon measurements (Figure 2.1B, ST2 vs ST3). StemΨ decreased significantly under drought stress ($P < 0.001$) to approximately -0.55 MPa (ST1, ST2 & ST5). Unlike the controls, stemΨ of drought plants was lower in the afternoon than the morning, reaching -0.7 MPa (ST2 vs ST3 & ST4). StemΨ was also significantly lower under heat stress ($P < 0.001$). In contrast to g_s, there was an additive effect (no interaction) of the two stresses, with the combined stress treatment having a lower stemΨ than either stress individually (Figure 2.1B, T3 vs T1 and T2, ST3 and ST4). After stress removal (ST5), the stemΨ of drought-stressed plants remained significantly lower ($P < 0.001$) than the control, while no significant difference was observed for heat-stressed plants. Similar to other physiological measurements, there were no significant effects of any former treatment on post-recovery period stemΨ (ST6), indicating a full recovery.

Leaf temperature (LT): No significant differences were observed in temperature between drought-treated and control plants before the initiation of any treatments (ST1) either for non-fully expanded or fully expanded leaves. Leaf temperature was not significantly affected by the initiation of drought treatment (ST2). During ST3 and ST4, the temperature of both non-fully expanded and fully expanded leaves was significantly higher under both heat ($P < 0.001$ in each case) and drought ($P = 0.025$ and $P < 0.001$, respectively) (Figures 2.1C, D). As with StemΨ, this effect was additive (no interaction), with the highest temperatures occurring in the combined stress treatment (Figures 2.1C, D). LTs of both the non-fully expanded leaf and first fully expanded leaf were higher at ST4 than ST3 ($P = 0.002$ and $P = 0.003$, respectively) in the heat treatment. For the non-

fully expanded leaves, there was only a small difference in LT between the heat (T2) and combined (T3) treatments, similar to the difference observed between drought and control leaves. For the fully expanded leaves, the difference was much larger and there was a marginally significant interaction between heat and drought (P=0.052), suggesting that the effect of heat on LT was greater in combination with drought (Figures 2.1C, D).

In measurements made around two hours after stress removal (ST5), LT for the previously heat-stressed plants were lower than the non-heat stressed plants in all cases except the droughted still expanding leaves. This would be expected where g_s was higher as there would be a higher transpiration rate. For the droughted vines not subject to heat stress LT remained higher than control. Following the period allowed for physiological recovery (ST6), the leaf temperatures for both leaves were fully recovered.

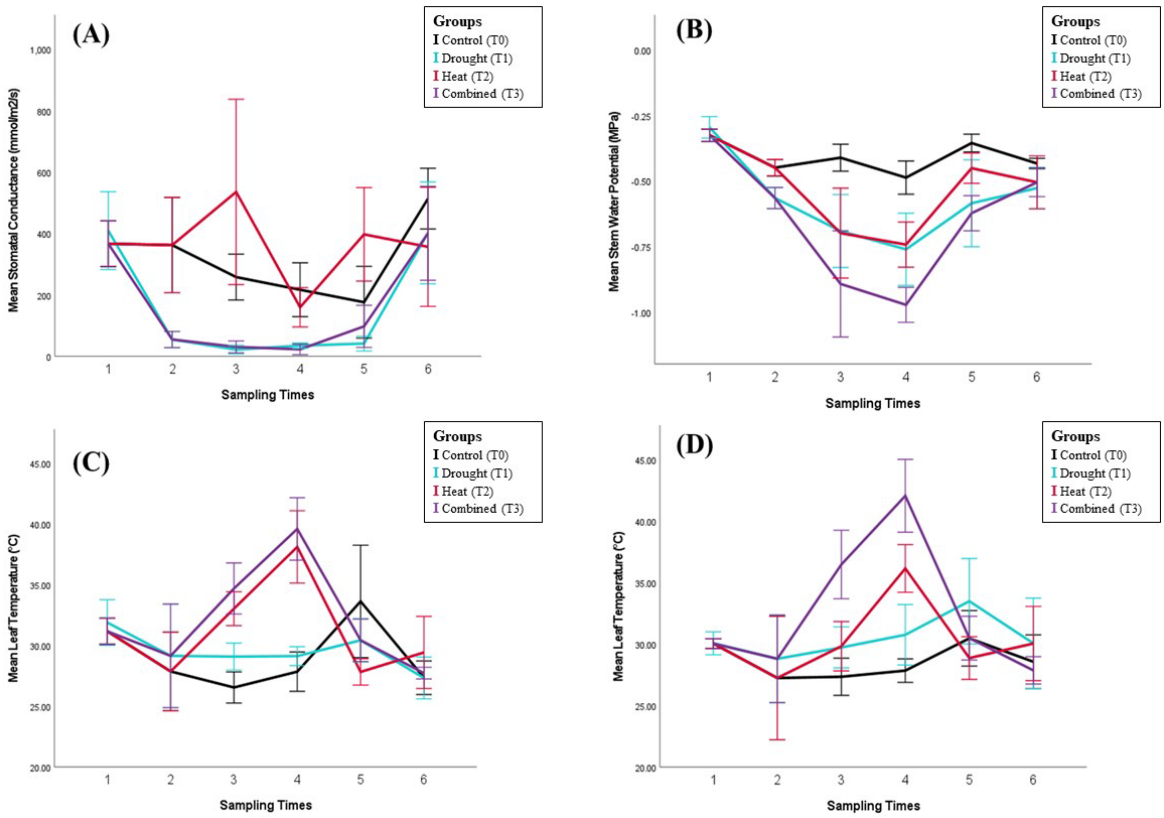


Figure 2.1 Physiological analysis results under different stress conditions.

Panels show collected physiological measurements for (A) Stomatal conductance (g_s). (B) Stem water potential (StemΨ). (C) Leaf temperature (LT) of the third young leaf (not fully expanded), and the first fully expanded leaf (D). Error bars indicate the standard error of means (n = 5).

2.4.3 Gene expression analysis

2.4.3.1 Next generation sequencing raw data

Transcriptome sequencing yielded a total of 3.3 billion reads, ranging from 2.66 to 9.56 Gbp of sequence per sample after quality filtering. The average number of mappable reads per sample after de-multiplexing was 23,631,104 (85%), ranging from 11,770,042 to 70,017,056 (75-91%) (Supplemental Table S2.1).

2.4.3.2 Identification of gene expression associated to physiological measurements using WGCNA and co-expressed gene cluster analysis

TPM counts of 30661 genes for 94 plants were calculated and used for gene expression analysis through WGCNA and clust (Supplemental Table S2.2).

Clust analysis generated a total of 9, 18 and 15 different co-expression clusters visually representing gene expression patterns for changes in given physiological parameters LT, g_s, and stemΨ of all 94 vine plants, respectively (Supplemental Figures S2.2-2.4). 11,250 genes were found in clusters showing either an increase or decrease in gene expression with increasing LT, g_s and stemΨ (Figure 2.2; Supplemental Table S2.3). In such clusters, biological regulation, response to stimulus, regulation of biological process and signaling were the most significant GO terms (Supplemental Figure S2.5). Pathway analysis revealed that genes involved in the seven most significantly enriched pathways, including thermogenesis, plant-pathogen interaction, cytosine and methionine metabolism, plant hormone signal transduction, MAPK signaling pathway in plants,

ubiquitin-mediated proteolysis and protein processing in the endoplasmic reticulum (Supplemental Figure S2.6).

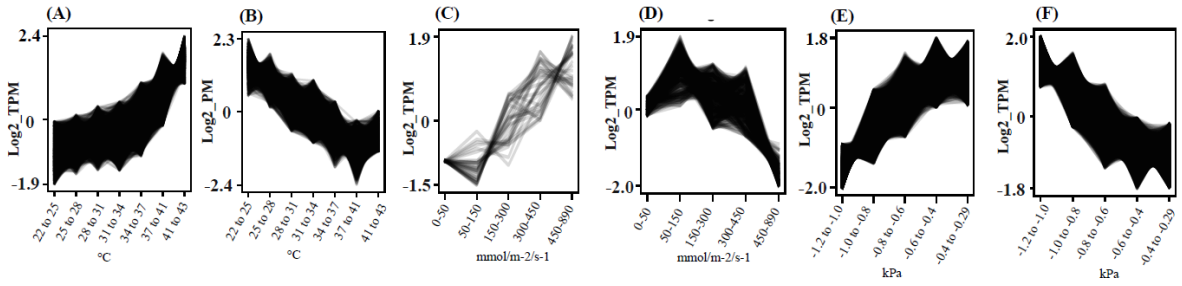


Figure 2.2 Identification of co-expressed genes in response to leaf temperature, stomatal conductance to water vapor, and stem water potential in grapevine.

Gene expression clusters were identified based on physiological and transcriptome data generated from 94 plants using *clust* v1.8.4. (A) Gene cluster showing positive correlation with temperature (°C) of non-fully expanded leaves, n = 3,513; (B) Gene cluster showing negative correlation with temperature (°C) of non-fully expanded leaves, n = 1,918; (C) gene cluster showing positive correlation with g_s (mmol/m^{-2}/s^{-1}), n = 36; (D) Gene cluster showing negative correlation with g_s (mmol/m^{-2}/s^{-1}), n = 401; (E) Gene cluster showing positive correlation with StemΨ (kPa), n = 3,824; (F) Gene cluster showing negative correlation with StemΨ (kPa), n = 1,006.

TPM values were clustered by Pearson's correlation and average linkage algorithms with the soft-thresholding power set to $\beta = 8$ (Supplemental Figure S2.7) to generate a scale-free gene co-expression network. 30 module eigengenes were generated by average linkage hierarchical clustering (Figure 2.3) (See Supplemental Table S2.4 for all genes, their respective modules and correlation values). Of these, 24 showed the same direction in correlation for g_s and stemΨ (Figure 2.3). Of these 24, 15 showed the opposite direction of correlation between LT and g_s or stemΨ. The only module deemed significant (i.e., correlation coefficient > 0.6 and p-value < 0.05), darkmagenta, showed a positive correlation with leaf temperature (*R=0.66, P < 1e-12*) and a negative correlation with stem water potential (*R= -0.61, P < 6e-11*).

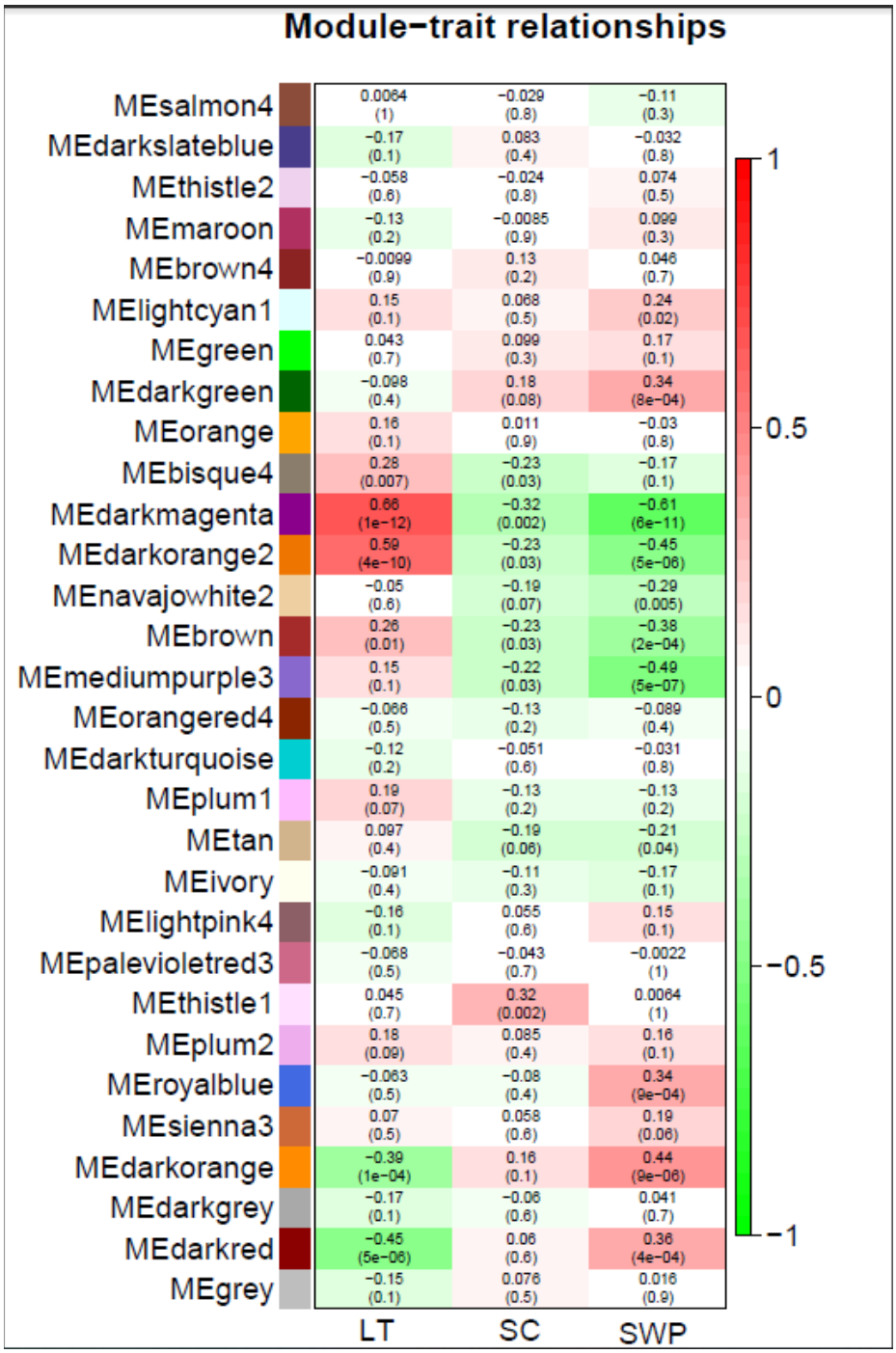
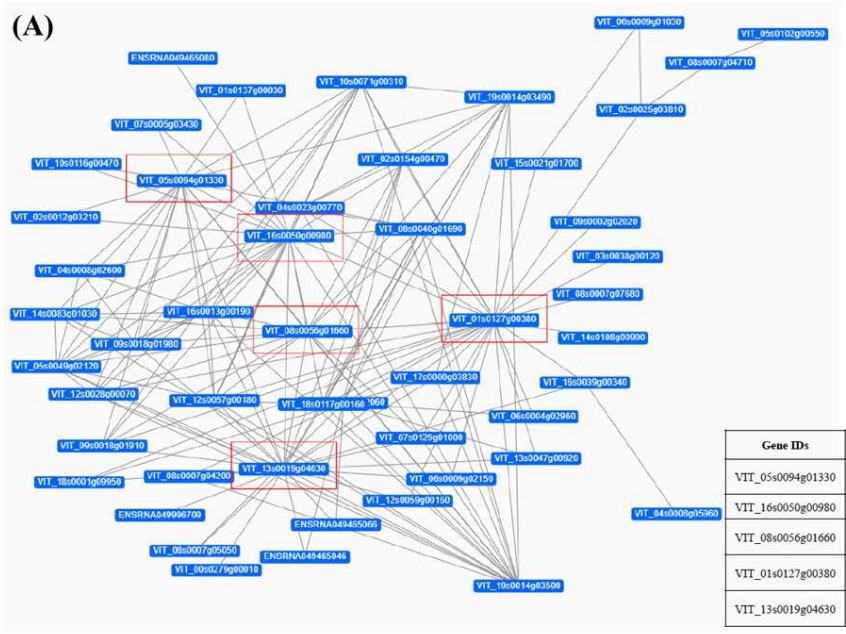


Figure 2.3 WGCNA module identification and correlation analysis of gene expression associated with leaf temperature, stomatal conductance to water vapor, and stem water potential in grapevine.

Red and green color denote positive and negative correlations with gene expression, respectively. The top number in each cell indicates the correlation coefficient, and the bottom number indicates the correlation significance (P-value).

Comparison of the genes forming the darkmagenta module (n = 252) to those contained in the cluster showing an increasing gene expression with increasing leaf temperature (n = 3513) (Figure 2.2A), and the cluster showing a decreasing gene expression with increasing stem water potential (n = 4451) (Figure 2.2F) showed that 79% (n = 200) and 77% (n = 195) of the genes forming the darkmagenta module overlapped with genes in clusters A and F, respectively.

Gene interaction network analysis of the top 50 genes in darkmagenta module revealed five important hub genes (genes with high correlation and connectivity in the module, with gene module membership > 0.5) in this network, namely Inositol Polyphosphate 5- phosphatase 12, Ferric reduction oxidase 2, Histone-lysine N-methyltransferase SUVR3, Pyrrolidone-carboxylate peptidase, and Root primordium defective 1 (Figure 2.4A). GO analysis of the 252 genes contained in the darkmagenta module identified a total of 41 significantly enriched GO terms. Of these, 27 were Biological Processes, 13 Cellular Components, and 1 Molecular Function terms (i.e., 'protein serine/threonine kinase activity' (Figure 2.4B)) (Supplemental Table S2.5). An overrepresentation of genes involved in the processes 'response to stimulus' and 'response to stress' (Supplemental Figure S2.8) was observed for the darkmagenta module in co-expression network analysis. Similarly, analysis of the top 50 genes in darkmagenta module revealed a total of 11 Cellular Components terms (Supplemental Table S2.5).



Gene IDs	NCBI gene description	Gene module membership
VIT_05s0094g01330	Inositol polyphosphate 5-phosphatase 12	0.68
VIT_16s0050g00980	Ferric reduction oxidase 2	0.56
VIT_08s0056g01660	Histone-lysine N-methyltransferase SUVR3	0.62
VIT_01s0127g00380	Pyrrolidone-carboxylate peptidase	0.61
VIT_13s0019g04630	ROOT PRIMORDIUM DEFECTIVE 1	0.60

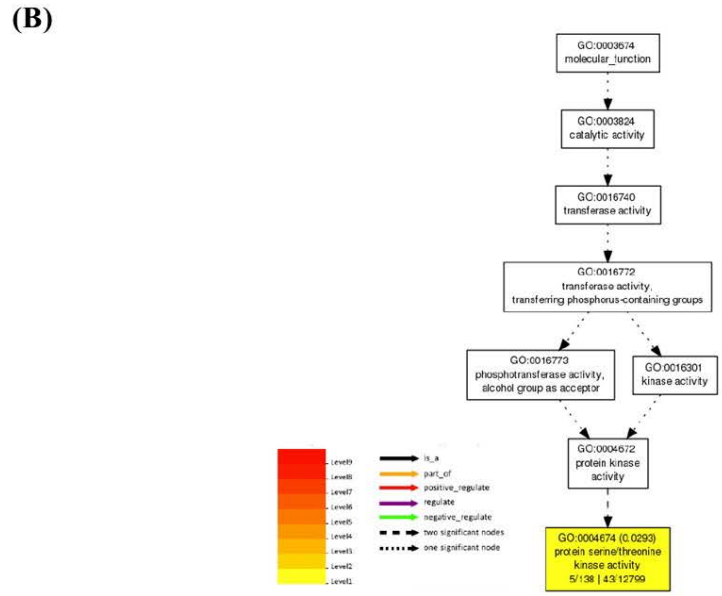


Figure 2.4 Gene interaction network of genes of module ‘darkmagenta’ associated with leaf temperature and stem water potential.

Gene interaction network of top 50 genes of darkmagenta module by Cytoscape. Each node represents a gene, and each line denotes the gene expression interaction between the two nodes. Hub genes are highlighted by red boxes, information about hub genes is given in insert table. (B) Gene Ontology molecular function analysis of the module.

2.4.3.3 Stress-induced differential gene expression

Differentially expressed genes (DEGs) identified between control and stressed plants (i.e., drought vs. control, heat vs. control, combined treatment vs. control) are summarized in Figure 2.5. In plants under drought stress, the number of identified DEGs peaked on the 11th day of drought treatment (ST3), with 161 up-regulated and 28 down-regulated genes, followed by the 12th day of drought treatment (ST4) with 141 DEGs, 48 up-regulated and 93 down-regulated. On the day of reinitiating normal irrigation and of heat stress removal (ST5), more genes were being down-regulated than up-regulated and no DEGs were detected at physiological recovery (ST6) (Figure 2.5A). Heat stressed plants produced most DEGs on the second day of stress (ST4, 54 DEGs) and at physiological recovery (ST6, 31 DEGs). The number of DEGs under heat stress was relatively small compared to drought and combined treatments. The majority of DEGs were detected in the combined treatment. The second day of heat stress in the combined treatment (ST4) had the most up-and down-regulated genes (671) and more genes were up-regulated (95) after physiological recovery (ST6) than were down-regulated (1).

The expression pattern of DEGs was visualized using a heat map to display the expression change and tendency (Figure 2.5). A small number of genes was differentially expressed at all sampling times (13, 0, and 4 genes for drought, heat, and combined treatments, respectively), with most DEGs only found at one sampling time (Figure 2.5 and Supplemental Table S2.6). A small number of DEGs (8/564, 2/867, and 4/304 for sampling times 3, 4, and 5, respectively) was observed to be common to all treatments.

A total of 163, 93, and 35 DEGs were common in drought and combined stress, for STs 3, 4, and 5, respectively. No common DEGs were found after physiological recovery (ST6) for drought and combined stress (Figure 2.6). At this stage, all DEGs in the heat treatment (31) were up-regulated and 95 of 96 DEGs were also up-regulated at physiological recovery in the combined treatment. None of the heat stress DEGs at physiological recovery had been differentially expressed during the treatment, and the small number of DEGs at physiological recovery (25) that overlapped with DEGs during treatment in the combined stress, were now up-regulated when they had previously been down-regulated (Supplemental Table S2.6).

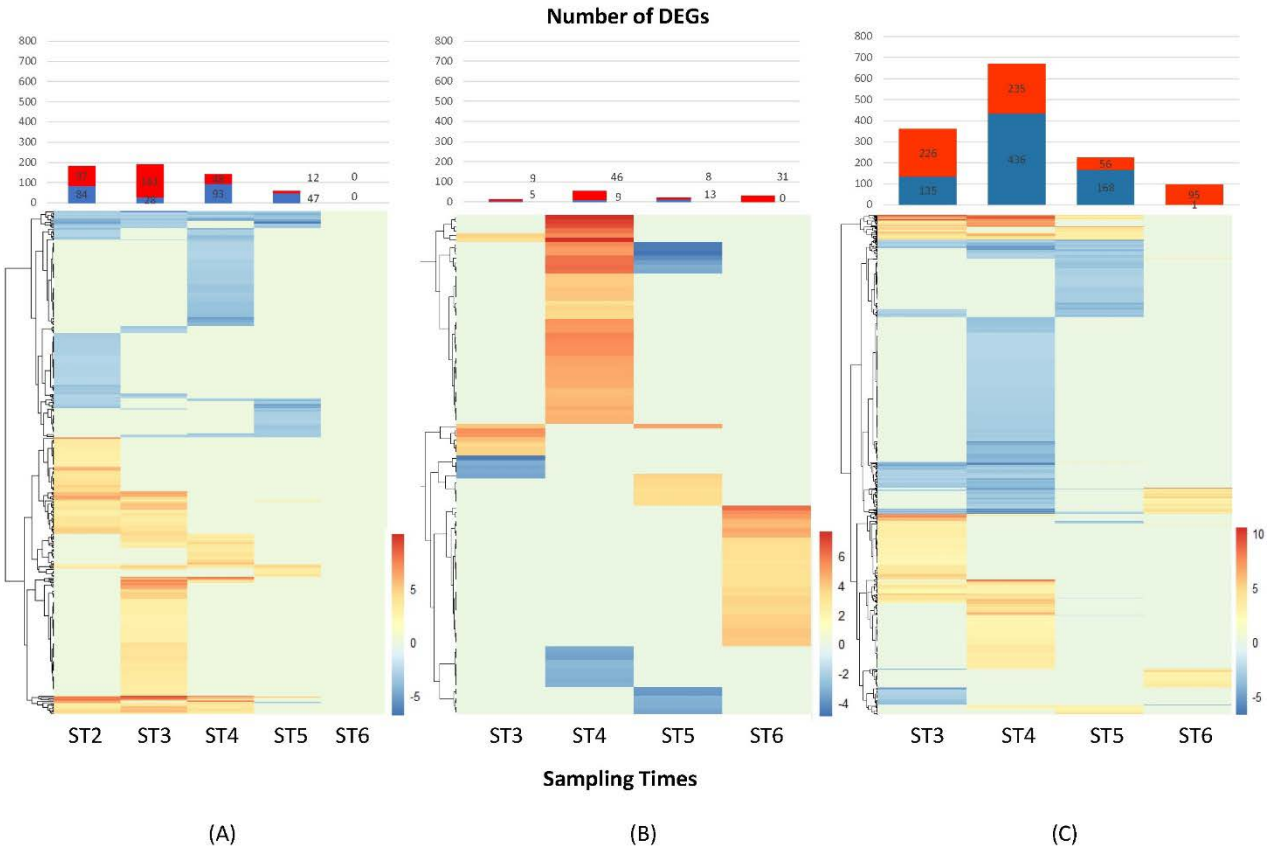


Figure 2.5 Differentially expressed genes (DEGs) identified under drought, heat, and combined treatments.

Bar plots indicate the number of DEGs (FDR adjusted P-val. < 0.05) identified per treatment and sampling point. Red and blue bars indicate the number of up-regulated and down-regulated genes, respectively. Heatmaps show the fold change of the identified DEGs. (A) DEGs identified under drought treatment, (B) Heat, (C) Combined (heat plus drought). Heat and combined stress had not been initiated at ST2; therefore, it is not included in here.

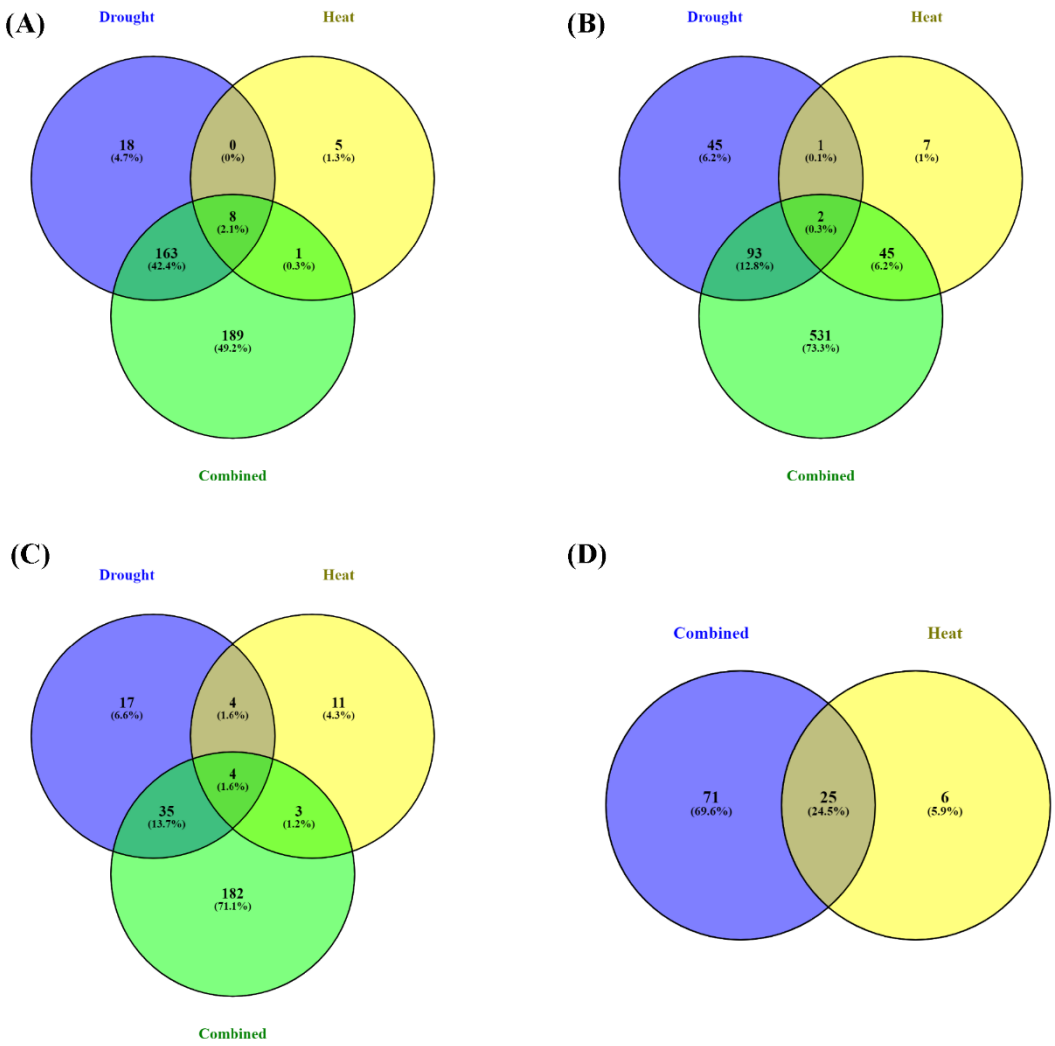


Figure 2.6 Identification of DEGs common for drought, heat, and combined treatment at each sampling time.

Number of DEGs identified for each treatment at (A) sampling time 3; 11th day of drought treatment and first day of heat treatment. (B) sampling time 4; 12th day of drought treatment and second day of heat treatment. (C) sampling time 5; day of stress removal. (D) sampling time 6; physiological recovery.

2.4.3.4 GO, network and KEGG pathway analysis of DEGs by treatment

A total of 342, 24, and 594 significant GO terms (P_{adj}-value ≤ 0.05) were identified for DEGs during drought, heat, and combined stress, respectively (Supplemental Figure S2.9). 107 of the 342 drought-induced GO terms were only identified early during drought stress (ST2). The network visualization of correlated GO terms seemed to follow a trend: while under individual stress, the gene regulation networks were relatively simple (Supplemental Figures S2.10, 2.11), under combined stresses, the gene regulatory networks were more complex and acted synergistically (Supplemental Figure S2.12), indicated by all the interacting GO terms. Seven biological process ontologies made up ~83 % of enriched categories in the combined treatment. Highly enriched categories were, histone modification (28.1%), regulation of the cell cycle (19%), response to stimulus (13.6%) and carbohydrate catabolic processes (10.5%) (Figure 2.7). Both the summary of GO terms and network visualization graph revealed the presence of DEGs associated with epigenetic and post-translational modifications during the latter stage of the combined stress treatment (ST4) and after stress removal (ST5), such as histone methylation, protein methylation, and protein alkylation (Figure 2.7). This was not observed in either individual drought or heat stress treatment (Supplemental Figure S2.13).

In the combined treatment, DEGs at ST3 were mostly involved in protein processing in the endoplasmic reticulum, galactose metabolism, plant hormone signal transduction and flavonoid biosynthesis. The same pathways, along with diterpenoid biosynthesis and glycosphingolipid biosynthesis were identified at ST4. The MAPK signaling pathway was significantly enriched at stress removal (ST5), while starch and

sucrose metabolism and pentose and glucuronate interconversion were enriched at physiological recovery (ST6) (Figure 2.8). KEGG pathway analyses of DEGs under individual drought and heat treatments at different sampling times can be found in Supplemental Figures S2.14 and S2.15, respectively. Different pathways were significantly enriched for heat and drought DEGs, although protein processing in the endoplasmic reticulum was still significantly enriched at specific sampling times (ST3 – ST5) in both treatments.

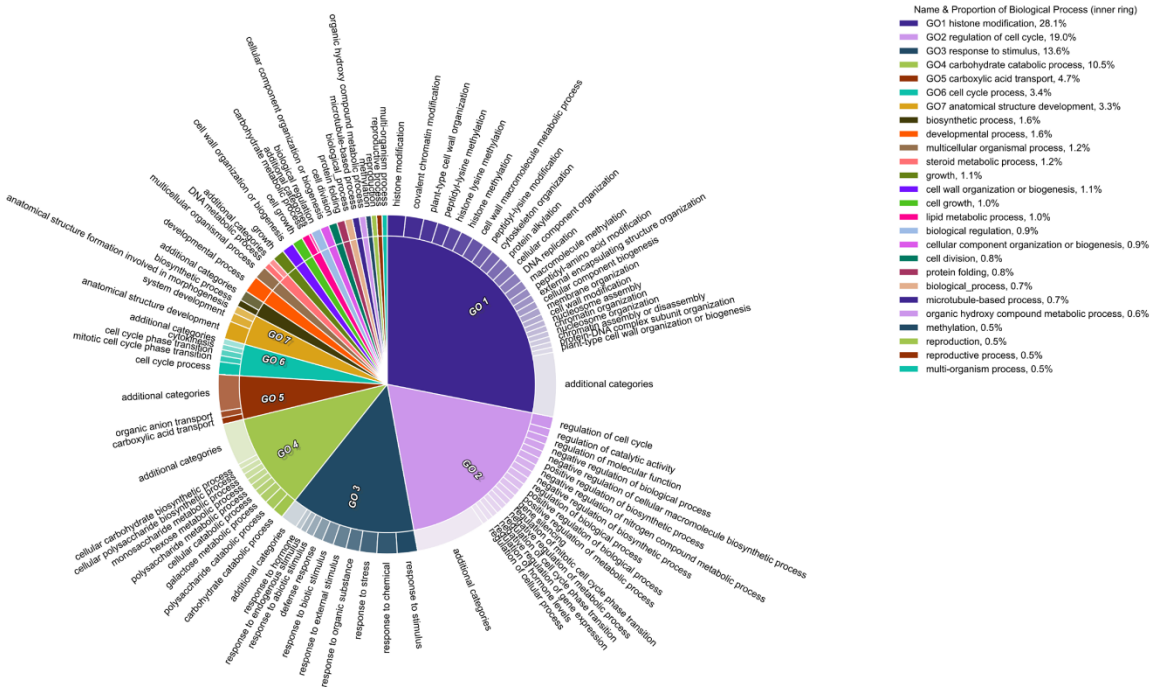


Figure 2.7 Gene ontology terms affected by combined stress.

Pie section is a single cluster representative. Different representatives are joined into a summarized section, visualized with different colors. Section size is associated to the P-value of that given GO term.

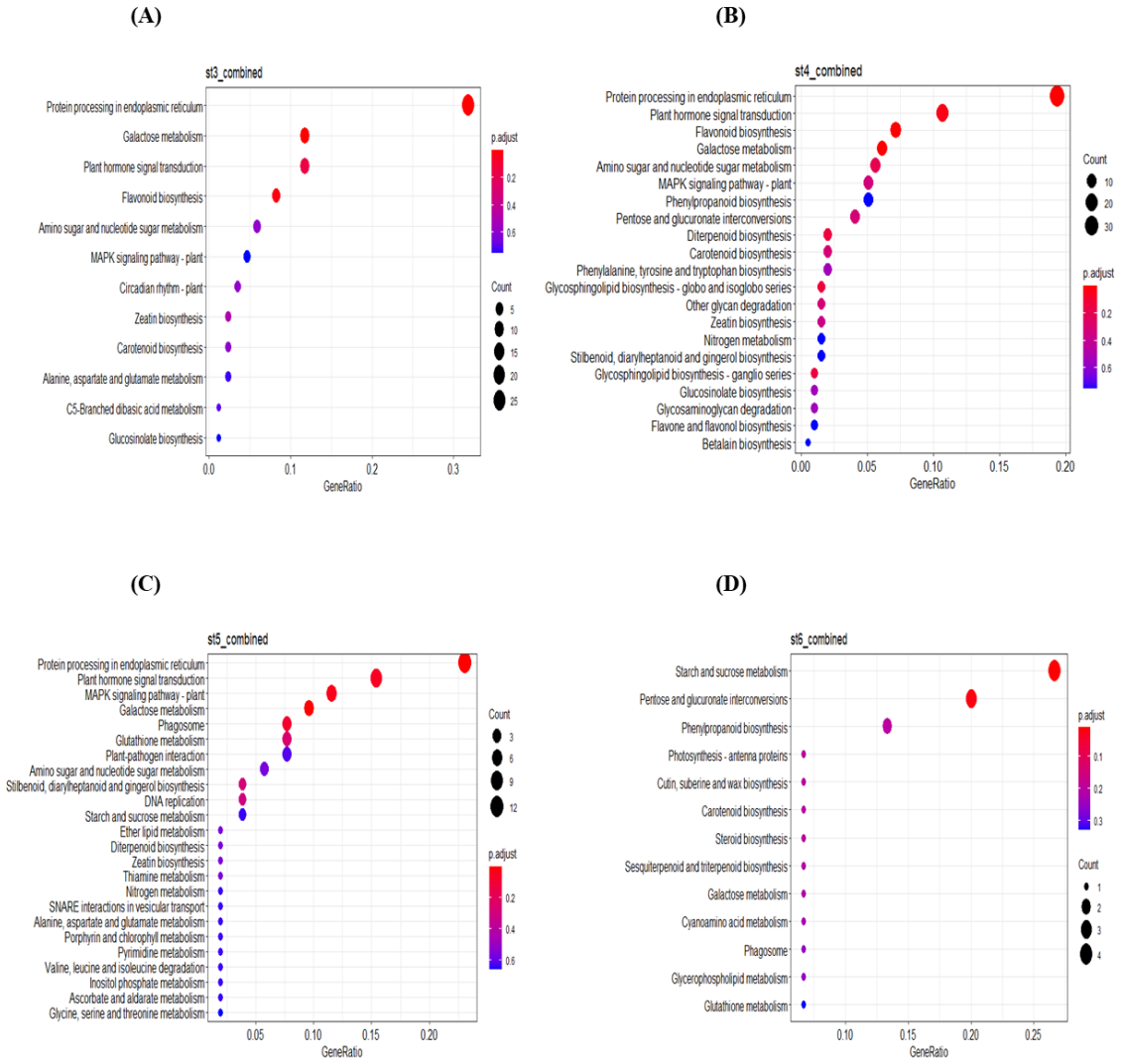


Figure 2.8 KEGG Functional enrichment analysis of DEGs identified.

KEGG functional enrich analysis of differentially expressed genes under combined treatment at different sampling time points; (A) sampling time 3; (B) sampling time 4; (C) sampling time 5; (D) sampling time 6. Significantly enriched pathways are with adjusted p-value < 0.05.

2.4.4 DNA methylation analysis

2.4.4.1 Global DNA methylation pattern induced by combined stress

Whole methylome sequencing yielded an average of 170 million reads per sample after quality filtering. Calculated enzymatic conversion efficiency showed a ratio of 0.2% and a 95.4% of unmethylated and methylated cytosines converted to uracils respectively.

The average percentage of mappable reads per sample after de-multiplexing was 50%, ranging from 47-53%. The average percentage of covered bases was 83.23% while the average sequencing depth achieved was 27X per sample (Supplemental Table S2.7).

Estimation of the average methylation percentage (methylated cytosines, mCs) for each sequence context (CG, CHG and CHH) showed the CG and the CHH contexts consistently presenting the highest and lowest levels of methylation respectively (Fig. 9A). Plants under combined stress (ST4) showed a significant 2.42% increase in mCG compared to control plants ($p \leq 0.05$), while no significant differences were observed for any context in at physiological recovery (ST6). Principal component analysis of methylation differences in all sequence contexts showed no clear separation between growing conditions or time points (Supplementary Figure S2.16). However, the dispersion of plants exposed to stress was lower than that of plants grown under control conditions (Supplementary Figure S2.16).

Differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) between control and stressed plants were identified for each of the time points indicated above (Figure 2.9). ST4 comparison produced 3,507 DMCs (1,192, 2,162 and 153 DMCs in CG, CHG and CHH context, respectively), while comparison at

physiological recovery produced 6,332 DMCs (2,090, 3,279 and 963 DMCs in CG, CHG and CHH context, respectively). In general, the majority of DMCs were associated to intergenic regions, regardless of context and comparison, and that identified DMCs were more likely to be hypomethylated than hypomethylated. A total of 2,148 and 1689 DMRs were identified for plants during stress exposure and at physiological recovery respectively. Among those identified in ST4, 1,094 DMRs are located in intergenic region, while 1,054 are in genic region (DMGs hereafter). For ST6, 880 and 809 DMRs are located in intergenic and genic regions, respectively. The majority of DMRs identified were located in intergenic regions (50-52%), followed by gene bodies (28-29%) and promoters (19-21%) (Figure 2.9B). Similar to the patterns observed for DMCs, the majority of the DMRs identified during ST4 and ST6 were hypomethylated (HypoDMRs). However, when considered independently, there were more DMRs hyper than hypomethylated DMRs during ST6 (Figure 2.9B). Of the 1746 unique DMGs identified, 117 were differentially methylated both during ST4 and ST6. Of those, 29 and 32 were hypermethylated or hypomethylated respectively at both time points, 22 were hypermethylated at ST4 and hypomethylated at ST6, and 34 were hypomethylated at ST4 and hypermethylated at ST6 (Figure 2.9A).

2.4.4.2 Association between DNA methylation and gene expression

For a better understanding of the potential functional roles of differential methylation on gene expression, we examined the relationship between the presence of DMGs (gene body and promoter) and transcriptional changes (Figure 2.10B, D). 14 DMRs were found to overlap with DEGs in plants under stress (6 in promoters, and 8 in gene bodies). Among those, 4 were hypermethylated and downregulated (2 in promoters, and 2

in gene bodies), 1 was hypermethylated and upregulated (promoter), 6 were hypomethylated and downregulated (2 in promoters, and 4 gene bodies), and 3 were hypomethylated and upregulated (1 in promoter, and 2 gene bodies) (Figure 2.9C). The identified DMR/DEG included two small heat shock proteins (sHSPs), a shikimate dehydrogenase associated gene, as well as a α/β hydrolase-1, a F-box, ripening regulated protein DDTFR8, an ABC transporter, and protein kinase domain-containing proteins associated genes (Table 2.1). At physiological recovery 5 genes were deemed differentially expressed and methylated (2 containing a DMR in their promoter, and 3 in their gene body). Among the 5, 3 located in gene body were hypomethylated and upregulated and 2 in promoter were hypermethylated and upregulated. Gene functional annotation of those DMR/DEG overlaps identified STAS domain-containing protein associated genes, as well as a MYB transcription factor associated gene (Table 2.1).

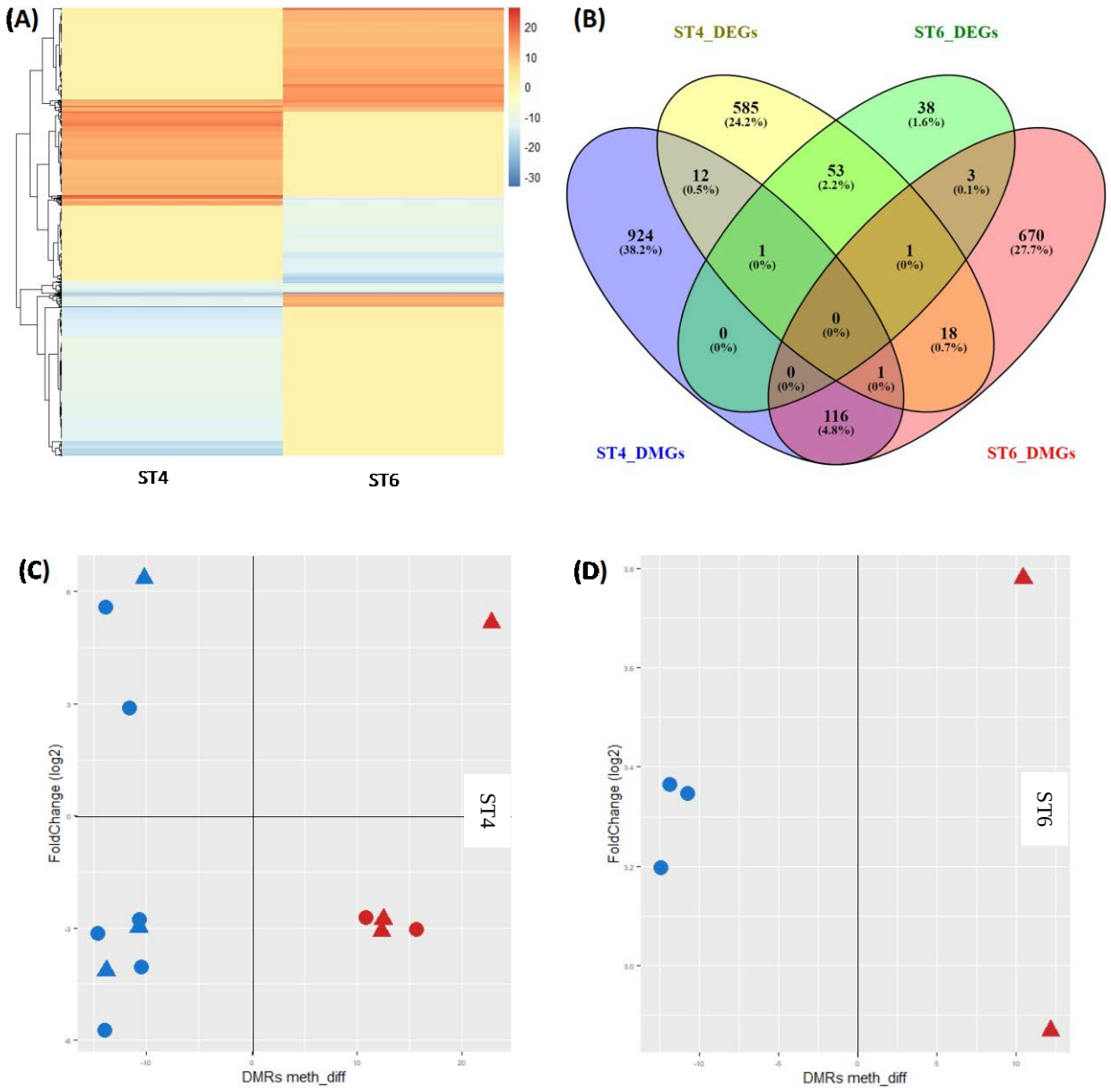


Figure 2.9 Graphical representation of DMGs and DEGs identified in ST4 and ST6.

(A) Heatmap of level of methylation changes for DMGs that were differentially methylated both during ST4 and ST6 (117). (B) Venn diagram of the DEGs and DMGs identified in ST4 and ST6. (C) Scatterplot of DEGs related with DMGs (located in both promoter and gene body) showing the relationship between transcript levels (fold change: log2) and DNA methylation (meth_diff) in ST4. Red: Hypermethylation. Blue: Hypomethylation. Circles: Gene Body. Triangles: Promoter. (D) Scatterplot of DEGs related with DMRs that located in promoter and gene body) showing the relationship between transcript levels (fold change: log2) and DNA methylation (meth_diff) in ST6. Red: Hypermethylation. Blue: Hypomethylation. Circles: Gene Body. Triangles: Promoter.

Table 2.1 Gene functional annotation of all overlapping DEGs and DMGs identified in ST4 and ST6.

Table consist of gene IDs, their respective sampling times, expression, methylation change direction and location, gene name, functional annotation and their involvement in plant stress response that have been previously studied.

		Expression	Methylation		Gene name	Functional annotation (PantherDB)	Stress Response
GENE ID	**Sampling Time**	**Stress vs. Control**	**Stress vs. Control**	**Genic context**			
VIT_02s0025g02620	ST4	Down	Hyper	Promoter	AB Hydrolase-1 domain-containing protein (Lysophospholipase bodyguard 3-related)	Serine protease	(Jiao and Peng, 2018)
VIT_04s0008g00300	ST4	Down	Hypo	Gene body	Clavata1 receptor kinase (CLV1)	Leucine-rich repeat receptor	(Hanemian et al., 2016)
VIT_04s0008g01520	ST4	Up	Hypo	Promoter	SHSP domain containing protein (18.0 KDA class II heat shock protein)	Response to stress/ protein folding	(Ji et al., 2019)
VIT_04s0008g01570	ST4	Up	Hypo	Promoter	sHSP domain-containing protein (18.0 KDA class II heat shock protein)	Response to stress / protein folding	(Ji et al., 2019)
VIT_06s0004g00990	ST4	Down	Hypo	Promoter	Dirigent protein (Dirigent protein 19)	Transporter	(Paniagua et al., 2017)
VIT_06s0004g02620	ST4	Down	Hypo	Gene Body	Phenylalanine ammonia-lyase (histidine ammonia-lyase)	flavonoid biosynthesis pathway	(Tu et al., 2022)
VIT_09s0002g02020	ST4	Up	Hypo	Gene Body	F-box domain containing protein	F-box	(Jiang et al., 2017; Li et al., 2018)
VIT_10s0003g01990	ST4	Down	Hypo	Gene Body	non-specific serine threonine protein kinase	Leucine-Rich Repeat-containing protein DDB	(Chen et al., 2021)
VIT_10s0003g03410	ST4	Down	Hypo	Promoter	Uncharacterized protein (protein, putative-related)	Transmembrane protein	
VIT_11s0016g05280	ST4	Down	Hyper	Gene Body	Peroxidase (peroxidase 25)	Response to stress	(Bela et al., 2015)
VIT_13s0047g00110	ST4	Up	Hypo	Gene Body	Ripening regulated protein DDTFR8	Hsp90 protein binding	(Liang et al., 2014)
VIT_14s0030g00650	ST4	Down	Hyper	Gene Body	Shikimate Dehydrogenase	Carboxylic acid biosynthetic process	(Díaz et al., 2001)
VIT_16s0050g01620	ST4	Down	Hyper	Promoter	ABC transporter domain-containing protein (ABC transporter G family member 8)	ATP-binding cassette (ABC) transporter	(Kang et al., 2011)
VIT_18s0001g01200	ST4	Down	Hypo	Gene Body	Uncharacterized protein		

VIT_05s0020g03930	ST6	Up	Hypo	Gene Body	STAS domain-containing protein	Sulfate transporter	(Varela et al., 2021)
VIT_10s0116g01780	ST6	Up	Hypo	Gene Body	Peroxidase (peroxidase 42)	Oxidoreductase /response to stress	(Liu et al., 2012)
VIT_10s0003g02100	ST6	Up	Hyper	Promoter	Uncharacterized protein (finger protein, putative-related)	GDSL-like lipase	(Ding et al., 2019)
VIT_14s0066g00120	ST6	Up	Hyper	Promoter	Uncharacterized protein (protein sieve element occlusion B)		(Froelich et al., 2011)
VIT_15s0046g00170	ST6	Up	Hypo	Gene Body	Uncharacterized protein (MYB51-like isoform X1)	Transcription cis-regulatory region binding	(Dal Santo et al., 2016; Xie et al., 2020)

2.5 Discussion

2.5.1 Physiological assessment of stress responses

Plant measurements of water status are usually destructive, so g_s was used as a proxy to monitor the extent of the drought stress imposed. This was then confirmed with measurements of stemΨ and pre-dawn water potential (data not presented) as direct measures of plant water status before imposing heat stress. The data confirmed the successful application of moderate to severe drought stress as intended, with stemΨ at -0.56 MPa, indicative of moderate stress in grapevines (Gambetta et al., 2020). As g_s was used to determine the level of drought stress, it was impacted by the drought treatment by definition. Nevertheless, it was still a useful measure of the relative effect of the treatments on leaf physiology. Leaf temperature is directly influenced by air temperature, but also by transpiration rate through evaporative cooling. As a result, although our physiological measurements were all obtained by independent methods, the results are linked by leaf processes, with stemΨ both influencing g_s and being influenced by g_s, while leaf temperature is also being influenced by g_s. This is supported by the observation in ST4, where stemΨ and g_s were well correlated, albeit with an offset with the heat treatment (r^2 = 0.68 and 0.44 for heat stress and control temperature respectively). The same was observed of g_s and LT of fully expanded leaves (r^2 = 0.80 and 0.51 for heat stress and control temperature respectively), stemΨ and fully expanded LT (r^2 = 0.84 and 0.61 for heat and control temperatures respectively) and the two LT measurements (fully expanded and developing leaves) across all treatments (r^2 = 0.84).

Such relationships are consistent with the literature, including for grapevines. They are linked by transpiration, with g_s determining transpiration rate at a given VPD and transpiration rate as a primary determinant for leaf temperature relative to air, as well as the difference between stemΨ and pre-dawnΨ which, in turn, is proportional to soil water availability (drought stress). It was beyond the capacity of this study to measure transpiration rates under ambient conditions, but differences between treatments can be inferred from g_s and VPD. A similar experimental system was used by Edwards et al. (2011) and reported a three-fold increase in transpiration in well-watered vines under heat stress.

The stemΨ measurements clearly demonstrated the interaction between the two stress treatments and the role of water and transpiration in the plant response. Drought stress alone lowered stemΨ relative to control, as the droughted plants were not able to obtain water from the soil at the rate to maintain the same water status as control plants. Heat stress alone also lowered stemΨ relative to control, as water loss via transpiration was increased due to the high VPD. The water uptake from the soil was not enough to compensate. The stemΨ of the combined stress was, however, lower than the drought stress alone; it is reasonable to assume that water loss via transpiration was higher in these plants. This is supported by the absence of a difference in g_s on day one of the heat stress treatment. The leaves subjected to the combined treatment would have been under greater stress than those subjected to the two stress treatments individually. Although g_s is typically well correlated with water deficits in grapevine leaves (e.g., Stevens et al., 1995; Cramer, 2010) and was used as an indicator of drought stress in this study (Figure 2.1A; Supplemental Figure S2.1), it did not reveal the impact of the heat stress on stemΨ. Furthermore, g_s

increased during the first day of heat stress. Such a response has previously been observed both in grapevine (Sommer et al., 2012) and other species (Reynolds-Henne et al., 2010; Marchin et al., 2022). This could be viewed as an adaptation to limit heat stress of the leaf when adequate water is available, as the combined stress treatment did not show a similar increase. Conversely, a study of 20 species found that a significant increase in g_s under combined heat and drought stress was more common than under heat stress alone (Machin et al., 2022). However, this was influenced by whether a species was classified as isohydric or anisohydric, where the observation is more common in the former group. Grapevine varieties vary significantly in this regard (Schultz, 2003). Anecdotally, the Cabernet Sauvignon cultivar used in this study is considered moderate between these two extremes.

Due to the destructive nature of some of the measurements, it was not possible to undertake all the measurements and sampling for gene transcription on the same leaf. Therefore, a younger leaf was used for the transcriptome samples. LT of the mature and younger leaf were highly correlated (e.g., Figures 2.1C, D, but the temperature increase of younger leaves under combined stress was less than that of fully expanded ones; this suggests a higher rate of water loss in the still expanding leaves, previously observed in grapevines (Hopper et al., 2014) and other species (Davis et al., 1977; Reich and Borchert, 1988). The observation may be explained by reduced stomatal function in the younger leaves compared with the fully expanded leaves, or possible differences in hydraulics or even the epidermal integrity of younger leaves, which do not appear to have been studied in detail in grapevine.

After the removal of stress, a rapid recovery was observed for all measured parameters in heat stress-treated plants (heat alone or in combination with drought). Leaf

temperatures and stem water potential also recovered rapidly in drought-stressed plants, although stomatal conductance was still reduced at the final sampling time in comparison with the controls.

2.5.2 Gene expression analysis

Analysis of the correlation between physiological parameters and gene expression levels identified clusters and networks of genes that were significantly positively and negatively correlated with measured physiological parameters across treatments. The expression of the largest number of genes was linearly correlated with increasing LT and decreasing stemΨ, and the majority and most significant of co-expression networks also showed this pattern. There were, however, more than 3000 genes strongly induced at water potentials below 1.0 MPa (e.g., Figure 2.2E, Supplemental Figure S2.4 clusters C9 and C10) or leaf temperatures above 34 ^{0}C (e.g., Figure 2.2A, Supplemental Figure S2.2 cluster C4), suggesting that these thresholds might be indicative of severe stress.

Several pathways where gene expression consistently correlated with physiological parameter measurements were also identified, including thermogenesis, plant-pathogen interaction, cytosine and methionine metabolism, plant hormone signal transduction, MAPK signaling, ubiquitin mediated proteolysis and protein processing in the endoplasmic reticulum. These are indicative of pathways that are important in drought, heat and combined stresses, where changes in gene expression are likely driven by changes in integrated plant physiology, regardless of the specific treatment (Supplemental Figure S2.6).

Quantitatively, transcriptomic changes were most pronounced in the combined treatment, as indicated by the larger numbers of genes being up- and down-regulated at each sampling time (Figure 2.5). Gene regulation and interaction networks for the combined drought and heat stress treatment were more complex than for either individual stress indicating not just that a larger number of genes is influenced (Figure 2.7, Supplemental Figures S2.9-13). The effect of combined stress on the grapevine transcriptome is more than simply additive, similar to what has been observed in other plants (Rizhsky et al., 2002; Rollins et al., 2013). The five hub genes in the network responding to combined drought and heat stress treatments appeared unique to the combined treatment and, to our knowledge, they have not been reported previously as regulators of gene expression networks in grapevine under either drought or heat stress.

Carvalho et al. (2015) reported differences in recovery of cellular redox status and metabolism following heat stress in two different grapevine varieties depending on whether they had acclimated to the stress and that were strongly dependent on genotype. In our experiment, with a limited number of physiological parameters measured and a short heatwave treatment, Cabernet Sauvignon appeared to recover immediately. There were generally fewer differentially expressed genes after recovery than during the treatments (Figure 5), as has previously been reported for Cabernet Sauvignon (Liu et al., 2012), and the shift to secondary metabolism following stress that has been reported as a general feature of grapevine (Carvalho and Amâncio, 2019) was indicated by the ontology of enriched DEGs.

2.5.3 Common stress response genes shared among heat, drought, and combined stress

A small number of DEGs was observed to be common to all treatments (Figure 2.6). More DEGs were shared among drought and combined stress than between heat and combined stress, suggesting that drought stress was the main driver of gene expression regulation for plants under combined stress. Despite the differences in DEGs observed at each sampling time, there were several genes common to all three treatments (Supplemental Table S2.6). DEGs shared by all three treatments included: (1) heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins, where their functions in drought and heat stress have previously been reported (Clément et al., 2011; Liu et al., 2012; Yang et al., 2012; Rocheta et al., 2016; Yu et al., 2018). (2) plant hormone signal transduction and transcription factor activation, as transcription factors are involved in signal transduction networks, regulating the expression of genes that encode proteins and that may act together to respond to multiple stresses (Mahajan and Tuteja, 2005; Bhatnagar-Mathur et al., 2008; Hu et al., 2010; Chen et al., 2012; Licausi et al., 2013; Zhang et al., 2014; Collin et al., 2020). (3) sucrose and starch metabolism and galactose metabolism pathway genes that has been shown altered expression in response to drought and heat stress (Taji et al., 2002; Greer and Weston, 2010; Pillet et al., 2012; Greer and Weedon, 2013; Thalmann and Santelia, 2017).

2.5.4 Differential gene expression exclusive to combined stress

2.5.4.1 Phenylpropanoids biosynthesis

The phenylpropanoids biosynthetic pathway and biosynthesis of flavonoids (anthocyanin, flavonols, and tannins) are important for wine composition and quality. In this study, DEGs associated with phenylpropanoids and flavonoids biosynthesis were identified in the combined stress treatment (Figure 2.8). Anthocyanin regulatory C1, which controls the expression of genes involved in anthocyanin biosynthesis (Cone et al., 1993) was exclusively down-regulated under combined stress during the stress period (ST3-ST4). Similarly, down-regulation of chalcone synthase, the first committed enzyme of the flavonoid biosynthetic pathway (Ferrer et al., 1999), was observed under combined stress during ST3-ST4. Previous studies have shown that the concentrations of flavonol and anthocyanin in berries and skins are negatively affected by heat stress (Mori et al., 2007; Movahed et al., 2016; Pastore et al., 2017). Conversely, anthocyanin biosynthesis is strongly up-regulated in grapevines under drought through the up-regulation of flavonoid biosynthetic genes such as chalcone synthase (Castellarin et al., 2007). It has been suggested that anthocyanin accumulation promoted by water-restricted cultivation could potentially alleviate the detrimental effect of excessive heat that causes reduced anthocyanin, although beneficial effects of water restriction may only occur at later growth stages when berries are ripening (reviewed in Scholasch and Rienth, 2019). We observed no differential expression of genes in these pathways under either drought or heat stress in leaves during this earlier developmental phase, but the downregulation of anthocyanin biosynthesis genes during the combined stress at this stage suggests that drought and heat were not able to offset one another, and that the severity of the stress will likely influence transcription of these genes pre-ripening. Overall, it is possible to hypothesize that combined stress will influence the biosynthesis and degradation of

phenylpropanoids/flavonoids and stilbene in grapevine differently from individual drought or heat stress through the regulation of important structural genes, such as chalcone synthase and anthocyanin regulatory C1 protein.

2.5.4.2 Epigenetic changes

The structure of chromatin is important in the regulation of gene expression (Struhl and Segal, 2013; Zentner and Henikoff, 2013), and depends upon several regulatory epigenetic marks, including DNA methylation, and histone modifications (Sahu et al., 2013). Here, the main category of DEGs found under combined stress was genes associated with histone modifications (Figure 2.7). Terms in this category included histone modification, histone lysine methylation, histone methylation and covalent chromatin modification, while the GO Methylation (*sensu lato*) made up a smaller portion. Upon further inspection, genes associated with histone-lysine methyltransferase appeared to be exclusively regulated in late-stage combined stress (ST4), while other methylation-associated genes were found at stress removal (ST5). Additionally, histone-lysine N-methyltransferase *SUVR3* was one of the five hub genes in the interaction network for combined stress (Figure 2.6B). SUVR3 catalyzes the transfer of one, two, or three methyl groups to lysine and arginine residues of histone proteins and plays a role in epigenetic gene regulation (Pontvianne et al., 2010). Studies have found that stress might induce changes in the epigenome and Bond and Finnegan (2007) proposed that modified chromatin is the basis for epigenetic memory. Some stress-induced modifications are reversed once the stress is over, while some may be stable and heritable, thus named the "stress memory" (Kinoshita and Seki, 2014). Although additional data and analyses are required to conclude whether the changes observed in this study are truly an event of

epigenetic memory formation, the alteration of the expression of those epigenetic change-related genes is potentially an indication of the establishment of epigenetic memory at the latter stage of combined drought and heat stress.

This study has generated valuable transcriptomic datasets on grapevines and provided useful resource for further targeted studies. However, to fully explore the causalities between gene regulation and physiological changes/stress conditions, future studies will need to carry out targeted studies testing the hypotheses linking the transcriptional regulation of individual genes to specific (preferentially different) physiological signals.

2.5.5 DNA Methylation changes

We observed an increase in global DNA methylation levels for combined stress plants compared to control. Genome-wide hyper-methylation have been associated with drought stress in cotton plants (Lu et al., 2017). On the contrary, loss of DNA methylation has been reported to associate with heat stress in other plants, such as maize (Qian et al., 2019). Further analysis at a gene level identified a relatively large number of genes showing differential methylation in stressed plants both during the combined stress event and after physiological recovery (1054 and 809 respectively). Interestingly, only a small fraction of the observed DMGs were also differentially expressed (1% at ST4 and 0.6% at ST6), and no clear correlation was found between the direction of change in methylation induced by the stress (i.e., hyper vs hypomethylation), the genic context in which the observed change in methylation occurred (promoter vs gene body), and the change in expression of the differentially methylated gene, which contradicts the stablished assumption that promoter hypermethylation is indicative of reduced gene expression, while gene body

hypermethylation is to constitutive gene expression (Zhang, Lang & Zhu, 2018). However, of the 17 genes that exhibited altered DNA methylation and gene expression at ST4 and ST6 in all three comparisons, including some well-known stress-responsive transcription factors and proteins, such as MYB, serine-threonine/tyrosine-proteins, pentatricopeptide repeat proteins, RING zinc finger proteins, F-box proteins, leucine-rich repeat proteins and tetratricopeptide repeat proteins (Table 2.1). Suggesting the changes in DNA methylation may be associated with the differential expression of some stress response genes. However, although this paper did not look into transposable elements explicitly, it is possible that changes in DNA methylation are more likely to control the expression of nearby transposable elements, rather than directly affecting the transcription of those stress response genes. As previously been observed in tomatoes under phosphate stress (Tian et al., 2021).

2.6 Conclusions

Differences in rates of stomatal conductance, stem water potentials, leaf temperatures and gene expression patterns were identified between different stress treatments. The combined drought and heat stress had more severe effects on the grapevines' physiology compared with individual stresses. Similarly, networks of genes co-expressing in the combined treatment were more complex than in either individual stress. The expression of a large number of genes was linearly correlated with increasing leaf temperatures or stem water potentials, but the overlap between genes commonly differentially expressed in all treatments and at all sampling times was small, and fewer genes were differentially expressed in the heat treatment than the drought or combined treatments. Of DEGs

common to all three stresses, many belonged to gene families previously implicated in abiotic stress responses. In contrast, the suppression of key regulators of the biosynthesis of phenylpropanoids/flavonoids was observed only under the combined stress. Histone modifying DEGs were also unique to the combined drought and heat stress treatment and genes in chromatin-modifying categories were significantly enriched in all analyses for this treatment. Following removal of stress and physiological recovery of the plants, a small number of DEGs remained in the heat and combined stress treatments, but no DEGs remained following drought. These remaining DEGs in the heat stress and combined treatments were almost exclusively up regulated and only at physiological recovery. They may be particularly important for grapevine acclimation to heat, combined drought and heat stress, or in any effect of encountered stress on the following season in these perennial plants. These results give a collective view of stress response and the similarities and differences in responses between individual and combined stress. They reveal differences in the transcriptomes of grapevine in combined drought and heat stress that are not simply additive of the two individual stresses but may be largely driven by physiological gradients and result in epigenetic modifications.

CHAPTER 3. TRANSCRIPTOME ANALYSIS REVEALS LONG-TERM SOMATIC MEMORY OF STRESS IN THE WOODY PERENNIAL CROP GRAPEVINE

3.1 Abstract

Plants have developed a suite of processes to endure stress conditions, including the ability to generate a molecular memory of stress that results in primed plants which are more resilient to subsequent stresses occurring days to weeks after the priming event. However, how such a priming effect is maintained over longer periods, and after dormancy cycle in perennial plants is less studied. Here, we used whole transcriptome and methylome sequencing of Cabernet Sauvignon grapevine plants over two growing seasons to characterize the vines' response to combined drought and heat stress in naïve and primed plants. Our results showed changes in expression of genes associated with epigenetic modifications during stress and after stress removal, suggesting the establishment of epigenetic memory of stress. This hypothesis was further supported by primed plants showing a small number of differentially expressed genes associated with stress response one year after the priming event and in the absence of second stress and presenting a stronger response than naïve plants when re-exposed to stress one year later. Additionally, we characterized stress responsive genes based on their transcription profile and function and propose a new comprehensive and intuitive classification model for stress memory genes in perennials. Our methylome analysis revealed an increase in DNA methylation in primed plants under combined stress, and that the methylation patterns are less variable among plants under stress than controlled plants. Interestingly, we did not observe a correlation between DNA methylation changes (hyper- or hypomethylation) and

transcription patterns (up- or down-regulation) of the overlapping genes. Suggesting the stress-induced expression changes are, at least partially, independent of DNA methylation. In conclusion, our two-year study revealed the potential role of different types of epigenetic regulation during stress response and stress memory establishment.

3.2 Introduction

Viticulture is highly dependent upon climatic conditions during the growing season. Climate determines the suitability to grow a particular variety, as the most desirable composition of grapes requires specific climatic conditions (Gladstones, 1992). Heat and drought are common abiotic stress factors often connected to grapevine yield losses (Vinocur and Altman, 2005). Although normally studied in isolation, such losses often result from both stresses acting in combination (Vogel et al., 2019). The grapevine responses to acute combined heat and drought stress have been studied and reported by Tan et al. (2023). But chronic and recurring stress are often observed in nature (Pagay et al., 2022), and responses to recurring stress are much less understood.

Extreme growth conditions that inhibit normal growth and development can trigger a priming response in plants. Priming has occurred when a plant has a modified response when re-exposed to stress than that of a naïve plant (unprimed) (Aranega-Bou et al., 2014). In general, priming is evidenced by positive effects like a stronger or faster response pattern (Bruce et al., 2007; Conrath, 2009; Crisp et al., 2016). Studies have shown that plants have a memory of the first (priming) stress and can retrieve the remembered information upon encounter with the later stress (triggering) when there is a prolonged period of no stress between the two stress events (Hilker and Schmülling, 2019). The maintenance of this

memory can be somatic (i.e., transmitted by somatic cells within the plant exposed to the stress) to inter- or transgenerational stress memory (transmitted to the offspring via the germline of the plant exposed to the stress) (Lämke and Bäurle, 2017). Studies have revealed mechanisms of the storage and retrieval of this stress memory, which include epigenetic regulation, transcriptional priming, the primed conformation of proteins, or specific hormonal or metabolic signatures (Crisp et al., 2016; Ding et al., 2012; Hake and Romeis, 2019; He and Li, 2018; Heil and Karban, 2010). Evidence suggests that stress memory is heavily epigenetic-based and involves mechanisms such as chromatin remodeling, DNA methylation, nucleosome position, histone modification, and noncoding RNA-mediated regulation (Liu et al., 2021). It is believed that stress induced epigenetic marks are the molecular basis for long-term and transgenerational maintenance of priming (Tricker et al., 2013a), and that this stress memory can be observed through the physiological, transcriptional, and biochemical modifications occurring when exposed to the stress factor in the future, hinting the plant has become more resistant (or sensitive) to the same (Alves de Freitas Guedes et al., 2019; Perrone and Martinelli, 2020) or different stress (Tricker et al., 2013b). The duration of stress memory will depend on the stability of the epialleles responsible for the stress memory, either mitotically or meiotically. In mitotically stable memory, it has been observed that plant epigenetic (e.g., DNA methylation) profiles are predictive of the environmental where the plant grows (Xie et al., 2017), and that such the changes are persistent during vegetative growth, throughout newly developing tissues, and along the lifetime of the plant (Deleris et al., 2016; Lämke and Bäurle, 2017).

From a transcriptional perspective, priming has been defined as a change in the expression of certain genes in primed plants when exposed to a second stress. According to this, stress responsive genes can be classified as: non-memory genes (i.e., those which expression is the same in primed and naïve plants when exposed to stress); and memory genes (i.e., those which expression is significantly different in primed and naïve plants). Two main memory gene classification systems have been proposed to date. Ding et al. (2014) defined 6 types of memory genes, i.e., (+/+), (-/-), (+/-), (-/+), (+/=), and (-/=); where the first symbol indicates the direction of the transcriptional changes occurring in plants exposed for the first time to stress compared to control plants (+ and - indicate an increase or decrease in expression of a given gene respectively), and the second symbol indicates the transcriptional changes of a primed plant compared to its naïve state response. On the other hand, Bäurle (2018), proposed a simpler classification system with non-memory genes (as defined above), and type I and type II memory genes. Type I genes maintain the alteration in transcription levels (upregulation or downregulation) passed the duration of the priming environmental stressor, while Type II genes present a modified response in expression after the triggering stress compared to the priming stress, following a lag phase of transcriptional inactivity. Although both models are complementary, they both fail to capture all possible types of memory genes (e.g., Ding et al do not include Type I genes, while Bäurle does not describe Type I gene expression patterns in response to a triggering stress) in a simple and intuitive manner.

Moreover, the majority of the studies exist on how this priming effect or the memory of stress is maintained in annual/model plants such as arabidopsis (e.g., Ding et al., 2012). How the memory of stress is maintained in perennial plants after dormancy cycle

is less studied. The few studies done on memories in perennials were on coffee plants (*Coffea canephora*) (de Freitas Guedes et al., 2018), wild strawberries (*Fragaria vesca*) (López et al., 2022) or perennial grass species such as tall fescue (*Festuca arundinacea*) (Bi et al., 2021). Grapevine has recently been proposed as a model plant to study epigenomics in perennial plants due to its unique characteristics (Fortes and Gallusci, 2017). Characteristics such as grape flower development is programmed one year in advance; and that the environmental conditions of the previous year affect flower and subsequent fruit development, suggesting that a memory of the environmental conditions is established every year in meristems committed to flowering. Therefore, making grapevine an interesting model to study how long-term somatic stress memory is maintained after dormancy cycle.

Multi-omics approaches such as transcriptomics, epigenomics, degradomics, proteomics, and metabolomics have been developed and deployed to study the mechanistic basis of plant stress memory (Liu et al., 2021). In this study, we used transcriptome and methylome sequencing to study the potential role of epigenetic regulation during stress response, stress memory establishment, and the maintenance of long-term somatic memory in grapevine, and to identify and characterize the expression patterns of genes associated to somatic memory of stress in grapevine.

3.3 Materials and methods

3.3.1 Plant materials and experimental design

To test the establishment, maintenance, and priming effect of long-term memory of stress in *V. vinifera*, a two-growing seasons experiment was carried out during 2016, 2017, and 2018 (Figure 3.1). Plant material and growing conditions used during the first growing season are described in detail in Tan et al. (2023). In short, 64 propagated dormant cuttings obtained from 6 donor vines (*V. vinifera* L. Cabernet Sauvignon) were randomly allocated into two different groups (i.e., control and combined drought and heat stress (T0 and T3 respectively hereafter)) and randomly divided into five replicate plots. Plants were then exposed to combined drought and heat stress as described in Tan et al. (2023). After stress treatment, all plants were maintained under control greenhouse conditions and left to enter dormancy cycle at the end of the 2016/17 season. Post-leaf fall, the vines were pruned to a single cane with four buds from the origin on the main stem. Prior to the spring of the second growing season, plants from each of the treatments (0, naïve plants hereafter; and 3, primed plants hereafter) were randomly assigned to two treatments (control or combined stress) and one of four blocks (each containing 4 groups of 4 plants randomly distributed within the block). This resulted in four groups depending on the first and second season groups: 0,0 refers to naïve plants grown under control conditions in season 2; 0,3 refers to naïve plants grown under combined stress in season 2; 3,0 refers to primed plants grown under control conditions on season 2; and 3,3 refers to primed plants grown under combined stress on season 2 (Figure 3.1a). Each block was placed on a separate bench in a glasshouse previously (CSIRO, Waite Campus, Adelaide, South Australia, Australia) set

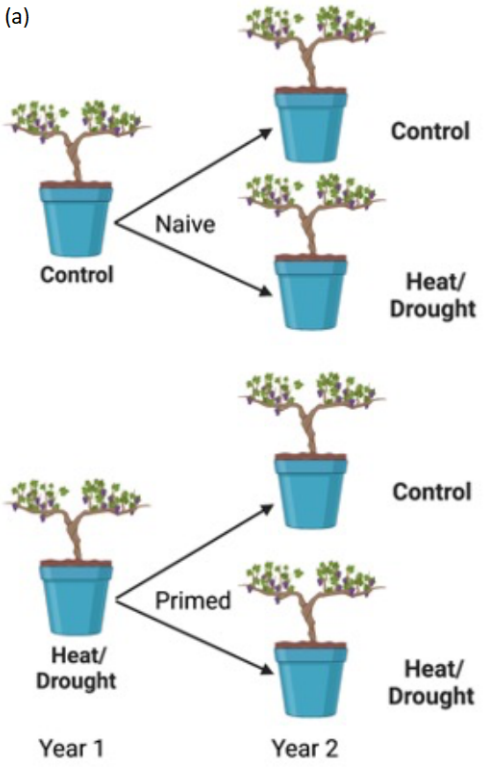
at an air temperature of 27°C day/20°C night, until stress treatments were applied. Humidity and light were uncontrolled. Air temperature and humidity were continuously recorded using a TinyTag Plus 2 logger in a small Stephenson shield (Hastings Data Loggers, Port Macquarie, NSW, Australia).

Water stress was imposed by removing drippers and monitoring stress using measurements of stomata conductance (g_s), with small additional water provided as required to maintain g_s in the 50-75 $mmol/m^{-2}/s^{-1}$ range. The water stress treatment started on 23/1/2018, with heat stress generated for two days using natural insolation, as in Tan et al. (2023), on 4-5/2/2018. On the second day of combined stress of year 2, g_s was measured on every vine (64 in total) at approximately 4 PM, then a single set of measurements (stem water potential (Ψs), leaf temperature, stomatal conductance (gs) and leaf sampling (snap-frozen leaves for DNA and RNA analysis) was undertaken (Figure 3.1b), as described in Tan et al. (2022), with the exception that it was not possible to take g_s measurements on every leaf (2-3 reps per 2017/2018 treatment).

The second and third of leaves counting from the apical meristem were sampled at four time points during the two seasons (Figure 3.1b). Four replicates were collected from each sampling time/treatment combination on season 1, and 8 replicates were collected from each sampling time/treatment combination in season 2. Samples were coded according to their sampling time (ST4, ST5, or ST6), season (1 or 2) and treatment (control (0) or heat and drought (3)). Season 1 samples are described using a five-character code, i.e., leaf samples collected at sampling time 4 of season 1 from plants under control conditions were coded $ST4_10$, while samples from season 2 are described using six

characters, i.e., a sample collected at sampling time 4 of season 2 from naïve plants under control conditions were coded $ST4_200$.

(a)



(b)

Sampling time	Description	Year 1	Year 2
ST1	Day before drought application	-	-
ST2	Day before heat application	-	-
ST3	10th day of drought treatment and 1st day of heat treatment	-	-
ST4	11th day of treatment and 2nd day of heat treatment	+	+
ST5	Day of stress removal	+	-
ST6	Plant physiological recovery, 16th day after stress removal	+	-

Figure 3.1 Experimental design.

(a) Scheme of stress treatment time course and plant assignment over two growing seasons. (b) Sampling and data collection times. Leaf samples collected at the time points indicated with a + were used for nucleic acid extractions to analyze gene expression and DNA methylation differences between plants grown under control and stress conditions.

3.3.2 Nucleic acid extraction

Collected leaves from each plant were frozen immediately after collection using liquid nitrogen and stored at -80°C. Frozen leaves were ground to a fine powder under liquid nitrogen using mortar and pestle. Samples were split into two subsamples and stored at -80°C until further use.

Total RNA was extracted from 100 mg of frozen and ground samples using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's Protocol A. Spectrophotometric analysis (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington, DE, USA) and Experion™ RNA StdSens Chips (BIO-RAD, USA) were used to determine RNA integrity. Only samples with a RNA quality indicator (RQI) above 7 and presenting 260/280 and 260/230 absorbance ratios between 1.8-2.2 were used for library preparation. 4ug of total RNA per sample was used for ribosomal RNA depletion using Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer’s instructions.

DNA extracted from leaf samples collected from three replicates randomly selected out the four available at each time point, using the DNeasy Plant kit (Qiagen). The concentration and integrity of the DNA were measured by Fragment Analyzer (Agilent Technologies).

3.3.3 RNA Sequencing (RNASeq)

5ul of ribosomal depleted RNA from each sample were used to prepare 64 individually barcoded RNA-seq libraries using the NEBNext® Ultra™ RNA Library Prep

Kit for Illumina (New England Biolabs, USA) following the manufacturer's instructions. The Illumina NextSeq 500 HighOutPut platform was used to produce 75bp single-end runs at the Australian Genome Research Facility (AGRF) in Adelaide, Australia.

3.3.4 Whole Methylome Sequencing (WMS)

WMS was performed on genomic Library preparations were done following the manufacturer instructions of the NEBNext Enzymatic Methyl-seq Kit (New England BioLabs). Each individual sample of genomic DNA was spiked with internal controls to determine the enzymatic conversion efficiencies and the abundance of false positives and negatives (i.e., 0% methylated Lambda DNA, and 100% CpG methylated pUC19 DNA). Spiked DNA samples were then fragmented to 200 – 300 bp using the Covaris S220 ultrasonicator. The resulting individually barcoded libraries were sequenced using Nova Seq 6000, and PE150 with a paired-end sequencing approach.

3.3.5 Bioinformatics Analyses

RNA-sequencing data analysis: Raw sequencing data were processed on the LipsComb Compute Cluster (LCC) platform at the University of Kentucky, United States. AdapterRemoval (Lindgreen, 2012) was used for removing adaptors of the raw reads. Sequence quality control was performed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (2015). The reads were mapped to a 12X grapevine reference genome (NCBI assembly ID: GCF_000003745.3) with the alignment tool (HISAT2) (Kim et al., 2015). The GTF reference of the *Vitis*

vinifera genome was downloaded from the *Ensembl Plants* website (http://plants.ensembl.org/Vitis_vinifera/Info/Index). Samtools (Li et al., 2011) was used to generate Binary Alignment Map (BAM) files after mapping the reads to the genome.

Identification of putative memory genes using co-expressed gene cluster analysis: Transcripts Per Million (TPM) of each plant sample were calculated from the BAM files using the TPMcalculator (Alvarez et al., 2019). Normalized data (calculated TPMs, log2 transformed) was used for the identification of gene expression clusters based on gene expression patterns during the following time point/treatment combinations: control plants sampled at season 1 ST4 ($ST4_10$), stressed plants sampled at season 1 ST4, ST5, and ST6 ($ST4_13$, $ST5_13$, and $ST6_13$), and primed plants under combined stress sampled at season 2 ST4 ($ST4_233$) using *clust* v1.8.4 (Abu-Jamous & Kelly, 2018). Resultant clusters were then classified according to three conditions: A) If the gene expression level in stressed plants at physiological recovery was significantly different than that presented by control plants ($ST4_10 \pm ST6_13$; T-Test p-val < 0.05) or not, b) if the change in expression in response to the triggering stress was significantly different than in response to the priming stress ($ST4_13 \pm ST4_233$; T-Test p-val < 0.05), and c) if the triggering stress induced a significantly different change in expression compared to the expression level at physiological recovery from the priming stress ($ST6_13 \pm ST4_233$; T-Test p-val < 0.05).

Differentially expressed genes (DEGs) analysis: Gene expression level was estimated using the *edgeR* package (Robinson *et al.*, 2010) on Rstudio. The raw mapped data of each sample was standardized by edgeR's trimmed mean of M values (TMM). This method estimates scale factors between samples to determine DEGs. Between control and treatment, a log2fold change(log2FC) of 2 and a false discovery rate adjusted P-value<0.05

using Benjamini and Hochberg's algorithm was adopted to indicate significance. This process was repeated for each group of comparisons.

Gene ontology (GO), DEGs visualization, and functional annotation: All differentially expressed genes of interest were subjected to ontology analysis through the usage of agriGO v2.0 (Tian et al., 2017). DEGs of each treatment were used to attain the significant GO terms with agriGO v2.0 with the following criteria: Fisher's statistical test method, Yekutieli (FDR under dependency) multi-test adjustment method, significance level <0.05, and selecting either complete GO or slim GO as the gene ontology type. The visualization of the expression level of selected DEGs was done through the built-in plot function of R. Functional annotation of DEGs was obtained from PantherDB (Mi et al., 2021). Plots were performed with R-package ggplot2.

Identification of differentially methylated cytosines and regions (DMCs and DMRs): Adaptor sequences, low-quality reads, and contaminants were removed from WMS reads using Adapter Removal V2 software. The enzymatic conversion efficiency of unmethylated and methylated cytosines was calculated using pipelines (https://github.com/nebiolabs/EM-seq/blob/master/em-seq.nf) and the methylation control sequences (https://github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa) provided by the NEBNext Enzymatic Methyl-seq Kit manufacturer.

Genome indexing was performed with Bismark using '--bismark_genome_preparation' option (Krueger and Andrews, 2011) using the C-to-T and G-to-A versions of the reference grapevine genome (PN40024 v.4) created with Bowtie2 (Langmead and Salzberg, 2012). Sequencing coverage and depth were estimated using Samtools coverage and depth toolkits (Li et al., 2009). Methylation calling was performed

with Bismark extractor (https://www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf) by calling '--comprehensive' and '--cytosine_report' option after the conversion to bedGraph. Both Differentially methylated cytosines and regions (DMCs and DMRs respectively) were determined using the 'Methylkit' package (Akalin et al., 2012) with default parameters (minimum coverage threshold of 10 and 5 for DMCs and DMRs, respectively; q-value $\leq$ 0.05; minimum differential methylation level of 10%); sliding window for DMRs was 1000 bp). Genes were deemed differentially methylated when a DMR overlapped with their promoter (defined here as 1000 bp upstream of the transcription starting site (TSS)), or with the body of the given gene.

3.4 Results

3.4.1 Gene expression analysis

Transcriptome sequencing data de-multiplexing yielded an average of 25 million reads per sample after quality filtering (QC 30). The average percentage of mappable reads per sample was 82%, ranging from 70-92% (Supplemental Table S3.1).

Identification of modified responses in gene expression as a result of priming: First the gene expression of $ST4_200$ and $ST4_203$ plants was compared to identify the genes differentially expressed under combined stress. This comparison served two functions, first, identify the genes differentially expressed by naïve plants when expose to a first stress event. Secondarily, these results would also serve as validation of the results presented by Tan et al. (2023), as the naïve plants priming state and growing conditions replicated those

in the aforementioned experiment. In this comparison, 176 genes were found to be up-regulated, and 431 were down-regulated (Figure 3.2a) in naïve plants grown under stress conditions ($ST4_203$), compared to naïve plants grown under control conditions ($ST4_200$). Pathway analysis revealed pathway enrichment similar to those during season 1 experiment, such as 'plant hormone signal transduction', 'protein processing in endoplasmic reticulum', and 'phenylpropanoid biosynthesis' (Supplemental Figure S3.1).

The existence of an epigenetic memory on plants exposed to stress on season one was then assessed using two different comparisons: First, the gene expression of primed plants was analyzed in the absence of a triggering stress event by determining differential gene expression between $ST4_200$ vs $ST4_230$ plants (i.e., naïve and primed plants in the absence of recurring stress respectively). In this comparison, 37 genes were found to be up-regulated, and 2 down-regulated in primed plants compared to naïve ones (Figure 3.2a). A histone lysine N-methyltransferase ATXR6-associated gene was found to be up-regulated among the identified DEGs. Other DEaGs identified included APETALA 2/Ethylene Responsive Factor (AP2/ERF), no apical meristem, Arabidopsis thaliana activating factor and cup-shaped cotyledon (NAC), WRKY, ATP-binding cassette (ABC) transcription factor family genes, as well as F-box domain-containing and cysteine-rich transmembrane module (CYSTM) domain-containing proteins (Supplemental Table S3.2). GO analysis of identified DEGs revealed 57 significantly enriched GO terms (Supplementary Table S2), with the top molecular function and biological process GO terms including nucleic acid/DNA binding, transcription factor activity, regulation of gene expression, and 'response to stress' (Figure 3.2b). Second, the number of DEGs in primed plants under a recurrent stress event (i.e., $ST4_200$ vs $ST4_233$) was calculated and compared

to those identified above in naïve plants exposed to stress on season 2 (i.e., $ST4_200$ vs $ST4_203$). Although the majority of DEGs found in both types of plants (543) were found to be commonly regulated, that is, they were up-or down-regulated in both primed and naïve plants (Figure 3.2c), primed plants showed a higher number of unique DEGs than naïve plants exposed to stress for the first time (i.e., 390 vs 64 DEGs respectively (Figure 3.2c)). GO analysis on naïve plant exclusive DEGs showed enrichment in histone methylation, covalent chromatin modification, and histone lysine methylation (Supplemental Table S3.3A). Similar GO terms were observed in first-year plants by Tan et al. (2022) under combined treatment. DEGs exclusive to primed plants exposed to a second stress showed enrichment in anatomical structure development and developmental process, GO terms associated with methylation were also identified (Supplemental Table S3.3B). The GO analysis on the 543 DEGs identified in naïve and primed plants exposed to stress showed enrichment of chromatin assembly, methylation, and chromatin organization (Supplemental Table S3.3C). To better understand the difference between naïve and primed plant responses to stress, and the effect of priming on gene expression, the magnitude of the expression changes in DEGs common between primed and naïve plants was compared (Figure 3.2d). For this, paired T-tests were performed for the individual fold changes (FC) of upregulated and down regulated genes, and the false discovery rates (FDR) of those genes (n=534). This analysis indicated that the fold change in expression of DEGs common to primed and naïve plants was larger and more significant (FDR) in primed plants (T-Test -FC and +FC, $p < 0.01$; T-Test FDR, $p < 0.01$).

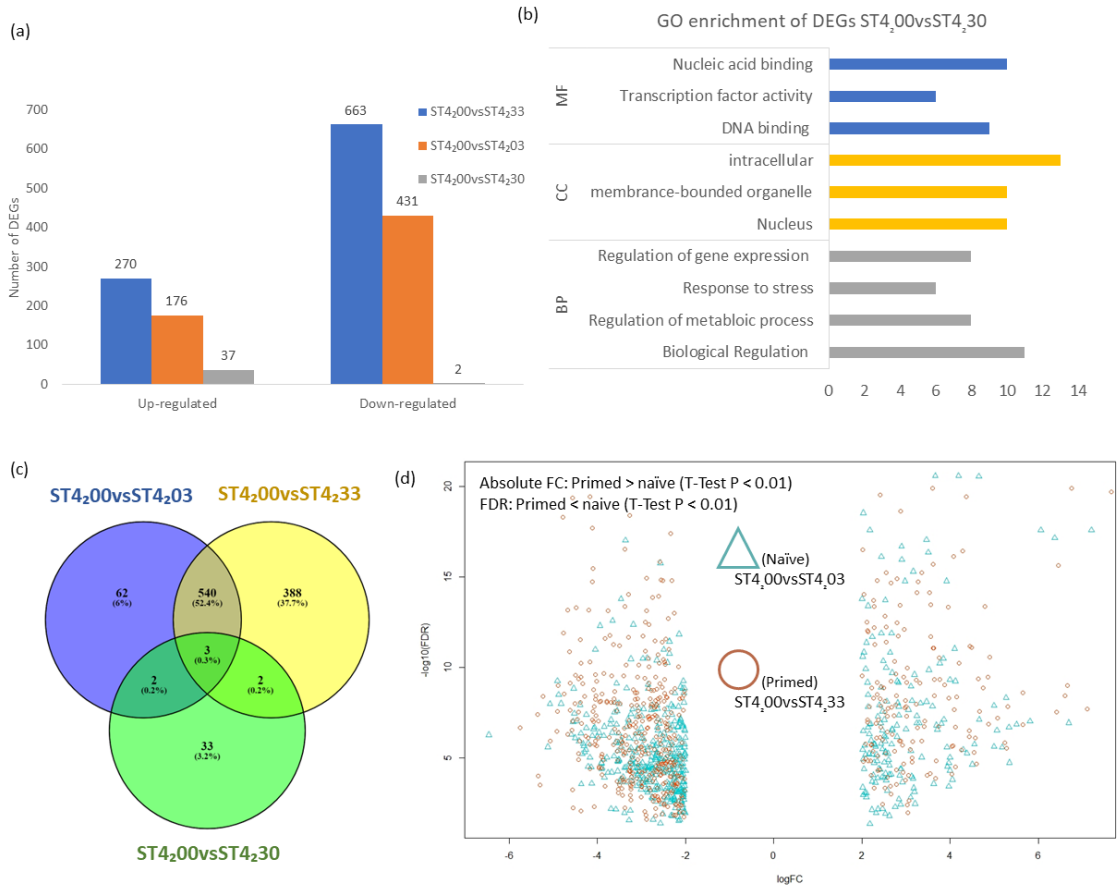


Figure 3.2 Analysis of differential gene expression between naïve and primed plants under stress or control conditions.

(a) Number of differentially expressed genes (DEGs) identified between naïve plants grown under control conditions on year 2 ($ST4_200$) and: primed plants exposed to stress on year 2 ($ST4_233$), naïve plants exposed to stress on year 2 ($ST4_203$) and primed plants exposed to control conditions on year 2 ($ST4_230$). (b) Significantly enriched GO terms for DEGs identified in $ST4_200vsST4_230$. (c) Common and unique DEGs for each of the comparisons described above. (d) Effect of priming on the magnitude of change in expression (i.e., log fold change (horizontal axis) and FDR (vertical axis) of the common 543 DEGs identified in primed and naïve plants exposed to stress on year 2. Brown circles represent the common DEGs found in $ST4_200$ (naïve plants under control conditions) compared to $ST4_233$ (primed plants under combined stress). Blue triangles represent the common DEGs found in $ST4_200$ compared to $ST4_203$ (naïve plants under combined stress).

Identification of putative stress memory genes: Memory genes are traditionally defined as those which response is different in primed than in naïve plants. To identify putative memory genes in grapevine, we used gene expression clustering analysis on combined stress-induced DEGs identified in year 1 (that is DEGs between control and stressed plants identified at season 1 sampling times $ST4_13$ (671 DEGs) and $ST5_13$ (224 DEGs) (ST4 and ST5 hereafter for simplicity). ST4 DEGs formed 10 clusters ($C0_{ST4}$ to $C9_{ST4}$) containing a total of 384 genes (Supplemental Figure S3.2), and ST5 DEGs formed 5 clusters ($C0_{ST5}$ to $C4_{ST5}$) containing 101 genes (Supplemental Figure S3.3). Among those, two clusters ($C0_{ST4}$: 96 genes, and $C6_{ST4}$: 20 genes) contained genes with similar expression levels in response to the priming and the triggering stress with an intermediate phase of no transcriptional differences (compared to the control plants) between stresses and were deemed non-memory genes (Figure 3.3 and Supplemental Figure S3.2). Nine clusters ($C1_{ST4}$: 21 genes; $C3_{ST4}$: 57 genes; $C4_{ST4}$: 26 genes; $C7_{ST4}$: 20 genes; $C8_{ST4}$: 71 genes; $C0_{ST5}$: 20 genes; $C2_{ST5}$: 12 genes; $C3_{ST5}$: 14 genes; and $C4_{ST5}$: 34 genes) (Supplemental Figure S3.2, 3) contained genes which expression was maintained at significantly different level from the control plants ($ST4_10$) during the time between the removal of the priming stress, and the triggering stress (i.e., ST6), so were deemed Type I memory genes. These clusters could be further divided into four different subgroups in relation to their response to the triggering stress. Genes in clusters $C4_{ST5}$ and $C7_{ST4}$ presented the same level of expression change in response to the priming and the triggering stress (Type $I^{=}$) (Figure 3.3). Genes in cluster $C0_{ST5}$ showed a significantly higher change in expression to the priming than the triggering stress (Type I^{+}) (Figure 3.3). Genes in clusters $C1_{ST4,}$ $C3_{ST4,}$ $C4_{ST4,}$ and $C3_{ST5}$ showed a significantly lower change in expression

to the priming than the triggering stress (Type I^-) (Figure 3.3). All these clusters presented a change in expression between physiological recovery to the priming stress and the triggering stress. Conversely, no change in expression was observed in response to the triggering stress in clusters $C2_{ST5}$ and $C8_{ST4}$ when compared to plants at physiological recovery (Type I^0) (Figure 3.3).

Three clusters ($C2_{ST4}$: 28 genes; $C9_{ST4}$: 26 genes; and $C1_{ST5}$: 21 genes) contained genes presenting a modified response to the triggering stress compared to the priming stress, following a lag phase of transcriptional inactivity, and so, were deemed Type II genes. As with Type I genes, these clusters separated into different subtypes. $C2_{ST4}$ and $C1_{ST5}$ presented an enhanced change in expression to the triggering stress compared to the priming stress (Type II^+) (Figure 3.3). Finally, $C9_{ST4}$ genes presented a diminished change in expression to the triggering stress compared to the priming stress (Type II^-) (Figure 3.3).

GO and functional analysis of all clusters containing memory genes as defined above was performed (Supplemental Table S3.4). Interestingly, the GO terms for 21 genes from cluster C1 of ST5 DEGs are enriched in methylation, including histone H3-K9 methylation and DNA methylation. Among those, a structural maintenance of chromosome protein-associated gene was identified through functional annotation.

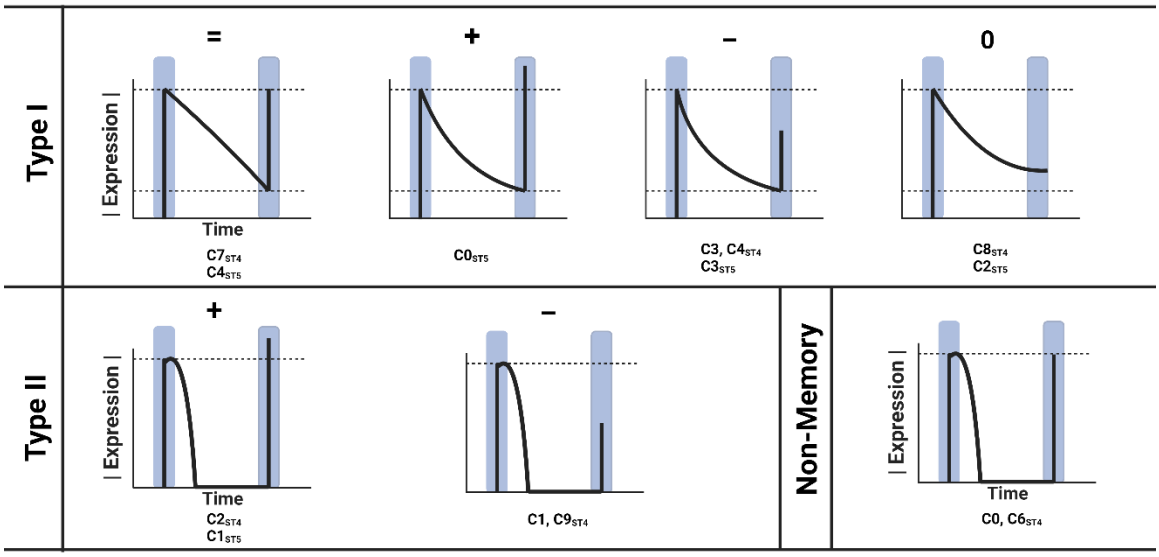


Figure 3.3 Stress memory gene models based on the expression patterns of DEGs found over two growing seasons.

Type I: genes that exhibited sustain expression after first stress encounter. Type $I^{=}$: expression changes upon second stress encounter was highly identical to the first. Type I^{+}: expression changes upon second stress encounter was higher than the first. Type I^{-}: expression changes upon second stress encounter was lower than the first. Type I^{0}: the expression changes upon second stress encounter was minimal compared to the sustained expression after the first stress encounter. Type II: genes that exhibited expression changes between first and second stress encounter, the expression level returned to basal after the first stress. Type II^{+}: the expression level of genes upon second stress encounter was higher than the first. Type II^{-}: the expression level of genes upon second stress encounter was lower than the first. Non-memory: genes that exhibited no expression changes between first and second stress encounter, the expression level returned to basal after the first stress.

3.4.2 DNA methylation analysis

An average of 69 million reads per sample were produced from the EM-seq library after quality filtering. The average percentage of mappable reads per sample to the PN40024 v.4 genomes was 54%. The average non-bisulfite conversion rate among the samples was 0.2%, and the average bisulfite conversion rate among the samples was 95.2%. The average percentage of covered bases was 81.22%, while the sequencing depth was 17X per sample (Supplemental Table S3.5).

Global DNA methylation pattern induced by recurrent combined stress in Grapevine: Analysis of the average methylation percentage (methylated cytosines, mCs) for each of the three contexts (CG, CHG, and CHH) showed that the CG context is the more methylated of the three, followed by CHG, and finally CHH (Figure 3.4a). Both naïve plants under stress conditions ($ST4_203$), and primed plants under control conditions ($ST4_230$) showed similar levels of DNA methylation to naïve plants under control conditions ($ST4_200$). Conversely, primed plants under stress conditions ($ST4_233$), presented a significant increase in mCG, mCHG, and mCHH (T-test, $p \leq 0.05$) (Figure 3.4a). PCA plot suggested that the global DNA methylation pattern in naïve plants ($ST4_200$ & $ST4_203$) appeared to be more variable compared to the primed plants ($ST4_230$ & $ST4_233$) (Supplemental Figure S3.4).

The effect that priming, and of stress on naïve and primed have on local DNA methylation was determined by identifying differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in the following comparisons $ST4_200$ vs $ST4_230$, $ST4_2$ 00vs $ST4_203$, $ST4_200$ vs $ST4_233$. Briefly, plants exposed to stress for the

first time presented a higher number of DMCs (1,254, 1,395, and 905 DMCs for CG, CHG, and CHH contexts, respectively), than primed plants in the absence of a triggering stress (938, 8, and 52 DMCs in CG, CHG, and CHH contexts, respectively), while primed plants under the effect of a triggering stress presented the largest number of DMCs across all three contexts (2,178, 1,779, and 2,637 for CG, CHG, and CHH, respectively) (Figure 3.4b). In general, the majority of DMCs were found in intergenic regions, regardless of context and comparison, and DMCs were more likely to be hypermethylated than hypomethylated (Figure 3.4b). The number of differentially methylated regions (DMRs) was assessed to study the dynamics of DNA methylation at specific loci. As with DMCs the total number of DMRs observed ranked from $ST4_200$ vs $ST4_233$ (2,312 DMRs), $ST4_200$ vs $ST4_203$ (1,749 DMRs), to $ST4_200$ vs $ST4_230$ (1,161 DMRs) (Figure 3.4c). Also, like the patterns observed for DMCs, the majority of DMRs identified were intergenic region (55-57%), followed by gene body (25-27%) and promoter (17-19%), and were more likely to be hypermethylated than hypomethylated, except the DMRs in intergenic and promoter regions for $ST4_200$ vs $ST4_230$, where more DMRs were hypomethylated (HypoDMRs) (Figure 3.4c).

As seen with DMCs and DMRs, the number of genes overlapping with a DMR (DMGs hereafter) was higher in primed plants under a triggering stress ($ST4_233$-DMGs = 1160), followed by naïve plants under stress ($ST4_203$-DMG = 969), and primed plants in the absence of a triggering stress ($ST4_230$-DMG = 584). Comparison of all DMGs identified showed that most of them were unique to each of the conditions ($ST4_230$, 03, and 33), while only 2.5% were common to all three conditions, 12.2% where common to $ST4_203$ and $ST4_233$ plants, and 7% to $ST4_230$ and $ST4_233$ plants (Figure 3.4d). The

magnitude of the methylation changes in DMGs common between primed and naïve plants was then compared (269 DMGs). Unlike what we observed for gene expression that we observed in primed plants, there was no significant difference in methylation level between common DEGs in primed and naïve plants under stress.

GO analysis performed on DMGs in naïve plants exposed to stress ($ST4_203$) showed similar enrichment terms regardless of their methylation change patterns (hyper- or hypo-methylated), such as 'developmental process', 'protein serine/threonine kinase activity', 'reproduction', and 'response to stress' (Supplemental Table S3.6A-B). Conversely, primed plants in the absence of a triggering stress ($ST4_230$) revealed a significant enrichment in GO terms such as 'transcription factor activity' and 'histone modification' both for hyper and hypomethylated DMGs. The term 'signal transduction' was unique to hyperDMGs, while 'pyrophosphatase activity' and 'post-transcriptional regulation of gene expression' were unique to hypoDMGs (Supplemental Table S3.6C-D). Finally, primed plants under a triggering stress event ($ST4_233$) showed a significantly enriched GO terms such as 'response to stress', 'chromatin modification', and 'gene silencing'. Terms 'mRNA metabolic process' and 'protein modification by small protein removal' were unique to hyperDMGs and hypoDMGs, respectively. (Supplemental Table S3.6E-F)

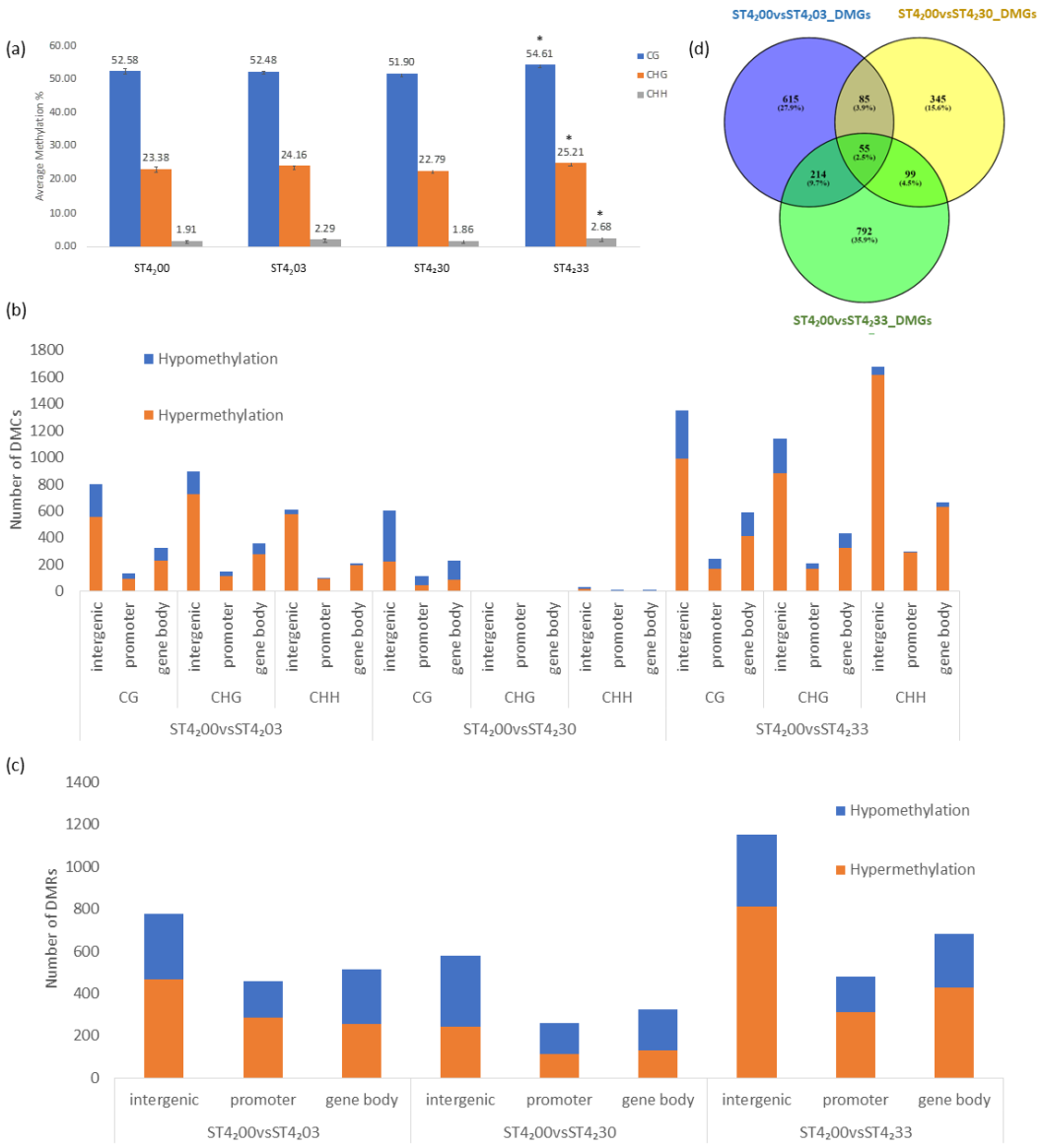


Figure 3.4 Effect of combined drought and heat priming and triggering stresses on grapevine DNA methylation.

(a) Average DNA methylation level for each cytosine context (CG, CHG, CHH) between plant groups: ST4$_2$33 (primed plants under combined stress), ST4$_2$03 (naïve plants under combined stress) and ST4$_2$30 (primed plants under control conditions) and ST4$_2$00 (naïve plants under control conditions) asterisks indicates the significance (Student's T test, $p \leq 0.05$) of the difference between ST4$_2$33, ST4$_2$03, and ST4$_2$30 compared to ST4$_2$00. (b) Number of hyper- (hyper-DMCs) and hypomethylated differentially methylated cytosines (hypoDMCs) separated by sequence context and group comparison. (c) distribution of hyper- (hyperDMRs) and hypo-methylated differentially methylated regions (hypoDMRs) and in genomic features: promoter, gene body, and intergenic regions. (d) Venn diagram of differentially methylated genes between different plant groups.

Changes in gene methylation overtime were examined by comparing DMGs identified in plants under combined stress ($ST4_13$), and during physiological recovery ($ST6_13$) in season 1, and in primed plants under a triggering stress ($ST4_233$) (Figure 3.5a). 20 genes were differentially methylated in all three time points. Among those, 4 were hypomethylated at all three time points, 2 were hypomethylated in $ST4_13$ and $ST6_13$ but hypermethylated in $ST4_233$. 2 were hypomethylated in $ST4_13$ and $ST4_233$ but hypermethylated in $ST6_13$, 7 were hypomethylated in $ST4_13$ but hypermethylated in both $ST6_13$ and $ST4_233$, 2 were hypermethylated in both $ST4_13$ and $ST4_233$ but hypomethylated in $ST6_13$, 1 was hypermethylated in $ST4_13$ but hypomethylated in $ST6_13$ and $ST4_233$. Lastly, 2 were hypermethylated in $ST4_13$ and $ST6_13$ but hypomethylated in $ST4_233$ (Figure 3.5b).

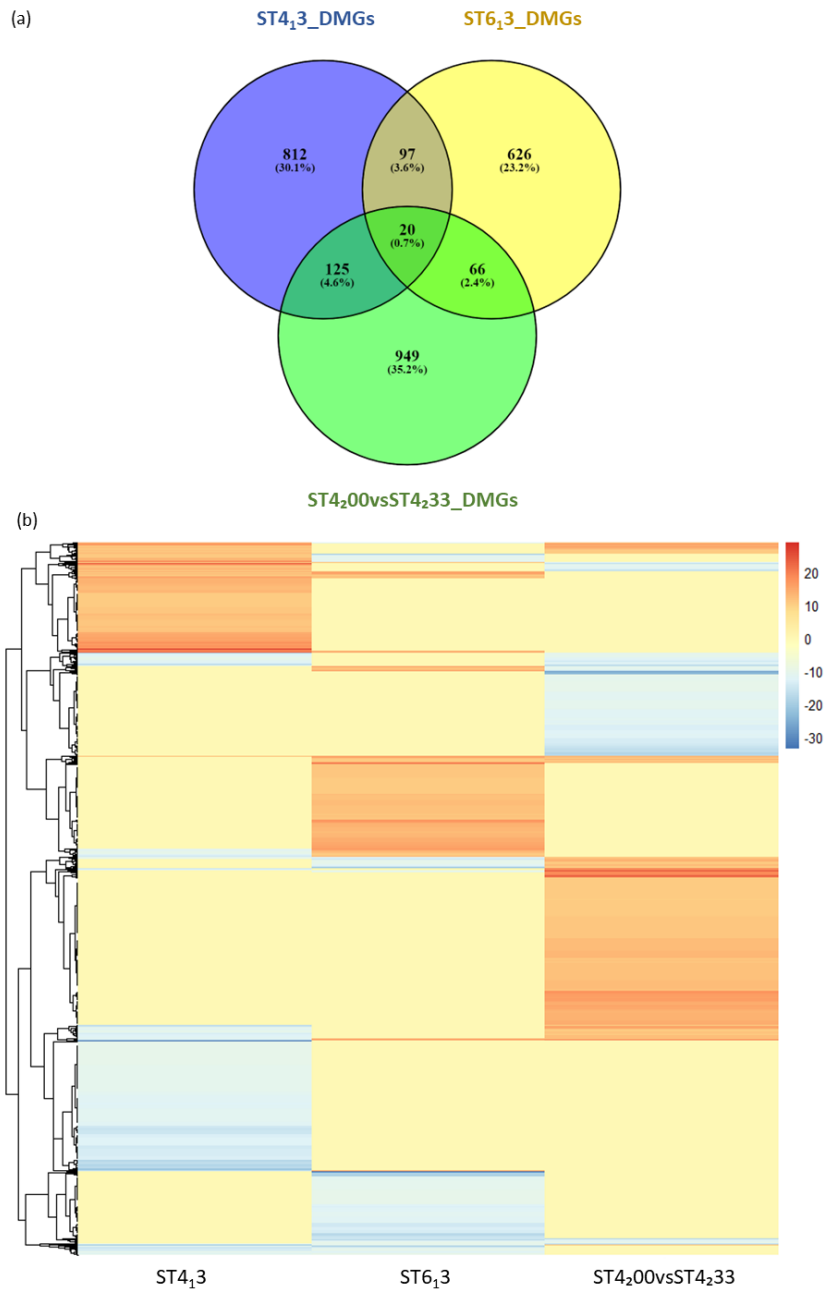


Figure 3.5 Changes in gene methylation over time.

(a) Venn diagram of DMGs identified in plants under combined stress ($ST4_13$), and during physiological recovery ($ST6_13$) in season 1, and in primed plants ($ST4_200vsST4_233$) under a triggering stress. (b) Heatmap of level of methylation changes for DMGs that were differentially methylated in $ST4_13$, $ST6_13$, and $ST4_200$ vs. $ST4_233$. Red: hypermethylation. Blue: hypomethylation. Yellow: not differentially methylated.

3.4.3 The potential relationship between DNA methylation and gene expression

For a better understanding of the potential functional roles of DNA methylation on gene expression, we focused on the DMRs overlapping with gene promoters and gene bodies (DMGs). The relationship between changes in gene methylation and transcriptional changes was examined (Figure 3.6a, b, top). For $ST4_233$ plants, 15 DEGs with DMGs located in the promoters and 8 DEGs with DMGs located in the gene body were identified. Among the 15 located in the promoter, 5 were found to be hypermethylated and down-regulated, 5 were hypomethylated and down-regulated, and 5 were hypomethylated and up-regulated. Among the ones located in the gene body, 4 were found to be hypermethylated and down-regulated, 1 was hypermethylated and up-regulated, 2 was hypomethylated and down-regulated and 1 was hypomethylated and up-regulated (Figure 3.6a, bottom). In $ST4_203$ plants, 11 DEGs with DMGs located in the promoter and 12 DEGs with DMGs located in the gene body were identified. Among the 11 located in the promoter, 4 were hypermethylated and down-regulated, 2 were hypermethylated and up-regulated, 3 were hypomethylated and down-regulated, while 2 were hypomethylated and up-regulated. For the 11 located in the gene body, 6 were hypermethylated and down-regulated, 2 were hypermethylated and up-regulated, 3 were hypomethylated and down-regulated, while 1 was hypomethylated and up-regulated (Figure 3.6b, bottom). No overlapping DEGs and DMGs were identified in $ST4_230$ plants. In general, the presence of hypo- or hyperDMGs did not correlate with the transcription patterns (up- or down-regulation) of those genes. Gene function annotation analysis revealed the involvement of sHSPs in both $ST4_203$ and $ST4_233$, interestingly, regardless of their methylation pattern, those sHSPs appeared to be all up-regulated (Supplemental Table S3.6-8). Other genes

associated with basic Helix-Loop-Helix (bHLH), NAC, B3 transcription factor family genes, as well as SUI1-domain, J-domain, tumor overexpressed genes (TOG)-domain, PMR5N domain, and WD-repeats-region domain-containing protein have been identified (Supplemental Table S3.7-8).

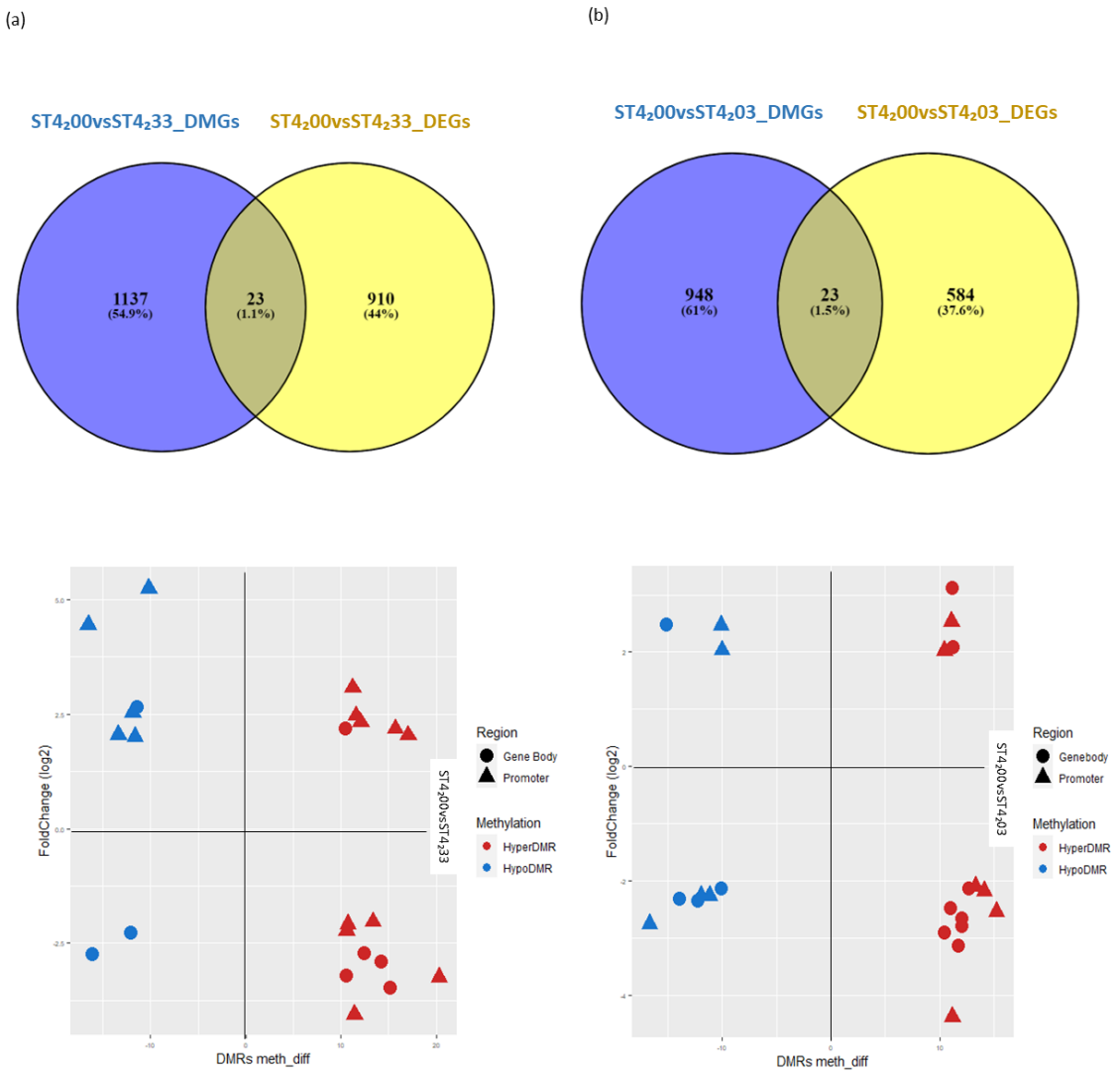


Figure 3.6 Graph representation of overlapping DEGs and DMGs based on group comparison.

(a) Top: venn diagram of overlapping genes between DEGs and DMGs in ST4$_2$00 vs ST4$_2$33. Bottom: scatterplot of DEGs related with DMGs that located in promoter and gene body, showing the relationship between transcript levels (fold change: log2) and DNA methylation (meth_diff) in ST4$_2$00 vs ST4$_2$33. (b) Top: venn diagram of overlapping genes between DEGs and DMGs in ST4$_2$00 vs ST4$_2$03. Bottom: scatterplot of DEGs related with DMGs that located in promoter and gene body) showing the relationship between transcript levels (fold change: log2) and DNA methylation (meth_diff) in ST4$_2$00 vs ST4$_2$03.

3.5 Discussion

3.5.1 Modified response in gene expression after priming

Plants have been shown to be able to establish a molecular memory of environmental stress (priming stress) that results in an enhanced response to subsequent stresses (triggering stress) (Crisp et al., 2016). Multiple studies suggest that the molecular basis of such memory is epigenetic in nature (Lämke et al., 2016; Liu et al., 2018). Most of this research has been done in annual plants, and the few examples of epigenetic memory of stress in perennials, studied epigenetic memory over short periods (days) between the priming and the triggering stress, and none of them, studied the effect of dormancy cycle on the maintenance of such memory. Our previous work showed that grapevines exposed to combined heat and drought stress express genes associated with epigenetic modifications during and after stress removal, and that GO terms associated to response to stress (i.e., starch and sucrose metabolism and pentose and glucuronate interconversion (Liu et al., 2020b) were enriched for differentially expressed genes after physiological recovery, (i.e., 16 days past the removal of the environmental insult) (Tan et al., 2023). Taken collectively, our results suggest the potential establishment and maintenance of epigenetic memory of stress in grapevine over multiple weeks within one growing season.

Here we studied the effect of a priming stress on the vine response (i.e., changes in gene expression and DNA methylation) to a triggering stress, of the same nature, occurring after a long period of no stress (approximately one year) and after dormancy cycle with the ultimate goals of identifying and describing grapevine genes associated with memory of stress and determining the potential contribution of DNA methylation towards its establishment and maintenance.

First, we compared the response observed in naïve plants under combined stress ($ST4_203$) to that observed in plants under the same conditions in season 1. Gene expression results suggested a degree of consistency in naïve plants' response to stress for the first time irrespective of the year of exposure in terms of the number of differentially expressed genes and their function. Similarly, KEGG pathway analysis revealed the involvement of 'plant hormone signal transduction', 'protein processing in endoplasmic reticulum', 'pentose and glucuronate interconversions', and 'phenylpropanoid biosynthesis pathway' genes. Which are consistent with the results previously observed. Confirming the importance of those pathways and pathway-associated genes in stress response in naïve plans (Tan et al., 2023).

The results observed in primed plants under combined stress ($ST4_233$) showed differences in expression compared to the results observed in naïve plants under combined stress ($ST4_203$). One common feature of the primed state is the reprogramming of the primed plant transcriptome. Such reprogramming results in differences in gene expression between naïve and primed plants in different temporal contexts. These include primed plants presenting 1) different transcriptional patterns than naïve plants, even in the absence of a triggering stress (Lämke and Bäurle, 2017); 2) different transcriptional patterns in response to a triggering stress (Ding et al., 2012); and 3) significant differences in the scale of expression change in response to the triggering than the priming stress (Lämke and Bäurle, 2017). Our results from season 1 showed that primed plants present different transcriptomes than naïve plants 16 days after the removal of the priming stress (i.e., at physiological recovery). Moreover, primed plants still showed different transcriptome profiles than naïve ones more than 11 months after the priming stress and after dormancy

cycle, and this was made evident by the identification of a small number of DEGs (39) between primed plants ($ST4_230$) and naïve plants ($ST4_200$) in the absence of second stress. Functional annotation of one of the up-regulated DEGs revealed that it was a histone-lysine N-methyltransferase ATXR6-associated gene. ATXR6 has been reported to deposit histone 3 lysine 27 mono-methylation (H3K27me1) (Jacob et al., 2009) to promote heterochromatin formation, which represses transposable elements (TEs), and control genome stability in arabidopsis (Ma et al., 2018). Interestingly, ATXR1, a gene from the same protein family, is necessary but not sufficient for transcriptional memory response (Ding et al., 2012). The involvement of transcription factor regulation in stress response has been well-studied, as they are required to reprogram stress-related genes (Ohama et al., 2017). Specific transcription factor families such as AP2/ERF, NAC, WKRY, and ABC identified among the 39 DEGs in $ST4_230$ have been shown to play important roles in response to abiotic stress such as heat and drought in plants (Chen et al., 2012; Hu et al., 2010; Licausi et al., 2013). More importantly, AP2/ERF and NAC families have been proven to involve in stress memory (Ding et al., 2014), although the transcriptional memory pattern of a transcription factor does not necessarily determine the memory pattern of its target gene (Ding et al., 2013; Jacques et al., 2021). Our results suggest that those transcription factors and ATXR6 could be contributing to the maintenance of the long-term somatic stress memory in the grapevine. Nevertheless, the establishment and maintenance of epigenetic memory of stress would not be of any biological significance if it did not alter the primed plant response to a recurrent stress. We observed that the exposure to a triggering stress led to not only a higher number of differentially expressed genes but also

a larger change in expression of those genes commonly expressed between naïve ($ST4_2 03$) and primed ($ST4_2 33$) plants.

The modified gene expression, even after a seemingly long period (~1 year) with no exposure to stress suggested this observed stress priming-induced somatic memory is long-term and relatively stable, contrary to the somatic stress memory found in annual plants, where it appeared to be transient (Feng et al., 2016; Singh et al., 2014). More importantly, GO analysis showed the heavy involvement of histone methylation and histone-lysine methylation in both naïve and primed plants response to stress (Supplemental Table S3.2). Suggesting the potential role of histone modification in establishing, maintaining, and retrieving this long-term stress-induced somatic memory, as previous research correlating histone methylation with somatic stress memory (Lämke et al., 2016).

3.5.2 Identification of putative stress memory genes

The expression patterns of certain genes in this study closely resemble the expression patterns of stress memory genes, which are stress-inducible genes that have been linked to stress memory establishment (Charng et al., 2007; Charng et al., 2006; Ding et al., 2012; Lämke et al., 2016; Liu et al., 2018). Previous studies have used different systems to classify memory genes based on their transcriptional profile. The first system, described in detail in Bäurle (2018), includes three types: type I, type II and non-memory genes. The expression patterns observed in $C7_{ST4}$, $C8_{ST4}$, and $C4_{ST5}$ could potentially be type I memory genes, as characterized by the gene expression that persists through the recovery phase. Whereas $C2_{ST4}$ and $C1_{ST5}$ could potentially be type II memory genes, where the response is modified, and usually stronger and faster during second exposure

(Bäurle, 2018). However, several clusters that do not resemble type I, type II, or non-memory gene expression patterns were also identified ($C1_{ST4}$, $C3_{ST4}$, & $C4_{ST4}$; $C2_{ST5}$ & $C3_{ST5}$). They displayed an opposite expression pattern upon the encounter of first and second stress. In the second classification system, presented in Ding et al. (2014), transcriptional changes are indicated by (+/+), (-/-), (+/-), (-/+), (-/=), and (+/=). Where first symbol indicates the transcriptional changes compared to control, and second symbol indicates the transcriptional changes compared to first stress response. Most of the clusters that could not be classified using the first system can now be put into a category. However, some of the clusters still could not be clearly classified (i.e., $C8_{ST4}$ and $C2_{ST5}$). Genes in both clusters showed a change in expression upon first stress exposure with an incomplete return to its original expression state at physiological recovery, and a lack of response upon encounter of a triggering stress. To account for this, we have proposed a modified system to classify the memory genes identified in our study using perennial plants. In general, it follows the type I and type II classification, but it also separates the different expression patterns based on the expression changes occurred upon second stress exposure (-/+/=/0). This modified classification system provides a simple and intuitive visual representation of how gene expression of potential memory genes changes upon the encounter of recurring stress signal and during the period of no stress/recovery.

GO and functional annotation analysis suggested that type I memory genes in grapevine are mainly involved in transcription regulator activity, catalytic activity, and binding, and mainly belong to chaperones. Moreover, the majority of type I memory genes are associated with sHSPs. The memory genes that displayed opposite regulation profile between first and second stress (Type I^-), belong to a wide range of groups such as protein

modifying enzyme and molecular function regulator. Interestingly, the well-characterized type II memory gene, heat stress transcription factor A-2 (HSAF2) associated gene (Charng et al., 2007) ortholog (VIT_04s0008g01110) in grapevine was found as part of the $C7_{ST4}$, which contains Type I^{-} memory genes. Suggesting that the expression pattern of memory genes may differ in perennial and annual plants.

Type II memory genes identified here, mainly belong to transporter, gene-specific transcriptional regulator, transmembrane signal receptor and chromatin/chromatin binding, or regulatory proteins. A number of chromatin -regulating enzymes and transcription factor associated genes have been identified in type II memory genes. In particular, GO analysis of $C1_{ST5}$ (containing type II^{+} memory genes) showed enrichment in methylation, including histone H3-K9 methylation and DNA methylation, suggesting a potential role of epigenetic chromatin based mechanisms in the regulation and maintenance of stress memory genes (Lämke and Bäurle, 2017).

3.5.3 Alteration of DNA methylation patterns under combined stress

A significant increase in global DNA methylation was observed only in primed plants under combined stress ($ST4_233$) (Figure 4a). Previous studies have reported a loss of global DNA methylation under heat stress (Li et al., 2016), while the increase in global DNA methylation is associated with drought stress in more drought-tolerant maize (Wang et al., 2021). The PCA analysis showed that the global DNA methylation pattern of plants under controlled conditions ($ST4_200$ and $ST4_230$), regardless of priming status, appeared to be more variable. While the global DNA methylation pattern of plants under stressed conditions ($ST4_203$ and $ST4_233$) is more conserved (Supplemental Figure S3.4). Suggesting that under control conditions cytosine methylation may have arisen

stochastically, whereas stress-induced DNA methylation is non-random (Feiner et al., 2022). Interestingly, when performing the DMC analyses, more DMCs have been identified in the CHH context for primed plants under combined stress, while more DMCs in CG and CHG contexts have been identified for both naïve plants under combined stress ($ST4_203$) and primed plants under control conditions ($ST4_230$) (Figure 4b). Methylated CHG and CHH are typically found in silenced regions of the genome such as transposons and repeats, whereas methylated CG is usually associated with gene expression regulation (Cokus et al., 2008). The high number of DMCs found in CHH context for primed plants under combined stress might be associated with the modified response to stress triggered by stress memory. For DNA methylation changes overtime, only a small amount of DMGs were commonly differentially methylated in our two-year experiment (Figure 3.5). The limited overlapping DMGs and no consistent patterns observed suggests that DNA methylation might be reset after stress is over (Viggiano and Pinto, 2017), and that the DNA methylation pattern induced by stress might vary.

3.5.4 Stress-induced transcriptional regulation partially independent of DNA methylation

Despite the general belief that DNA methylation in the promoter region of genes usually inhibits gene expression by influencing the binding of transcription activators or repressors (Zhang, Lang & Zhu, 2018), and gene body methylation (GbM) is positively correlated with expression (Yang et al., 2014). We did not observe the correlation between DNA methylation changes (hyper- or hypomethylation) in different genic regions (gene body or promoter) and transcription patterns (up- or down-regulation) of the overlapping genes. Moreover, only around 2% of the DMGs are differentially expressed (23/1160, and 23/971 for $ST4_200vsST4_233$ and $ST4_200vsST4_203$, respectively) (Figure 3.6). This is

considerably a small portion of the total DMGs. A similar portion and no correlation between methylation changes and transcriptional patterns have been observed in previous studies (López et al., 2022; Rambani et al., 2020).Taken together, our results suggest that stress-induced transcriptional regulation might be, at least partially, independent of DNA methylation. Similar to what has been observed in tomatoes for the flower-to-fruit transition, where the variation in the expression of the majority of genes was associated with a change in histone mark distribution, only a minor fraction of differentially expressed genes were associated with DNA methylation (Hu et al., 2021).

The gene functional annotation of those overlapping genes revealed the involvement of sHSPs, and basic Helix-Loop-Helix (bHLH), NAC, B3 transcription factor family genes, as well as SUI1-domain, J-domain, tumor overexpressed genes (TOG)-domain, PMR5N domain, and WD-repeats-region domain-containing protein have been identified (Supplemental Table S3.8). As mentioned before, the involvement of transcription factors in transcriptional memory has been characterized (Ding et al., 2014; Jacques et al., 2021), and whether there is an epigenetic basis for such involvement has seldom been studied. In our study, we observed that a small amount of differentially expressed sHSPs and transcription factors were also differentially methylated. DNA methylation may play a role in regulating the expression of those genes, which then contributes to somatic stress memory, however, due to the overwhelming small portion (2%), it will be difficult to determine whether this association was random or not. Therefore, it might be safe to say that the establishment, maintenance, and retrieval of stress-induced long-term somatic memory in grapevine through priming appeared to require more than DNA methylation alone. Histone modification might be the key player

in those processes. A study has shown that the reprogramming of genes is correlated with their histone marking status but not with changes in cytosine methylation, indicating that histone posttranscriptional modifications rather than DNA methylation is associated with the remodeling of the epigenetic landscape (Hu et al., 2021). Although this study did not address histone modification explicitly, the presence of many stressed-induced DEGs (primed or naïve) that are histone/chromatin modifications associated might be an indication of the importance of histone modification. Consistent with previous research where specific histone modification marks have been shown to not only play a role in drought memory establishment and retrieval but also in heat and salinity stress memory (Ding et al., 2012; Lämke et al., 2016; Sani et al., 2013).

3.6 Conclusions

Plant priming, and subsequent stress memory establishment, maintenance, and retrieval are seldom studied in woody perennial species. Our two-year study showed that the establishment of memory in grapevine is epigenetic-related, and that the established somatic memory can be maintained through dormancy cycle. The memory allowed the grapevine to employ a modified transcriptional response upon encountering second stress, which is reflected in more DEGs and the magnitude of the expression. Moreover, we have identified potential key factors, such as sHSPs and transcription factor families AP2/ERF and NAC in the maintenance of this somatic memory by examining primed plants that never experience second stress. We have also identified and characterized potential stress memory genes in grapevine based on their transcription patterns. In addition to the modified transcriptional response, we have observed an increase in global DNA

methylation level for primed plants, and the global DNA methylation profile appeared to be more variable for plants under controlled conditions compared to plants under combined stress, regardless of their priming status. The lack of consistent methylation pattern and small number of overlapping differentially methylated genes before and after stress and after dormancy cycle suggest that the DNA methylation induced by stress varies upon each stress encounter. We also observed changes in DNA methylation and gene expression changes do not necessarily coincide with second stress exposure. Suggesting that stress memory establishment, maintenance, and retrieval might be more complex and involves multiple epigenetic mechanisms such as histone modification. It remains to be tested if such epigenetic changes can be inherited during clonal propagation, which is common in grapevine, and if such changes could contribute to adaptation to changing environments. To conclude, stress-induced memory appeared to be more consistent on transcriptional level rather than on DNA methylation level.

CHAPTER 4. MAINTENANCE OF LONG-TERM SOMATIC MEMORY OF STRESS IN GRAPEVINE IS DEPENDENT ON THE VEGETATIVE PROPAGATION SYSTEM

4.1 Abstract

Maintenance of epigenetic memory of abiotic stress in plants has been shown to act as a priming effect that offers adaptive advantage both to the stress exposed plant and its offspring, by improving their response to subsequent stress. Our previous research has shown that in the perennial woody plant *Vitis vinifera* (grapevine), this memory of stress is maintained at least one year after the priming event and over dormancy cycle. However, whether memory of stress is maintained during clonal vegetative propagation and the potential effect of different vegetative propagation systems is less studied. Understanding the effect of vegetative propagation on epigenetic priming is paramount in order to take full advantage of this biological process to generate more resilient crops, especially when this is the main propagation system at a commercial level. Here, we used whole transcriptome and methylome sequencing of 64 Cabernet Sauvignon ramets generated from naïve and primed ortets using callused cuttings and layering to characterize their molecular response to combined drought and heat stress. Our results showed plants propagated from primed mother plants using callused cuttings showed more differentially expressed genes than plants propagated using layering. However, the scale of change in expression of those commonly differentially expressed genes in cutting propagules appeared to be smaller than the ones found in layered propagules. Moreover, only primed layered propagules showed differentially expressed genes in the absence of a second stress event. Additionally, analysis of DNA methylation changes showed that the changes in

DNA methylation cannot be stably inherited through hardwood cutting compared to layered plants. Taken collectively, our results indicate that both transcriptional and epigenetic memory of stress established in the ortets, is, at least partially, lost during callused cutting propagation, while it seems to be faithfully maintained in layered propagules. In conclusion, our two-year study revealed how the priming and methods of propagation affect this stress response and stress memory establishment in grapevine.

4.2 Introduction

Stress and environmental stimulus can induce adaptation strategies in plants. As the global climate continues to warm, the large variation in temperature will affect both natural plant populations and crop production. The production of grapes for example, is particularly vulnerable to environmental stress as the environmental conditions occurring during one growing season contribute to the quality of the next vintage (Edwards and Clingeleffer, 2013; Martínez-Lüscher and Kurtural, 2021; Mullins et al., 1992). Therefore, a better understanding in mechanisms that plants use to rapidly adapt and become more resilient is crucial when facing the climate change-related stresses. Advancement in genome sequencing showed that both genetic and epigenetic mechanisms can contribute to the variation of the genome under stress, where the epigenetic variation in the genome can lead to phenotypic plasticity (Fortes and Gallusci, 2017). Where different phenotypes can arise from the same genome due to alterations in the epigenetic marks to enhance the transcriptional regulation associated with environmental acclimation (Asensi-Fabado et al., 2017).

The acclimation and response process are thought to be related to the development of stress memory in plants. Stress memory is often associated with a phenomenon called stress priming, where it is trigged by extreme conditions that inhibit normal growth and development. The plants that are primed will show a modified response for future stress exposure as compared to a plant in the naïve (unprimed) state, after the initial exposure to a stimulus (Aranega-Bou et al., 2014). The modified response can be changed in the speed of the response or the magnitude of the response (Baldwin and Schmelz, 1996). Studies have shown that plants have memory of the first (priming) stress and are able to retrieve the remembered information upon encounter with the later stress when there is a prolonged period of no stress between the two stress events (Hilker and Schmülling, 2019). Additionally, studies have shown that priming is effective at various stages of the plant life cycle, starting from seed (i.e., seed priming) to seedlings and to subsequent adult stages (Mozgova et al., 2019). The establishment and maintenance of this stress memory in plants often involve increased metabolite levels, signaling molecules and transcription factor activation/repression, and more importantly, alteration of epigenetic marks to coordinated changes in gene expression pattern (Crisp et al., 2016; Galviz et al., 2020; Lämke and Bäurle, 2017; Perrone and Martinelli, 2020).

Multiple studies have suggested that the memory of stress can remain days to weeks or months for somatic memory, however, it may also be stable and inherited by the offspring. This stable and inherited memory is termed inter- or trans-generational memory depending on how many offspring generations have inherited the memory (Crisp et al., 2016; Galviz et al., 2020; Lämke and Bäurle, 2017; Weinhold, 2018). The duration of stress memory will depend on the stability of the epialleles responsible for the stress memory,

and they can be either mitotically or meiotically stable. Interestingly, grapevines are commonly clonally propagated, how this vegetative propagation contributes or affects the stableness and inheritance of the memory is less studied. More importantly, studies have shown that epigenetic mechanisms may provide mechanistic basis for the memory formation (Bruce et al., 2007) and changes in the epigenome play a fundamental role in memory responses to recurrent stress. Previous research has indicated that sexual reproduction, where the transmission of epigenetic marks to meiotic descendants is unstable (Danchin et al., 2019), because some DNA methylation changes, and histone modifications are often reset during meiosis. In contrast, clonally propagated plants do not undergo meiosis and gametogenesis, and the transmission of epigenetic marks through mitosis appears to be stable (Latutrie et al., 2019). Therefore, the transmission of epigenetic variants to the next generation in clonally propagated plants (e.g., cuttings, in vitro propagation) is stable. Some evidence showing that this stability lasting up to five rounds of clonal propagation, such as genome-wide DNA methylation modifications associated with biomass changes induced by maternal stress (drought, soil contamination, and shading) in the clonal plant *Trifolium repens* L. (Rendina González et al., 2018) and global demethylation associated with early flowering in the clonally propagated plant *Fragaria vesca* (Xu et al., 2016). However, the specificity of DNA and chromatin marks, and their persistence and stability during mitosis, and thus the maintenance of stress memory remains to be studied (Hilker and Schmülling, 2019). Moreover, it has been proposed that the propagation through cuttings is a desirable way to identify whether the epigenetic modifications is stable or transient (Perrone and Martinelli, 2020). Therefore, making

grapevine a fitting model to study the effects of vegetative propagation on the establishment and maintenance of long-term somatic stress memory in a perennial plant.

It is important to consider, however, that a transcriptional response is usually triggered when plants are exposed to stress. Some among those stress-inducible genes are linked to establishing a memory of stress, and therefore is termed stress memory genes (Charng et al., 2006, 2007; Ding, Fromm & Avramova, 2012; Lamke et al., 2016; Liu et al., 2018). In our previous study, we have identified stress-inducible genes in grapevine after dormancy cycle and proposed a new classification system based on their transcriptional profiles. Getting a deeper understanding of these memory genes after propagation will provide new insights in stress memory in perennials.

In this study, we used transcriptomic data generated by the NGS platforms, and epigenomic data generated by whole methylome sequencing to study the potential role of epigenetic regulation during stress response and stress memory inheritance, and the effect of vegetative propagation methods on the maintenance of long-term somatic memory in woody perennial grapevine.

4.3 Materials and methods

4.3.1 Plant materials and experimental design

A two-growing season experiment was designed to study the effect of vegetative propagation in the long-term maintenance of epigenetic memory in grapevine. Briefly, during growing season 1 (2016-2017), as part of a larger experiment, a set of grapevine plants were exposed to combined heat and drought stress (T3 or primed hereafter), while

others were maintained under non-stress conditions (T0 or naïve hereafter). A full description of the experimental design and plant material used the first growing season can be found in detail in Tan et al. (2023). In summary, and for the purpose of this experiment, 65 hardwood cuttings propagated from 6 donor grapevine (*V. vinifera* L. cv. Cabernet Sauvignon) plants were assigned randomly two treatment groups: (T0, n=35), and (T3, n=30). Of these, a total of 32 plants (16 per treatment group) were randomly selected for the second season experiment and, at the end of the 2016-2017 season, post-leaf fall, were propagated via hardwood cutting or layering (8 per propagation system and season 1 treatment group). For the hardwood cuttings, the single cane was cut four buds from the origin on the main stem and trimmed to sections containing buds five to nine, callused and then potted as for the vines in experiment 1. For the layering, empty pots were prepared as for experiment 1, then the cane of the mother vine, bent over and pegged into the empty pot, with at least two buds below the soil surface. After the layered vines had viable shoots, the cane was cut between the two pots and the layered vine trimmed to a single shoot four buds from the origin on the main stem. Propagules were then randomly assigned to one of four blocks and maintained under control greenhouse conditions as for experiment 1 until use. T0 and T3 Propagules were then split into two treatments (control or combined stress) (n=4 per season 2 treatment, propagation system, and season 1 treatment group). Plants were labeled based on their previous group and current group. An alphanumeric coding system was used to uniquely identify propagules based on their original ortet, and growing conditions on each of the seasons. The second and third of leaves counting from the apical meristem were sampled at four time points during the two seasons. Samples were coded according to their sampling time (ST4, ST5, or ST6), season (1 or 2), treatment (control

(0) or heat and drought (3)), and the propagation techniques (layered (L) or hardwood cutting (D)). Season 1 samples are described using a five-character code, i.e., a leaf samples collected at sampling time 4 of season 1 from plants under control conditions were coded $ST4_10$, while samples from season 2 are described using seven characters, i.e., a sample collected at sampling time 4 of season 2 from naïve layered plants under control conditions were coded $ST4_200L$ (Figure 4.1).

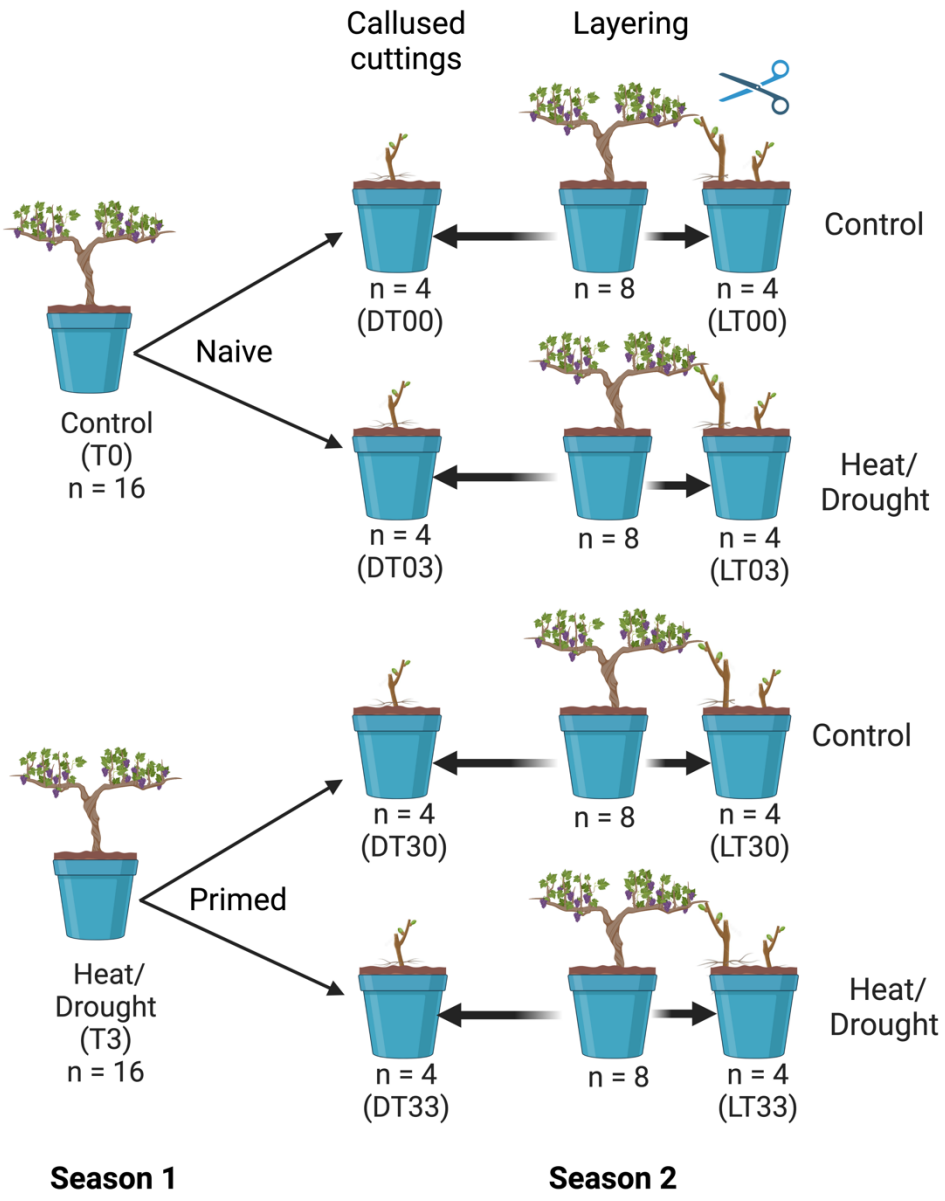


Figure 4.1 Experimental design.

Schematic representation of growing conditions, and propagation systems using during this experiment. Cabernet sauvignon plants were exposed to control or heat and drought conditions during seasons 1. After growing season 1, dormant plants were propagated either by layering or callused cuttings. Resulting ramets were randomly allocated into two growing conditions (i.e., control or combined heat and drought). N indicates number of replicates per group. Alphanumeric codes indicate growing condition (T0 = control conditions; T3 = heat and drought, where the first and second number indicate growing condition during season 1 and 2 respectively), and propagation method (D = Dormant cutting; L = Layering).

4.3.2 Nucleic acid extraction

Collected leaves from each plant were frozen immediately after collection using liquid nitrogen and stored at -80°C. Frozen leaves were ground to a fine powder under liquid nitrogen using mortar and pestle. Samples were split into two subsamples and stored at -80°C until further use.

Total RNA was extracted from 100 mg of frozen and ground samples using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's Protocol A. Spectrophotometric analysis (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington, DE, USA) and Experion™ RNA StdSens Chips (BIO-RAD, USA) were used to determine RNA integrity. Only samples with a RNA quality indicator (RQI) above 7 and presenting 260/280 and 260/230 absorbance ratios between 1.8-2.2 were used for library preparation. 4ug of total RNA per sample was used for ribosomal RNA depletion using Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer’s instructions.

DNA extracted from leaf samples collected from three replicates randomly selected out the four available at each time point, using the DNeasy Plant kit (Qiagen). The concentration and integrity of the DNA were measured by Fragment Analyzer (Agilent Technologies).

4.3.3 RNA Sequencing (RNASeq)

A total of 5ul ribosomal depleted RNAs were used to prepare 64 individually barcoded RNA-seq libraries using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, USA) following the manufacturer’s instructions. The

Illumina NextSeq 500 HighOutPut platform was used to produce 75bp single-end runs at the Australian Genome Research Facility (AGRF) in Adelaide, Australia.

4.3.4 Whole Methylome Sequencing (WMS)

WMS was performed on genomic DNA extracted from leaf samples using DNeasy kit (Qiagen). The concentration and quality of the DNA were measured by Fragment Analyzer (Agilent Technologies). Library preparations were done following the manufacturer instructions of the NEBNext Enzymatic Methyl-seq Kit (New England BioLabs), genomic DNA spiked with internal controls to determine the enzymatic conversion efficiencies and the abundance of false positives and negatives (i.e., 0% methylated Lambda DNA, and 100% CpG methylated pUC19 DNA). Spiked DNA samples were then fragmentated to 200 – 300 bp using Covaries S220. The resulting individually barcoded libraries were sequenced using Nova Seq 6000, and PE150 with a paired-end sequencing approach.

4.3.5 Bioinformatics Analysis

RNA-sequencing data analysis: Raw sequencing data were processed on the LipsComb Compute Cluster (LCC) platform at the University of Kentucky, United States. AdapterRemoval (Lindgreen, 2012) was used for removing adaptors of the raw reads. Sequence quality control was performed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/**fastqc**/) (2015). The reads were mapped to a 12X grapevine reference genome (NCBI assembly ID: GCF_000003745.3) with the alignment tool (HISAT2) (Kim et al., 2015; Khalil-Ur-Rehman et al., 2017). The GTF reference of the *Vitis vinifera* genome was downloaded from the *Ensembl Plants*

website (http://plants.ensembl.org/Vitis_vinifera/Info/Index). Samtools (Li et al., 2011) was used to generate Binary Alignment Map (BAM) files after mapping the reads to the genome.

Differentially expressed genes (DEGs) analysis: The gene expression level was estimated using the *edgeR* package (Robinson *et al.*, 2010) on Rstudio. The raw mapped data of each sample was standardized by edgeR's trimmed mean of M values (TMM). This method estimates scale factors between samples to determine DEGs. Between control and treatment, a log2fold change(log2FC) of 2 and a false discovery rate adjusted P-value<0.05 using Benjamini and Hochberg's algorithm was adopted to indicate significant. This process is repeated for each group of comparisons.

Co-expressed gene cluster analysis: Transcripts Per Million (TPM) of each plant sample were calculated from the BAM files using the TPMcalculator (Alvarez et al., 2019). Normalized data (calculated TPMs, log2 transformed) was used for the identification of gene expression clusters based on patterns during the following time point/treatment combinations: control plants sampled at season 1 ST4 ($ST4_10$), stressed plants sampled at season 1 ST4 ($ST4_13$), ST5 ($ST5_13$), and ST6 ($ST6_13$), and primed plants under combined stress at season 2 for both dormant cutting and layered plants using *clust* v1.8.4 (Abu-Jamous and Kelly, 2018). Resultant clusters were then classified according to three conditions: A) If the gene expression level in stressed plants at physiological recovery was significantly different than that presented by control plants ($ST4_10 \pm ST6_13$; T-Test $p < 0.05$) or not, b) if the change in expression in response to the triggering stress was significantly different than in response to the priming stress ($ST4_13 \pm ST4_233L/D$; T-Test $p < 0.05$), and c) if the triggering stress induced a significantly different change in

expression compared to the expression level at physiological recovery from the priming stress ($ST6_13 \pm ST4_233L/D$; T-Test $p < 0.05$).

Gene ontology (GO), DEGs visualization, and functional annotation: All differentially expressed genes of interest were subjected to ontology analysis through the usage of agriGO v2.0 (Tian et al., 2017). DEGs of each treatment were used to attain the significant GO terms with agriGO v2.0 with the following criteria: Fisher's statistical test method, Yekutieli (FDR under dependency) multi-test adjustment method, significance level <0.05, and selecting complete GO as the gene ontology type. The visualization of the expression level of selected DEGs were done through the built-in plot function of R. Functional annotation of DEGs were obtained from PantherDB (Paul et al., 2003).

Identification of differentially methylated cytosines and regions (DMCs and DMRs): Adaptor sequences, low-quality reads and contaminants were removed using Adapter Removal V2 software. Enzymatic conversion efficiency of unmethylated and methylated cytosines was calculated using pipelines (https://github.com/nebiolabs/EM-seq/blob/master/em-seq.nf) and the methylation control sequences (https://github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa) provided by the NEBNext Enzymatic Methyl-seq Kit manufacturer.

Genome indexing was performed with Bismark using '--bismark_genome_preparation' option (Krueger and Andrews, 2011) using the C-to-T and G-to-A versions of the reference grapevine genome (PN40024 v.4) created with Bowtie2 (Langmead and Salzberg, 2012). Sequencing coverage and depth were estimated using Samtools coverage and depth toolkits (Li et al., 2009). Methylation calling was performed with Bismark extractor

(https://www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf) by calling '--comprehensive' and '--cytosine_report' option after the conversion to bedGraph. Both differentially methylated cytosines and regions (DMCs and DMRs, respectively) were determined using the 'Methylkit' package (Akalin et al., 2012) with default parameters (minimum coverage threshold of 10 and 5 for DMCs and DMRs, respectively; q-value $\leq$ 0.05; minimum differential methylation level of 10%); sliding window for DMRs was 1000 bp). Genes were deemed differentially methylated when a DMR overlapped with their promoter (parameters being 1000 bp upstream of the transcription starting site (TSS)), or with the body of the given gene.

4.4 Results

4.4.1 Gene expression analysis

Transcriptome sequencing yield an average of 25 million reads per sample after quality filtering. The average percentage of mappable reads per sample after de-multiplexing was 82%, ranging from 62-91% (Supplemental Table S4.1).

Identification of modified response in gene expression: we tested the effect of the type of plant propagation (i.e., hardwood cuttings and layering) on the establishment of a memory of stress and its effect on primed plants response to a subsequent stress. To test the presence of a memory of a previous stress we compared gene expression of naïve and primed propagules in the absence of a second stress ($ST4_200L$ vs. $ST4_230L$, and $ST4_200D$ vs. $ST4_230D$). Such comparison identified a total of six genes downregulated in primed layered plants (Figure 4.2). Gene functional annotation revealed that all 6 genes belong to

small heat shock protein family (sHSPs) (Table 4.1). Conversely, no DEGs were observed in primed hardwood cutting propagules in the absence of a second stress event.

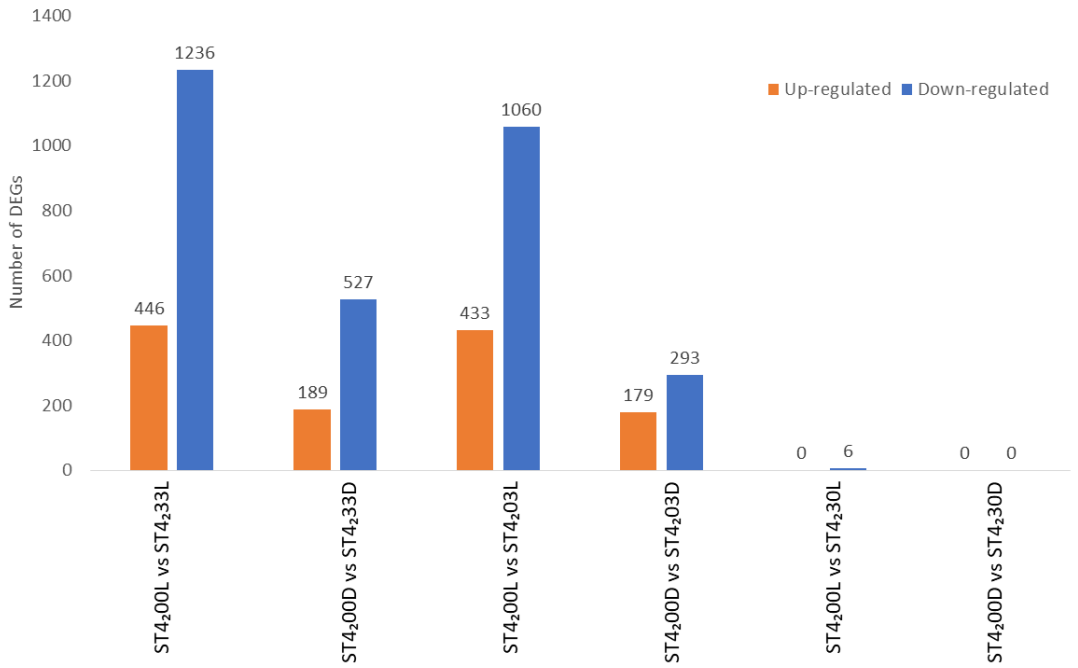


Figure 4.2 Analysis of differential gene expression in naïve and primed propagule plants under stress or control conditions.

Orange bars represent differentially expressed genes (DEGs) that were upregulated. Blue bars represent DEGs that were downregulated. The letter "L" and "D" were used to classified layered plants and hardwood cuttings, respectively. The number represent DEGs between $ST4_233$ (primed plants under combined stress), $ST4_203$ (naïve plants under combined stress) and $ST4_230$ (primed plants under control conditions) compared to $ST4_200$ (naïve plants under control conditions), respectively.

Table 4.1 Gene functional annotation of six differentially expressed genes identified in $ST4_200L$ vs. $ST4_230L$.

Gene ID	Gene Functional Annotation
VIT_04s0008g01580	18.0 KDA class II heat shock protein; sHSP domain containing protein
VIT_04s0008g01590	18.0 KDA class II heat shock protein; sHSP domain containing protein
VIT_04s0008g01510	18.0 KDA class II heat shock protein; sHSP domain containing protein
VIT_04s0008g01530	Uncharacterized protein; 18.0 KDA class II heat shock protein
VIT_13s0019g03090	sHSP domain containing protein
VIT_04s0008g01570	18.0 KDA class II heat shock protein; sHSP domain containing protein

Gene functional annotation done using PantherDB.

Two approaches were used to test if such memory of stress would induce a stronger response during a subsequent stress event in primed propagules. Frist, the number of DEGs induced by combined stress in naïve (i.e., $ST4_200L$ vs. $ST4_203L$, and $ST4_200D$ vs. $ST4_203D$) and primed propagules ($ST4_200L$ vs. $ST4_233L$, and $ST4_200D$ vs. $ST4_233D$) was compared. Both types of primed propagules ($ST4_233L$ and $ST4_233D$) presented more DEGS (1682 and 746 respectively) than naïve ones ($ST4_203L$ and $ST4_203D$) (1493 and 472 respectively). Then, we compared the intensity of the change in gene expression on those DEGs common to primed and naïve propagules during a second stress event. Primed and naïve layered plants shared 1,248 DEGs (Figure 4.3A), and the scale and significance of change in gene expression of common DEGs in layered plants was significantly higher in primed than naïve plants (T-test of absolute fold change for both up-regulated and down-regulated genes, $P < 0.001$, and T-test of FDRs, $P < 0.001$) (Figure 4.3B). GO analysis showed significant enrichment in chromosome organization, response to stimulus and transcription factor activity for the commonly expressed genes of primed and naïve layered plants. While similar enrichments were observed for DEGs exclusive to naïve layered plants with addition of defense response and protein serine/threonine kinase activity. DEGs exclusive to primed layered plants also showed similar GO term enrichment, with the addition of the term regulation of catalytic activity (Supplemental Table S4.2).

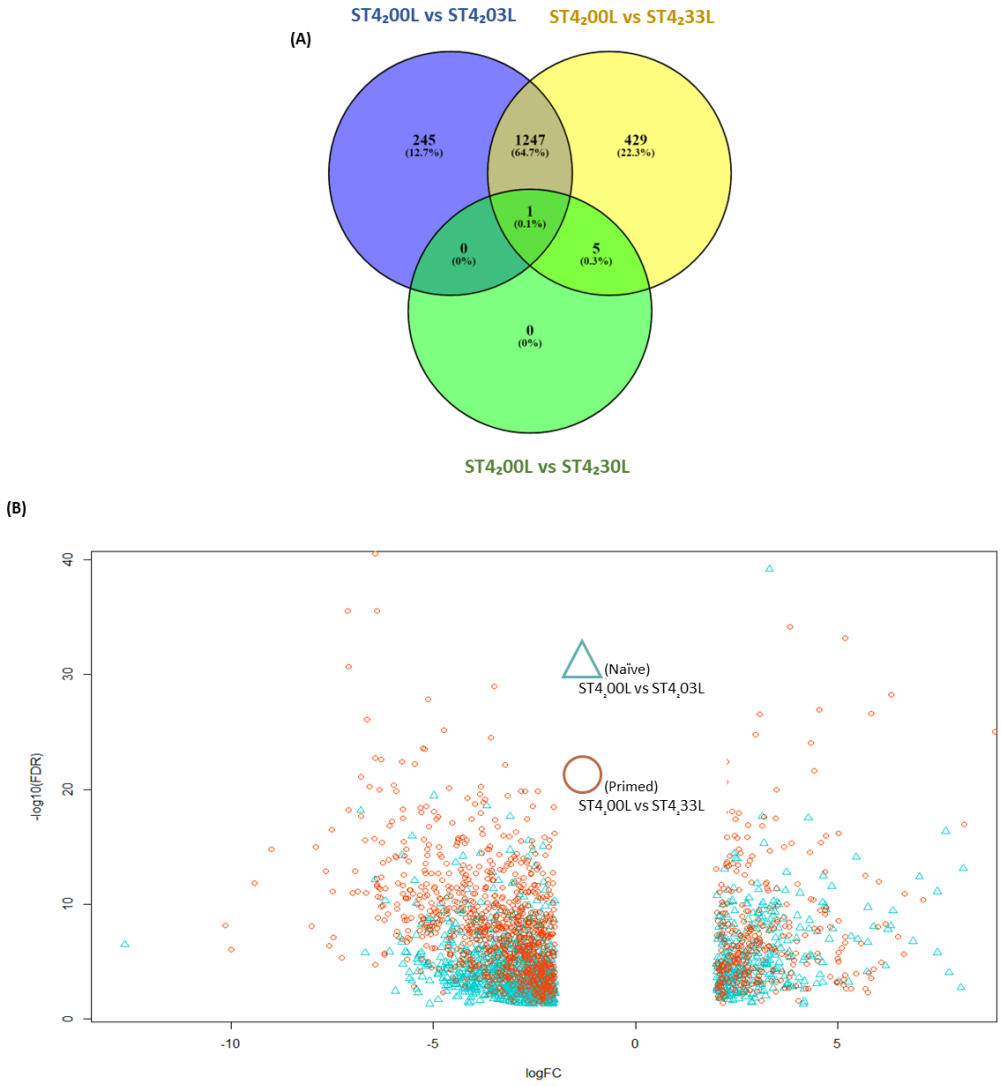


Figure 4.3 Analysis of differential gene expression between ramets propagated using layering from naïve or primed ortets.

(A) Number of DEGs identified in all comparisons. (B) Comparison of Log Fold-change (logFC) and -log10FDR of 1248 common differentially expressed genes (DEGs) identified in naïve ($ST4_200L$ vs. $ST4_203L$) and primed ($ST4_200L$ vs. $ST4_233L$) layered ortets under a triggering stress. Red circles represent the common DEGs found in $ST4_200L$ (naïve layered plants under control conditions) compared to $ST4_233L$ (primed layered plants under a triggering combined stress). Blue triangles represent the common DEGs found in $ST4_200L$ compared to $ST4_203L$ (naïve layered plants under a triggering combined stress). Gene expression shows a significantly higher or lower logFC, and a significantly lower FDR in primed than naïve ramets for up- or down- regulated DEGs, respectively (both $p < 0.001$).

Plants propagated using hardwood cuttings shared 363 DEGs (Figure 4.4A). Unlike to the pattern observed in the layered plant plot, only the down-regulated common genes in primed hardwood cuttings appeared to be more regulated (lower FC and smaller FDR, both $p < 0.01$). The up-regulated common genes in primed callus cuttings appeared to be less regulated than in naïve plants (lower FC and higher FDR, $p < 0.01$ and $p < 0.05$, respectively) (Figure 4.4B). The GO analysis for common DEGs between primed and naïve hardwood cutting plants showed significant enrichment in chromatin organization, cell cycle and histone modification. While naïve hardwood cutting exclusive DEGs showed significant enrichment in photosynthesis, generation of precursor metabolites and energy, and cell communication. Primed hardwood cutting plants exclusive DEGs are enriched in mitotic cell cycle, transcription factor activity (Supplemental Table S4.3).

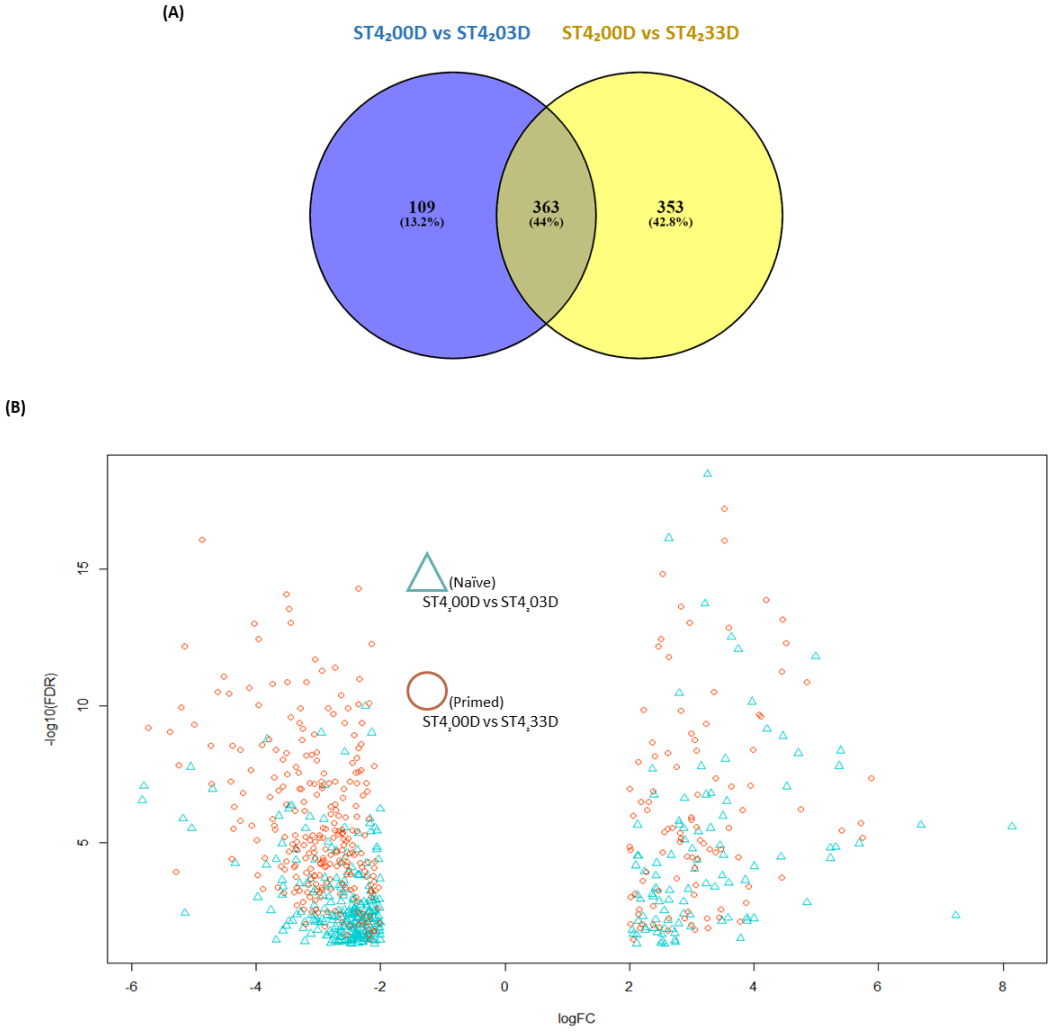


Figure 4.4 Analysis of differential gene expression between ramets propagated using hardwood cuttings from naïve or primed ortets.

(A) Number of DEGs identified in all comparisons. (B) Comparison of Log Fold-change (logFC) and -log10FDR of 363 common differentially expressed genes (DEGs) identified in naïve ($ST4_200D$ vs. $ST4_203D$) and primed ($ST4_200D$ vs. $ST4_233D$) hardwood cutting ortets under a triggering stress. Red circles represent the common DEGs found in $ST4_200D$ (naïve hardwood cutting plants under control conditions) compared to $ST4_233D$ (primed hardwood cutting plants under combined stress). Blue triangles represent the common DEGs found in $ST4_200D$ compared to $ST4_203D$ (naïve hardwood cutting plants under combined stress). Only the down-regulated common genes in primed hardwood cuttings appeared to be more regulated (lower FC and smaller FDR, both $P < 0.01$). The up-regulated common genes in primed callus cuttings appeared to be less regulated than in naïve plants (lower FC and higher FDR, $P < 0.01$ and $P < 0.05$, respectively).

Identification of putative stress memory genes: Gene clustering analysis were performed on combined stress induced DEGs from the first season (i.e., identified DEGs ST4: second day of combined stress; ST5: day of stress removal). To achieve this, the normalized expression (calculated TPMs, log2 transformed) of those genes of the same season 1 plants used for propagation was clustered and plotted using *clust* package with three time points selected. 671 DEGs from ST4 formed 10 and 9 clusters for layered and dormant cutting plants (C0-C9$_{ST4}$:L and C0-C8$_{ST4}$:D), respectively (Supplemental Figure S4.1-2).

Among the 10 clusters found in layered plants, based on our newly proposed classification system, six clusters exhibited gene expression level in stressed plants at physiological recovery that was significantly different than that presented by control plants ($ST4_10 \pm ST6_13$, T-Test $p < 0.05$). Those clusters have been assigned as Type I memory genes. Further classification of this type is based on the magnitude and directionality of changes in expression in response to the triggering stress compared to the priming stress ($ST4_10 \pm ST4_233L/D$; T-Test $p < 0.05$), and that includes Type I^+, Type I^- and Type $I^=$ genes. The classification of Type I^0 genes refer to triggering stress does not induced a significantly different change in expression compared to the expression level at physiological recovery from the priming stress. Two clusters showed expression patterns that resembles Type I^+ genes (C3$_{ST4}$:L, 73 genes and C4$_{ST4}$:L, 15 genes), where the magnitude of expression at the triggering stress was significantly higher than that of the priming stress. Genes from C6$_{ST4}$:L (75 genes) resembles Type I^- genes, as the expression changes in response to the triggering stress was significantly lower than that of the priming stress. C8, C9$_{ST4}$:L (13, 19 genes, respectively) were classified as Type I^0 genes, where the

expression changes at the triggering stress was not significantly different from the expression changes at ST6. No cluster resembles Type $I^{=}$ genes (the magnitude of change in expression in response to the triggering stress was not significantly different from the priming stress) (Figure 4.5). Three clusters exhibited change in expression in response to the triggering stress was significantly different than in response to the priming stress ($ST4_10 \pm ST4_233L/D$; T-Test $p < 0.05$), those clusters have been assigned as Type II memory genes. Further classification depends on the magnitude of changes in expression in response to triggering stress compared to response to the priming stress, and that includes Type II^+ and Type II^-. Genes from $C2_{ST4}$:L (28 genes) showed expression pattern of Type II^+ genes, where the change in expression in response to the triggering stress was significantly higher than the priming stress. Genes from C1, $C7_{ST4}$:L (37 and 13 genes, respectively) were classified as Type II^- genes, where the change in expression in response to the triggering stress significantly lower than the priming stress (Figure 4.5). Genes from $C0_{ST4}$:L (98 genes) resembles non-memory genes, where there are no significant difference between the changes in gene expression in response to triggering and priming stress, and no significant difference between changes in gene expression for stressed plants in physiological recovery and control plants (Figure 4.5).

Among the 9 clusters identified for dormant cutting plants, genes from five of those clusters were classified as Type I memory genes. Where genes from C5, C6 and $C7_{ST4}$:D (17, 20, and 74 genes, respectively) were Type I^+ genes. Genes from $C2_{ST4}$:D and $C3_{ST4}$:D (29 genes and 59 genes, respectively) were Type I^- genes, and genes from $C8_{ST4}$:D were Type I^0 genes. Genes from $C1_{ST4}$:D (16 genes) was classified as Type II^+ genes. Lastly, genes from C0 and $C4_{ST4}$:D resembles non-memory genes (Figure 4.5). 224 DEGs from

ST5 generated 6 and 2 clusters for layered and hardwood cutting plants, respectively (Supplemental Figure S4.3-4). Among the 6 clusters generated for layered plants, genes from 3 of those clusters were Type I genes, $C5_{ST4}$:L resembles the expression pattern of Type $I^{=}$ genes, genes from $C4_{ST5}$:L were Type I^{+} genes, while genes $C2_{ST5}$:L closely resembles Type I^{-} memory genes (Figure 4.5). The only cluster of genes that resembles the Type II memory genes was $C1_{ST5}$:L, the expression pattern classified it as Type II^{+} memory genes. And genes from $C3_{ST5}$:L are considered non-memory genes. Interestingly enough, the two clusters generated from dormant cutting plants exhibited expression patterns that cannot be classified using the three classification criteria mentioned here.

The functional annotation of clusters within the sample group have been performed to analyze the similarities and differences in memory genes by plant propagation types. In the cases where only one cluster was identified, the genes of that cluster were still functionally annotated. For Type $I^{=}$ genes ($C5_{ST4}$:L), the involvement of chaperones such as sHSPs, C2H2-Type domain containing protein, Bcl-2-associated athanogene (BAG) family chaperone regulator, tryptophan-aspartic acid (WD) repeats region domain containing protein and hatpase_C domain containing protein have been identified (Supplemental Table S4.4A). For some common Type I^{+} memory genes among all clusters (C3, $C4_{ST4}$:L; $C4_{ST5}$:L; and C5, C6, $C7_{ST4}$:D) include basic helix-loop-helix (BHLH) transcriptional factor, transcription repressor ovate family protein 3 (OFP3), non-receptor serine/threonine protein kinase associated genes, and sucrose synthase 6 (Supplemental Table S4.4B). Some common memory genes identified among layered plants (C3, $C4_{ST4}$:L and $C4_{ST5}$:L) include ethylene responsive transcription factor WIN1 and SHINE2, remorin family protein, and agglutinin domain containing protein (Supplemental Table S4.4B).

While the common genes identified among dormant cutting plants (C5, C6, $C7_{ST4}$:D) include cytochrome B561, dirigent protein containing, and DNA damage repair protein DRT100 (Supplemental Table S4.4B).

Functional annotation revealed the commonly expressed Type I^- memory genes between all clusters ($C6_{ST4}$L; $C2_{ST5}$:L and C2, $C3_{ST4}$:D) include F-box domain containing protein (FBA-3), basic leucine zipper (bZIP) domain containing protein, phytocyanin domain containing protein, and homeobox-leucine zipper protein ATHB-51 associated genes (Supplemental Table S4C). The commonly expressed Type I^- memory genes among layered plants ($C6_{ST4}$L and $C2_{ST5}$:L) include chaperone protein DNAJ 11, Chorismate synthase, and dehydrin RAB18 associated genes (Supplemental Table S4.4C). The commonly expressed Type I^- memory genes among dormant cutting plants (C2, $C3_{ST4}$:D) include transcription factor EFL-3 and TCP10, ethylene-responsive transcriptional factor CRF2, and histone-lysine N-methyltransferase SUVR3 (Supplemental Table S4.4C). No commonly expressed Type I^0 memory genes among all clusters (C8, $C9_{ST4}$:L and $C8_{ST4}$:D) were observed. However, gene functional annotation revealed commonly expressed genes among layered plants (C8, $C9_{ST4}$:L) include B-Box type zinc finger family protein, plant organelle RNA recognition (PORR) domain containing protein, and plants and prokaryote conserved (PPC) domain containing protein associated genes (Supplemental Table S4.4D). Type I^0 memory genes in dormant cutting plants include expansin, shikimate dehydrogenase and peroxidase associated genes (Supplemental Table S4.4D).

For Type II^+ memory genes ($C2_{ST4}$:L; $C1_{ST5}$:L, and $C1_{ST4}$:D), the functional annotation of commonly expressed genes revealed the involvement of WRKY transcriptional factors related protein, serine proteinase 1 and SANT domain containing

protein associated genes (Supplemental Table S4.4E). The commonly expressed Type II^{+} memory genes in layered plants ($C2_{ST4}$:L; $C1_{ST5}$:L) include Gibberellin 2 beta dioxygenase 1, RAC like GTP binding protein ARAC9, and phosphoethanolamine methyltransferase associated genes (Supplemental Table S4.4E). And the 3 commonly expressed Type II^{-} memory genes found in dormant cutting plants were protein casparian strip integrity factor 1, protein radialis like 4, and alpha/beta hydrolases domain containing protein associated genes (Supplemental Table S4.4E). And lastly, gene functional annotation of Type II^{-} memory genes (C1, $C7_{ST4}$:L) revealed the involvement of NAC domain containing protein, histone-lysine N-methyltransferase SUVR3 and SAUR like auxin responsive family protein associated genes (Supplemental Table S4.4F).

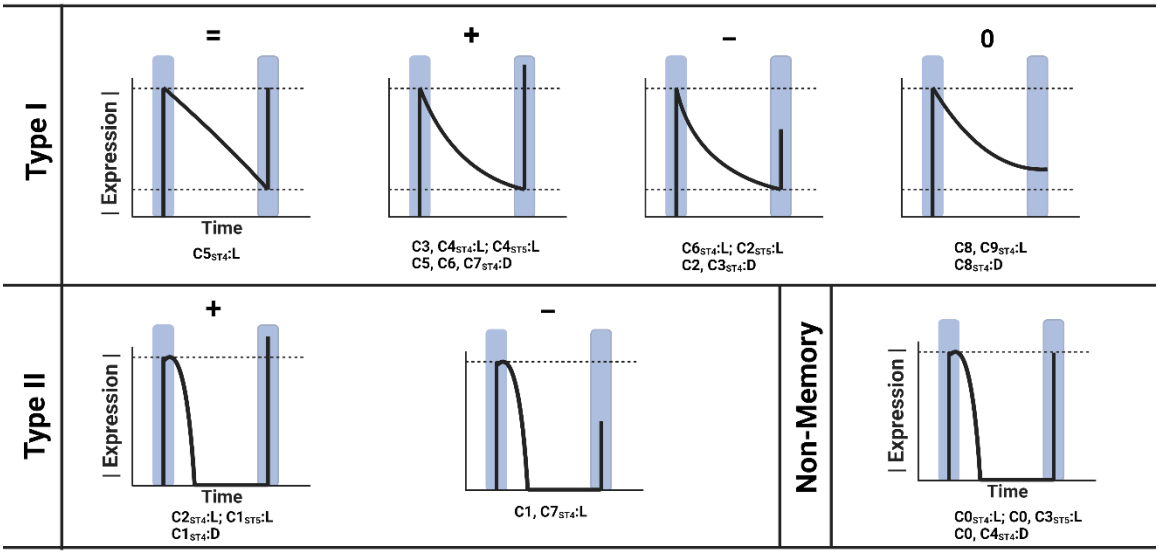


Figure 4.5 Gene clusters grouped based on expression patterns of DEGs found over two growing seasons.

Type I: genes that exhibited sustain expression after first stress encounter. Type $I^{=}$: expression changes upon second stress encounter was highly identical to the first. Type I^{+}: expression changes upon second stress encounter was higher than the first. Type I^{-}: expression changes upon second stress encounter was lower than the first. Type I^{0}: the expression changes upon second stress encounter was minimal compared to the sustained expression after the first stress encounter. Type II: genes that exhibited expression changes between first and second stress encounter, the expression level returned to basal after the first stress. Type II^{+}: the expression level of genes upon second stress encounter was higher than the first. Type II^{-}: the expression level of genes upon second stress encounter was lower than the first. Non-memory: genes that exhibited no expression changes between first and second stress encounter, the expression level returned to basal after the first stress.

4.4.2 DNA methylation

An average of 92 million reads per sample were produced from the EM-seq library after quality filtering. The average percentage of mappable reads per sample to the PN40024 v.4 genomes was 54%. The average non-bisulfite conversion rate among the samples was 0.2%, and the average bisulfite conversion rate was 96%. The average percentage of covered bases was 82%, while the sequencing depth was 23X per sample (Supplemental Table S4.5).

Global DNA methylation changes by propagation methods: The effect of the type of plant propagation (i.e., dormant cuttings and layering) on the genome-wide DNA methylation level under combined stress have been assessed. The average methylation percentage (methylated cytosines, mCs) per three contexts (CG, CHG and CHH) for both layered and dormant cutting plants have been calculated (Figure 4.6). The majority of global DNA methylation changes have been observed in CG context for both layered and hardwood cutting plants, followed by CHG, then CHH. No significant difference in global DNA methylation level have been detected between plant group ($ST4_230L/D$) by comparing to naïve plants under control conditions ($ST4_200L/D$) for both layered and hardwood cutting plants in CG, CHG, and CHH. There was, however, a significant increase in average global methylation level for $ST4_203L$ and $ST4_233L$ compared to $ST4_200L$ in the CHG context (Student's T-test, $p \leq 0.05$).

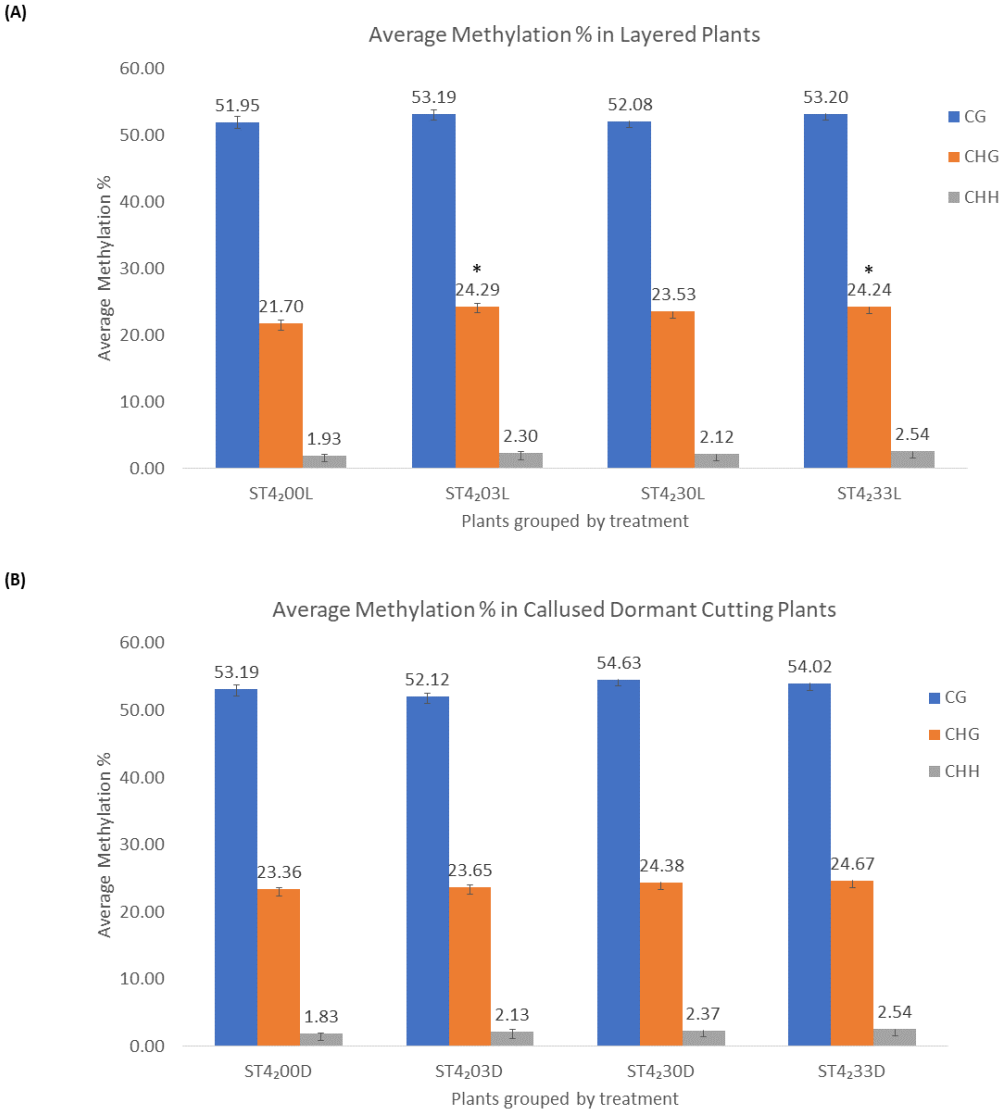


Figure 4.6 Effects of combined stress on genome-wide DNA methylation levels based on propagation methods and plant groups.

(A) Average DNA methylation level for each cytosine context (CG, CHG, CHH) between plant groups in layered plants: $ST4_233L$ (primed layered plants under combined stress), $ST4_203L$ (naïve layered plants under combined stress) and $ST4_230L$ (primed layered plants under control conditions) and $ST4_200L$ (naïve layered plants under control conditions). (B) Average DNA methylation level for each cytosine context (CG, CHG, CHH) between plant groups in hardwood cutting plants. Statistical significance between $ST4_233L/D$, $ST4_203L/D$, and $ST4_203L/D$ compared to $ST4_200L/D$ will be indicated by asterisks (Student's T test, $p \leq 0.05$).

Differentially methylated cytosines (DMCs) were identified for layered and hardwood cutting plants by comparison indicated above (Figure 4.7A-B: Top). When compared to naïve layer plants under control condition ($ST4_200L$), naïve layered plants under combined stress ($ST4_203L$) produced 5,976 DMCs (2171, 2,883 and 922 DMCs in CG, CHG and CHH context, respectively). While the same comparison in dormant cutting plants ($ST4_200D$ vs. $ST4_203D$) produced 4,203 DMCs (1,805, 2,118, and 280 DMCs in CG, CHG and CHH context, respectively). The comparison between $ST4_200L$ and primed layerd plants under control conditions ($ST4_230L$) resulted in 3,861 DMCs (1,322, 2,261 and 278 DMCs in CG, CHG and CHH context, respectively). The same comparison in dormant cutting plants ($ST4_200D$ vs. $ST4_230D$) produced less total DMCs (1,911), where 630, 851 and 430 DMCs are found in CG, CHG and CHH context, respectively. The last comparison of $ST4_200L$ and primed layered plants under combined stress condition ($ST4_233L$) generated 8,116 DMCs (2,824, 3,162 and 2130 DMCs for CG, CHG and CHH, respectively). This comparison in dormant cutting plants resulted in similar number of total DMCs (8,322). 1,358, 2,782 and 4,182 DMCs for CG, CHG, and CHH context, respectively.

In general, the majority of DMCs are rich in intergenic region, regardless of context and comparison, and that identified DMCs were more likely to be hypermethylated than hypomethylated.

In addition to DMCs, the number of differentially methylated regions (DMRs) were assessed to study the dynamics of DNA methylation at specific loci (Figure 4.7A-B: Bottom). Using the same comparison groups, $ST4_200L$ vs. $ST4_203L$ produced 2,191 DMRs, with 1,092 DMRs found in the intergenic region and 1,099 DMRs in genic region.

$ST4_200D$ vs. $ST4_203D$ generated less DMRs (775), where 407 DMRs in intergenic region and 368 in genic region. 1,790 DMRs have been identified in $ST4_200L$ vs. $ST4_230L$, with 861 and 929 DMRs found in intergenic and genic regions, respectively. The same comparison in dormant cutting plants ($ST4_200D$ vs. $ST4_230D$) generated 1,140 DMRs, 569 in the intergenic region and 571 in the genic region. $ST4_200L$ vs. $ST4_233L$ produced the most DMRs, with a total of 2,938 DMRs, 1,451 in intergenic region and 1,487 in the genic region. 1,391 DMRs have been identified in $ST4_200D$ vs. $ST4_233D$, where 642 and 749 DMRs are located in intergenic and genic regions, respectively. The majority of DMRs identified in layered plants were rich in the intergenic region (48-50%), followed by gene body (29-30%) and promoter (21-23%) (Figure 4.7A: Bottom). Similarly, DMRs identified in hardwood cutting plants were enriched in intergenic region (46-53%), followed by gene body (27-30%) and promoter (18-24%) (Figure 4.7B: Bottom). The identified DMRs in layered plants are more likely to be hypermethylated (HyperDMRs) compared to dormant cutting plants, where the trend seemed to be hypomethylation (HypoDMRs).

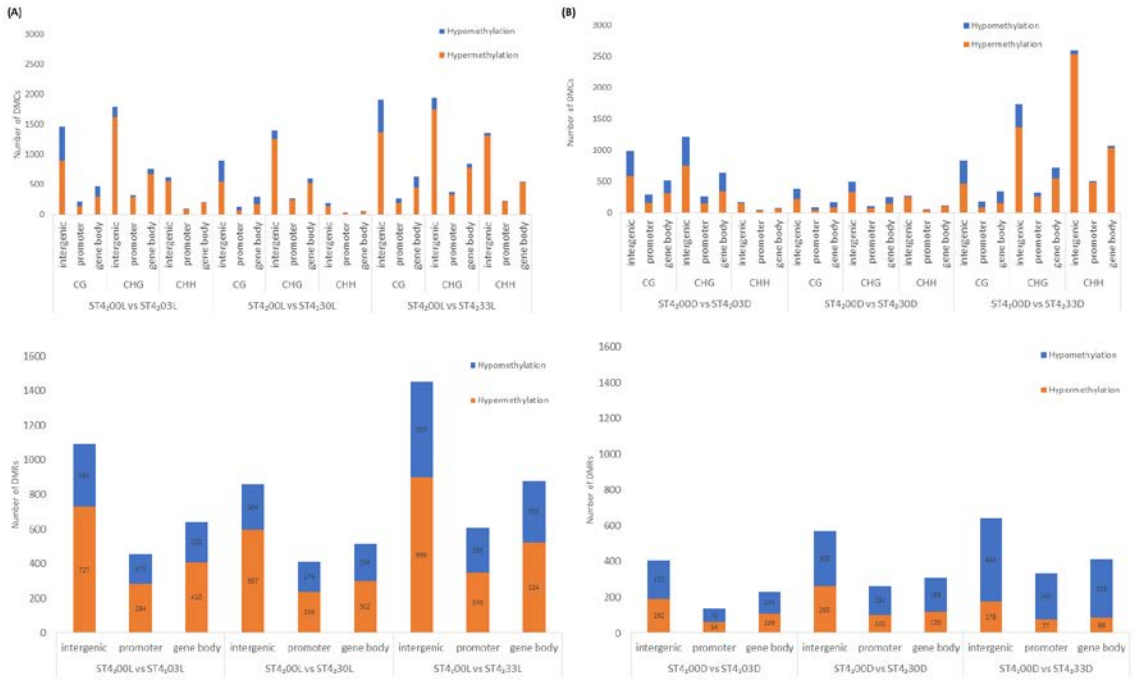


Figure 4.7 The distribution of differentially methylated cytosines/regions based on propagation methods and plant groups.

(A) Top: Number of hyper- (hyper-DMCs) and hypomethylated differentially methylated cytosines (hypoDMCs) separated by sequence context and group comparison in layered plants. Bottom: Distribution of hyper- (hyperDMRs) and hypo-methylated differentially methylated regions (hypoDMRs) and in genomic features: promoter, gene body, and intergenic regions in layered plants. (B) Top: Number of hyper- (hyper-DMCs) and hypomethylated differentially methylated cytosines (hypoDMCs) separated by sequence context and group comparison in hardwood cutting plants. Bottom: Distribution of hyper- (hyperDMRs) and hypo-methylated differentially methylated regions (hypoDMRs) and in genomic features: promoter, gene body, and intergenic regions in hardwood cutting plants.

The pattern of methylation changes over time was examined by comparing DMGs identified in our first-year study under combined stress, during physiological recovery and in the primed plants under combined stress ($ST4_200L$ vs. $ST4_233L$ and $ST4_200D$ vs. $ST4_233D$) (Figure 4.8). Only 20 and 18 differentially methylation genes were commonly differentially methylated in all three time points for layered and hardwood cutting plants, respectively (Figure 4.8A). No consistent methylation pattern has been observed for those DMGs (Figure 4.8B-C).

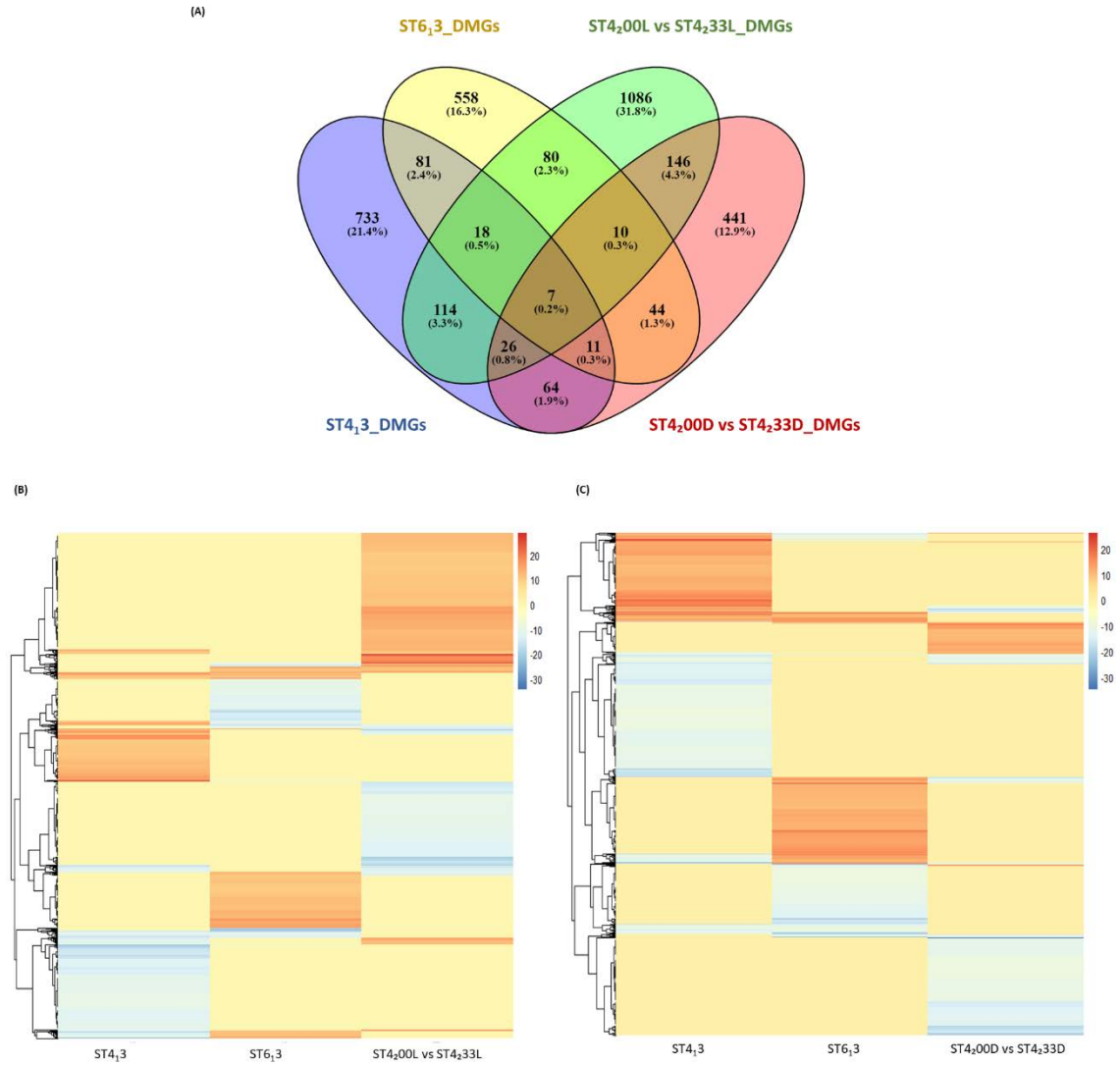


Figure 4.8 Methylation changes of differentially methylated genes (DMGs) over time. (A) Venn diagram of DMGs identified in ST4 (ST4$_1$3, first stress) and ST6 (ST6$_1$3, recovery of first year), ST4$_2$00L vs. ST4$_2$33L (second stress/layering propagation), and ST4$_2$00D vs. ST4$_2$33D (second stress/hardwood cutting propagation). (B) Heatmap of level of methylation changes for DMGs that were differentially methylated in ST4$_1$3, ST6$_1$3, and ST4$_2$00L vs. ST4$_2$33L. (C) Heatmap of level of methylation changes for DMGs that were differentially methylated in ST4$_1$3, ST6$_1$3, and ST4$_2$00D vs. ST4$_2$33D. Red: hypermethylation. Blue: hypomethylation. Yellow: not differentially methylated.

4.4.3 The relationship between DNA methylation and gene expression

For a better understanding of the potential functional roles of the DNA methylation on gene expression, we focused on the DMRs located within promoters and gene bodies (DMGs). The relationship between the presence of DMGs in genic regions (gene body and promoter) and transcriptional changes was examined (Figure 4.9). A total of 53 and 3 overlapping DEGs and DMGs have been identified for naïve plants ($ST4_200L$ vs. $ST4_203L$ and $ST4_200D$ vs. $ST4_203D$), respectively (Figure 4.9A-B).

Among the 53 overlapping DEGs and DMGs for naïve layered plants, 24 were hypermethylated and downregulated (12 located in promoter, and 12 in gene body). 5 were hypermethylated and upregulated (3 in promoter and 2 in gene body). 15 were hypomethylated and downregulated (4 in promoter, and 11 in gene body). 8 were hypomethylated and upregulated (3 in promoter and 5 in gene body) (Figure 4.9A). 2 out of the 3 overlapping DEGs and DMGs for dormant cutting plants were located in the promoter region and were hypermethylated and downregulated. The remaining 1 was hypomethylated and down regulated, located in the gen body region (Figure 4.9B). A total of 70 and 13 overlapping DMGs and DEGs have been identified in primed plants ($ST4_200L$ vs. $ST4_233L$ and $ST4_200D$ vs. $ST4_233D$) respectively (9C-D). Among the 70 overlapping DMGs and DEGs identified in primed layered plants, 15 were hypermethylated and upregulated (6 in promoter and 9 in gene body). 31 were hypermethylated and downregulated (15 in promoter and 16 in gene body). 7 were hypomethylated and upregulated (3 in promoter and 4 in gene body). 17 were hypomethylated and downregulated (7 in promoter and 10 in gene body) (Figure 4.9C).

For primed dormant cutting plants, 1 overlapping DEG and DMG located in promoter and was hypermethylated and upregulated. 5 were hypermethylated and downregulated (2 promoter and 3 gene body). 6 were hypomethylated and downregulated (5 promoter and 1 gene body). And 1 was hypomethylated and upregulated (gene body) (Figure 4.9D). For primed plants that were not under stress, only layered plants contained overlapping DMG and DEG ($ST4_200L$ vs. $ST4_230L$), the 1 was hypermethylated and downregulated (promoter), gene functional annotation showed that was a SHSP associated gene (Supplemental table S4.6A). There was a minimal overlap between DMGs/DEGs identified in hardwood cutting and layered plants under their respective groups (2 for $ST4_233L/D$ and 0 for $ST4_203L/D$).

Functional annotation showed that overlapping DMGs/DEGs found in $ST4_200L$ vs. 33 consist of leucine-rich repeat (LRR) protein kinase, WRKY transcription factor, cytochrome P450, sHSPs, histone regulatory, MYB transcription factor family associated genes (Supplemental table S4.6B). While HMG box domain containing protein, xyloglucan hydrolase, chitinase, patellin, and a/b hydrolases_5 domain containing protein associated genes were found in $ST4_200D$ vs. 33 (Supplemental table S4.6C). MAD2L1 binding protein, AP2/ERF transcription factor, sHSPs, histone regulatory proteins, and BHLH transcription factor associated genes have been identified in $ST4_200L$ vs. 03 (Supplemental table S4.6D), and cyclin-dependent protein kinase inhibitor SMR4 and peroxidase 25 associated genes were for $ST4_200D$ vs. 03 (Supplemental table S4.6E).

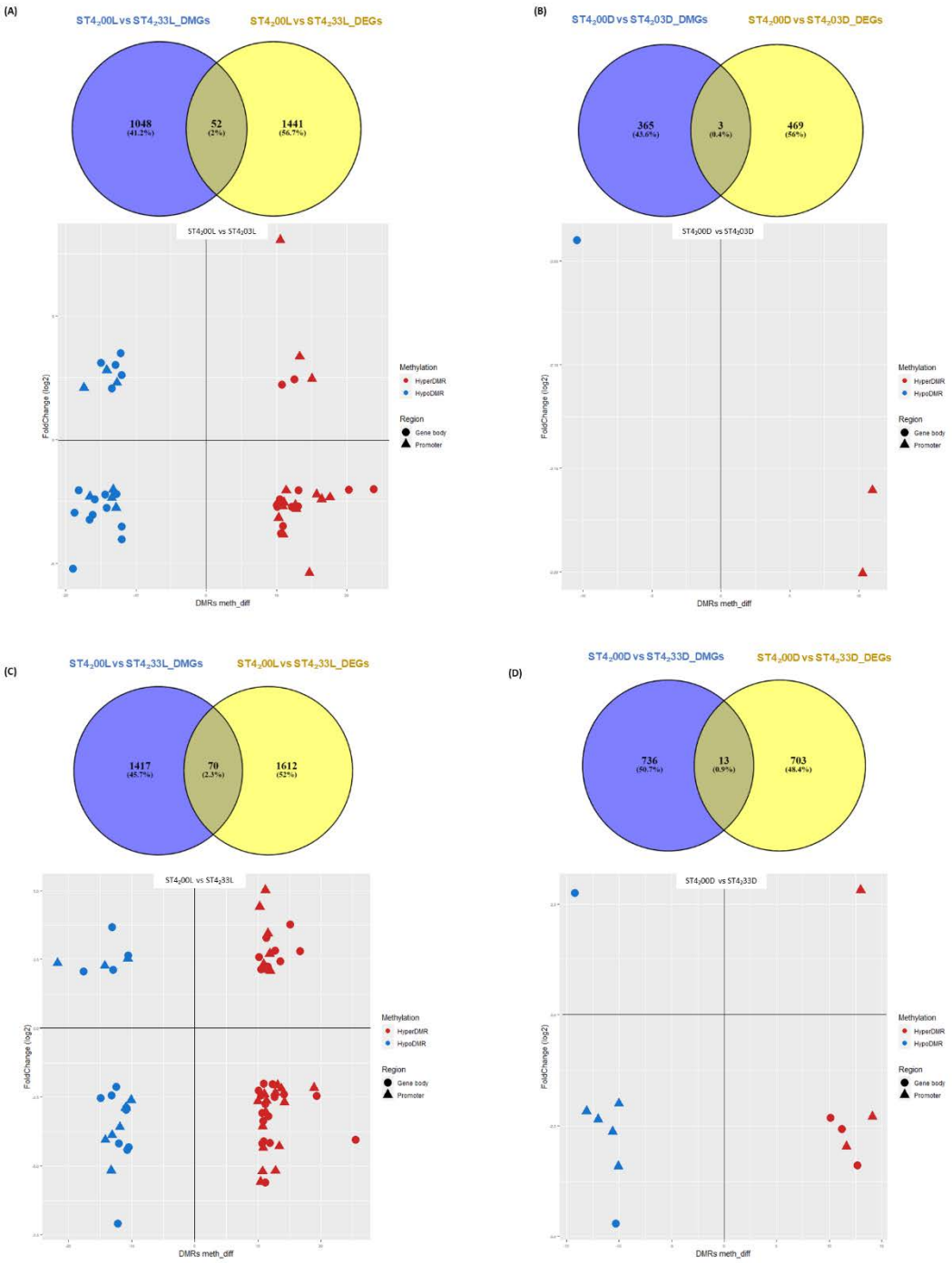


Figure 4.9 Graphical representation of overlapping DEGs and DMGs based on propagation methods and group comparison.

(A) Top: Venn diagram of DEGs that are also DMGs in ST4200L vs. ST4203L. Bottom: scatterplot showing the relationship between transcript levels (fold change: log2) and DNA methylation (meth_diff) of those DEGs/DMGs. (B) DEGs/DMGs in ST4200D vs. ST4203D. (C) DEGs/DMGs in ST4200L vs. ST4233L. (D) DEGs/DMGs in ST4200D vs. ST4233D.

4.5 Discussion

4.5.1 Transcriptional memory of stress after vegetative/clonal propagation

Similar to our previous experiments, the plants that were treated with combined stress has been selected to better understand how stress primes the plant and the maintenance of the memory through vegetative/clonal propagation methods in grapevine. To examine the difference in stress response between primed and naïve plants, we looked at the commonly differentially expressed genes in primed and naïve plants under stress ($ST4_200L/D$ vs. 03 and $ST4_200L/D$ vs. 33). For layered propagules, GO analysis showed significant enrichment in chromosome organization, response to stimulus and transcription factor activity for the commonly expressed genes of primed and naïve layered plants (Supplemental Table S4.2). Similarly, commonly differentially expressed genes in hardwood cutting primed and naïve propagules showed significant enrichment in chromatin organization, cell cycle and histone modification (Supplemental Table S3). Suggesting the potential role of chromatin modification and transcriptional factor activation in establishing, maintaining, and retrieving the stress memory after vegetative propagation. When examining primed propagules under controlled conditions ($ST4_230L/D$). The presence of DEGs are only found in primed layered plants ($ST4_200L$ vs. $ST4_230L$). Interestingly, all six genes appeared to be sHSP family associated genes (Table 4.1). The involvement of sHSPs in stress response have been well studied (Ji et al., 2019). The downregulation of those genes in the absence of a recurring stress might be an indication of the maintenance of the acquired stress memory.

Taking collectively, on transcriptional level, we did not observe a complete, but a partial, reset or erase of the acquired stress memory after first stress event in either layered

or hardwood cutting propagules. Compared to the previous observations, primed layered propagules ($ST4_233L$), although they had a slightly less total number of increases for DEGs compared to primed hardwood cutting propagules (Figure 4.2). The presence of DEGs in primed layered propagules that are not exposed to a recurrent stress event (Figure 4.3) suggest the maintenance of that stress-induced memory. Moreover, the magnitude of expression level were significantly higher in both directions compared to the naïve layered propagules ($ST4_203L$) that are experiencing stress for the first time even after propagation (Figure 4.4). Primed hardwood cutting ($ST4_233D$) propagules in the other hand, are more similar to their mother plants in the total number of increases for DEGs (Figure 4.2). Suggesting a part of stressed induced memory is still being maintained. However, we did not identify DEGs in primed hardwood cutting propagules that were not under a recurrent stress, and the magnitude of expression level only significantly increased in commonly down-regulated genes compared to the naïve hardwood cutting propagules (Figure 4.4). Hinting that some part of the memory might have been reset or erased, on transcriptional level.

4.5.2 Classification of stress memory genes

In the previous study, we have created a new classification system for stress memory genes in grapevine. Differ from last study, this study have taken vegetative/clonal propagation as a factor. For Type I memory genes, the transcriptional profile of many of the transcription factors and sHSPs associated genes have been similarly observed in both layered and hardwood cutting plants (Figure 4.5; Supplemental Table S4.4). Many of those transcription factors and sHSPs involvement in stress response have been studied, namely bZIP (Kim, 2006) and BHLH (Sun et al., 2018),suggesting that the expression pattern of

those genes might be consistent under stress conditions irrespective of propagation methods. Interestingly, transcription repressor OFP3 (Xiao et al., 2020), non-receptor serine/threonine protein kinase (Rudrabhatla et al., 2006) associated genes also shared similar expression pattern irrespective of propagation methods (Supplemental Table S4).

Furthermore, for Type II memory genes, the involvement of transcriptional factors such as WKRY (Phukan et al., 2016) and SANT (AbuQamar et al., 2009) associated genes is a good indication of their role in stress response (Figure 4.5; Supplemental Table S4.4). In addition, we have also identified stress memory genes that are unique to plants propagated through different propagation methods. For Type I memory genes that are exclusively expressed in layered plants, we see the involvement of specific ethylene responsive transcription factors such as WIN1 and SHINE2 (Kannangara et al., 2007), BAG family chaperone regulator (Irfan et al., 2021), and dehydrin RAB18 (Lång and Palva, 1992) associated genes (Figure 4.5, Supplemental Table S4.4), their role in plant stress response have been well studied. While DNA damage repair protein DRT100 (Fujimori et al., 2014), transcriptional factor TCP10 (Liu et al., 2020a) and specific ethylene responsive transcription factor CRF2 (Xie et al., 2019) and a histone lysine N-methyltransferase SUVR3 (Zhou et al., 2020) are exclusively expressed in hardwood cutting plants. For Type II memory genes, NAC,(Hu et al., 2010), RAC like GTP binding protein (Gu et al., 2004), and SAUR (Ren and Gray, 2015) associated genes are found to be exclusively expressed in layered plants (Figure 4.5, Supplemental Table S4.4).

Interestingly, SUVR3 exhibited a type II memory gene expression pattern in layered plants, where it appeared to be a type I in hardwood cutting plants. Only 3 type II memory genes have been identified that expressed exclusively in hardwood cuttings,

including casparian strip integrity factor (Barbosa et al., 2019), protein radialis like (Yang et al., 2018), and alpha/beta hydrolases (Liu et al., 2014) associated genes (Supplemental Table S4.4). Taken together, our results suggested that the stress response is a complex interaction between stress memory genes within the examined methods of propagation. The many that are shared between the two propagation methods might indicate their essentialness in stress response.

4.5.3 The transmission of epigenetic marks in clonally propagated grapevine

Overall, no significant increase or decrease in global methylation level has been observed for either layered or hardwood cutting plants, with the exception of $ST4_203L$ and $ST4_233L$ compared to $ST4_200L$ in CHG context (Figure 4.6). It has been previously reported that drought stress induced both mCG and mCHG hypermethylation in mulberry (Li et al., 2020). On the contrary, heat stress is normally associated with loss of global DNA methylation (Li et al., 2016). Interestingly, when performing the DMC analyses, more DMCs are identified in the intergenic regions of CG and CHG contexts for layered plants in all three comparisons (Figure 4.7A top). While the overwhelmingly large amount of DMCs is identified in the intergenic regions of CHG and CHH contexts in primed hardwood cutting plants under stress ($ST4_200D$ vs. 33). In DMR analysis, we observed the majority of DMRs are located in the intergenic region for both hardwood cutting plants and layered plants. It is possible that the grapevine methylome under stress induces methylation that are mainly found in silenced regions of the genome such as transposons and repeats. Moreover, a small amount of DMGs were commonly differentially methylated in our two-year experiment for both layered (25/2978 DMGs) and hardwood cutting (18/2333 DMGs) plants (Figure 8A). The limited overlapping DMGs and no consistent

patterns observed suggests that either DNA methylation are reset after the stress is over (Viggiano and Pinto, 2017), or that the DNA methylation marks induced by stress have not been stably inherited through clonal propagation. Although previous studies suggested the transmission of epigenetic marks to the next generation in clonally propagated plants is stable in *Trifolium repens* L. (Rendina González et al., 2018) and *Fragaria vesca* (Xu et al., 2016) using cutting. However, the cutting propagation method used in viticulture involves callus formation (Pratt, 1974). It has been shown that in grapevine, callus cutting alters the DNA methylation patterns (Grigg, 2017).

4.5.4 Stress-induced transcriptional regulation and DNA methylation changes after clonal propagation

In this study, we observed only a small number of DMGs that are also differentially expressed in both layered and hardwood cutting plants (Figure 4.9). For naïve plants under combined stress, only 5% (52/1100) and 0.9% (3/368) DMGs are also differentially expressed in layered ($ST4_200L$ vs. 03) and hardwood cutting ($ST4_200D$ vs. 03) plants, respectively (Figure 4.9A-B). For primed plants under combined stress, only 5% (79/1487) and 2% (13/749) DMGs are also differentially expressed in layered ($ST4_200L$ vs. 33) and hardwood cutting ($ST4_200D$ vs. 33) plants, respectively (Figure 4.9C-D).

Similar to previously observed in mother plants and other studies, there is no clear correlation between methylation changes (hyper- or hypomethylation), region of methylation (gene body or promoter), and the transcriptional changes (up- or downregulation). Moreover, this holds true for both plants experiencing stress for the first time and for the second time. Suggesting that stress induced transcriptional regulation might be, at least partially, independent of DNA methylation.

Interestingly enough, many of the DMGs that are also differentially expressed found in both layered and hardwood cutting plants (both $ST4_200L/D$ vs. 03 and $ST4_200L/D$ vs. 33) are well characterized stress response genes (Supplemental table S4.8). This has also been observed in studies conducted in season 1 and mother plants. Further supporting the hypothesis that the changes in DNA methylation may be associated with the differential expression of some stress response genes. However, instead of directly affecting the transcription of those stress response genes, changes in DNA methylation are more likely to control the expression of nearby transposable elements (Tian et al., 2021). It is interesting to consider the difference not only in the numbers of DMGs but also in the numbers of DMGs/DEGs in layered and hardwood cutting plants. Previous reports have suggested stress-induced DNA methylation changes can be stably inherited through cutting (Latutrie et al., 2019); however, it is not reflected in our study as layered plants presented more stress induced DMGs and DMGs/DEGs. Grapevines propagated through hardwood cutting behaves more similar to annuals and biennials, where the memory of stress are often erased in offspring for needs to overwinter again before flowering in spring (He and Li, 2018). Although in this case, the memory of stress (both transcriptional and epigenetically) is not completely, but partially, erased in hardwood cutting grapevines used in our study.

4.6 Conclusions

In this study, we have examined both stress-induced transcriptional memory and stress-induced epigenetic memory in clonally propagated grapevines. We observed the partial loss of transcriptional memory in plants propagated via hardwood cuttings. Where the transcriptional memory was faithfully maintained in layered plants, as primed layered plants showing a small number of differentially expressed genes associated with stress

response even in the absence of a second stress and also presenting a stronger response than naïve plants when re-exposed to stress one year later. Putative stress memory genes have been identified in both layered and hardwood cutting plants, and they have been classified based on our proposed classification system. When looking at the DNA methylation changes, we did not observe common pattern between the two propagation methods. No strong evidence to show that epigenetic marks, at least on DNA methylation level can be stably inherited through hardwood cutting compared to layered plants. Moreover, no clear correlation between changes in DNA methylation and gene expression changes have been observed, irrespective of propagation methods and the number of stresses. Lastly, we believe that it should be important to consider the difference in propagation techniques used in viticulture than common clonal propagation. Callus formation appeared to have more of a resetting effect on both transcriptional memory and epigenetic modifications.

CHAPTER 5. SUMMARY AND FUTURE DIRECTION

Upon writing this dissertation, a quick google search with the keyword “plant memory” returned more than three million results, including but not limited to peer-reviewed journal articles, books and book chapters, and conference presentations. Needless to say, there is a consolidated literature conceptualizing and characterizing memory in plants (e.g. Demongeot et al., 2019; Michmizos and Hilioti, 2019; Thellier and Lüttge, 2013). This doctoral work merely scratched the surface of understanding this intriguing process and its underlying mechanisms.

What exactly is ‘memory’? This was one of my very first questions. According to the Merriam-Webster Dictionary, the very broad definitions of memory are (i) “the power or process of reproducing or recalling what has been learned and retained especially through associative mechanisms”, (ii) “the store of things learned and retained from an organism’s activity or experience as evidenced by modification of structure or behavior or by recall and recognition” or, in its simplest form, (iii) “capacity for storing information”. It is hard to imagine, plants possess the ability to remember a past event and are capable of using it to adapt to the environment they grow in. But they can.

Plants are sessile organisms; therefore, they cannot simply run away from punctual and/or repeated stimuli like we do, whether they are abiotic or biotic. Plants rely on the ability to establish, store, and retrieve the memory induced by stimuli, which then can adjust their metabolic, growth, and morphogenetic behavior through phenotypic plasticity (Crisp et al., 2016). This process is referred to as priming or acclimation (Conrath, 2009). However, we should not simply assume that the modified response will always improve a

plant's metabolism or growth under stress. Rather, to just know that memory is just a basic capacity to store and eventually recall information, which can also result in disruptive effects and maladaptation (Galviz et al., 2022).

Similar to how memory functions in other living organisms, multiple molecular mechanisms are involved in the perception, transduction, storage, and recall of information in plants (Galis et al., 2009). Epigenetic mechanisms have been proposed to mediate the formation and maintenance of memory (Bruce et al., 2007). The majority of the available literature on this area concentrates on model species such as *Arabidopsis* and other annual plants. Perennials seemed to have always fallen under the radar due to long-life span and costly maintenance. However, it will make the most sense to study memory in perennials, because they constantly face recurrent, sometimes chronic stress events (Fortes and Gallusci, 2017). Grapevine was chosen for this doctoral work because of its economic importance and the intrinsic characteristics that make it a model to study epigenomics in perennial woody crops. After many intriguing, novel, and sometimes confounding results, we proposed a comprehensive model integrating plant response to stress, the establishment of transcriptional and epigenetic memory of stress, and its maintenance, over time and during vegetative propagation in perennial plants.

Results from Chapters 2-4 discussed this model in detail, starting with the most basic building block of all, the response of naïve vines to stress. I then delved into the response of grapevine plants to a triggering stress one year after the priming event, and after dormancy cycle. Finally, I studied the effect of two vegetative propagation systems used in commercial viticulture on the maintenance of memory of stress. Contrary to most previous studies, which focus on the effect of plant response to single abiotic stress (e.g.,

drought. heat, cold stress, etc.), **chapter 2** compared the effect of a more realistic combined drought and heat stress (the grapevine growing season in Mediterranean climate regions, where viticulture is more commonly practiced, is characterized by recurrent heat waves with no rainfall) to each stressor on its own, while **Chapters 3 and 4** compared the effect of a combined stress priming stress on the response of grapevines to subsequent combined stress.

Chapter 2 results show that, unsurprisingly, combined drought and heat stress had more severe effects not only on the grapevine's physiology but also at a transcriptional level. We observed a large difference in the number of differentially expressed genes found under combined stress treatment and each individual stress. Many of those DEGs have been previously shown to be stress response genes. It piqued our curiosity when a histone-lysine N-methyltransferase SUVR3 was identified as one of the five hub genes in the interaction network for combined stress. This marked the first, but not the last time, the observation of epigenetic regulation associated with genes involved in grapevine response to stress. Several other histone and chromatin modification associated genes were also identified as unique to combined stress in subsequent analyses. These results hinted at the association between epigenetic mechanisms and the formation/establishment of stress memory (Friedrich et al., 2019; He and Li, 2018; Tan et al., 2023). Therefore, a genome-wide DNA methylation study was carried out. We observed a genome-wide increase in DNA methylation level under combined stress. To our surprise, and contrary to what previous literature has suggested, no clear correlation between DNA methylation changes (hyper- or hypo-methylation), genic location (promoter or gene body), and expression pattern (up- or down-regulation) was observed. But the few genes that exhibit altered

methylation and gene expression levels appeared to be well-characterized stress response genes.

Chapter 3 focuses was on the differential response of naïve and primed grapevines when exposed to combined stress one year after the priming stress and after dormancy cycle. We observed a clear modified transcriptional response upon the encounter of second stress. Primed plants were more transcriptionally active than naïve ones, that is, they presented a higher number of DEGs, and the magnitude of the change in expression of common DEGs was larger in primed plants. Moreover, primed plants presented DEGs even in the absence of a second stress event. Suggesting that memory of stress was indeed established in response to stress, and that grapevine is capable of maintaining that memory over a year and through dormancy cycle. This memory of stress was deemed somatic memory (without sexual reproduction), even when somatic memory was not supposed to last this long (~1 year) (Bäurle, 2018; Bäurle and Trindade, 2020; Tricker et al., 2013b). When we study primed plants that never experience second stress, we identified several DEGs such as sHSPs and transcription factor families AP2/ERF and NAC associated genes, and the ATXR6 gene, which we believed might be key genes in the maintenance of the long-term somatic memory (Jacob et al., 2009). We were unable to classify all of the transcriptional patterns observed in primed plants in response to triggering stress using the existing classification systems for plant memory genes (Bäurle, 2018; Ding et al., 2014). Therefore, we proposed a new classification system for grapevine that is inclusive and intuitive. Insofar this long-term somatic memory was only studied and reflected on the transcriptional level, it is clear that transcriptional memory does exist. But what about the epigenetic side of the story? We observed more variability in the global DNA methylation

for plants grown under non-stress conditions compared to plants under combined stress, regardless of their priming status. Such stress-induced loss of DNA methylation variability suggests that stress-induced methylation changes are not random. This is a subject that needs to be explored in more detail in future research, since conflicting results can be found in the literature (e.g. Konate et al., 2020), the authors proposed that at least part of the stress-induced epigenetic variability, previously thought to be stochastic, is linked to environmental micro-variations exerted on the experimental population by the experimental design. However, we did not see a clear correlation between DNA methylation and gene expression changes in the first stress encounter, and changes in DNA methylation and gene expression changes do not necessarily coincide with second stress exposure either. Perhaps it is an indication of stress memory establishment, maintenance, and retrieval in grapevine might be more complex and involves multiple epigenetic mechanisms. At this point, the memory of stress appeared to be more consistent on a transcriptional level than DNA methylation level.

In **Chapter 4**, we tested if the observed transcriptional and epigenetic changes can be inherited by clonal propagation systems most commonly used in commercial viticulture. We saw a partial loss of transcriptional memory in plants propagated using hardwood cuttings, while the transcriptional memory was faithfully maintained in layered plants, as primed layered plants showed a small number of differentially expressed genes associated with stress response even in the absence of second stress and also presented a stronger response than naïve plants when re-exposed to stress one year later. We also used our newly proposed classification system to classify stress memory genes found in both hardwood cutting and layered plants. When we look into the DNA methylation changes, no common

pattern was observed between the two propagation methods. Contrary to previous studies (e.g. Latutrie et al., 2019), we found no strong evidence to show that epigenetic marks, at least on the DNA methylation level can be stably inherited through hardwood cutting compared to layered plants. We hypothesize that the reason for this observation is due to the formation of callus tissue during callused cutting propagation of grapevines. Once again, there was no clear correlation between changes in DNA methylation and gene expression changes, irrespective of propagation methods and the number of stresses.

In conclusion, this dissertation used a two-year experiment to study grapevine response to abiotic stresses, from a transcriptome and methylome perspective and proposed a comprehensive model integrating response to stress, the establishment of transcriptional and epigenetic memory of stress, and its maintenance, over time and during vegetative propagation in perennial plants. (1) Upon the encounter of a priming stress, grapevine transitions from a naïve state to a prime state after a priming response. This priming response is reflected on both transcriptional and epigenetic levels. (2) After reaching physiological recovery, that memory of stress, as a result of priming, is maintained through dormancy cycle. The divergence starts here: (3.1) After dormancy cycle, primed plants encounter triggering stress, such memory of stress induces a modified/enhanced response on both transcriptional and epigenetic levels. (3.2) After dormancy cycle and with vegetative propagation, depending on the propagation method, propagules present a different memory of stress. Hardwood cutting propagation resulted in a partial loss of transcriptional and epigenetic memory. While layered grapevines maintain that memory.

While this doctoral work laid foundational research in understanding stress-induced memory and the underlying mechanisms in grapevine, there is still much to be done. Future

research will need to explore stress-induced DNA methylation changes and stress-induced transcriptional changes more, including the study of transposable element (TE) superfamilies and/or cis-regulatory elements (Tian et al., 2021). Other areas that require attention are histone modification and small RNAs expression in response to stress (Lewsey et al., 2016). For DNA methylation, stress-induced DNA methylation changes may be more likely to control the expression of nearby TEs or affect the conformation of distal or proximal regulatory elements outside of the gene, rather than directly affecting the transcription of stress-induced genes. Moreover, researchers have used machine learning to predict tissue-specific gene expression with DNA methylation profiles (N'Diaye et al., 2020), whether the same can be applied to genome-wide in perennials or perennials under stress remains to be tested. Previous research has suggested the importance of chromatin-based mechanisms in the establishment of stress memory (Bäurle and Trindade, 2020). And we have observed the involvement of histone/chromatin modification associated genes throughout this doctoral work. Exploring this epigenetic mechanism will provide more insights into the establishment, maintenance, retrieval, and the reset of stress-induced memory in grapevine. Similar reasoning can apply to small RNAs research. That additional information will greatly complement our proposed model.

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Tan, J. W., Kester, S. T., Su, K., Hildebrand, D. F., & Geneve, R. L. (2022). Seed Priming and Pericarp Removal Improve Germination in Low-Germinating Seed Lots of Industrial Hemp. *Crops*, *2*(4), 407-414.

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Publication in review:

Tan, J.W., & Rodriguez-Lopez, C.M. (2023). Epigenomic: A New Tool for the Generation of Climate Resilient Grapevines. *Frontier in Horticulture.* Under review.

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Gender Identification of Dry *Cannabis sativa* Seeds. U.S Patent Application No. 62/877,535

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Tan, J.W. 2022. Plant Propagation and Epigenetic Priming Modulates Grapevine Responses to Subsequent Stress: A Transcriptomic Study.
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2022 Manuscript Publication Award.

2021 Excellent Graduate Research Assistant Award.

2021 Manuscript Publication Award.

2017 Undergraduate Summer Research Award.

2013 Hamline Academic Excellence Scholarship.