UNIVERSITY OF UDINE

Department of Agricultural, Food, Environmental, and Animal Sciences

PhD School "Agricultural Science and Biotechnology"

XXXIV Cycle - Coordinator: Prof. Francesco Nazzi

PhD Thesis

Bud dormancy in *Vitis vinifera*: molecular regulation and response to temperature changes

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2018 - 2022

Say to them,

say to the down-keepers,

the sun-slappers,

the self-soilers,

the harmony-hushers,

"Even if you are not ready for day

it cannot always be night."

You will be right.

For that is the hard home-run.

Live not for battles won.

Live not for the-end-of-the-song.

Live in the along.

Speech To The Young: Speech To The Progress-Toward

by Gwendolyn E. Brooks

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ABSTRACT

Environmental conditions are paramount for optimal grapevine development and fruit ripening, and consequently for wine production. Temperature, water status and radiation are all influencing factors. On these premises, climate change is an obvious threat to viticulture as it is currently practiced, and adaptation measures are required to maintain productivity levels and wine tipicity. Due to higher average surface temperatures that accelerate bud phenological development, spring frost damage risk cannot be overlooked in the future in several areas of the world, making the identification of effective adaptive measures an issue of the present. Understanding the molecular mechanisms underlying cold acclimation and freezing tolerance acquisition, deacclimation and budbreak is essential to improve crop sustainability and directing breeding efforts. On this regard, the potential of wild Vitis varieties to bear favorable traits, starting from changing chilling requirements and budburst rates, needs to be considered. As a first step in elucidating grapevine dormancy regulation, cultivar Fleurtai and selection UD 31-103, characterized by different tolerance to winter minima, were studied. It is known that, paradoxically, grapevine hybrids most resistant to freezing temperatures are also the most vulnerable to spring frost damage due to higher responsiveness to warm temperatures. This evidence was confirmed by differential thermal analysis (DTA) carried out on Fleurtai and UD 31-103 buds during the 2019-2020 winter season, with cv. Fleurtai, characterized by a greater winter freezing tolerance compared to UD 31-103, being also the fastest to deacclimate. Carbohydrate metabolism was analyzed in both hybrids due to the relevance of soluble sugars as osmoprotectants and metabolic substrates in grapevine buds, and the role as signaling molecules in several developmental processes. Evidence on multiple sugar-related responses taking place inside buds was detected, with soluble sugars content appearing to be greatly influenced by warm spells during winter. The involvement of hexose transporter VvHT5 in the tolerance of physiological water reduction, connected to cold hardiness acquisition or resistance to freezing-induced dehydration stress in grapevine buds, was hypothesized. Moreover, the first evidence on *VvMSA* expression in buds, sole member of ASRs proteins in grapevine, was presented and a role in bud phenological advancement towards budbreak speculated. Lastly, two *DEMETER-like* DNA

demethylases (*DMLs*) homologs were identified in Fleurtai and UD 31-103 and a role for *DEMETER*-dependent DNA demethylation in the regulation of dormancy-growth cycle was suggested.

To further inspect the transcriptomic landscape of dormancy release, two cultivars characterized by contrasting budbreak timing were considered, early-budbreak hybrid Sauvignon Nepis and late-budbreak Cabernet Sauvignon. Total RNA sequencing and gene ontology analyses performed on cold acclimating, dormant and deacclimating buds of both cultivars allowed to hypothesize that a plethora of developmental and reproductive processes are differentially activated in winter buds of the early-budbreak cultivar during dormancy, possibly anticipating the late-budbreak one due to an early chilling requirement fulfilment. In addition, new insight has been provided regarding the potential participation of specific transcription factors in dormancy release regulation, with possible genotype-specific roles. In detail, particular interest was raised by dormancy-associated MADS-box *VvDAM3-SVP* and positive regulator of budbreak *VvEBB1*, whose expression was significantly different in the two cultivars. Ongoing Whole Genome Bisulfite Sequencing analyses will provide information regarding possible *VvDMLs* targets during deacclimation and dormancy release.

Lastly, a preliminary insight into early molecular responses of actively deacclimating bud tissues to a spring frost occurrence was provided by the analysis of several genes of interest in buds of field-grown plants of Sauvignon Nepis after a spring frost which took place on April 7th, 2021, in Friuli Venezia Giulia (Northern Italy). The results highlighted differential responsiveness of buds in persisting BBCH 00 stage as compared to more advanced phases.

Gaining an integrative understanding of dormancy regulation is necessary to guide future breeding efforts towards the generation of more sustainable varieties in the context of a changing climate. Cultivars capable of spring frost avoidance thanks to reduced reactivity to warm spells and delayed budbreak timings might in fact be an environmentally safe solution as opposed to artificial heating and irrigation systems. This work contributes to this purpose by bringing to the light several key elements to hopefully aid and inspire further research.

PhD-RELATED ACTIVITIES

Publications

*correspondence

De Rosa V.*, Falchi R., Vizzotto G. (2022). Early molecular response of cold-deacclimated grapevine buds to late frost occurrences.

Acta Horticulturae (under review).

De Rosa V., Falchi R.*, Moret E., Vizzotto G. (2022). Insight into Carbohydrates Metabolism and Signaling in Grapevine Buds during Dormancy Progression.

Plants, 11:1027. doi: 10.3390/plants11081027.

De Rosa V.*, Falchi R., Peressotti A., Vizzotto G. (2022). Expression patterns of DEMETER-like demethylases homologs hint at potential involvement in grapevine dormancy release.

BIO Web of Conferences, 44:04001. doi: 10.1051/bioconf/20224404001.

De Rosa V., Vizzotto G.*, Falchi R.* (2021). Cold Hardiness Dynamics and Spring Phenology: Climate-Driven Changes and New Molecular Insights Into Grapevine Adaptive Potential.

Frontiers in Plant Science, 12:644528. doi:10.3389/fpls.2021.644528.

Conferences

XIth International Symposium on Grapevine Physiology and Biotechnology

October 31st – November 5th, 2021 (Stellenbosch, South Africa). Poster presentation + flash talk.

VIII CONAVI – 8° Convegno Nazionale di Viticoltura

July 5th – 7th, 2021 (Udine, Italy). Poster presentation.

XIII Giornate Scientifiche SOI

"I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana"

June 22nd – 23rd, 2021 (Catania, Italy). Poster presentation.

2nd Joint Meeting of Agriculture-Oriented PhD Programs at UniCT, UniFG, and UniUD

"Higher Education for Sustainable Food Production"

September 14th –19th, 2020 (Online meeting). Oral presentation.

1st Joint Meeting of Agriculture-oriented PhD Programs at UniCT, UniFG and UniUD

"Higher Education for Sustainable Food Production"

June 17th – 21st, 2019 (Catania, Italy). Oral presentation.

♦ CHAPTER I

State of the art: introduction to spring frost tolerance in fruit crop *Vitis vinifera* in the context of a changing climate

INTRODUCTION TO THE RESEARCH TOPIC

The topic of spring frost tolerance has recently sparked interest due to the high impact of late frost damage on grapevine production, documented in several recent occurrences in multiple areas of the world. The ongoing increase of average surface temperatures registered all around the globe might appear in contrast with the threat posed by spring frosts; however, the rhythm of plant development, including grapevine's, is highly dependent on temperature regimens. Higher temperatures are usually connected to faster phenological advancement, which also applies to budbreak timings. In a context in which grapevine buds break earlier and spring frost occurrences remain supposedly fixed events, spring frost damage risk is expected to increase. Budburst has in fact been delineated as a critical stage in plant development, in which tissue vulnerability to freezing damage is substantial.

Current spring frost avoidance approaches are based on the adoption of training systems and cultural practices, aimed at delaying budbreak, or wind machines and other instruments that force warm air towards the ground. These approaches are not guaranteed to work in every environment, and present sustainability issues. For these reasons, the topic of long-term resilience to spring frost occurrences needs to be tackled, keeping genetic improvement in mind as an adaptive strategy. Such strategies must be applied both to the breeding of new varieties and the improvement of traditional ones. The first step towards this goal is reaching a deep molecular understanding of freezing tolerance, cold acclimation and deacclimation dynamics, chilling requirement and budbreak regulation.

A summary of an in-depth analysis of previously published studies surrounding these topics was included in a literature review titled '*Cold hardiness dynamics and spring phenology: climate-driven changes and new molecular insights into grapevine adaptive potential*', published by *Frontiers in Plant Science* journal in 2021.

Cold hardiness dynamics and spring phenology: climate-driven changes and new molecular insights into grapevine adaptive potential[1]

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Abstract

Climate change has become a topic of increasing significance in viticulture, severely challenged by this issue. Average global temperatures are increasing, but frost events, with a large variability depending on geographical locations, have been predicted to be a potential risk for grapevine cultivation. Grape cold hardiness encompasses both mid-winter and spring frost hardiness, while the avoidance of spring frost damage due to late budbreak is crucial in cold resilience. Cold hardiness kinetics and budbreak phenology are closely related and affected by bud's dormancy state. On the other hand, budbreak progress is also affected by temperatures during both winter and spring. Genetic control of bud phenology in grapevine is still largely undiscovered, but several studies have recently aimed at identifying the molecular drivers of cold hardiness loss, and the mechanisms that control deacclimation and budbreak. A review of these related traits and their variability in different genotypes is proposed, possibly contributing to develop the sustainability of grapevine production as climate-related challenges rise.

[1] The topics covered in this chapter are the subject of a literature review published by *Frontiers in Plants Science* journal.
(De Rosa *et al.*, 2021 – *Front. Plant. Sci.* 12:644528, doi:10.3389/fpls.2021.644528.)

1 INTRODUCTION

Climate change is a proven reality whose consequences on human activities and natural systems have reached an undeniable magnitude all around the world (IPCC, 2014). Global mean surface temperatures are predicted to increase by 0.3°C to 4.8°C by the end of the 21st century, depending on the trend of anthropogenic greenhouse gas emissions, compared to the reference time-frame 1986-2005 (IPCC, 2014). Many plant species are expected to be unable to shift their geographical range quickly enough to keep up with these changes, and production will be negatively impacted if no adaptation occurs. Rainfall changes are likely to differ depending on the region, whereas radiation and extreme weather events are expected to increase (IPCC, 2019). Agriculture, and viticulture in particular, greatly depend on thermal regimen, soil composition and water availability, in terms of fruit yield and metabolite composition (van Leeuwen and Darriet, 2016). Grapevine holds great economical value since it can be used fresh (table grape), dry (raisin) and for winemaking (Delrot *et al*., 2020). Climate variations in wine-producing regions induce the so-called “vintage effect”, the year-to-year variations in yield, quality and typicity (van Leeuwen and Darriet, 2016). Grape berry composition also depends on “terroir”, defined as the complete natural environment in which a wine is produced, in which climate plays a major role, with the interplay of human activity (Santos *et al*., 2020; Delrot *et al*., 2020). Grapevine phenology and fruit ripening are greatly affected by temperature conditions. Berry composition is key in determining the subsequent quality of wines. The increase of temperature has been shown to cause a rise of berry sugar concentration (Coombe, 1987), whereas some secondary metabolites, such as malic acid or anthocyanins (Kliewer and Torres, 1972), are negatively affected. Higher temperatures produce an advance of phenology, causing earlier harvest dates (van Leeuwen and Darriet, 2016) and decoupling sugar and phenolic compound accumulation at maturity, thus leading to unbalanced wines (Sadras and Moran, 2012; Bonada *et al*., 2015). High temperatures during the final stages of berry growth, together with high precipitations, can also be the cause of cracks and rots (Molitor *et al*., 2016). Although rainfall tendencies are difficult to predict, the increase of evapotranspiration caused by temperature increase will cause plants to experience water stress even when rainfall does not directly decrease (van Leeuwen and Darriet, 2016).

The new climate change scenario will lead to increasing difficulty in the production of traditional wines in their areas of origin if no adaptation occurs. Therefore, adaptation measures are necessary since wine quality greatly depends on ripening conditions (Bonada *et al*., 2015), which in turn are a direct consequence of the timing of several phenological phases starting with budbreak.

Although the impacts of climate change are expected to be diverse in different wine-making regions (Santillán *et al*., 2019), and among cultivars with different phenological rhythms (McIntyre *et al*., 1982), several adaptation practices may be able to cope with the short-term effects of climate change and maintain wine typicity, and new training systems could be developed for the middle-term (Duchêne, 2016). Remarkably, several variations in training systems and cultural practices have been adopted and tested in recent times with the aim to lower the risk of freezing damage in spring. Trimming, hedging or pruning have been evaluated in order to mitigate the short-term impacts of climate change (Herrera *et al*., 2015; Frioni *et al*., 2016; Palliotti *et al*., 2017; Abad *et al*., 2019). In the past, late winter pruning was shown to be effective in delaying bud burst in cool climate areas (Trought *et al*., 1999), although it could not be applied for grapevine grown in different environments, in which both yield increase (Friend and Trought, 2007) and loss (Frioni *et al*., 2016) were observed. Recently, a double-pruning approach has shown a potential budburst delay of up to 4 weeks depending on the timing of the second pruning (Palliotti *et al*., 2017). As regard to the direct avoidance of spring frost damage, several methods, encompassing active and passive types, have been used in the past (Liu and Sherif, 2019). Active approaches include the use of wind machines and helicopters to force the warmer air towards the ground, or heaters and irrigation, to exploit the fusion heat of water. Efficacy of such methods depends greatly on external factors and cannot guarantee a complete avoidance of damage. Moreover, these approaches are costly and environmentally unsustainable, and require coordinated action by growers to avoid the rise of production costs and to assure the effectiveness in the short-term (Unterberger *et al*., 2018). Additionally, the application of chemicals (e.g Amigo oil, FrostShield, ProTone) and Plant Growth Regulators (PGR) (i.e. ethephon) has been shown to delay budbreak although these results remain inconsistent (Qrunfleh and Read, 2013; Centinari *et al*., 2018; Kovaleski and Londo, 2019; Liu and Sherif, 2019; Wang and Dami, 2020).

In this context, the genetic improvement of grapevine has been taken into consideration to cope with the effects of climate change in the long run. Cultivated grapevines all around the world are usually grafted and this adds a layer of complication to the understanding of plant-environment interactions. Moreover, the communication between scion and rootstock is often unclear or unexplored since the connection that is immediately established at grafting may evolve as the plant ages (Delrot *et al*., 2020). Therefore, despite the numerous aspects to consider, the investigation of unexploited varieties in germplasm collections, for both rootstock and scion, could be an interesting opportunity, strengthened by the continuous evolution of sequencing technologies and gene mapping approaches. Efficient phenotyping methods also need to be developed to assess the effectiveness of varietal selection and the plasticity of the phenotype in different scion-rootstocks combinations (Warschefsky *et al*., 2016). However, the possibility that the variability within clones of the *V. vinifera* species might be insufficient to compensate the phenological shifts caused by climate change must be contemplated; the need to introduce new varieties with the abandonment of the traditional ones will eventually arise if no measure is taken (Duchêne, 2016). Moreover, in addition to the already existing varieties, new ones could be generated through traditional breeding approaches or even genetic engineering. In any case, the comparison and analysis of different Vitis species could, firstly, help in clarifying the molecular regulators and drivers of cold hardiness, deacclimation and budbreak, and secondly allow the identification of targets to optimize clone selection and breeding efforts.

In this review spring frost frequency and trends for different geographical regions are reported, together with the recent findings about the potential pathways involved in cold deacclimation and budbreak. We aim to provide an update on current status of research regarding the effects of climate change on grapevine phenology, with a focus on cold hardiness dynamics, budbreak and the key molecular players involved in these processes. This will hopefully help in developing new ways to face current and future climate-related contingencies to allow berry ripening and harvest to be achieved in favorable conditions.

2 Effects of Climate Change on Grapevine Phenology

Several studies have assessed the impact of climate change on grapevine phenology and viticulture in the past and in the present (Biasi *et al.*, 2019), and numerous models have been tested to predict future consequences (Caffarra and Eccel, 2011; Bonfante *et al.*, 2017; Costa *et al.*, 2019; Alikadic *et al.*, 2019; Ramos and de Toda, 2020). Agro-climatic indices are considered more reliable than individual climatic variables to describe climate change effects (Santos *et al.*, 2020); these tools allow to closely follow and simulate plant development in different scenarios and can be used to evaluate the potential of different areas for viticulture (Molitor *et al.*, 2014; Blanco-Ward *et al.*, 2019). Redistribution of wine production within continents is a likely perspective and the change in viticultural suitability for different geographic regions has been calculated, showing agreement among a 17 global climate models. Wine-producing regions will possibly decrease by 2050 (mainly in Mediterranean climate area), whereas expanding suitability has been predicted for New Zealand, western North America, and Northern Europe (Hannah *et al.*, 2013).

However, commonly bioclimatic indices used in viticulture (e.g. Huglin Index, Winkler Index, Dryness Index, Cool Night Index) are arguably replaced by dynamic crop models (e.g. STICS, BRIN), which combine several indices and integrate phenotype, soil, weather data and management practices into a more comprehensive picture (Cortázar-Atauri *et al.*, 2009; Moriondo *et al.*, 2013; Fraga *et al.*, 2016).

Heat requirements, determined in terms of growing-degree days (GDD), represent the climatic constraint that allows grape to successfully complete its annual cycle when met. Distinct phenological phases need different climatic conditions to take place (e.g. release from ecodormancy) (Ruml *et al.*, 2016). Higher temperatures lead to an acceleration of plant development, being a potential cause of premature loss of bud cold hardiness (Londo and Kovaleski, 2017; Kovaleski *et al.*, 2018; Pagter and Arora, 2012). In fact, early events like budbreak and flowering have been shown to be the most sensitive to temperature-driven variations as compared to later phases (Jones *et al.*, 2005). This increases the chances of vulnerable green tissues to be exposed to late spring frost events, which have been known to be the cause of great yield losses in the past (Gu *et al.*, 2008). The timing of budbreak is strictly linked to

the end of dormancy, a genetically programmed state of self-arrest in which the bud stops its development to avoid breaking at unfavorable times (Lang *et al.*, 1987; Horvath *et al.*, 2003). Whether the risk of damage due to spring frosts is globally increasing or not is up to debate, although recent reports suggest the relevance of this phenomenon in several locations (Augspurger, 2013; Ma *et al.*, 2018; Sgubin *et al.*, 2018). Effects are expected to vary depending on the geographical position, and changes in seasonal water availability need to be taken into account together with temperature variations. Great attention has been always given to budbreak timing since early dormancy release in cold winter regions can cause significant crop losses, and frost-protecting measures represent a notable cost for producers. To the contrary, warmer regions can be affected by low rates of budburst and lower productivity due to insufficient chilling during winter, making the use of artificial dormancy-breakers a necessity. On the other hand, increasing average temperature has been predicted to have positive outcomes on winemaking regions in central and Western Europe, and to allow the extension of viticultural areas in the north and east (Gaal *et al.*, 2012; Cardell *et al.*, 2019). This will favor the introduction of new currently inaccessible varieties in colder areas, since frost is expected to decrease and optimal ripening temperatures to be reached (e.g. Northern Europe, North America) (Santillán *et al.*, 2019); moreover, wine-producing suitable areas are expected to develop up to the 55°N by 2070 (Fraga *et al.*, 2016).

2.1 Cold hardiness variations

Dormancy encompasses endodormancy, determined by internal factors, which allows buds to cold acclimate and reach a state of hardiness to survive freezing temperatures during winter. Cold acclimation is a process in which physiological, biochemical and epigenetic changes driven by cold temperatures confer freezing tolerance (Wisniewski *et al.*, 2018). Exposure to chilling temperatures, with difference depending on cultivar (Anzanello *et al.*, 2018), is required to resume bud responsiveness to environmental signals and avoid growth start if mild temperatures occur during winter (Rohde and Bhalerao, 2007). Internal signals also prevent growth resumption in late summer or early autumn, which would cause the death of the bud in unfavorable environmental conditions (Lang *et al.*, 1987; Horvath *et al.*, 2003).

The productivity of grapevine and temperate plants is related to the capability of buds, both reproductive and vegetative, to tolerate freezing temperatures. Cold hardiness correlation with winter temperatures has been measured (Kovaleski *et al*., 2018). In general, sudden or recurring warm spells in winter can endanger the survival of woody perennials to freezing temperatures because the deacclimation process, during which cold tolerance is lost, is relatively fast (Pagter and Arora, 2012). Although deacclimation and acclimation cycles seem possible and efficient in several herbaceous plants (Vyse *et al*., 2019), it appears diverse for woody perennials with cold acclimation being restored only in part (Shin *et al*., 2015). Various grapevine species have been shown to be differently responsive to temperature variations during dormancy, likely related to the dissimilar chilling requirements that allow the transition from endodormancy to ecodormancy, at distinct timings. In addition, maximal cold hardiness is not reached automatically and a cold sustained winter is needed (Londo and Kovaleski, 2017). Depending on the species, grapevine buds' cold hardiness can reach temperatures below -30°C (Londo and Kovaleski, 2017). However, once buds begin to swell and deharden during the deacclimation process, their freezing tolerance quickly reduces, and the observed advancements in phenological timings may possibly increase the exposure of vulnerable plant structures to late frost events.

2.2 Spring frost risk

Late spring frosts have often resulted in great damage to cultivated fruit trees and in important economic losses (Gu *et al*., 2008; Marino *et al*., 2011; Ault *et al*., 2013, Vitasse and Rebetez, 2018). In the bigger picture, these phenomena can alter the ecosystem and evolution of entire populations because of competition among species and parasite opportunism (Inouye, 2000; Reineke and Thiéry, 2016). As previously stated, the vulnerability of plant structures to freezing temperatures differs depending on their level of cold hardiness, which varies seasonally, and on their intrinsic ability to sustain lower temperatures. Green tissues, flowers and fruit are in fact significantly more susceptible to lower temperatures than wooden tissues as their hydration levels are considerably higher and their supercooling capabilities lower (Fennell, 2004). Budburst and leafout have been delineated as the most critical, as several trees have been shown to be the most vulnerable at that specific time

(Vitasse *et al*., 2014; Lenz *et al*., 2016). Moreover, a lower temperature stability is expected during winter in the future, which will require the use of cultivars with a lower response to so-called "false springs" (Londo and Kovaleski, 2019). A "false spring" can be empirically defined as a period of warm temperatures with premature rapid vegetative growth, followed by a freeze (Gu *et al*., 2008; Ault *et al*., 2013); several mathematical approaches to evaluate these phenomena have been attempted (Marino *et al*., 2011). Freezing temperatures following a "false spring" can culminate in more serious damage, which affects photosynthetic tissue and reproductive tissue alike with consequences spread on multiple years of development (Carmona *et al*., 2008). In general, the influence of climate change on late frost events frequency and distribution remains unclear, and whether risk is increasing for temperate trees remains up for debate. The analysis of remote-sensing data showed that frost days in which the temperature drops below 0°C during the growing season have increased in the Northern Hemisphere (Liu *et al*., 2018). Concerning Europe, phenological and climate records were used to analyze the evolution of spring frost risk as regards to several tree species, between 1950 and 2013, with a focus on determining variations in the frequency of the phenomenon (Ma *et al*., 2018). These results showed that species whose phenology is more responsive to temperature increases tend to experience a higher risk of being subjected to frost occurrences and damage. Maritime areas in Europe were also more exposed to frost compared to continental ones (Ma *et al*., 2018). Besides, high-altitude areas could experience varying risk since the rate of warming seems to be amplified with elevation (Pepin *et al*., 2015). The effects of late frosts on the distribution of grapevine in Europe were analyzed (Leolini *et al*., 2018). The results, simulated under future scenarios, described in the AR5 IPCC report (2014), show that budbreak and flowering advancement are more pronounced in Northeastern Europe compared to the Southwest. The simulations showed that changes in the phenology stages of grapevine might expose it to higher frequency of extreme events, with the effects being strictly linked to the phenological cycle of the considered variety (Leolini *et al*., 2018). An increased risk of spring frost damage is also predicted in several regions of France, supported by two budburst day simulation models (Sgubin *et al*., 2018). Similarly, a high probability of spring frost damage for several woody species in Illinois (USA) was reported, by integrating field observations of temperature, phenology, and frost damage over long timeframes (Augspurger,

2013). “False spring” occurrences were reviewed across the USA over the 1920-2013 interval by taking into consideration the trends of vegetation start dates, spring freezes and a sensitivity analysis, which indicated a decrease of spring frost exposure (Peterson and Abatzoglou, 2014), pointing out distinct tendencies for different geographical locations.

3 LONG-TERM RESILIENCE TO CLIMATE CHANGE

3.1 Breeding approaches

Passive spring frost damage avoidance approaches are used preemptively and are suited to work on the long-term, and include breeding and selection of new fitter varieties (Liu and Sherif, 2019). Traditional breeding approaches have been successfully used in the past to select new cultivars with characteristics of economic interest, and in a perennial crop such as grapevine the entire traditional breeding procedure and evaluation process can take many years to be completed (Eibach and Töpfer, 2015). Since cultivated grapevines are propagated clonally to fix and maintain specific production parameters, somatic variations that can accumulate during clonal propagation are almost the only source of genetic diversity (Carbonell-Bejerano *et al*., 2017; van Houten *et al*., 2020), greatly lower than inter-varietal diversity (Roach *et al*., 2018). Clone collections exist and are available worldwide and represent a source that should be accessed to search for interesting genotypes (Duchêne, 2016). A possible adaptation for the current grape-growing areas should consist in the selection of varieties with a later ripening period; such varieties can be obtained from germplasm collections or through breeding processes (Duchêne, 2012).

Fruit trees must fulfill a chilling requirement to transition from endodormancy to ecodormancy, a phase of dormancy in which buds are responsive to growth-promoting conditions. The amount of chilling hours required to do so depends on the genotype, and genotypes that require less chilling have been shown to deacclimate earlier. In any case, the models describing winter chill accumulation are purely empirical or based on experiments in controlled conditions, and the physiological processes occurring in plants during winter are still poorly understood (Luedeling, 2011). The most popular chilling hours accumulation models estimate effective

chilling temperatures are to be included in the 0 - 7.2°C interval (Dokoozlian, 1999), although different models attribute varying effectiveness to specific temperatures or even negative impacts of higher temperatures on previously accumulated chill (Darbyshire *et al.*, 2011). The widely applied, and possibly most accurate, Dynamic Model also suggests that the same temperatures might have inconsistent effectiveness depending on which time of the season they are registered, making it difficult to transfer available information from one location to another (Luedeling, 2012). Even if cultivated grapevines are generally considered low-chilling-requiring species compared to other woody perennials, however chilling requirements can differ significantly in high- and low-chill varieties, and fast- or slow-burst phenotypes (Londo and Johnson, 2014). Production located at higher latitudes could benefit from the use of grapevines characterized by higher chilling requirements and slower budburst rates, which would allow lowering the risk of spring frost damage (Londo and Johnson, 2014). Wild grapevines presented a continuous range of chilling requirements and budburst rates, making them an interesting source of variability. In detail, *Vitis amurensis*, *Vitis labrusca* and *Vitis riparia* were classified as low-chill and fast-burst species, whereas *Vitis rupestris*, *Vitis aestivalis* and *Vitis vulpina* showed higher chilling requirements (> 1000 hours) and longer budburst timings (> 14 days). Different latitudes were also proposed as seemingly having an adaptive effect. In fact, North-distributed genotypes (*V. riparia*, *V. labrusca* and *V. amurensis*) were all classified as low-chill, fast-bursting species. On the contrary, southern varieties (*V. aestivalis*, *V. cinerea*, *V. rupestris* and *V. vulpina*) were all characterized by higher chilling requirements and slower budburst timings (Londo and Johnson, 2014).

Hybrid crosses were shown to allow lowering the deepest level of cold hardiness, although this could also introduce enhanced midwinter responsiveness in areas where climate warming produces mild winter temperatures (Londo and Kovaleski, 2019). Deacclimation rates were also observed to be much faster in wild varieties *V. riparia* and *V. amurensis*, commonly used by breeders to increase freezing-tolerance in cultivated varieties. This could contribute to increased risks of deacclimation during warmer winters and of spring frost damage (Kovaleski *et al.*, 2018). These phenomena could be explained by the evolutionary necessity of these varieties to develop rapidly during short growing seasons typical of their area of origin (Ferguson *et al.*, 2014). Paradoxically, this would make the varieties with the

deepest levels of cold hardiness also the most vulnerable to spring frost damage (Ferguson *et al*., 2014) and considering the observed advancement of spring phenology, winter-hardy varieties could display unwanted phenotypes. For these reasons, focusing breeding efforts on the production of delayed growth-start cultivars could be an alternative favorable approach. A prerequisite for this strategy is the gaining of a comprehensive understanding of the biochemical and molecular mechanisms responsible for dormancy establishment and release in grapevine buds.

Rootstocks are traditionally used to protect scions from soil-borne pests and to improve tolerance to various abiotic stresses, however their effects on the entirety of the plant often remain obscure (Ollat *et al*., 2016). The breeding of new rootstocks needs to be considered as a long-term strategy to cope with the consequences of climate change since the substitution of traditional scions with new ones is not going to be accepted as easily. The genetic background of commonly used rootstocks can be difficult to understand since their heritage is often mixed (Poczai *et al*., 2013), but efforts to improve breeding by enhancing the knowledge of genetic markers has been attempted in recent years (Riaz *et al*., 2019; Migliaro *et al*., 2019). This information is important and needs to be exploited to improve marker-assisted selection of new rootstocks, since their influence on scion signaling molecules, response to several stresses and even berry quality has been observed (Tramontini *et al*., 2013; Martin *et al*., 2020; Pagliarani *et al*., 2017; Zombardo *et al*., 2020). Moreover, rootstocks can alter scion development rate possibly due to their different abilities to take up nutrients and water from the soil (Zhang *et al*., 2016). Additionally, messenger RNA molecules and hormones have been reported to pass through the graft site in a possibly environment- and genotype-dependent manner (Yang *et al*., 2015; Nikolaou *et al*., 2000). Putative rootstock effects on grapevine phenology, and in particular on its heat requirements, have also been described (Miele, 2019).

A great boost in breeding effort can be attributed to the identification of molecular markers, the introduction of genetic mapping and genotype-phenotype associations, considerably facilitated by the release of the complete sequence of the *V. vinifera* genome (Jaillon *et al*., 2007; Velasco *et al*., 2007). MAS (Marker Assisted Selection) can help the identification of sequences with different genetic

backgrounds, aiding the potential exploitation of wild Vitis species carrying traits of interest (Daldoul *et al*., 2020).

3.2 Molecular mechanisms involved in deacclimation and budbreak

Monitoring dormancy status of the bud in real time appears really challenging, due to the absence of visual changes during the bud dormancy cycle (Or *et al*., 2009) and the use of GDD as a proxy for spring phenology is not always reliable. Therefore, a better knowledge base of the physiological mechanisms underpinning dormancy induction and release can be an important part of predicting the potential effects of global warming on grapevine. A strict correlation between budbreak and loss of winter cold hardiness (deacclimation) has been recently hypothesized, pointing out that a temperature-controlled interplay underpins these phenological changes (Kovaleski and Londo, 2019).

In this context, recent advances in the understanding of cold hardiness and spring budburst mechanisms may contribute to enhance the sustainability of viticulture, especially when acute cold weather events are expected to increase (Kovaleski and Londo, 2019). On the other hand, traditional breeding is also empirical and requires a deep knowledge of the physiological characteristics of the selected cultivars in past and present cultivated areas. Recently introduced molecular approaches allowed new methods of "molecular breeding" to be applied, allowing speedier and refined crosses (Delrot *et al*., 2020).

Unfortunately, phenological traits, such as budburst, are often regulated by many QTLs which are highly responsive to environmental factors. For this reason, the mapping and cloning of genes related to phenological traits is really challenging, and the reproducibility of these QTLs remains low (Delrot *et al*., 2020).

Recently, several works have identified QTLs associated with budbreak. For example, two independent QTLs on chromosomes 4 and 19 were identified using a genetic map build with microsatellites markers on varieties Riesling and Gewurztraminer (Duchêne *et al*., 2012). The WRKY transcription factor *VvWRKY3* was found within the confidence interval on chromosome 19; a similar transcription factor, *AtWRKY2* from Arabidopsis, was shown to mediate ABA (abscisic acid) control on seed germination (Jiang and Yu, 2009). Moreover, several genes encoding

glutathione S-transferases (GSTs) were also identified on both chromosomes 4 and 19. Increased levels of expression of these genes was registered after both HC (hydrogen cyanamide) application (Ophir *et al*., 2009), a dormancy-breaking agent, and after the natural fulfilment of chilling requirements (Pacey-Miller *et al*., 2003). Similarly, SSRs and SNPs were used to map another QTL related to budburst on chromosome 15, overlapping on QTLs related to véraison (Grzeskowiak *et al*., 2013). Genes on chromosome 15 included several transcription factors involved in bud and fruit development (Grzeskowiak *et al*., 2013).

With regard to cold hardiness control, the progeny resulting from the cross between cold-vulnerable cv. Cabernet Sauvignon and the cold-tolerant hybrid Zuoyouhong was used for the construction of a high-density genetic linkage map on which cold hardiness-related QTLs were mapped (Su *et al*., 2020). Six QTLs located on chromosomes 2, 3, and 15 were identified and four cold-responsive candidate genes were proposed. In detail, a dehydration-responsive protein containing a cis DRE (dehydration responsive) element was identified. CRT (C-repeat)/DRE elements, containing a core CCGAC sequence designated as C-repeat, are present in single or multiple copy in the promoter regions of plant COR (Cold-Responsive) genes which are induced by low temperatures exposure (Stockinger *et al*., 1997). The COP9 signalosome (CSN) subunit 1 was also individuated; CSN was shown to be required for the expression of COR genes in Arabidopsis (Schwechheimer *et al*., 2002). Additionally, an RRM (RNA Recognition Motif)-containing protein was found to be putatively involved in cold hardiness as well. RRM modules were found in cold-responsive RNA-binding proteins from cyanobacteria (Maruyama *et al*., 1999). Lastly, a MYB-related gene's expression was also reported to be enhanced by cold exposure. Its overexpression in Arabidopsis was previously shown to confer increased tolerance to cold (Sun *et al*., 2018).

Transcriptomic tools have led to new insights into the gene expression processes that take place in dormant tissues. Dormancy release is regulated by a multitude of independent genes whose mechanisms of action are still unclear, together with their conservation among species (Table 1). Growth resumption happens simultaneously with cold deacclimation, although most hardiness is already lost when new tissue is visible (Kovaleski and Londo, 2019). Growth start is also

subordinate to the fulfillment of the chilling requirement and the transition from endodormancy to ecodormany, in which the bud becomes sensitive to favorable environmental conditions. CBFs/DREBs (C-repeat Binding Factors/Dehydration Responsive Element Binding) are important cold response regulators stimulated by low temperatures. These transcription factors act as a part of a signaling cascade in which they are induced by ICEs (Inducers of CBF Expression) and activate *COR* genes by binding to the CRT/DRE *cis*-elements in their promoter regions and thus conferring freezing tolerance to the plant (Chinnusamy *et al*., 2006; Thomashow, 2010). Another cold-responsive transcription factor, bHLH, was characterized in both *V. vinifera* cv. Cabernet Sauvignon and wild *V. amurensis* with aproposed putative regulatory role in cold stress response in a CBF-dependent way (Xu *et al*., 2014). Changes in expression levels and timing of *VvbHLH* and *VabHLH* were observed, possibly caused by differences in the cis-regulatory elements in their sequence (Xu *et al*., 2014). CBFs/DREBs have been identified in several woody species as well as Arabidopsis and their functions are highly conserved (Wisniewski *et al*., 2014). Several CBFs/DREBs are known in grapevine (Xiao *et al*., 2006; Tillett *et al*., 2012; Rubio *et al*., 2019) and show increased mRNA expression following exposure to freezing temperatures (Xiao *et al*., 2006; Xiao *et al*., 2008). The most well-known targets of CBFs/DREBs are DHNs (dehydrins), part of the LEA (Late Embryogenesis Abundant) proteins. DHNs accumulate during dormancy induction and cold acclimation and protect cells from dehydration damage (Wisniewski *et al*., 2014). Four grape DHNs have been identified (Yang *et al*., 2012). *DHN*s were reported to be differently expressed in wild *Vitis riparia* and in cultivated variety Chardonnay following cold exposure (Xiao and Nassuth, 2006). Increased freezing tolerance is also observed in case of *VvCBF*s overexpression (Tillett *et al*., 2012). Moreover, the synergistic effect of low temperatures and ABA application in stimulating the expression of CBFs/DREBs in grapevine dormant buds has been recently assessed (Rubio *et al*., 2019a). ABA has a key role in plant dormancy regulation since ABA variations have been correlated to different degrees of seed dormancy (Nambara *et al*., 2010). ABA's role in bud dormancy in woody perennials has been hypothesized, although the regulation mechanism is complex and is still obscure. Recently, several studies showed that the highest levels of ABA were reached at the maximum depth of dormancy and started decreasing at the end of endodormancy in grapevine buds

(Kovaleski and Londo, 2019; Rubio *et al*., 2019b). ABA was also observed to promote starch synthesis in dormant buds, thus promoting their sink capacity and regulating dormancy depth this way (Rubio *et al*., 2019b). Changing ABA balance in the buds is also the mechanism by which dormancy-breaking agents, such as HC, seem to accomplish their effect (Zheng *et al*., 2015; Rubio *et al*., 2019b). In detail, the budbreaking effect of HC in grapevine was reported to be exerted by the stimulation of the ABA-degrading enzyme ABA 8'-hydrolase (A8H), encoded by the *VvA8H-CYP707A4* gene (Zheng *et al*., 2015). A8H and ABA catabolites increase was also observed during natural dormancy release (Zheng *et al*., 2015). Moreover, the reversible ability of ABA to prevent loss of cold hardiness and deacclimation after several days of prolonged application on grapevine buds was observed (Kovaleski and Londo, 2019). Together these results suggest an important role of ABA in endodormancy maintenance and dormancy release, but not in its induction. More recent studies showed that transgenic vines overexpressing *VvA8H-CYP707A4* show both a higher catabolism of ABA as well as an enhancement of budbreak. Hypoxia and ethylene, which are both considered dormancy release stimulants, enhance the expression of *VvA8H-CYP707A4* (Zheng *et al*., 2018a). Multiple studies have shed light on the role of other hormones in dormancy release and budbreak; for example, a recent work focused on the expression of several genes involved in the gibberellins (GA) biosynthetic pathway and the interaction of GAs with cytokinins (CK) in grapevine buds (Zheng *et al*., 2018b). Although further studies are required, the authors propose an inhibitory effect of GA on budbreak that would give account of the low levels of this hormone registered during dormancy. Authors also hypothesize that this inhibition results from the antagonistic effect of GAs on CK responses, which are required for bud meristem reactivation; only following meristem activation higher levels of GA could be required to sustain growth and budbreak (Zheng *et al*., 2018b). In addition to this, the effects of cold temperatures on the concentration of salicylic acid (SA) and the expression of genes in its biosynthetic pathway in dormant grapevine buds were also explored (Orrantia-Araujo *et al*., 2020). Buds exposed to longer periods of chilling hours showed a higher content of endogenous SA once transferred in forcing conditions. The expression of genes *ICS2* (Isochorismate Synthase 2), *NPR1* (Non-Expressor of PR genes 1) and *WRKY70* showed variations in buds subjected to cold treatment compared to control ones. ICS2 takes part in the

biosynthesis pathway of SA, NPR1 is a master regulator of SA-mediated defense signaling, and WRKY70 participates in both positive and negative regulation of SA signaling. These results indicate that cold accumulation could stimulate the synthesis of SA in grapevine buds, and introduce the possibility of a role of SA-mediated defense signaling in bud dormancy release (Orrantia-Araujo *et al*., 2020).

The discovery and characterization of the EBB1 gene with a role in shoot growth resumption after winter has been carried out both in Populus (Yordanov *et al*., 2014) and in peach, where RNA-seq analysis confirmed that EBB1 is involved in budbreak by taking part into the regulation of several pathways that act synergistically and involve hormones, cell division, and cell wall modifications (Zhao *et al*., 2020). The conservation of this AP2/ERF family transcription factor was evidenced by the identification of several homologs in various woody perennial species among which also *V. vinifera* (Busov *et al*., 2016). Consistently with the *EBB1* expression in Poplar, *VvEBB1* resulted greatly downregulated during dormancy and upregulated before budbreak.

It is well-known that genomic DNA methylation is a mechanism that influences gene expression. In plants a subgroup of DNA glycosylase-lyases, known as DEMETER-LIKE DNA demethylases (DMLs), can actively demethylate DNA and have been shown to be involved in abiotic stress responses in Arabidopsis (Le *et al*., 2014), developmental transitions in tomato (Liu *et al*., 2015) and nodule development in *Medicago truncatula* (Satgé *et al*., 2016). A *Populus trichocarpa* DML, PtaDML10, was proposed to be responsible for DML-mediated demethylation at the shoot apical meristem (SAM) in budbreak regulation (Conde *et al*., 2017). A loss of function analysis confirmed the chilling-responsive demethylation performed by DML10 in proximity to dormancy release. RNA-seq combined with methylome data analysis revealed that the DML10 gene targets are genetically associated with budbreak (Conde *et al*., 2017). Moreover, no overlap was found between the targets of DML10-mediated demethylation and EBB1 targets in poplar. This seemingly confirms that these genes act on separate pathways (Conde *et al*., 2017). No evidence on the role of DML genes on grapevine dormancy release currently exists, although several DML demethylases have been identified (Shangguan *et al*., 2020).

Table 1. Genes with putative involvement in cold deacclimation and budbreak regulation.

Gene	Physiological role	Reference
CBFs/DREBs	Low temperatures response	Xiao *et al*., 2006
bHLH		Tillett *et al*., 2012
		Xu *et al*., 2014
		Rubio *et al*., 2019
		Su *et al*., 2020
VpERF2		Zhu *et al*., 2013
VpERF3		Gibbs *et al*., 2014
VvA8H	ABA regulation	Duchêne *et al*., 2012
VvWRKY3		Zheng *et al*., 2015
		Zheng *et al*., 2018a
VvICS2	Defense mechanisms	Zheng *et al*., 2018b
VvNPR1		Orrantia-Araujo *et al*., 2020
VvWRKY70		
VaCPK20	Ca^{2+} transport	Dubrovina *et al*., 2013
CNGCs		Kovaleski and Londo, 2019
FAD5	Membrane fluidity	Kovaleski and Londo, 2019
GSTs	Hypoxia response and oxidative stress	Duchêne *et al*., 2012
ERF-VIIs		Grzeskowiak *et al*., 2013
RBOHF		Meitha *et al*., 2018
		Kovaleski and Londo, 2019
EBB1	Growth resumption	Busov *et al*., 2016
DMLs	Chilling-responsive demethylation	Conde *et al*., 2017
		Shangguan *et al*., 2020

Additionally, regulated hypoxia has been found to be a development signal in several stages of plant life (Gibbs *et al*., 2014; Abbas *et al*., 2015) and many responses to hypoxia are regulated by group VII of ETHYLENE RESPONSIVE TRANSCRIPTION FACTORS (ERF-VIIs) (Gibbs *et al*., 2014). For these reasons, the role of oxygen-dependent signaling in transcriptional and metabolic reactivation during budburst in grapevine was investigated (Meitha *et al*., 2018). The data support that oxygen-

dependent signaling through grape ERFs is involved in the transition from dormancy to budburst. Moreover, approximately 20% of grapevine genes presenting a HRPE (hypoxia-responsive promoter element)-motif in their promoter were differently expressed in the first 24 hours of budburst (Meitha *et al*., 2018). These results strongly suggest an important developmental function of oxygen-dependent signaling through VvERF-VIIs in determining timing and coordination of budburst in grapevines. Further support of the role of oxidative stress response pathways in grapevine budbreak regulation, is provided by Kovaleski and Londo (2019) proposing the expression of RBOHF (RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN F) as a marker for budbreak. RBOHF is involved in ABA and ethylene signaling through H2O2 production (Kwak *et al*., 2003). In addition to this, two ERF genes from Chinese wild *Vitis pseudoreticulata*, *VpERF2* and *VpERF3*, were reported to be involved in abiotic stress response pathways including cold exposure (Zhu *et al*., 2013). Overexpression studies also pointed out a role of these transcription factors in pathogenesis-related proteins accumulation. Moreover, ABA-dependent expression of *VpERF2* and SA-dependent expression of *VpERF3* was shown through exogenous hormone application on leaves (Zhu *et al*., 2013).

Recently, dormant buds of several Vitis genotypes, belonging to different species, were observed to sense the stimulus for dormancy release and deacclimation simultaneously when put into the same forcing conditions (Kovaleski and Londo, 2019). The observed differences in budbreak timings would then be attributed to the ability of the specific genotypes to restart growth. In fact, temperature sensing is believed to be the first step towards bud growth. Among the first sensors, membrane CNGCs (Cyclic Non-Gated Ion Channels) are very responsive to temperature changes. These non-selective Ca^{2+} channels are placed as very first components of the thermosensing pathways in Arabidopsis and Physcomitrella (Finka *et al*., 2012) and possibly have the ability to sense membrane fluidity changes caused by temperature shifts (Finka and Goloubinoff, 2014). Synchronous downregulation of nuclear-localized *CNGC15* and *FAD5* (Fatty Acid Desaturase 5) was reported, suggesting a role of nuclear Ca^{2+} signaling during dormancy in grapevine buds (Kovaleski and Londo, 2019). A role in cold and water stress response of Ca^{2+} flux sensor VaCPK20 (Calcium-dependent Protein Kinase) from wild *V. amurensis* vines was also suggested (Dubrovina *et al*., 2013).

4 CONCLUDING REMARKS

Spring frost damage risk cannot be overlooked in the future in several areas of the world, making the identification of effective adaptive measures an issue of the present. Understanding the molecular mechanisms underlying cold hardiness loss/deacclimation and budbreak is essential for improving crop sustainability and adaptation in the future changing climate. The observations gathered so far on cold deacclimation and dormancy release regulation in grapevine outline a very complex scenario in which many pathways are involved (Figure 1).

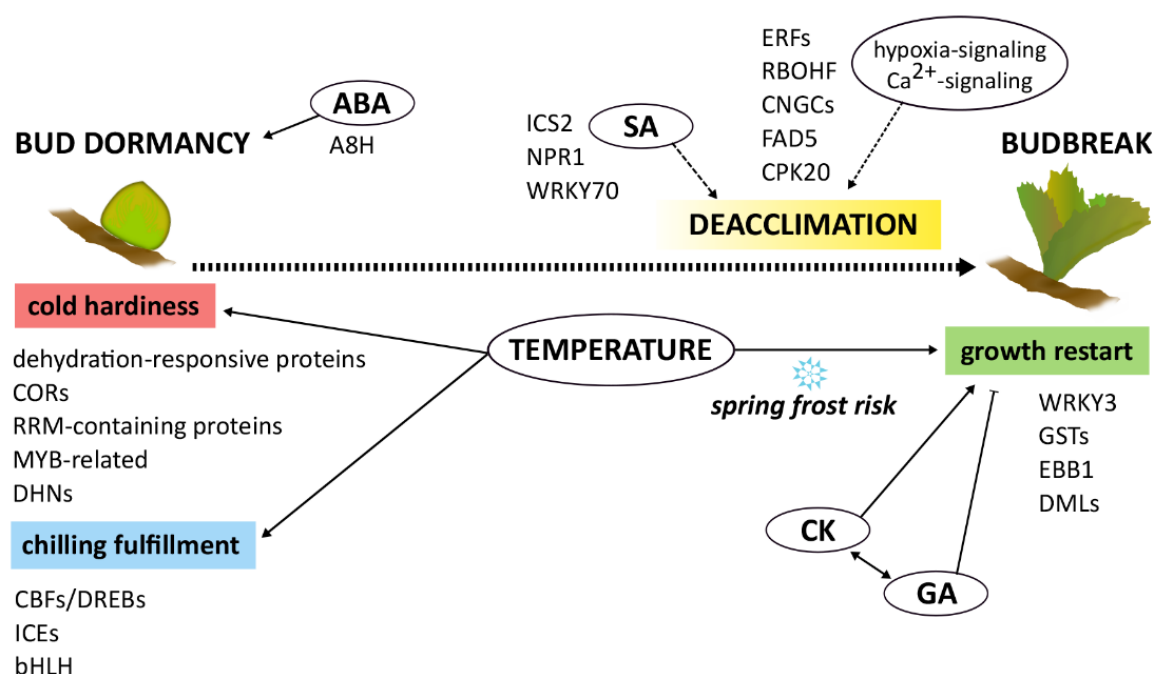

Figure 1. Schematic representation of the current knowledge on the molecular control of bud dormancy-budbreak transition. Temperature plays a key role in influencing both phenological stages. Most of the gene functions involved at each phenological stage are reported, as well as their interplay with other metabolic and hormonal signaling pathways.

Since chilling requirement, deacclimation dynamics and budbreak timing appear tightly connected, a major regulatory role can be ascribed to temperature-sensing related genes, common among different genotypes. Hormonal interplay, at times synergistic as well as antagonistic or seemingly independent, should also draw great attention since not only ABA's expected involvement seems ascertained, but also growth reactivation-related, defense-related and oxidative stress-related hormones putatively perform actively in the regulation of these phenomena. A third

valuable and worthy of notice opportunity concerns epigenetics and epigenetic regulators, which add an extra layer of complexity. Defining the extent of the role and significance of each component of this intricate net of regulators requires further studies.

Breeding efforts need to focus on the potential of wild Vitis varieties to bear peculiar traits, starting from changing chilling requirements and budburst rates. In this regard, the accuracy of all most popularly used chilling hours accumulation models needs to be standardized in order to select varieties suitable to changing conditions is specific areas. An intense application of genetic mapping approaches is required to locate and isolate the genetic loci that are responsible for the phenotypic expression of these characteristics so that traditional or new plant breeding techniques can be carried out more swiftly and purposefully (Figure 2). Despite the complexity of the full picture and the uncertainties about the connections among the players, the variety of elements involved allows to tackle the problem through a multitude of approaches and should be considered encouraging.

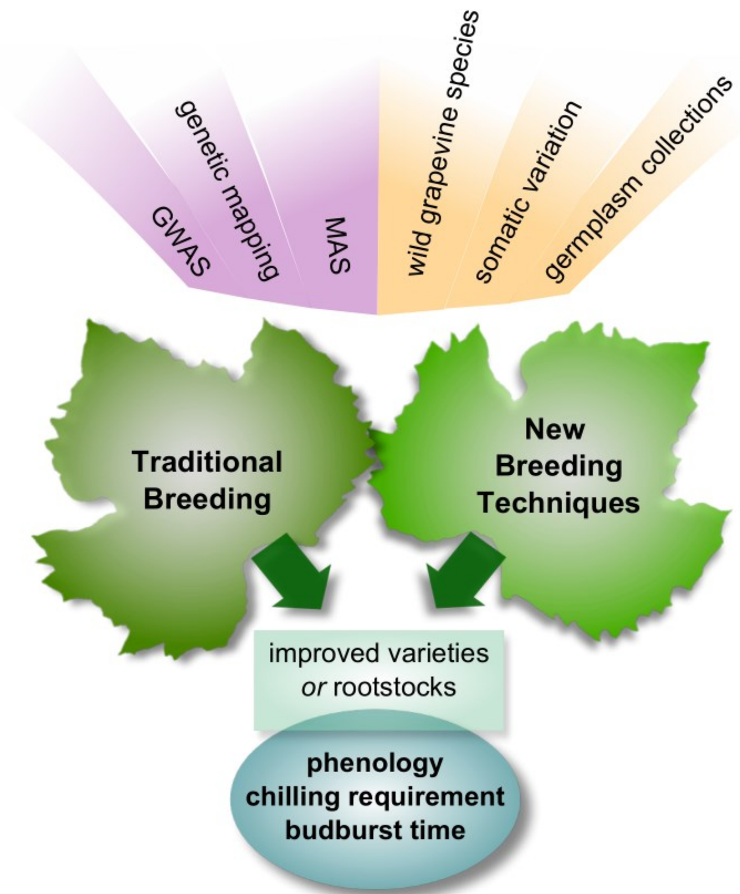

Figure 2. Schematic overview of traditional and new breeding approaches to cope with climate change issues. Natural variability and genetic knowledge are important building blocks of breeding; phenology-related traits are the main target. GWAS, genome-wide association studies; MAS, Marker Assisted Selection.

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♦ CHAPTER II

Aim of the study

A SUSTAINABLE FUTURE

Available information in the literature regarding pathways and regulatory networks involved in freezing tolerance, chilling requirement, cold deacclimation and dormancy release highlighted that the identification of suitable targets to genetically improve grapevine's tolerance to spring frost damage is no easy task.

To address this challenge, the phenological behavior of dormant buds of grapevine cultivars characterized by different developmental parameters was examined. Moreover, hybrid crosses with wild *Vitis* species have also been included to check the presence of favorable traits.

In detail, two differently cold-resistant hybrids were firstly chosen as object of study (Figure 1):

cv. Fleurtai (Friulano × Kozma 20-3);

selection UD 31-103 (Merlot × Kozma 20-3).

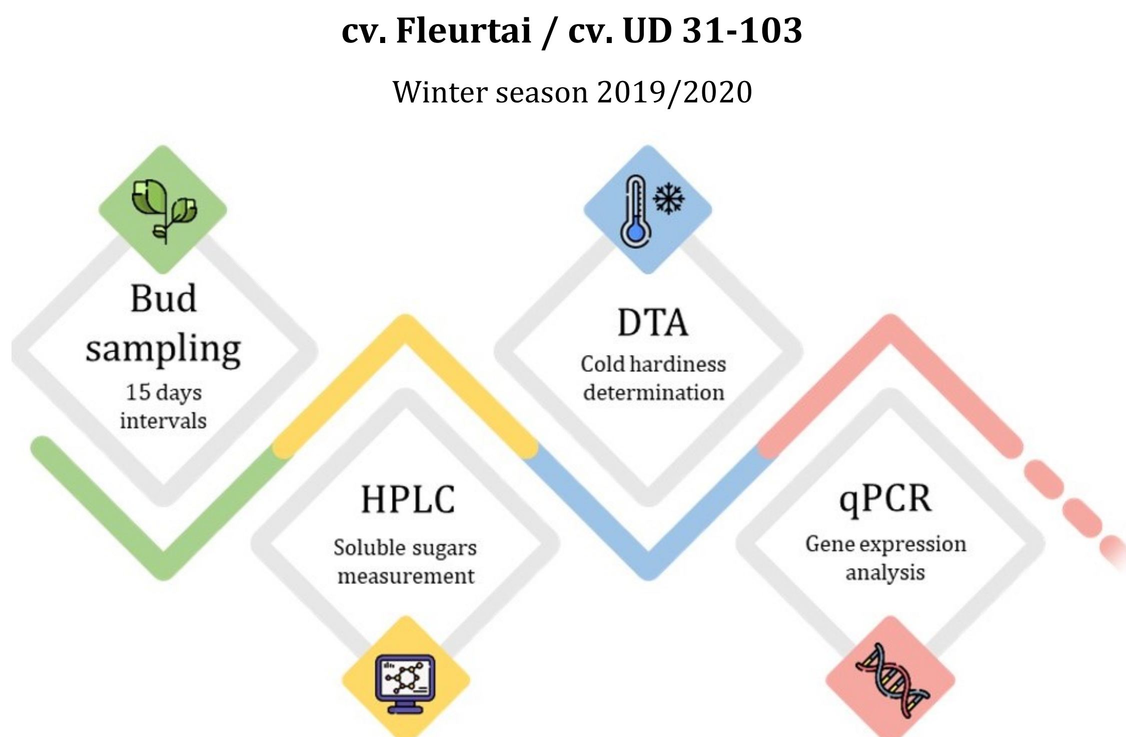

Figure 1. Schematic view of the experiments performed in order to characterize two differently cold-tolerant Vitis varieties, cv. Fleurtai and cv. UD 31-103 (field-grown plants). Icons: Flaticon.com.

Hybrids Fleurtai and UD 31-103 are a white- and red-berry variety, respectively, connected by a common parent resulting from a Vitis interspecific crossing. Cv. Fleurtai is characterized by good levels of winter hardiness with resistance up to -23°C, whereas UD 31-103 is sensitive to winter temperatures below -20°C. Both genotypes were produced in the context of a breeding program aimed at the selection of powdery mildew- and downy mildew-resistant cultivars, capable of reducing pesticides use and improving viticulture sustainability (Foria *et al*., 2019).

Secondly, two cultivars were selected to study dormancy release regulation based on their greatly different budbreak precocity (Figure 2):

cv. Sauvignon Nepis (Sauvignon Blanc × Bianca);

cv. Cabernet Sauvignon (Cabernet Franc × Sauvignon Blanc).

Cv. Sauvignon Nepis, a disease-resistant white berry variety, is an early-budbreak cultivar, in contrast to the globally renowned cv. Cabernet Sauvignon which is a late-budbreak variety.

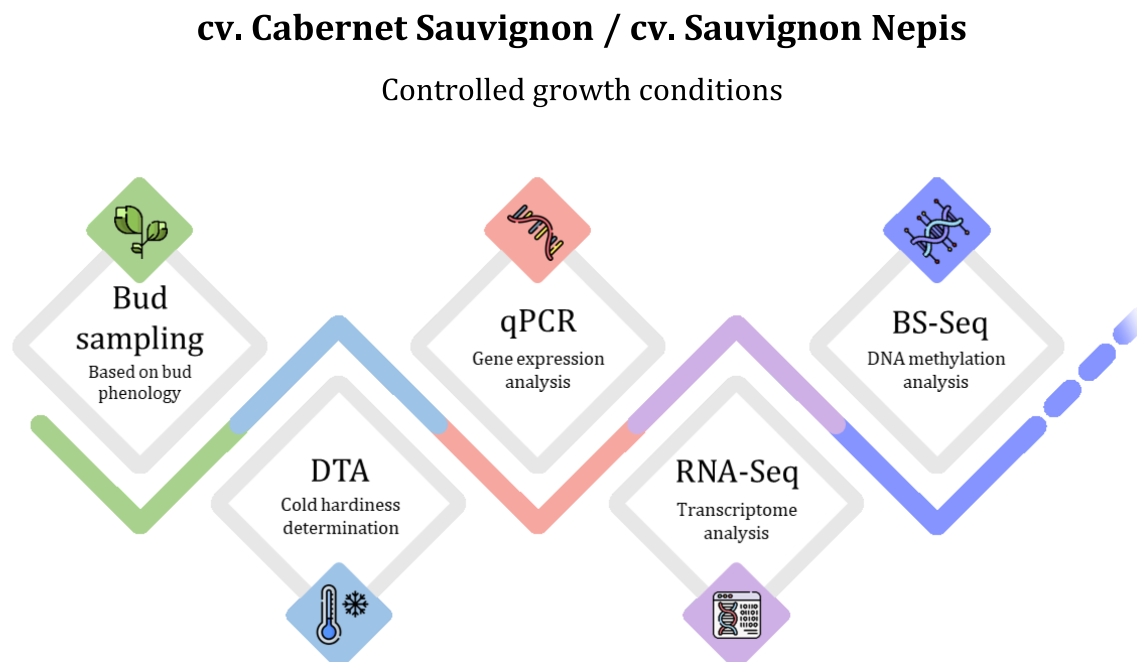

Figure 2. Schematic view of the experiments performed in order to characterize two *Vitis* varieties distinguished by different budbreak timings, cv. Cabernet Sauvignon and cv. Sauvignon Nepis (potted plants). Icons: Flaticon.com.

In this thesis, a multidisciplinary approach was adopted (Figure 1, 2) to elucidate bud dormancy regulation in its many facets, and answer a few of the many questions that the literature poses (Figure 3).

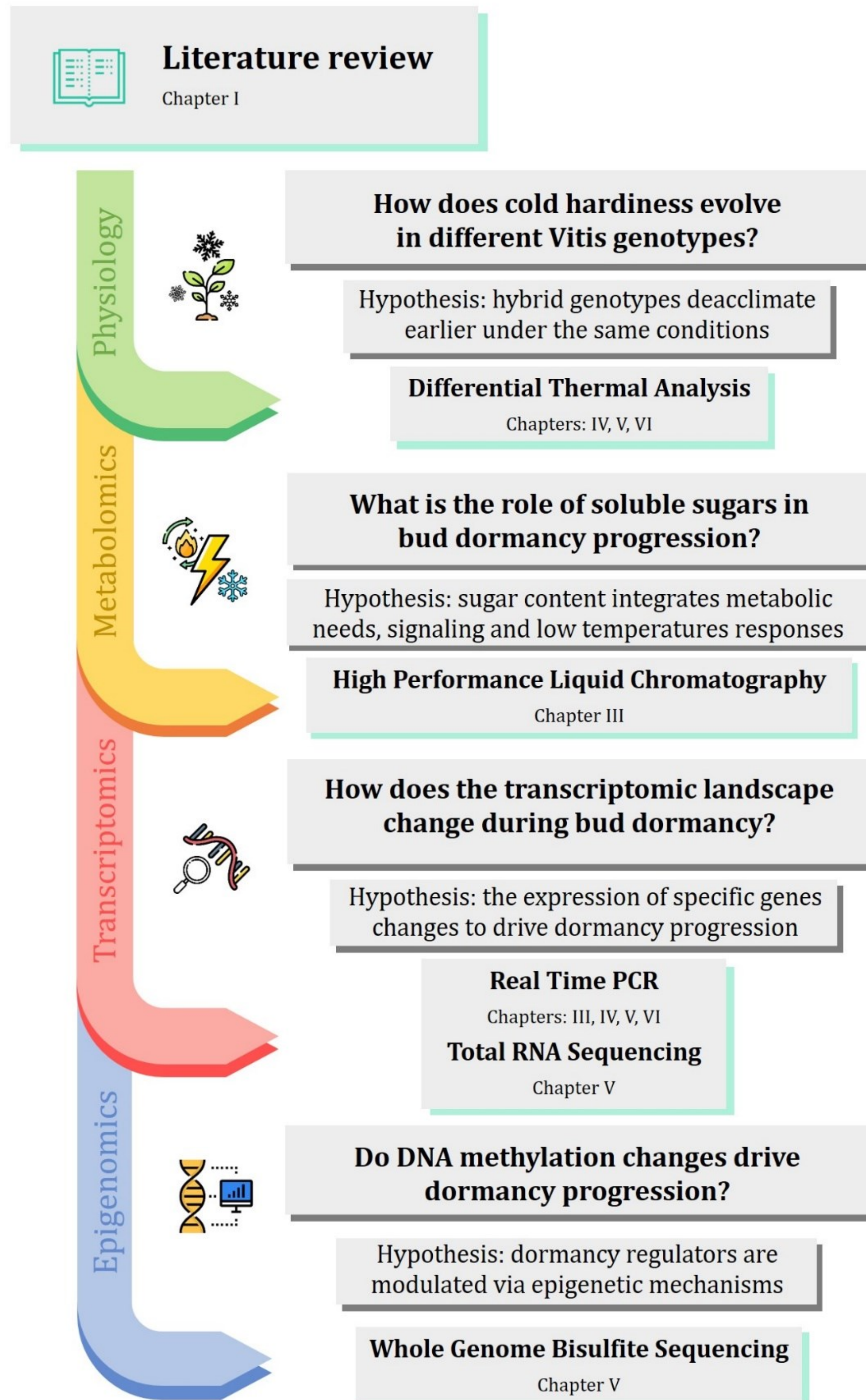

Figure 3. Logic flow of the multidisciplinary approach adopted in this thesis. Icons: Flaticon.com

Understanding the principles of dormancy progression is key to maintain grapevine productivity in the face of a changing climate, in a sustainable way.

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♦ CHAPTER III

Characterization of sugar metabolism in Vitis hybrids Fleurtai and UD 31-103 during dormancy progression

INTRODUCTION TO THE STUDY

Non-structural carbohydrates have been found to be fundamentally connected to many aspects of plant development and stress tolerance. In the context of grapevine dormant buds, the osmoprotectant role of glucose, fructose, sucrose and raffinose accumulation, participating in winter cold hardiness acquisition, has been documented multiple times. Particularly interesting is the role of raffinose, which has been found to be most correlated to freezing tolerance. Besides, soluble sugars represent a substrate for bud baseline metabolism throughout dormancy progression and are connected to growth resumption following dormancy release. Non-structural carbohydrates are players involved in signalling pathways that integrate internal and external stimuli driving different developmental stages or stress responses. The dynamics of sugar signalling in dormancy progression are rather obscure, and the multiple roles interpreted by these molecules make it difficult to shed light on this specific aspect.

Based on these premises, we analysed the soluble sugar content in hybrid cultivars Fleurtai and UD 31-103, characterized by different tolerances to winter minima, throughout an entire winter season. We focused our attention on dehydration-responsive hexose transporters *VvHT1* and *VvHT5*. Our evidence suggests VvHT5 could participate in tolerance to physiological water reduction by supporting signalling pathways connected to stress adaptation and recovery. We also present, to our knowledge, a first example of *VvMSA* expression in grapevine buds. *VvMSA* is the only member of ASRs (*ABA- Stress- and Ripening-induced*) proteins in grapevine. A double regulation by sugars and abscisic acid, both highly relevant in dormancy contexts, has been previously documented in grapevine tissues, making *VvMSA* an interesting target. Our data suggest a possible role for VvMSA in dormancy release.

Preliminary results were first presented in poster form at the XIII Giornate Scientifiche SOI in June 2021 in Catania (Italy). Final results are available in the article '*Insight into Carbohydrates Metabolism and Signaling in Grapevine Buds during Dormancy Progression'*, recently published by journal *Plants* of MDPI.

Role of non-structural carbohydrates in grapevine buds during dormancy progression[2]

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Abstract

Perennial fruit crops enter dormancy to ensure bud tissue survival during winter. However, a faster phenological advancement caused by global warming exposes bud tissue to higher risk of spring frost damage. Tissue dehydration and soluble sugars accumulation are connected to freezing tolerance, but non-structural carbohydrates act also as metabolic substrates and signaling molecules. A deepened understanding of sugar metabolism in the context of winter freezing resistance is required to gain insight into adaptive possibilities to cope with climate changes. In this study, soluble sugar content was measured in grapevine hybrid cultivars throughout a winter season. Moreover, the expression of drought-responsive *VvHT1* and *VvHT5*, *VvRS* and *VvMSA* was analyzed. Results suggest a differential role of sugars in short-term cold response in the two cultivars, with raffinose being the most correlated to freezing tolerance. *VvHT5* expression suggests a potential involvement of VvHT5 in tolerance to physiological water reduction related to cold hardiness, or resistance to freezing-induced dehydration. The first evidence of *VvMSA* expression in grapevine buds allows to speculate a role in bud phenological transition towards budbreak. The multifaceted role of sugars on the intricate phenomenon, which is the response of dormant buds to changing temperature, is discussed.

[2] The finalized version of part of the results presented in this chapter was recently published by *Plants* of MDPI (De Rosa *et al.*, 2022 - *Plants*. 11:1027, doi: 10.3390/plants11081027).

1 INTRODUCTION

Woody perennials have developed several adaptive measures to endure yearly temperature fluctuations, such as bud dormancy and cold hardiness acquisition, to survive winter freezing conditions (Wisniewski *et al*., 2014). In the context of climate change, the observed increase of average surface temperatures causes and acceleration of plant phenology progression, exposing vulnerable green bud structures to higher risk of late frost damage (IPCC, 2021). This is especially true for grapevine, whose development rate is very sensitive to temperature variations (Alikadic *et al*., 2019). In several perennial species non-structural carbohydrates have a crucial role in budbreak process (Tixier *et al*., 2017; Perez and Noriega, 2018; Tixier *et al*., 2019), but they are also required for basal metabolism during winter dormancy (Bonhomme *et al*., 2005; Zwieniecki *et al*., 2015; Beauvieux *et al*., 2018). Moreover, soluble sugars are tightly connected to plant tolerance to cold temperatures (Jiang *et al*., 2014, Ershadi *et al*., 2016), due to their role as osmolytes and cryoprotectants, by reducing ice nucleation within the apoplast and limiting freezing-induced dehydration (Grant and Dami, 2015). Intracellular ice formation results not only in water subtraction but also in mechanical stress for the plasma membrane, likely lethal for cells (Yamazaki *et al*., 2009). In grapevine, variations of concentration levels of several sugars such as sucrose, glucose, fructose, raffinose and stachyose have been associated with freezing tolerance (Fennell, 2004; Pedryc *et al*., 2004; Badulescu and Ernst, 2006; Grant and Dami, 2009; Jiang *et al*., 2016; Grant and Dami, 2015). In particular, raffinose has been shown to be the most connected to cold resistance, and was shown to accumulate earlier in cold-tolerant cultivars compared to cold-sensitive ones (Grant and Dami, 2015). Moreover, freezing tolerance enhancement has also been associated with bud water content reduction (Fennell, 2004).

Monosaccharides are delivered to sink tissues, such as buds, by hexose transporters (HTs). Fifty-nine putative grapevine HTs have been identified using protein sequences of Arabidopsis monosaccharide transporters (Afoufa-Bastien *et al*., 2010). Only six HTs (VvHT1 to VvHT6) have been functionally studied and three of these, namely VvHT1, VvHT4 and VvHT5, are located on the plasma membrane (Vignault *et al*., 2005; Hayes *et al*., 2007). VvHT1 and VvHT5 were characterized as high affinity H^+-dependent glucose transporters (Vignault *et al*., 2005; Hayes *et al*.,

2007) and were shown to be responsive to water stress, with *VvHT1* being downregulated and *VvHT5* upregulated (Medici *et al*., 2014). *VvHT5* is also considered a general stress response-related gene, possibly due to its role in enhancing sink strength under stress conditions (Hayes *et al*., 2010, Medici *et al*., 2014]. In this regard, VvHT5 regulation by abscisic acid (ABA) signaling has been proposed (Medici *et al*., 2014). VvHT1 was recently shown to be a target of VvMSA protein (*Maturation, Stress, ABA*), the only identified member of ASRs (*ABA-, stress- and ripening-induced*) in grapevine, hypothesized to act as a transcriptional regulator connecting sugar and ABA signaling (Çakir *et al*., 2003; Saummoneau *et al*., 2012), although many aspects of its action remain to be elucidated. ASR proteins have recently sparked interest because of their role as transcriptional regulators, suggested by their DNA-binding activity and nuclear localization (Çakir *et al*., 2003), and as candidates for direct protein protection due to their hydrophilic nature (Battaglia *et al*., 2008). A role in plant response to various environmental cues is strongly suggested by ASR induction following several stresses (Maskin *et al*., 2001; Hsu *et al*., 2011; Park *et al*., 2020; Lin *et al*., 2021). An interaction of VvMSA and a dehydration-responsive element-binding protein, named VvDREB, mostly involved in osmotic stress and dehydration responses, was also observed in the nucleus of grape cells (Saummoneau *et al*., 2008). Surprisingly, to our knowledge, no data on *VvMSA* expression and interactions in buds are available.

Wild grapevine species are typically more cold-hardy compared to cultivated *Vitis vinifera*. However, they are also early in terms of budbreak timing, which paradoxically puts them at a higher risk of spring frost damage, with differences depending on cultivar (Kovaleski and Londo, 2019). In fact, for putative evolutionary reasons, deacclimation was observed to proceed much faster in wild species such as *Vitis riparia* and *Vitis amurensis*, routinely used by breeders to introduce resistant phenotypes in *V. vinifera*. As a better strategy for spring frost avoidance, breeding efforts should focus on the production of late-budbreak varieties instead of cold hardy ones. Increasing knowledge on dormancy regulation and its release is fundamental for these purposes (De Rosa *et al*., 2021).

On these premises, this work aims to explore soluble sugars metabolism in the context of low temperature-induced responses during endo- and ecodormancy in

buds of two Vitis hybrids characterized by different winter minima tolerance. This investigation will provide new insights to deepen our understanding of the regulation of dormancy and budbreak phenology, paving the way for effective frost mitigation strategies.

2 MATERIALS AND METHODS

2.1 Plant material

Two grapevine interspecific hybrids, characterized by different resistance to winter freezing temperatures were selected: white cv. Fleurtai (Friulano × Kozma 20-3), tolerant to minima of -23°C, and red selection UD 31-103 (Merlot × Kozma 20-3), tolerant to minima of -20°C. Field-grown plants were located at the Experimental Farm "A. Servadei" (46° 04′ N 13° 14′ E, University of Udine, Northern Italy). During the 2019-2020 winter season, buds were regularly collected at 9-10 AM every ~15 days from October to March, in proximity to budbreak. Buds were stored at -80°C for gene expression analysis and soluble sugars measurements.

2.2 Soluble sugars extraction

Soluble sugar extraction was performed based on a previously tested protocol (Grant *et al*., 2009). For each biological replicate, 10 buds were ground in liquid nitrogen and subsequently freeze-dried for 72 h. Forty ± 5 mg of ground sample powder were moved to 2 mL tubes, and 1 mL of 75% ethanol (v/v) at room temperature was added for incubation of 3 h. Samples were continuously shaken during incubation and vortexed at maximum speed for 1’ every 30’. After 5’ centrifugation at 6700 x g, supernatants were collected and dried inside a centrifugal vacuum concentrator. All steps were repeated twice for each sample.

2.3 HPLC analysis

Soluble sugars were separated using a 250 mm long Ultra Amino column (Restek S.r.l., Cernusco sul Naviglio, Italy) with 4.6 mm internal diameter and 5 µm particle size, equipped on a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) with autosampler, quaternary pump and refractive index detector.

Acetonitrile 70% was used as mobile phase (1 mL·min^{-1}). Oven and detector were set at 30°C. Before injection, dry soluble sugars extracts were added 300 µL of mobile phase and thoroughly mixed using a vortex for 30''. To ensure complete sample solubilization, sonication in ultrasound bath was performed for 5'. Finally, 50 µL of sample were injected following filtration.

Standard solutions of glucose, fructose, sucrose and raffinose (Sigma-Aldrich, St. Louis, MO, USA) were used for sugar detection and quantification. Calibration curves were constructed injecting each sugar standard at concentration ranges: 25000 – 25 µg/mL glucose, 20000 – 25 µg/mL fructose, 25000 – 25 µg/mL sucrose, and 1990 – 7.5 µg/mL raffinose. Sugar quantification was calculated from peak area using Agilent OpenLab CDS ChemStation Edition (Version C.01.03) software (Agilent Technologies).

2.4 Gene expression analysis

For each sampling time, RNA extraction was performed from 3 biological replicates of 10 buds using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized with QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany), and real-time PCR was carried out with SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) as described in Sivilotti *et al.*, 2017. Primers used to detect gene expression were either found in the literature or built with Primer-BLAST tool on NCBI (Table 1). Statistical analyses were performed using SigmaPlot 12.0 (https://systatsoftware.com/).

Table 1. List of primers used for qPCR analysis. *VvMSA* = Maturation, Stress, ABA; *VvHT1* = Hexose Transporter 1; *VvHT5* = Hexose Transporter 5; *VvRS* = Raffinose Synthase.

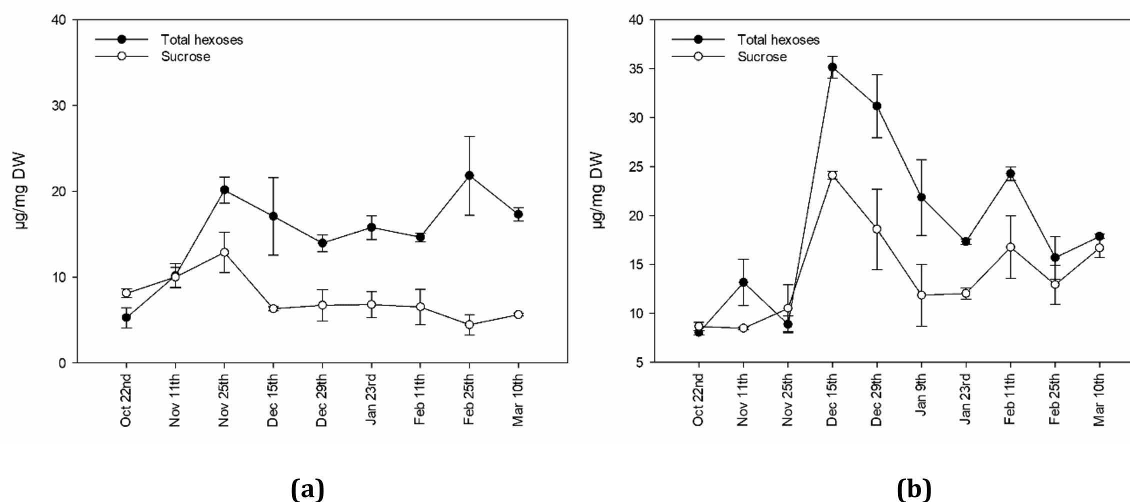
Gene target		Primer sequence (5'-3')	Accession
VvHT5[1]	F	CTTTCCATGCTTTGCCATTT	Vitvi05g00468
	R	ACCAATGCTTCTTCCACACC	
VvHT1[2]	F	TCAACGATGGTTCTTACAGC	Vitvi10g00358
	R	AACCGATAGTATTGTATTCG	
VvINV[3]	F	GCAGCAGAAATGGGGTTGAA	Vitvi04g00094
	R	AGGTATCGGTTTCAGGCACA	
VvMSA[4]	F	GCATGTGTGCTTGTTGTGTAA	Vitvi18g02973
	R	TCACAAGGACACACAGAGAGA	
VvRS	F	CTCTCCCGGGGAAATCTGTT	Vitvi14g01717
	R	GATCTTGGTTTCTCGGCTGC	

[1]Pérez and Noriega, 2018; [2]Afoufa-Bastien *et al.*, 2010; [3]Liang *et al.*, 2019; [4]Parrilla, 2015

3 RESULTS

3.1 Soluble sugars accumulation dynamics

Total hexoses and sucrose content were successfully detected and quantified by HPLC analysis in buds of hybrids Fleurtai (Figure 1a) and UD 31-103 (Figure 1b) throughout the 2019-2020 winter season. Additionally, raffinose concentrations were also analyzed, although it appeared to be stably less concentrated, in all samples, as compared to sucrose and hexoses (Figure 2).



(a) **(b)**

Figure 1. Accumulation dynamics of total hexoses (•) and sucrose (°) in buds of hybrids Fleurtai (**a**) and UD 31-103 (**b**) throughout the 2019-2020 winter season. Results are expressed as mean of 3 biological replicates of 10 buds ± standard error.

In cv. Fleurtai (Figure 1a) total hexoses' content appeared more stable as compared to selection UD 31-103 (Figure 1b), and overall present in lower concentrations. Nevertheless, two tendential increases in their accumulation were measured in late November and late February, whereas the lowest total hexoses content was registered in late October, and from the end of December until the first half of February. As for sucrose dynamics, its levels started increasing in late October up to the second half of November, representing the highest concentration of the disaccharide measured throughout the season. Thereafter, sucrose levels decreased in the first half of December and remained stable in all other measurements. In selection UD 31-103, total hexoses and sucrose variations appeared more pronounced as compared to cv. Fleurtai, exhibiting the same pattern of accumulation. In detail, all sugars sharply peaked on December 15th reaching seasonal maximum levels. Following this, sugar levels were observed to gradually decrease up to the

second half of January. A second peak, smaller than the first, was detected on February 11th.

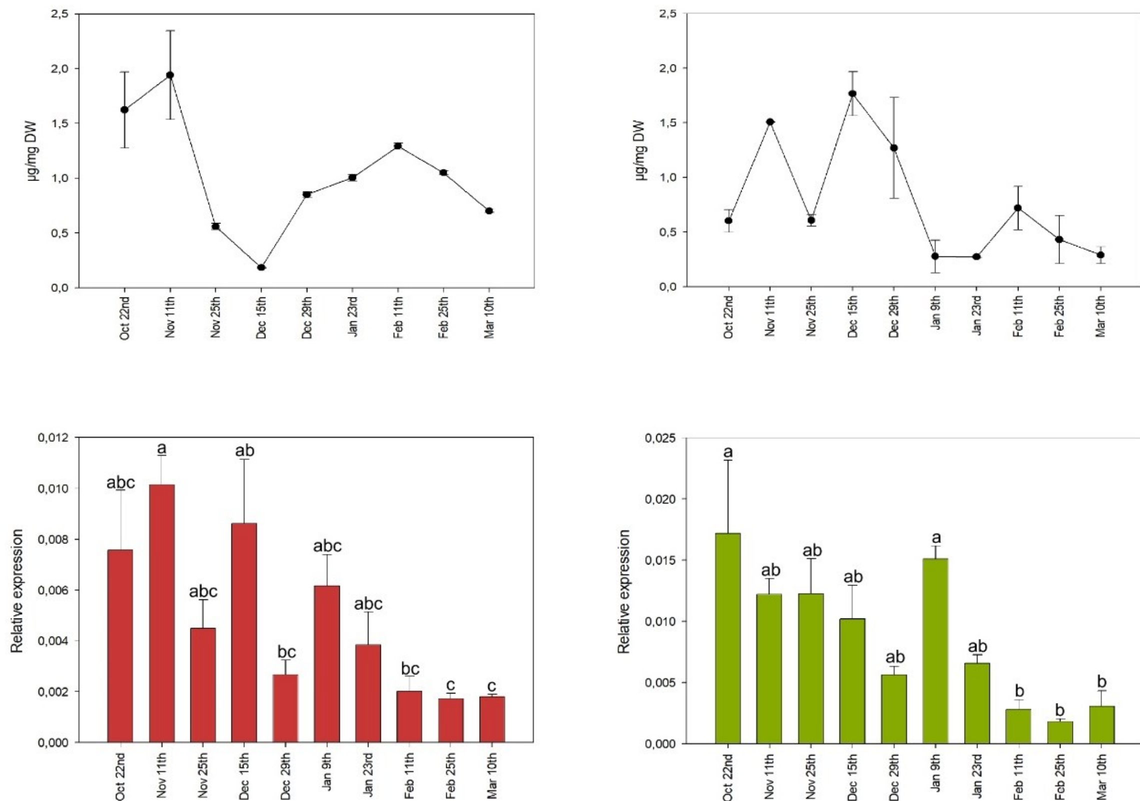


Figure 2. Raffinose accumulation dynamics and *VvRS* (raffinose synthase) expression in hybrids Fleurtai (left) and UD 31-103 (right) buds collected during the 2019-2020 winter season. Results are expressed as mean of 3 biological replicates of 10 buds ± standard error.

Raffinose and *VvRS* expression, monitored in buds of hybrids Fleurtai and UD 31-103 throughout the 2019-2020 winter season, are both shown in Figure 2. Although maximum raffinose levels appeared similar in the two cultivars, different accumulation trends were observed. In detail, the trisaccharide concentration in cv. Fleurtai showed the highest levels in late October and in the first half of November, then sharply decreased and reached a minimum on December 15th. A second more gradual increase was detected from late December to early February. In cv. Fleurtai, *VvRS* showed higher expression levels up to the end of January as compared to February and March. Interestingly, a significant highest upregulation was measured in the first half of November, in accordance with raffinose content. Following that, *VvRS* expression significantly decreased in late December to reach a minimum in February and March.

As far as selection UD 31-103 is concerned, raffinose concentration appeared more modulated as compared to cv. Fleurtai, showing a transient increase at early November, followed by the highest concentration on December 15th. Raffinose content then decreased until January 9th and remained stable for the entire month, until a less pronounced peak registered on February 11th. In this cultivar *VvRS* expression levels appeared higher and variably regulated, although not always in a significant manner. In fact, a general down-regulation of the gene has been observed in February and March, in accordance with raffinose dynamics.

3.2 Gene expression variations during the winter season

The gene encoding the hexose transporter HT5, *VvHT5*, showed similar expression trends in buds of both hybrids Fleurtai and UD 31-103, with a peak on January 9th and a decrease to a new significant minimum in late February and March.

On the contrary, *VvHT1* transcription exhibited fairly complementary dynamics compared to *VvHT5*, and similar in the two hybrids. In detail, *VvHT1* expression displayed its minimum from November to January and seemed tendentially upregulated from the beginning of February onwards. In addition, the expression of cell wall invertase *VvINV* was also monitored in buds throughout the winter season. The gene appeared regulated in a very complex way in both hybrids, even if similar trends can be identified. Notably, a downregulation was observed from December to January-February, while the highest *VvINV* transcription was detected in February and March.

VvMSA expression was successfully detected in grapevine buds of both hybrids Fleurtai and UD 31-103. Results show that *VvMSA* was significantly and sharply upregulated in both genotypes in spring. In detail, *VvMSA* expression increased more gradually in cv. Fleurtai as compared to selection UD 31-103. Minima expression levels were registered in the second halves of November and December in cv. Fleurtai. No substantial difference was observed in *VvMSA* expression from October to the second half of February in selection UD 31-103.

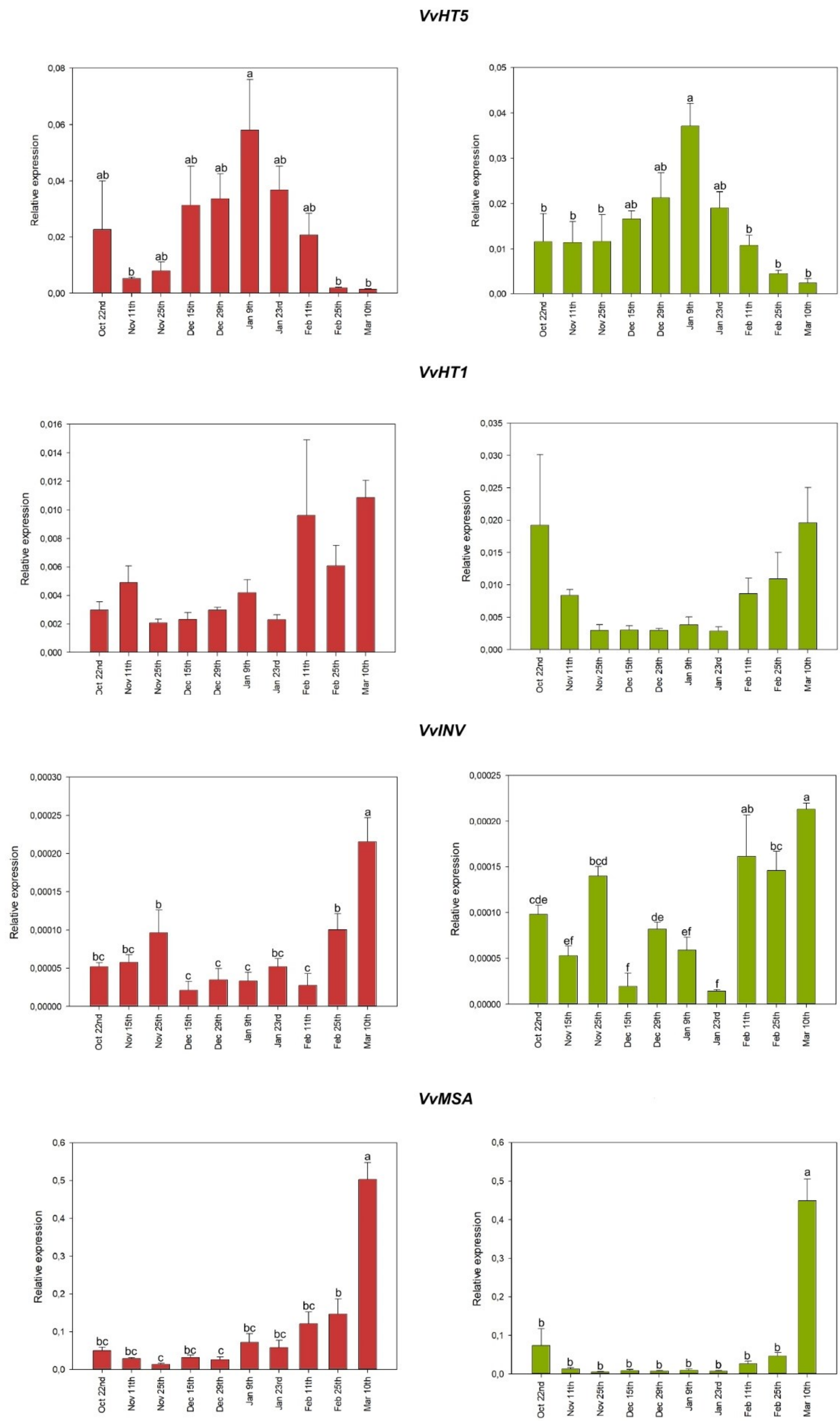


Figure 2. qPCR analysis of buds of hybrids Fleurtai (left) and UD 31-103 (right) throughout the 2019-2020 winter season. Results are expressed as mean of 3 biological replicates of 10 buds ± standard error. *VvHT5* = Hexose Transporter 5; *VvHT1* = Hexose Transporter 1; *VvINV* = Cell Wall Invertase; *VvMSA* = Maturation, Stress, ABA. Statistical analyses were performed using one-way ANOVA and Tukey HSD as *post hoc* test for all pairwise comparison procedures.

4 DISCUSSION

Climate change is not only registered as torrid summers, but also warmer winters or acute cold weather episodes (IPCC, 2021). Recently, dormancy regulation has gained additional interest due to global warming and unpredictable temperature fluctuations. Cold hardiness kinetics and budbreak phenology are strictly connected and affected by dormancy state (Londo and Kovaleski, 2019). In fact, early cold hardiness loss often implies higher frequencies of late frost damage in sensitive species (Inouye, 2008). Grapevine cultivars have been shown to be differentially sensitive to warm spells during winter; however, sustained cold winters are necessary for high cold hardiness levels to be maintained (Londo and Kovaleski, 2017). Soluble sugars play a well-documented role in freezing tolerance acquisition by accumulating during cold acclimation (Zhang *et al.*, 2018). However, sugars are also known as signaling molecules involved in the combination of internal and external stimuli in different developmental stages and stress responses (Ljung *et al.*, 2015; Li and Sheen, 2016), as well as a source of energy for primary metabolism (Tixier *et al.*, 2019). The role of sugar metabolism in bud dormancy transitions is still largely unknown (Horvath *et al.*, 2003). A better understanding of the mechanisms underpinning dormancy progression and the key players in this process could be helpful in increasing the sustainability of grapevine cultivation as climate variation increases.

Cold hardiness levels of buds collected from hybrids Fleurtai and UD 31-103 during the 2019-2020 winter season have been recently documented (De Rosa *et al.*, 2022, see Chapter IV). The observed raffinose accumulation dynamics appear to confirm its greater connection to freezing tolerance compared to other soluble sugars (Grant and Dami, 2015). In support of this, *VvRS* expression in both hybrids is generally higher in colder months, and its downregulation from the start of February corresponds to the increase of average daily temperatures during the 2019-2020 winter season following the coldest interval (Figure S1, see Appendix). On the other hand, the accumulation pattern of sucrose and hexoses appears more difficult to be directly related to low temperatures occurrences. This is specifically due to the difficulties in distinguishing the signaling role of these molecules from their contribution as mere building blocks for reserve and/or osmolites (Rosa *et al.*, 2009).

However, taken together, results suggest that Fleurtai and UD 31-103 could be characterized by different levels of reactivity to seasonal temperature fluctuations (Figure S1). In particular, total hexoses and sucrose content variations shown in this study allow to speculate a relation between sugar content and different sensitivities to winter minima, possibly connected to a differential use of sugars in short-term cold response.

In regard to hexose transport-related evidences, *VvHT1* is tendentially downregulated during winter months, while VvHT5 is significantly upregulated, almost complementarily around the same time period. These results seem coherent with previously reported drought-induced *VvHT5* upregulation and *VvHT1* downregulation (Medici *et al*., 2014). In addition to this, recent evidence documented the upregulation of an unspecified hexose transporter in cold-treated *Vitis amurensis* seedlings, while showing no obvious difference in *V. vinifera* (Xin *et al*., 2013). Given the similarities of plants' adaptation mechanisms to cold and drought stress (Du *et al*., 2012; Bao *et al*., 2017; Li *et al*., 2017; Hussain *et al*., 2018), our results allow to speculate that the hexoses transporters-encoding genes examined in this work display comparable behavior as observed in drought-stress responses; *VvHT5* upregulation, in particular, could be related to freezing-induced dehydration stress.

Ice nucleation events in plant tissues take place outside living cells, namely in the apoplast, from where freezing can spread to the symplast (Lintunen *et al*., 2017). Cell wall invertases are located in the apoplast, where they hydrolyze sucrose into glucose and fructose, doubling their osmoprotective function. *VvINV* expression in hybrids Fleurtai and UD 31-103 peaks in November together with total hexose content. Recent evidence collected in tomato has indicated that cell wall invertases play an important role in chilling tolerance by regulating sugar content (Xu *et al*., 2017). On these premises, the evidence collected in this study allows to hypothesize that an influx of invertase-generated hexoses from the apoplast could participate in reaching the optimal hexoses content to establish freezing tolerance in grapevine buds in low temperature conditions. In this view, a correct understanding of sugars concentration will be possibly obtained only if subcellular compartmentation will be considered.

In addition, to our knowledge, the results presented in this study are the first example of documented *VvMSA* expression in grapevine buds. Expression patterns

shown in Figure 3 suggest a role for VvMSA in the context of dormancy release. Cv. Fleurtai is presented as an early-budburst variety, whereas selection UD 31-103 is classified as an average-budburst variety (Italian *Vitis* Database, 2022; Technical Booklet VCR 18, 2022). In support of VvMSA possible role in bud phenological advancement, *VvMSA* upregulation starts at least 15 days earlier, although a gradual increase is detected even before that time in Fleurtai compared to UD 31-103. Although an immediate connection between soluble sugars and *VvMSA* expression in grapevine buds cannot be derived from these data, as seen in other grapevine tissues (Çakir *et al*., 2003; Saummoneau *et al*., 2012), its double regulation by both ABA and sugars makes it an interesting target for future investigations, being both ABA and sugars highly relevant in dormancy of grapevine buds.

5 CONCLUSIONS

In conclusion, this work presents evidence of multiple sugar-related responses taking place inside buds of differently cold-tolerant Vitis hybrids. Soluble sugars content appears to be greatly influenced by warm spells during winter, with detectable differences in cultivars characterized by different tolerance to winter minima. Hexose transporter VvHT5 could be involved in the tolerance of physiological water reduction connected to cold hardiness acquisition, or resistance to freezing-induced dehydration stress in grapevine buds, by supporting signaling pathways designated to stress adaptation or recovery (Secchi *et al*., 2016). The evidence on *VvMSA* expression in grapevine buds indicates a possible role in bud phenological advancement towards budbreak.

While spring frosts remain a threat in the present, deepening our understanding on grapevine soluble sugars-induced freezing tolerance could help to identify target genes for future breeding endeavors. To this aim, an integrative understanding of dormancy regulation is required, in which soluble sugars are potentially involved not only as osmoprotectants and metabolic substrates, but also as signaling molecules.

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♦ CHAPTER IV

First steps into dormancy release regulation: an introduction to grapevine *DEMETER-like demethylases*

INTRODUCTION TO THE STUDY

In order to further characterize dormancy progression in buds of hybrids Fleurtai and UD 31-103, we explored a new frontier of plant development regulation, namely epigenetics. This includes all post-replication modifications in the genome, such as DNA methylation, that result into specific chromatin accessibility states influencing gene activity and expression. Epigenetic modifications concerning the DNA sequence are modulated by DNA methylases and demethylases. Among them, DEMETER-like demethylases have been demonstrated to be involved in stress responses and plant development. In relation to winter dormancy and budbreak in woody perennials, recent evidence collected in *Populus trichocarpa* documented the involvement of chilling responsive *PtaDML10* in dormancy release regulation. Specifically, its upregulation in correspondence of budbreak was detected.

We investigated whether *DML* homologs are present in grapevine genome using both *Arabidopsis DML1* and *Populus trichocarpa PtDML10* sequences. A BLAST search revealed three putative grapevine *DML* homologs, although only two genes, *DEMETER 1* (*VvDEM1*) and *DEMETER 3* (*VvDEM3*) appeared expressed in grapevine buds. Our results resemble those found in poplar, with low *VvDMLs* expression during the deepest phase of dormancy, and higher expression in proximity to budbreak. This makes it possible to speculate a potential involvement of *VvDMLs* in dormancy release regulation.

This work was presented in poster form at the VIII CONAVI (Convegno Nazionale di Viticoltura) in July 2021 and is now included in the conference paper '*Expression patterns of DEMETER-like DNA demethylases homologs hint at potential involvement in grapevine dormancy release*', published by *BIO Web of Conferences* journal in 2022.

Expression patterns of DEMETER-like DNA demethylases homologs hint at potential involvement in grapevine dormancy release[3]

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Abstract

Climate change represents an undeniable threat to traditional viticulture in many areas of the world. Although an increase of average surface temperatures is expected in the future, late frost occurrences are predicted to be an actual challenge, being budburst the critical stage during which cold hardiness levels dramatically decrease and frost vulnerability is at its maximum. Genomic DNA methylation is known as an important mechanism for gene expression regulation. A sub-group of epigenetic regulators named DEMETER-like DNA demethylases (DMLs) has been shown to be involved in budbreak regulation in *Populus trichocarpa*. In this study, buds of two Vitis genotypes, Fleurtai (Friulano × Kozma 20-3) and UD 31-103 (Merlot × Kozma 20-3), characterized by different levels of resistance to winter freezing temperatures, were sampled during the 2019/2020 winter season. Cold hardiness dynamics were monitored with differential thermal analysis (DTA) at regular intervals. Expression levels of two putative grapevine *DEMETER* homologs were investigated during natural dormancy conditions. Results show dissimilar deacclimation rates in the two varieties. As observed in other woody species, putative grapevine *DEMETER*s show downregulation and upregulation trends hinting at a potential involvement in grapevine dormancy release.

[3] The data presented in this chapter were recently published by *BIO Web of Conferences* journal. (De Rosa *et al.*, 2022 - *BIO Web of Conferences*. 44:04001, doi: 10.1051/bioconf/20224404001).

1 INTRODUCTION

Climate change represents a multifaceted phenomenon that threatens crop productivity all around the globe (IPCC, 2014). Grapevine phenology is greatly responsive to environmental conditions, with temperature being the most critical factor (van Leeuwen and Darriet, 2016). Higher temperatures produce an acceleration of grapevine development, with consequent earlier budbreak, flowering and harvest dates. Faster development rates not only impact ripening conditions, which greatly affect berry composition and wine quality, but also compromise the survival of buds and shoots to potential late frosts (Kliewer and Torres, 1972; Coombe 1987; Bonada *et al*., 2015).

Green tissues are significantly more vulnerable to freezing damage due to the high hydration levels combined with low supercooling capabilities (Fennell, 2004). Therefore, budburst and leafout have been described as most vulnerable phases in several woody species (Vitasse *et al*., 2014; Lenz *et al*., 2016). Consequently, sudden occurrences of late freezing temperatures following a period of premature vegetative growth, known as *spring frosts*, can greatly damage bud tissues thus impairing fruit trees production and causing great economic losses (Ault *et al*., 2013; Gu *et al*., 2008). Late frosts are expected to remain a continuing challenge in several areas of the world (Augspurger, 2013; Ma *et al*., 2018; Sgubin *et al*., 2018; Vitasse and Rebetez, 2018). Moreover, frost damages occurring at budbreak negatively affect grapevine photosynthetic and reproductive tissues alike with consequences that spread on multiple years of development (Carmona *et al*., 2008).

Bud dormancy in woody perennials is defined as a state of temporary cessation of all visible growth. It can be induced by environmental conditions such as low temperatures and short photoperiod (ecodormancy), or endogenous signals originating from inside (endodormancy) or outside the bud (paradormancy) (Lang *et al*., 1987). Woody perennials need to fulfil a chilling requirement during winter to shift from endodormancy to ecodormancy, when the tissues become responsive to growth-promoting external conditions. Chilling requirements are not thoroughly understood and can currently be predicted only by using empirical models (Luedeling, 2012). Cultivated grapevines are generally considered low-chill

compared to other fruit trees, however considerable diversity can be found among species and cultivars (Londo and Johnson, 2014). Hybrid crosses have been useful in the past to enhance cold resistance to winter freezing temperatures in grapevine; however, wild Vitis species have been observed to be more responsive to mid-winter warm temperatures, leading to earlier budbreak and to a consequent susceptibility to spring frost damage (Londo and Johnson, 2014).

Gaining a comprehensive understanding of chilling requirement and dormancy release regulation is essential to direct breeding efforts towards the production of delayed-growth cultivars; such an approach could be favourable to lower spring frost damage risk in the face of a changing climate (De Rosa *et al*., 2021).

Epigenetic regulation of processes such as cold stress response, cold acclimation or dormancy regulation has been an object of interest in plants (Ríos *et al*., 2014; Liu *et al*., 2017; Park *et al*., 2018; Rothkegel *et al*., 2020), although the understanding is still limited. Similarly, very little is known about deacclimation, which naturally proceeds into dormancy release, in woody perennials (Vyse *et al*., 2019).

DNA methylation taking place at the 5' position of cytosines is a renowned conserved epigenetic mechanism involved in both gene expression regulation and genomic stability (Zhang *et al*., 2018). DNA demethylation occurs passively during DNA replication, or actively through enzymatic removal of methylated cytosines. In Arabidopsis thaliana, demethylation is carried out by four DEMETER-like DNA demethylases (DMLs) (Zhu, 2009), which activate gene expression in response to abiotic or biotic stresses (Yu *et al*., 2013; Le *et al*., 2014). DMLs have also been associated with fruit development in other species such as tomato (Liu *et al*., 2015) and grapevine (Shangguan *et al*., 2020), in addition to nodule development in Medicago truncatula (Satgé *et al*., 2016). Poplar DML *DEMETER-like 10* (*PtaDML10*) was recently described as a potential chilling-responsive regulator of budbreak. It putatively enables the reactivation of genes controlling meristem activity, and the downregulation of dormancy-related genes (Conde *et al*., 2017).

This work aims to ascertain the presence of grapevine DMLs homologs in differently cold tolerant cultivars, and to monitor their expression to understand

whether, as observed in poplar, grapevine DMLs could be involved in budbreak regulation.

2 MATERIALS AND METHODS

2.1 Plant material

Two differently cold tolerant Vitis hybrid cultivars were selected for the study: the white cv. Fleurtai (Friulano × Kozma 20-3), tolerant to freezing temperatures up to -23°C, and the red hybrid UD 31-103 (Merlot × Kozma 20-3), tolerant to freezing temperatures up to -20°C. Plants were located at the Experimental Farm "A. Servadei" (University of Udine, Northern Italy) and grown in the field. During the 2019/2020 winter season, buds were collected at 15 days' intervals and immediately used for cold hardiness determination, or stored at -80°C for subsequent gene expression analysis.

2.2 Cold hardiness monitoring

For each sampling time, 3 biological replicates of 5 buds each were used for cold hardiness determination with differential thermal analysis (DTA) using thermoelectric modules (TEM) and temperature probes placed in a T700BXPRO temperature-controlled freezing chamber (FDM, Rome, Italy). Temperature was quickly lowered to 7°C for 1 hour and subsequently lowered to -25°C at a rate of -2.5°C·h^{-1}. A CR1000 data-logger (Campbell Scientific, Logan, UT, USA) was used for data recording. Temperature and voltage signals were analyzed using RStudio software (https://www.r-project.org/).

2.3 Gene expression analysis

Grapevine DMLs homologs were BLAST-searched in the grapevine whole genome using poplar *PtDML10* sequence (Phytozome accession: Potri.010G234400.1) and *A. thaliana DML1* (AT2G36490). Three sequences were found and putatively named *VvDEMETER1* (*VvDEM1*, GSVIVT01034713001), *VvDEMETER2* (*VvDEM2*, GSVIVT01031400001) and *VvDEMETER3* (*VvDEM3*,

GSVIVT01033777001). Primers to detect *VvDEM* expression were built with Primer-BLAST tool on NCBI (Table 1).

For each sampling time, RNA extraction was performed from 3 biological replicates of 10 buds using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized with QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany), and real-time PCR was carried out with SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) as described in Sivilotti *et al.*, 2017 by primers listed in Table 1. Statistical analyses were performed using SigmaPlot 12.0 (https://systatsoftware.com/).

Table 1. List of primers used for *VvDEM* cDNA amplification.

Target gene		*Sequence (5'-3')*	*Expected product size*
VvDEM1	**F**	AATGAGGGGAGAGTGCAGAC	247 bp
	R	TATGCATTGCGGTTCTGGTG	
VvDEM2	**F**	CACATCTCCTCGACCCAAGT	197 bp
	R	GTGTAGGGATGGAAGTGGCT	
VvDEM3	**F**	CGCACTGTGTACTTTGGGAC	170 bp
	R	GTCAACCTGCTTGCTGGAAA	

3 RESULTS

3.1 Cold hardiness determination

DTA analysis was successful in determining cold hardiness levels of Fleurtai and UD 31-103 buds throughout the 2019/2020 winter season. Low temperature exotherms (LTEs), which represent temperatures corresponding to lethal freezing events of intracellular water, are shown in Figure 1. Cold acclimation appeared to have already started in buds of both hybrids by the first half of December and sharply reached its maximum level by the second half of December, with a deviation of about 10°C, consistently with the lowest temperatures registered in the area (Figure 2). Deacclimation also started concomitantly in the two cultivars during the first part of

January, but proceeded more slowly in buds of UD 31-103 compared to cv. Fleurtai. In fact, in March the two varieties reached the greatest divergence, with cv. Fleurtai proceeding into deacclimation and reaching the same cold hardiness levels observed in November, while UD 31-103 LTEs remaining stable. Detailed LTEs values are listed in Table 2.

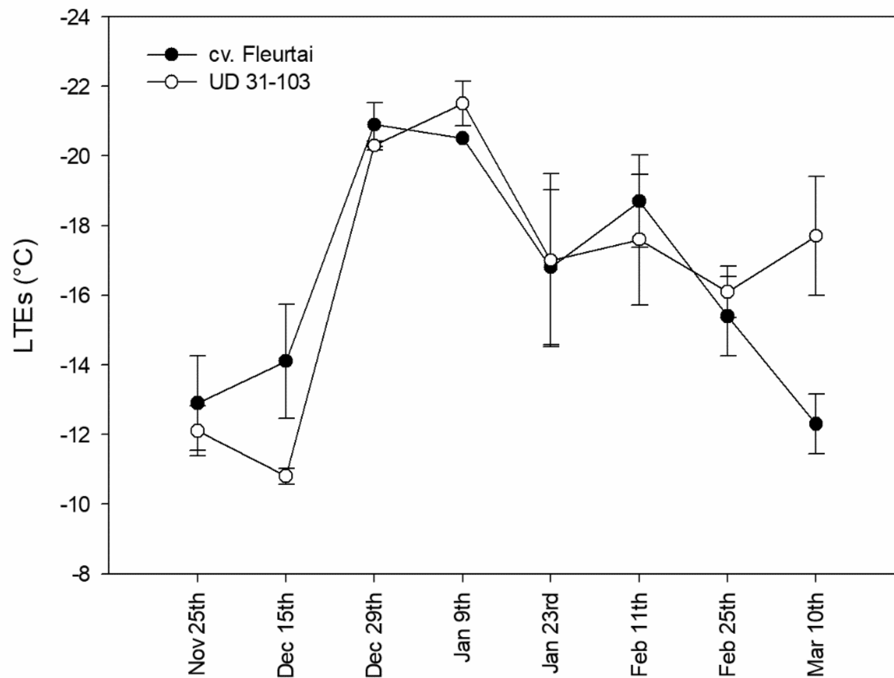


Figure 1. Low-temperature exotherms (LTEs) of grapevine buds during the 2019/2020 winter season. Results are expressed as mean of 3 biological replicates ± standarderror.

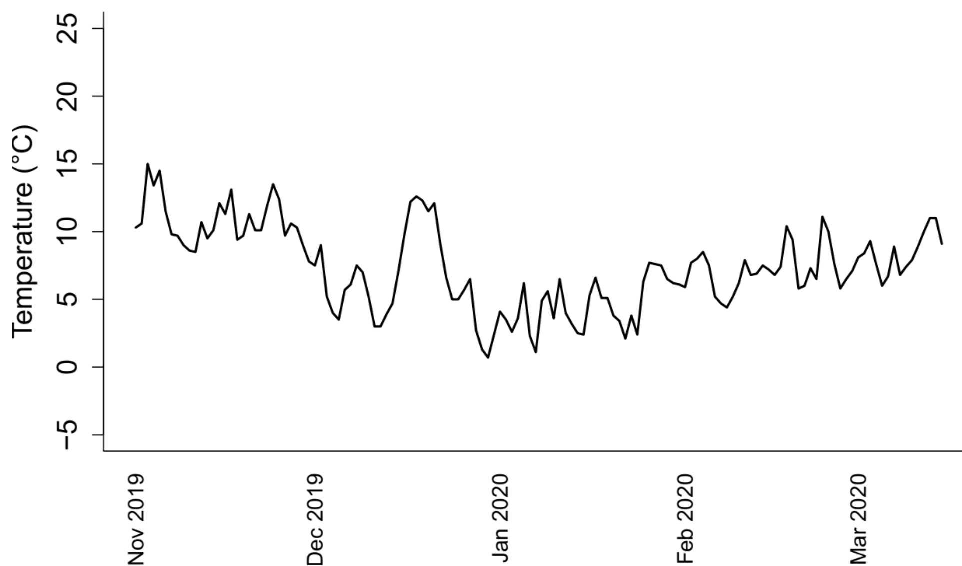


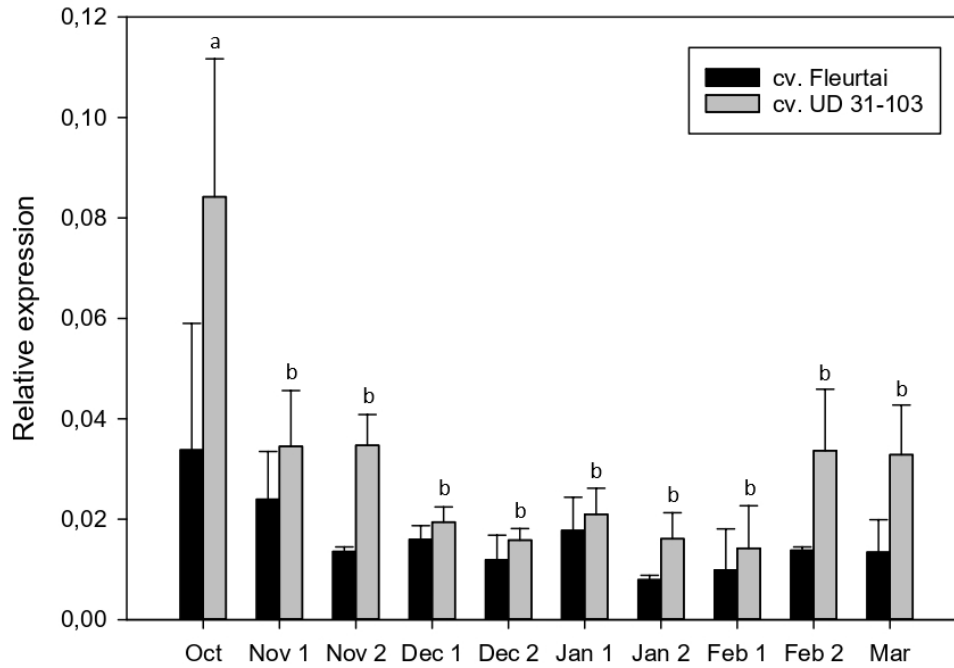
Figure 2. Average daily temperatures during the 2019/2020 winter season. Data recorded by the S. Osvaldo (Udine, Italy) weather station managed by ARPA FVG.

Table 2. List of measured LTEs in buds of hybrids Fleurtai and UD 31-103.

Sampling date	*cv. Fleurtai*	*cv. UD 31-103*
Nov	-12,9 °C	-12,1 °C
Dec 1	-14,1 °C	-10,8 °C
Dec 2	-20,9 °C	-20,3 °C
Jan 1	-20,5 °C	-21,5 °C
Jan 2	-16,8 °C	-17,0 °C
Feb 1	-18,7 °C	-17,6 °C
Feb 2	-15,4 °C	-16,1 °C
Mar	-12,3 °C	-17,7 °C

3.2 Grapevine *DEMETER* expression

DMLs expression was tested in both grapevine hybrids Fleurtai and UD 31-103 plants. No expression of *VvDEM2* was detected in bud tissue, thus excluding it from further analysis. Expression levels of *VvDEM1* and *VvDEM3* are shown in Figure 3A and 3B, exhibiting similar trends in the considered varieties. In detail, *VvDEM1* expression remained stable throughout the season in both cultivars with the exception of a statistically significant upregulation in October in UD 31-103 (Figure 3A). *VvDEM3* expression underwent significant variations in both genotypes. In particular, a significant downregulation was detected during the coldest winter months, consistent with the highest registered levels of freezing tolerance (Figure 1). A statistically significant upregulation of *VvDEM3* took place during the deacclimation process in UD 31-103. An upregulation tendency is also observable in cv. Fleurtai in the same phase (Figure 3B).



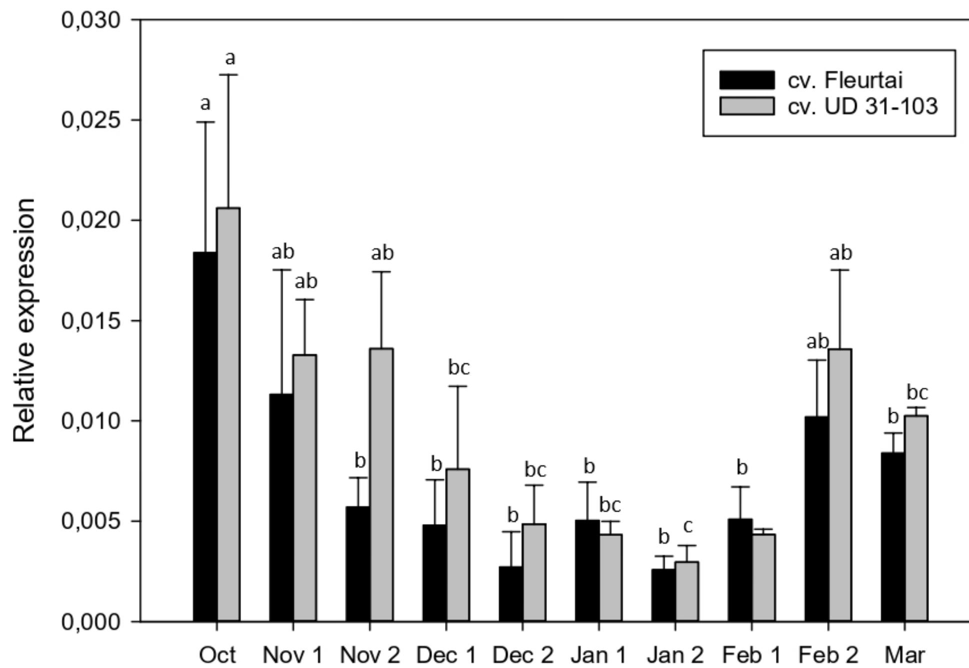


Figure 3. Expression patterns of *VvDEM1* (A) and *VvDEM3* (B) in buds of field-grown grapevine hybrids Fleurtai and UD 31-103. Results are expressed as mean of 3 biological replicates ± standard deviation. Statistical analyses were performed within each variety using one-way ANOVA and Tukey HSD as *post hoc* test for all pairwise multiple comparison procedures.

4 DISCUSSION

Cold acclimation and deacclimation are driven by both genetic factors and environmental conditions (Wisniewski *et al*., 2018). In this context, species within the Vitis genus represent a valuable source of variability with a wide range of chilling requirements and budburst rates (Londo and Johnson, 2014). In terms of cold acclimation dynamics, grapevines are differently responsive to temperature fluctuations during dormancy and require sustained low temperatures to reach their respective maximum levels of cold hardiness (Londo and Kovaleski, 2017). As for deacclimation, paradoxically, grapevine hybrids most resistant to freezing temperatures have also been observed to be the most vulnerable to spring frost damage due to higher responsiveness to warm temperatures (Ferguson *et al*., 2014). While DTA carried out on Fleurtai and UD 31-103 buds does not highlight substantially different maximum levels of cold hardiness, possibly due to the higher temperatures observed in December 2019 (Figure 2), cv. Fleurtai, characterized by a greater winter freezing tolerance compared to UD 31-103, is the fastest to deacclimate as expected. *VvDEM*3 expression patterns suggest a role in dormancy release comparable to the observations carried out in *P. trichocarpa* (Conde *et al*., 2017).

Taken together our results allow hypothesizing that a *DEMETER*-dependent DNA demethylation could be involved, also in a tree crop such as grapevine, in the regulation of dormancy-growth cycle. Further experiments, including methylome and functional studies, involving early-budbreak and late-budbreak varieties, are needed to confirm and further reinforce *VvDEM*s putative role as transcriptional regulators in the context of grapevine dormancy release.

If confirmed, these preliminary results may have important implications for both research and breeding programs.

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♦ CHAPTER V

Delving deeper: transcriptomics and epigenomics of dormancy regulation of early- and late-budbreak Vitis cultivars

INTRODUCTION TO THE STUDY

The resemblance between the evidence collected on *PtDML10* expression in poplar and the results on *VvDMLs* expression in cv. Fleurtai and selection UD 31-103 appeared very encouraging. To better understand the role of *VvDEM1* and *VvDEM3* in grapevine dormancy release and budbreak, a trial on two new cultivars was planned: renowned late-budbreak Cabernet Sauvignon, and early-budbreak hybrid Sauvignon Nepis. Potted plants of these cultivars, characterized by different budburst timing, were grown in controlled conditions to first induce cold acclimation and chilling requirement fulfilment, and later deacclimation and bud development up to budbreak.

DTA analysis clearly underlined the different cold acclimation levels and deacclimation timings of Cabernet Sauvignon and Sauvignon Nepis, with the hybrid cultivar exhibiting higher cold hardiness levels while also being faster to deacclimate. *VvDEM1* and *VvDEM3* expression patterns appeared uncompromised by forced development, as compared to open field conditions, confirming the results included in Chapter IV. In both cultivars, total RNA sequencing provided a large amount of information regarding the main biological processes involved in dormancy progression of grapevine buds, highlighting several differential timings in the mechanisms related to cold hardiness acquisition, and possibly new evidence regarding transcription factors involved in dormancy exit. In particular, differences regarding carbohydrate metabolism, cell wall-related metabolism, oxidative stress responses, tissue development and photosynthesis were detected, suggesting late responses to low temperatures in buds of Sauvignon Nepis, associated to early reactivation of bud activity in forcing conditions. Whole-genome bisulfite sequencing, a method providing single-base resolution of methylated cytosines in the genome, performed on Cabernet Sauvignon buds did not suggest pivotal genome wide changes in the methylation profiles of genes during dormancy progression. Further inspection of methylation differences at single locus level has allowed the identification of three demethylated gene targets, possibly connected to auxin and flavonoid metabolism. Further analyses are under way.

Transcriptome and methylome analyses of early- and late-budbreak genotypes provide new insight on grapevine dormancy progression

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Abstract

The ongoing global increase of average surface temperatures does not only translate into greater heat and water stress in summer, but also in warm winters that compromise cold hardiness acquisition and dormancy depth of fruit crops. Grapevine bud phenological development is greatly influenced by temperature regimens, and faster deacclimation rates peaking in premature budbreak timings expose vulnerable bud tissues to higher risk of late frost damage. An integrative understanding of dormancy regulation and release dynamics is required to define fitting adaptive measures. For this purpose, late-budbreak Cabernet Sauvignon and early-budbreak hybrid Sauvignon Nepis were studied at transcriptomic and epigenetic level from cold acclimation to budbreak in controlled growth conditions. Particular attention was paid to grapevine *DEMETER-like* DNA demethylase (*VvDMLs*), active during dormancy release. Bud monitoring highlighted higher cold hardiness levels reached in hybrid Sauvignon Nepis, together with faster deacclimation rates in forcing conditions. Transcriptomic analyses support diverse cold hardiness and dormancy-related processes in the two genotypes, and an earlier deacclimation in Sauvignon Nepis, with several biological processes differentially active between cultivars. Evidence regarding specific transcription factors potentially involved in dormancy release regulation is also described, with putative genotype-specific roles. Results allow to speculate that the early-budbreak cultivar anticipates several developmental processes compared to late-budbreak one due to early chilling requirement fulfilment. *VvDMLs* expression is consistent with a putative role in dormancy release, and ongoing genome methylation analyses will contribute to define targets of their activity.

1 INTRODUCTION

Climate change has forced viticulture to confront itself with new challenges that endanger fruit crop production (IPCC, 2021). The global increase of average surface temperatures not only exposes plants to higher heat and water stress risks (van Leeuwen and Darriet, 2016), but also accelerates grapevine phenological development causing major difficulties for traditional wine production (Bonada *et al*., 2015). Faster development rates, therefore also early budbreak, can lead to higher spring frost damage risk during the most vulnerable phase of bud development (Fennell, 2004). Late freezing occurrences can thus greatly damage vegetative and reproductive tissues (Gu *et al*., 2008; Vitasse and Rebetez, 2018), potentially compromising grapevine production for multiple years (Carmona *et al*., 2008). Various agricultural practices might be adopted to mitigate climate change effects in the short term (Duchêne, 2016), while heaters and wind machines represent an environmentally unsustainable approach (Liu and Sherif, 2019). Additionally, traditional and new breeding technologies must be taken into consideration for obtaining new grapevine cultivars better suited to spring frost tolerance. A broad range of budburst rates and chilling requirements can be found in wild Vitis species, making them an interesting subject for breeding efforts (Londo and Johnson, 2014). Chilling requirement, cold deacclimation and budburst timing are connected by several pathways, but the relationships between the different processes remain to be clarified. Therefore, defining the relevance of each pathway is crucial for breeding to obtain spring frost-tolerant varieties, and dedicated studies are required. See Chapter I, available in *Frontiers in Plant Science*, 12:644528 (De Rosa *et al*., 2021), for a more in-depth review on these topics.

An additional level of complexity is represented by epigenetic regulation. In fact, phenotypic variations of multicellular organisms cannot be attributed exclusively to direct mutations in genomic sequences, but also to stable alterations of gene expression patterns conferred by epigenetic modifications. Epigenetic regulation includes all post-replication modifications in the genome that result into specific chromatin accessibility, states which in turn determine gene activity and expression (Jaenisch and Bird, 2003). These changes, namely DNA methylation, post-translational histone modifications and RNA interference, play a strategic role in

controlling plant development and genome-defense processes, and are often inheritable (Henderson and Jacobsen, 2007; Rigal and Mathieu, 2011). DNA methylation consists in the transfer of a methyl group onto cytosines to form a 5-methylcytosin (5-mC) (Zhang *et al.*, 2018). As opposed to mammals, DNA methylation in plants can be found in all cytosine sequence contexts: CG, CHG and CHH (H being A, T or C) (Law and Jacobsen, 2010). Since cell division leads to the formation of hemi-methylated DNA molecules, 5-mCs must be reestablished by several context-specific mechanisms with the recruitment of distinct players. In *Arabidopsis thaliana*, CG methylation is maintained by DNA Methyltransferase 1 (MET1); CHG methylation sites are preserved by Chromomethylases (CMTs); methylation maintenance at CHH sites is ascribed to Domain Rearranged Methyltransferases (DRMs) (Law and Jacobsen, 2010). DNA demethylation is not only the passive product of genomic replication, but can also be an active process performed at specific loci during development-related DNA reprogramming (Li *et al.*, 2018). In *A. thaliana*, active demethylation is carried out by four DNA glycosylases: Repressor of Silencing 1 (ROS1, also known as DEMETER-like 1, DML1), DEMETER (DME), and two DEMETER-like demethylases, DML2 and DML3 (Zhu, 2009). With the exception of DME, which has been implicated in endosperm imprinting and seed viability (Choi *et al.*, 2002), *Arabidopsis* DMLs are thought to have a housekeeping function of the overall methylation profile of the genome by avoiding 5-mC accumulation at 5' and 3' ends of genes (Penterman *et al.*, 2007). AtROS1, AtDML2 and AtDML3 are believed to be responsible for all demethylation activity in somatic tissues, with AtROS1 being the dominant DNA demethylase (Schumann *et al.*, 2017). A *ros1, dml2* and *dml3* mutant line (*rdd*) shows no obvious developmental defects under normal growth conditions (Penterman *et al.*, 2007).

In regard to DMLs involvement in stress responses and plant development, *AtROS1* constitutive overexpression in *Nicotiana tabacum* was shown to enhance salt stress tolerance by reducing methylation in the promoter regions of enzymes belonging to the flavonoid biosynthetic and antioxidative pathways (Bharti *et al.*, 2015). Evidence collected in the *rdd* mutant line, characterized by higher susceptibility to *Fusarium oxysporum* infection, implicates *AtDMLs* action in the activation of defense-related genes through demethylation of transposable elements promoters (Le *et al.*, 2014). Besides *Arabidopsis*, tomato *SlDML2* knockdown resulted

in ripening inhibition due to hypermethylation of ripening-related transcription factors and enzymes (Liu *et al*., 2014).

The role of *DMLs* genes has been also related to winter dormancy and budbreak regulation in woody perennials: an upregulation of both chestnut (*Castanea sativa*) *CsDML* and poplar (*Populus trichocarpa*) *PtaDML6* was observed in the context of growth cessation-promoting conditions, such as short day length and low temperatures (Conde *et al*., 2017a). *CsDML*-overexpressing poplar plants showed enhanced flavonoid biosynthesis, implicated in apical bud maturation, and decreased expression of cell proliferation-related genes (Conde *et al*., 2017a). Recently, evidence collected in *P. trichocarpa* documented the involvement of another DNA demethylase, *PtaDML10*, in dormancy release regulation (Conde *et al*., 2017b). In particular, transcriptome and methylome analyses highlighted a reduction of DNA methylation before the reactivation of the shoot apical meristem, correlating with the induction of *PtaDML10* expression. *PtaDML10* expression peaked at budbreak following its lowest levels recorded in endodormant buds, suggesting a chilling-responsive demethylation mechanism. Moreover, loss of function studies in *PtaDML10* knockdown lines confirmed a delayed budbreak phenotype (Conde *et al*., 2017b).

The data collected in poplar encouraged a recent study, reported in Chapter IV and available in *BIO Web of Conferences* journal, which analyzed the expression patterns of two putative grapevine *DML*s throughout dormancy progression in differently cold-tolerant cultivars (De Rosa *et al*., 2022a). Results closely resemble those observed in poplar (Conde *et al*., 2017b), suggesting a potential involvement of *VvDML*s in dormancy release regulation. On these premises, this work aims to further explore *VvDML*s involvement in dormancy release by comparing two Vitis cultivars, Cabernet Sauvignon and the hybrid Sauvignon Nepis, grown in controlled conditions, and characterized by diverging budbreak timings. To this purpose, RNA-Seq was performed to monitor the transcriptomic landscape of both cultivars at crucial timings in dormancy progression. Additionally, to scrutinize *VvDML* activity, genome bisulfite sequencing (WGBS) was carried out on Cabernet Sauvignon buds to analyze genome wide changes in methylation profiles of genes and their immediate genomic context.

2 MATERIALS AND METHODS

2.1 Plant material and growing conditions

Two Vitis cultivars characterized by different budbreak timings were selected: the early-budbreak, white cv. Sauvignon Nepis (Sauvignon Blanc × Bianca), and the late-budbreak cv. Cabernet Sauvignon (clone VCR8 provided by Vivai Cooperativi Rauscedo, Italy) both grafted on SO4 rootstocks. Potted plants, located at the Experimental Farm "A. Servadei" (University of Udine, Northern Italy), were moved in September 2021 from open field conditions to a 4°C dark chamber for 45 days to ensure chilling requirement fulfillment. To force growth, all plants were subsequently transferred to a greenhouse at the same site at 15 ± 2 °C, under natural light conditions. Buds were collected during the cold acclimation phase at regular time intervals, whereas subsequent samplings were synchronized with Sauvignon Nepis bud phenological transitions as they progressed from BBCH 0 (winter bud) to BBCH 09 (green tip) (Lorenz *et al*., 1994). Buds were immediately used for cold hardiness determination or stored at -80 °C for subsequent analysis.

2.2 Cold hardiness monitoring

For each sampling time, 3 biological replicates of 5 buds each were used for cold hardiness determination with differential thermal analysis (DTA) using thermoelectric modules (TEM) and temperature probes placed in a T700BXPRO temperature-controlled freezing chamber (FDM, Rome, Italy). Temperature was quickly lowered to 7°C for 1 hour and subsequently lowered to -25°C at a rate of -2.5°C$\cdot$h^{-1}. A CR1000 data-logger (Campbell Scientific, Logan, UT, USA) was used for data recording. Temperature and voltage signals were analyzed using RStudio software (https://www.r-project.org/).

2.3 RNA extraction and total RNA sequencing

For each sampling time, RNA extraction was performed using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). RNA quality was checked using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quantity was determined with Qubit™ RNA Broad-Range Assay kit (Thermo Fisher Scientific) using Qubit™ 4 Fluorometer (Thermo Fisher Scientific). Total RNA

of both varieties from three time-points, days -98, 0, and 33 (Figure 1), chosen in accordance to Sauvignon Nepis phenological development from BBCH 00 to BBCH 09, was used for library preparation with TruSeq RNA Library Prep Kit v1 rev. A (Illumina, San Diego, CA, USA) and sequenced on Illumina NovaSeq™ 6000 platform (Illumina). RNA-Seq library construction and sequencing were performed by IGA Technology Services Srl (https://igatechnology.com/). Universal Plus mRNA-Seq kit (Tecan Genomics, Redwood City, CA) was used for library preparation following the manufacturer's instructions (library type: fr-secondstrand). RNA samples were quantified, and quality tested by 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA). Final libraries were checked with both Qubit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Bioanalyzer DNA assay.

2.4 Read mapping and analysis of differential gene expression

RNA sequencing producing 150 bp paired-end reads. Sequencing adapters and low-quality bases were masked using the Trimmomatic tool (Bolger *et al.*, 2014). Quality check was performed on masked reads with FastQC (Andrews, 2010), showing an average Phred quality per read equal to 36 for all libraries. Reads were mapped to the V1 version of the PN40024 12X reference genome (Jaillon *et al.*, 2007) using STAR software package v2.7.9a (Dobin *et al.*, 2013) with default parameters and the v2.1 gene annotation (https://phytozome-next.jgi.doe.gov/info/Vvinifera_v2_1). Read counting per gene was carried out with STAR using the "--quantMode GeneCounts" option, to produce counts coinciding with those produced by htseq-count with default parameters. Differential gene expression analysis was performed with the DESeq2 R package (Love *et al.*, 2014) with default settings. Differentially expressed genes (DEGs) between timepoints and between varieties at the same timepoint were identified based on |log2FoldChange| ratios > 1 and adjusted p-value < 0.01. Homoscedastic gene counts, with variance stabilized across the mean, were produced with the variance stabilizing transformation (VST, Anders and Huber, 2010) and the regularized-logarithm transformation implemented in DESeq2 (rlog, Love *et al.*, 2014) and applied in the following analyses. Principal Component Analysis was performed using the standard R prcomp function with STAR gene counts normalized by the regularized-logarithm transformation method of the DESeq2 R package (Love

et al., 2014). The resulting principal component matrix was plotted using the ggplot2 R package (Wickham, 2016).

2.5 Functional annotation of total DEGs

Gene Ontology term enrichment was carried out using the topGO R package (Alexa and Rahnenfuhrer, 2021) with Fisher's exact test.

2.6 qPCR validation of DEGs

cDNA was synthesized with QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany), and real-time PCR was carried out with SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) as described in Sivilotti *et al.*, 2017. Primers used for cDNA amplification are described in Chapter IV (De Rosa *et al.*, 2022a). Statistical analyses were performed using SigmaPlot 14.0 (https://systatsoftware.com/).

2.7 DNA extraction and Whole Genome Bisulfite Sequencing analysis

For 3 sampling times (Figure 1), 3 biological replicates of 7 buds each of Cabernet Sauvignon were used for DNA extraction according to the Doyle and Doyle (1990) protocol. DNA quality was checked using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific), whereas DNA quantity was determined using Qubit™ dsDNA Broad-Range Assay kit (Thermo Fisher Scientific) with Qubit™ 4 Fluorometer (Thermo Fisher Scientific). Bisulfite treatment, BS-Seq library construction and sequencing were performed by IGA Technology Services Srl (https://igatechnology.com/). Ultralow Methyl-Seq System (Tecan/NuGEN, Redwood City, CA) was used for library preparation following the manufacturer's instructions. The system produces directional bisulfite-converted libraries. The forward sequencing reads correspond to a bisulfite-converted version of either the original top or the original bottom strand (the C-to-T reads) and the reverse sequencing reads correspond to the complement of the original top or the complement of the original bottom strand (the G-to-A reads). DNA samples were quantified with Qubit™ 2.0 Fluorometer (Invitrogen). Final libraries were checked with both Qubit™ 2.0 Fluorometer (Invitrogen) and Bioanalyzer DNA assay (Agilent technologies). Libraries were then prepared for sequencing and sequenced on paired-end 150 bp mode on NovaSeq™ 6000 (Illumina).

Raw reads were trimmed using TrimGalore (Krueger, 2021) and quality controlled using FastQC (Andrews, 2010). Read mapping against the V1 version of the PN40024 12X reference genome and conversion to cytosine-specific DNA methylation levels were performed using the bisulfite sequencing alignment tool Bismark v0.19.0 (Krueger and Andrews, 2011). DNA methylation-based PCA and Differential Methylation Analysis over single cytosines were performed using the methylKit R package (Akalin *et al*., 2012) with default parameters and a Q-value cutoff of 0.01.

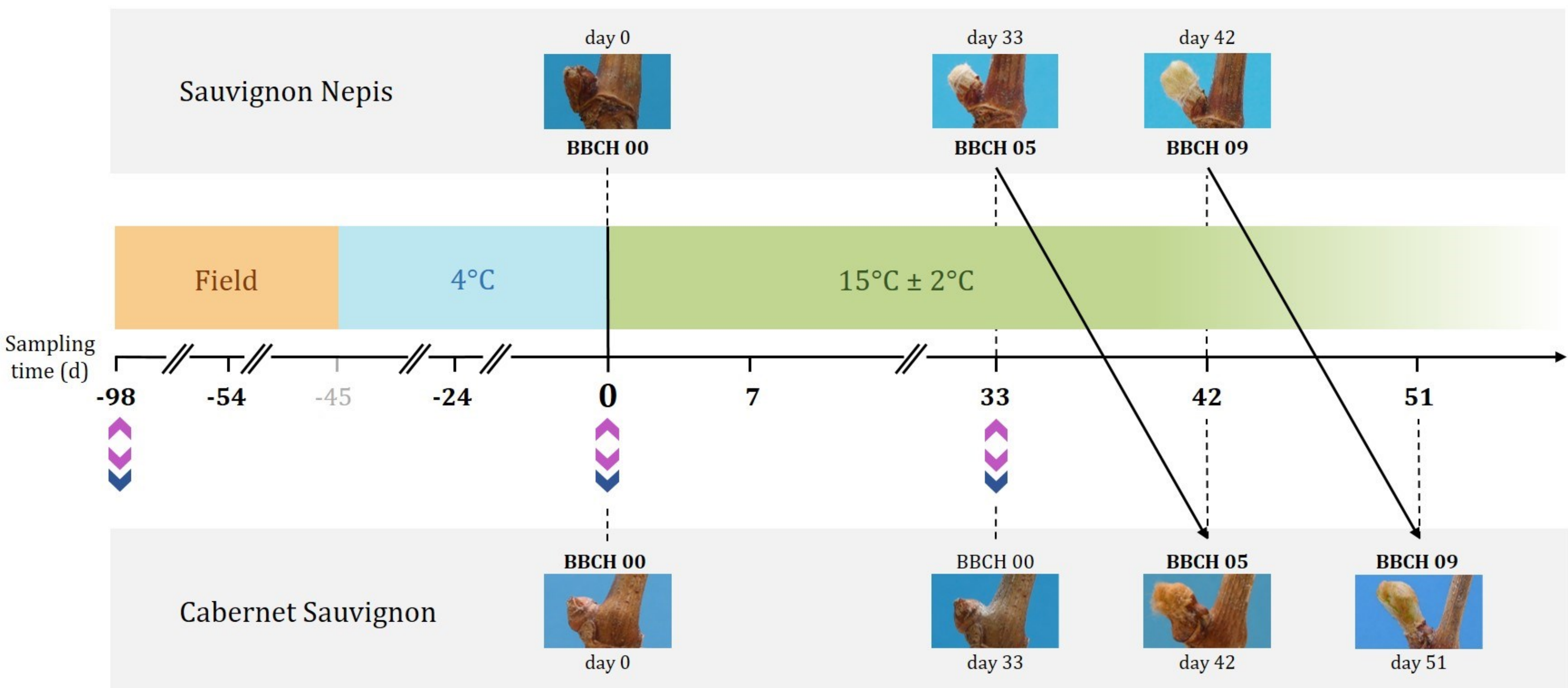


Figure 1. Graphic representation of timeline of analysis in Cabernet Sauvignon and Sauvignon Nepis potted plants. Time-points are expressed as days before and after expected chilling requirement fulfillment (day 0) following 45 days of cold exposure at 4°C (blue area). Bold numbers on the timeline delineate sampling times for DTA and qPCR analysis; purple arrows indicate RNA-Seq performed on buds of both cultivars; blue arrows indicate BS-Seq performed on Cabernet Sauvignon buds. Buds' pictures show the differential phenological progression of Sauvignon Nepis and Cabernet Sauvignon from BBCH 00 (winter bud) to BBCH 05 (wooly bud) and BBCH 09 (green tip) stage.

3 RESULTS

3.1 Cold hardiness determination and phenology advancement monitoring

DTA allows to detect lethal freezing temperatures for grapevine buds, and is a consolidated method to measure bud cold hardiness (Mills *et al*., 2006; Kovaleski and Londo, 2019). DTA successfully allowed to follow cold hardiness dynamics in cvs. Cabernet Sauvignon and Sauvignon Nepis buds throughout the experiment (Figure 2).

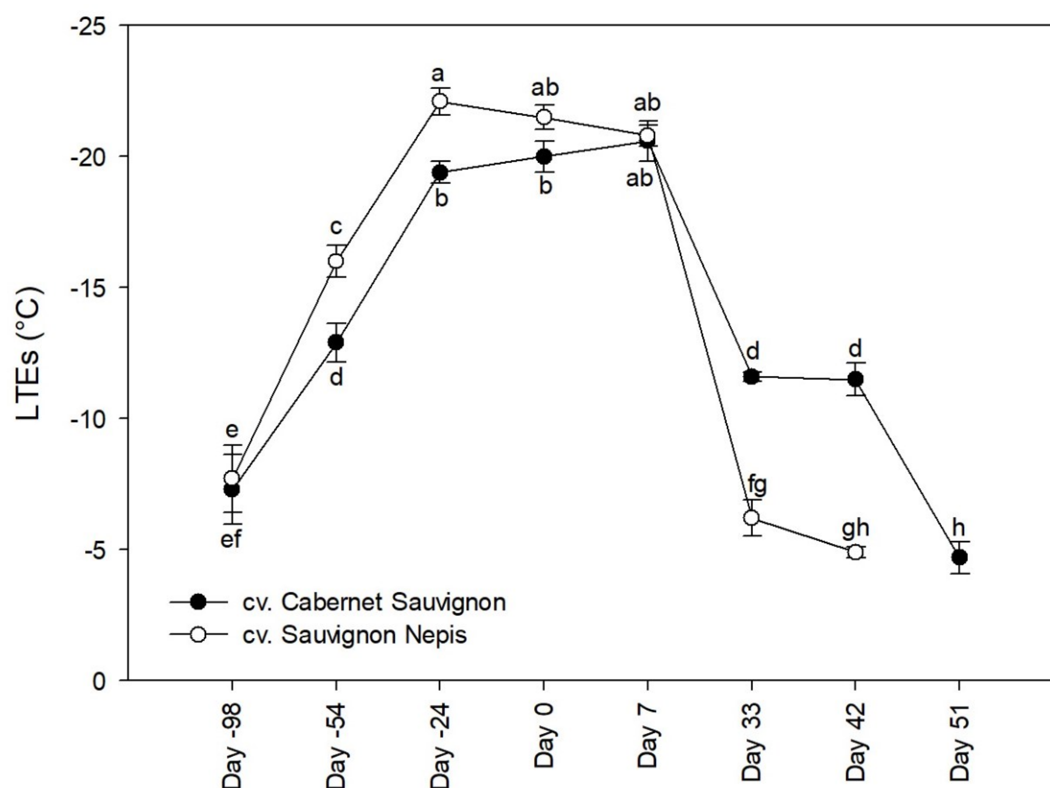


Figure 2. Low-temperature exotherms (LTEs) of grapevine buds. Results are expressed as mean of 3 biological replicates ± standard deviation. X-axis delineates time of sampling in relation to expected chilling requirement fulfillment (day 0). Statistical analysis was performed using one-way ANOVA and Tukey HSD as *post hoc* test for all pairwise comparison procedures.

Cabernet Sauvignon and Sauvignon Nepis buds showed similar acclimation dynamics, slightly faster in Sauvignon Nepis, and reached their respective maximum cold hardiness levels simultaneously (24 days before the expected chilling requirement fulfillment). Lowest mean low-temperature exotherms (LTEs) were -22.1°C for Sauvignon Nepis and -20.6°C for Cabernet Sauvignon. Cold hardiness levels of both cultivars dropped 33 days following the transfer to the greenhouse at 15°C ±

2°C and natural light conditions, with registered LTEs of -6.2°C for Sauvignon Nepis and -11.6°C for Cabernet Sauvignon. These results possibly mirrored the different deacclimation rhythms of the two varieties, since day 33 also coincided with the wooly bud stage (BBCH 05) of Sauvignon Nepis, as opposed to Cabernet Sauvignon which appeared still in the winter bud stage (BBCH 00). Day 42 marks the transition of Sauvignon Nepis buds to the BBCH 09 (green tip) stage, with mean LTEs reaching the minimum value of -4.9°C. On the other hand, Cabernet Sauvignon buds shifted to the BBCH 05 stage at day 42, although recorded mean LTEs remained stable (-11.5°C). The green tip stage was reached at day 51, 9 days later as compared to Sauvignon Nepis, at day 51.

3.2 *VvDMLs* expression trends

Two grapevine *VvDMLs* homologs, *VvDEM1* and *VvDEM3*, were recently detected and found to be downregulated during dormancy and upregulated in proximity to budbreak in field-grown grapevine plants (De Rosa *et al*., 2022a, see Chapter IV). Due to their hypothesized role as transcriptional regulators in the context of dormancy release, *VvDML* expression was analyzed in both cvs. Sauvignon Nepis (Figure 3A) and Cabernet Sauvignon (Figure 3B), with similar expression trends detected in the two cultivars. In general, *VvDMLs* expression declines of during the 4°C phase and increases during forced growth conditions.

In detail, Sauvignon Nepis *VvDEM1* expression was significantly downregulated at days 0 and 33 compared to all other time-points, with day 33 representing the lowest expression level (Figure 3A). A similar downregulation was observed in Cabernet Sauvignon *VvDEM1*, with a significant minimum at day 7 (Figure 3B).

Meanwhile, *VvDEM3* expression showed a clearly delineated trend of upregulation during both field and greenhouse growth conditions and downregulation during the chilling period in both cultivars. In particular, *VvDEM3* expression in Sauvignon Nepis dropped at day -24, its lowest level being recorded at day 0 (Figure 3A). *VvDEM3* downregulation was slightly postponed in Cabernet Sauvignon buds, significantly dropping at day 0 and remaining low until day 7.

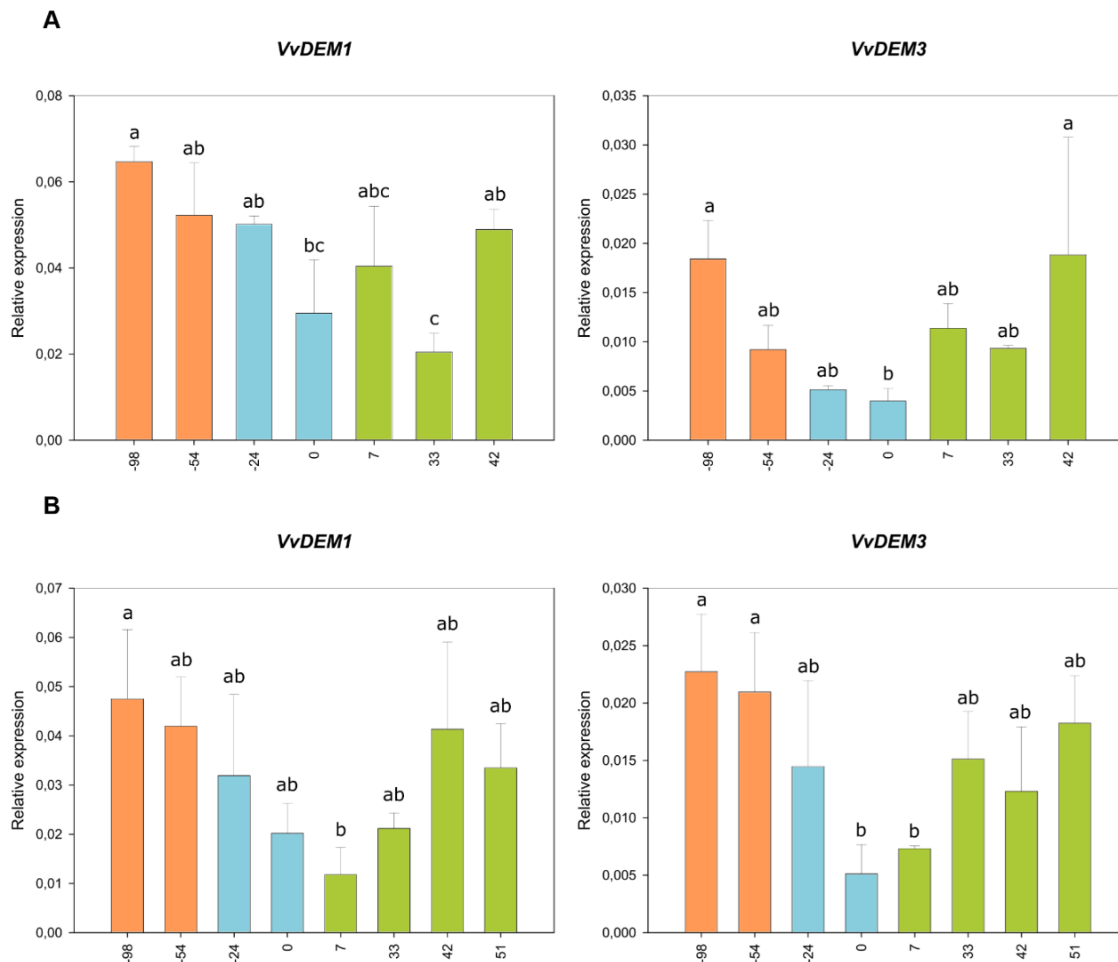


Figure 3. Expression patterns of *VvDEM1* and *VvDEM3* in buds of cvs. Sauvignon Nepis (**A**) and Cabernet Sauvignon (**B**) potted plants. X-axis indicates time of sampling in relation to expected chilling requirement fulfillment (day 0). Results are expressed as mean of 3 biological replicates ± standard deviation. Statistical analyses were performed within each variety using one-way ANOVA and Tukey HSD as *post hoc* test for all pairwise multiple comparison procedures.

3.3 Analysis of buds' transcriptome at different stages of dormancy progression

We used high-throughput RNA sequencing to investigate the transcriptome dynamics of both Cabernet Sauvignon (CS) and Sauvignon Nepis (SN) buds over three time points. Specifically, we analyzed gene expression at T1, which corresponds to cold acclimation for both varieties at -98 days before expected chilling requirement fulfillment; T2, corresponding to deep dormancy in both cultivars at day 0, when chilling requirement is expected to be fulfilled; T3, which corresponds to deacclimation in both varieties at 33 days after expected chilling requirement fulfillment, with SN at wooly bud stage (BBCH 05) and persisting winter bud stage (BBCH 00) for CS.

3.3.1 Principal Component Analysis

A Principal Component Analysis (PCA) of the 36 samples showed good overlapping of the triplicates of each time point for each variety (Figure 4). Considering the three time-points one at a time, the two genotypes tend to segregate separately. The greatest differences between CS and SN can be appreciated at T3, in alignment with the marked differences in phenological stage (BBCH 00 for CS, BBCH 05 for SN, see Figure 1).

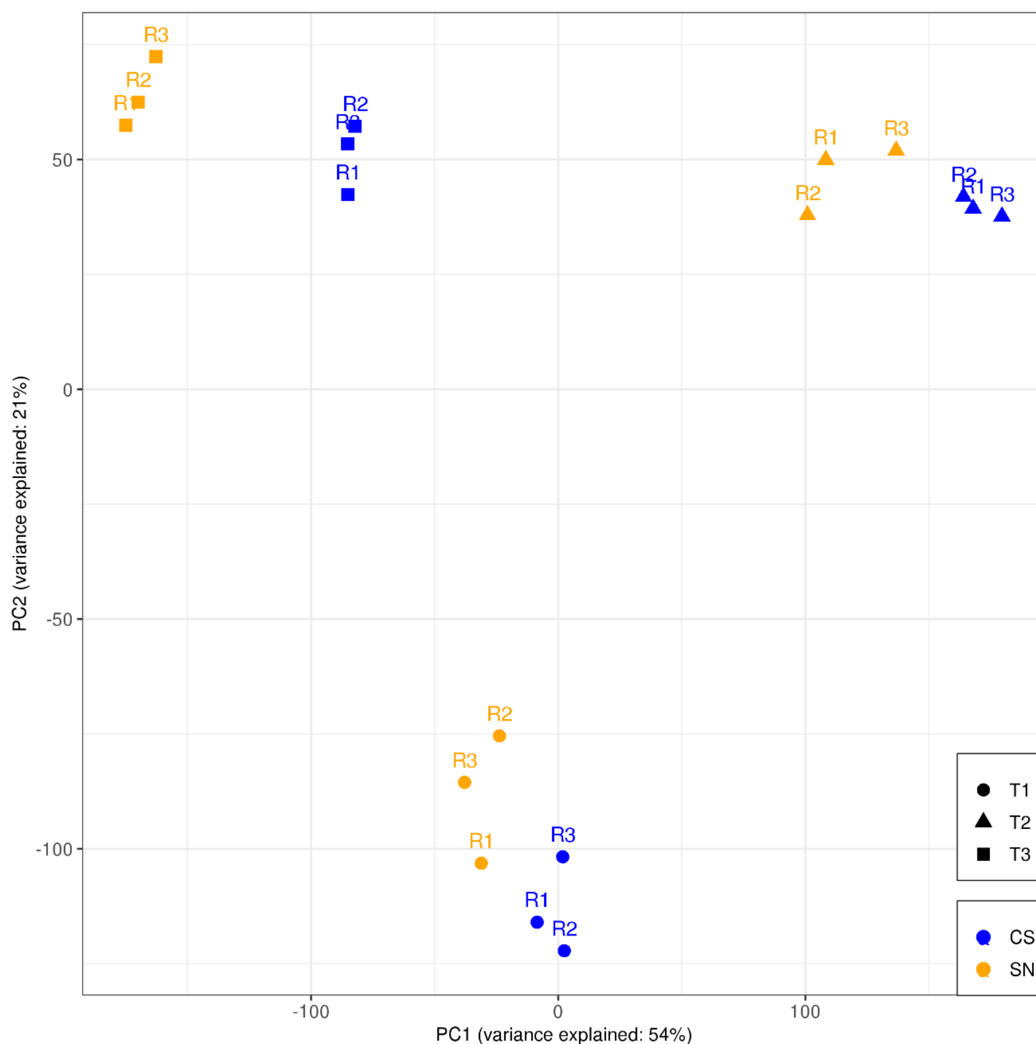


Figure 4. Principal Component Analysis (PCA) of RNA-Seq data of CS and SN buds from T1 to T3.

PC1 (x-axis) and PC2 (y-axis) explained 54% and 21% of the variance, respectively. PC1 suggests that gene expression profiles of T1 (cold acclimation) of both cultivars are equally different to both T2 (expected ecodormancy) and T3 (deacclimation) ones. PC1 also indicates that T2 (ecodormancy) and T3

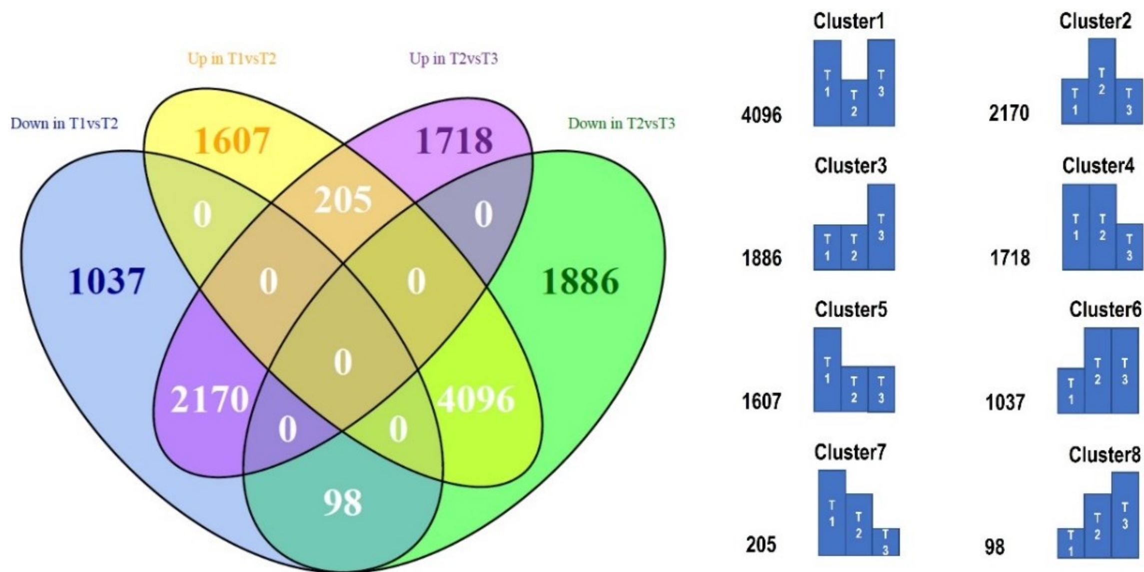
(deacclimation) expression profiles are the most divergent between all time-points comparisons. Lastly, PC2 hints to a smaller component of genes that contribute to further differentiating T1 from both T2 and T3.

3.3.2 Clustering analysis of DEGs

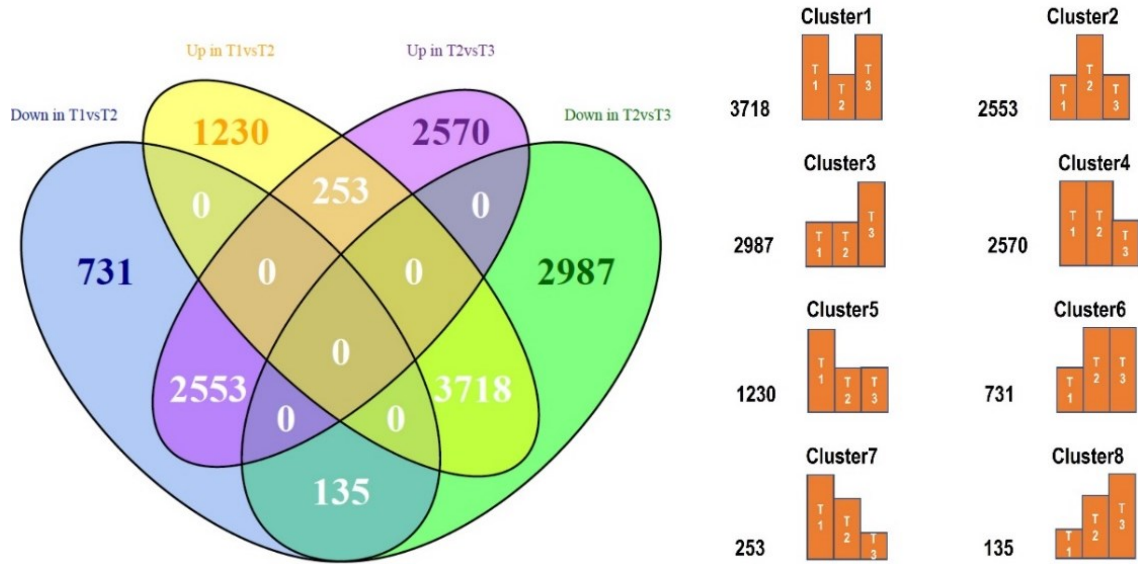
To identify total Differentially Expressed Genes (DEGs), the three time points were subjected to pairwise comparison: differential gene expression within each variety among the three time points was examined using |log2(FoldChange)| > 1. A total number of 25836 DEGs was found from the pairwise comparisons of the time points in CS, whereas 26966 DEGs were found for SN.

In CS (Figure 5a), 5908 upregulated DEGs and 3305 downregulated DEGs were found in the T1 vs T2 comparison, while 4093 upregulated DEGs and 6080 downregulated DEGs were found in the T2 vs T3 comparison. In detail, 205 DEGs, out of the 5908 upregulated in T1 vs T2 comparison, were found to be also upregulated in the T2 vs T3 comparison, whereas 4096 of them were also downregulated in T2 vs T3. Moreover, in CS, 2170 out of the 3305 DEGs downregulated of T1 vs T2 were also upregulated in T2 vs T3 and 98 DEGs were downregulated in T2 vs T3 as well. In the given comparisons, 1718 upregulated DEGs and 1886 downregulated DEGs were unique to the T2 vs T3 comparison and 1607 upregulated DEGs and 1037 downregulated DEGs were found to be exclusive of T1 vs T2.

As for SN (Figure 5b), 5201 upregulated DEGs and 3419 downregulated DEGs were found in the T1 vs T2 comparison, while 5376 upregulated DEGs and 6840 downregulated DEGs were found in the T2 vs T3 comparison. In particular, 253 DEGs upregulated in T1 vs T2 were also found to be upregulated in the T2 vs T3 comparison. On the other hand, 3718 upregulated in T1 vs T2 were found downregulated in T2 vs T3. Moreover, 2553 DEGs downregulated in T1 vs T2 were upregulated in T2 vs T3, and 135 DEGs downregulated in T1 vs T2 were also downregulated in T2 vs T3. Finally, 1230 upregulated DEGs and 731 downregulated DEGs were found exclusive of the T1 vs T2, in our comparisons. Similarly, 2570 upregulated DEGs ad 2987 downregulated DEGs were found only in the T2 vs T3 comparison (Figure 5b).



(a)



(b)

Figure 5. Venn diagrams of identified differentially expressed genes (DEGs) in Cabernet Sauvignon (**a**) and Sauvignon Nepis (**b**) buds in T1 vs T2 and T2 vs T3 comparisons (left). DEGs characterized by similar expression patterns were grouped in eight clusters (right)

In order to better describe the DEGs functions and allow to formulate hypothesis about their role in dormancy progression, DEGs of each variety were further classified into 8 clusters, based on their expression patterns in the transition from T1 to T3 (Figures 5a, 5b). In detail, clusters 1 and clusters 2 of CS and SN grouped genes whose expression in T2, as compared to T1 and T3, is lower or higher, respectively. clusters

3 encompassed genes upregulated in T3; clusters 4 include genes downregulated at T3; clusters 5 contain genes upregulated at T1; clusters 6 contain genes downregulated at T1; clusters 7 and cluster 8 included genes whose expression gradually decreases or increases, from T1 to T3, respectively.

3.4 Functional annotation of DEGs during grapevine dormancy progression

To better understand the biological functions of DEGs involved in dormancy evolution (Figure 5), gene ontology (GO) enrichment analysis was undertaken. Enriched GO terms related to biological process (BP) taking place in the transition from T1 to T3 were identified for CS and SN buds. Taking advantage of the previously defined clusters (section 3.3), a timescale was built and a chart was provided for each cultivar, aiming to visualize the relevance of the identified GO annotations in the different stages of dormancy progression (Figures 6 and 7).

In CS (Figure 6), DEGs whose expression is highest at T1 are found in cluster 5 and 7 (Figure 5a). BP terms enriched in these clusters are mainly involved in abiotic stress responses and defense responses, such as 'GO:1901698 response to nitrogen compound', 'GO:1901700 response to oxygen-containing compound'. Cluster 4 contains genes whose expression is similarly high in both T1 and T2 (Figure 5a), and terms related to abiotic stress responses appeared to be the most significant also in this context. In more detail, 'GO:1901700 response to oxygen-containing compound', 'GO:0042221 response to chemical' and 'GO:0010033 response to organic substance' stood out distinctly as most enriched. Moving to cluster 2, whose DEGs are highly expressed at T2 (Figure 5a), BP terms related to abiotic stress response, gene expression and metabolic processes were found ('GO:0009628 response to abiotic stimulus', 'GO:0015996 chlorophyll catabolic process', 'GO:0044248 cellular catabolic process', 'GO:0046149 pigment catabolic process'). Cluster 1, showing the opposite pattern, with DEGs downregulated in T2 (Figure 5a), encompassed BP terms related to cell organization and growth and development (Figure 6). In particular, the most significant terms were 'GO:0048509 regulation of meristem development', 'GO:0005975 carbohydrate metabolic process' and 'GO:0071554 cell wall organization or biogenesis'. Cluster 6, with genes upregulated in both T2 and T3 (Figure 5a), included BP terms related to cell organization, gene expression and metabolic processes. In particular, the most relevant terms were 'GO:0006996

organelle organization', 'GO:0006139 RNA processing' and 'GO:0006139 nucleobase-containing compound metabolic process'. Moving to cluster 8, which contains DEGs whose expression gradually increases from T1 to T3 (Figure 5a), BP terms belonged to cell cycle and cell replication, and epigenetic processes. In detail, the most significant terms were 'GO:0007049 cell cycle', 'GO:0008283 cell proliferation', 'GO:0051276 chromosome organization' and 'GO:0006325 chromatin organization'. Lastly, relevant BP terms related to cluster 3 DEGs, i.e. upregulated at T3 (Figure 5a), were related to gene expression, cell organization and metabolic processes. In particular, 'GO:0022613 ribonucleoprotein complex biogenesis' and 'GO:0042254 ribosome biogenesis' and 'GO:0006412' were the most significant terms, followed by 'GO:0043604 amide biosynthetic process', 'GO:0071840 cellular component organization or biogenesis', and several terms related to epigenetic processes.

As for SN (Figure 7), BP terms enriched in cluster 5 (Figure 5b) were related to metabolic processes and abiotic responses. In particular, the most relevant terms in these categories were 'GO:0009698 phenylpropanoid metabolic process', 'GO:0009808 lignin metabolic process' and 'GO:0042493 response to drug'. Similar BP terms were found moving to cluster 7 (Figure 5b), related to metabolic processes and abiotic responses (Figure 7). In detail, most enriched terms were 'GO:0046271 phenylpropanoid catabolic process', 'GO:0046274 lignin catabolic process' and 'GO:0046688 response to copper iron'. Enriched BP terms in cluster 4 (Figure 5b) were related to gene expression regulation and abiotic responses, such as 'GO:0000956 nuclear-transcribed mRNA catabolic process', 'GO:0006402 mRNA catabolic process' and 'GO:1901700 response to oxygen-containing compound'. Cluster 2 (Figure 5b) presents BP terms enriched in the metabolic process and gene expression categories, the most relevant terms are 'GO:0009889 regulation of biosynthetic process', 'GO:0031326 regulation of cellular biosynthetic process' and 'GO:0010468 regulation of gene expression'. Moving to DEGs lowly expressed at T2, BP terms enriched in cluster 1 (Figure 5b) appeared connected to growth and development and several metabolic processes. In detail, most significant terms were 'GO:0048589 developmental growth', 'GO:0005975 carbohydrate metabolic process', 'GO:0005976 polysaccharide metabolic process', 'GO:0006694 steroid biosynthetic process' and 'GO:0006629 lipid metabolic process'. As far as cluster 6 is concerned (Figure 5b), relevant BP terms belonged mostly to cell organization, cell trafficking

and gene expression. Among them, ‘GO:0006996 organelle organization’, ‘GO:0009451 RNA modification’ and ‘GO:0006913 nucleocytoplasmic transport’ were the most relevant terms. Finally, DEGs gradually more expressed from T1 to T3 in cluster 8 (Figure 5b) showed BP terms connected to epigenetic processes and cell cycle, division and organization. In detail, ‘GO:0051276 chromosome organization’, ‘GO:0006325 chromatin organization’, ‘GO:0051567 histone H3-K9 methylation’, ‘GO:0006996 organelle organization’ and ‘GO:0008283 cell proliferation’ were among the most relevant terms. Lastly, cluster 3 of DEGs upregulated at T3 was characterized by BP terms related to gene expression, metabolic processes, photosynthesis and respiration, cell organization and division: ‘GO:0015979 photosynthesis’, ‘GO:1901566 organonitrogen compound biosynthetic process’, ‘GO:0044085 cellular component biogenesis’, ‘GO:0043043 peptide biosynthetic process’ and ‘GO:0006412 translation’ were most relevant.

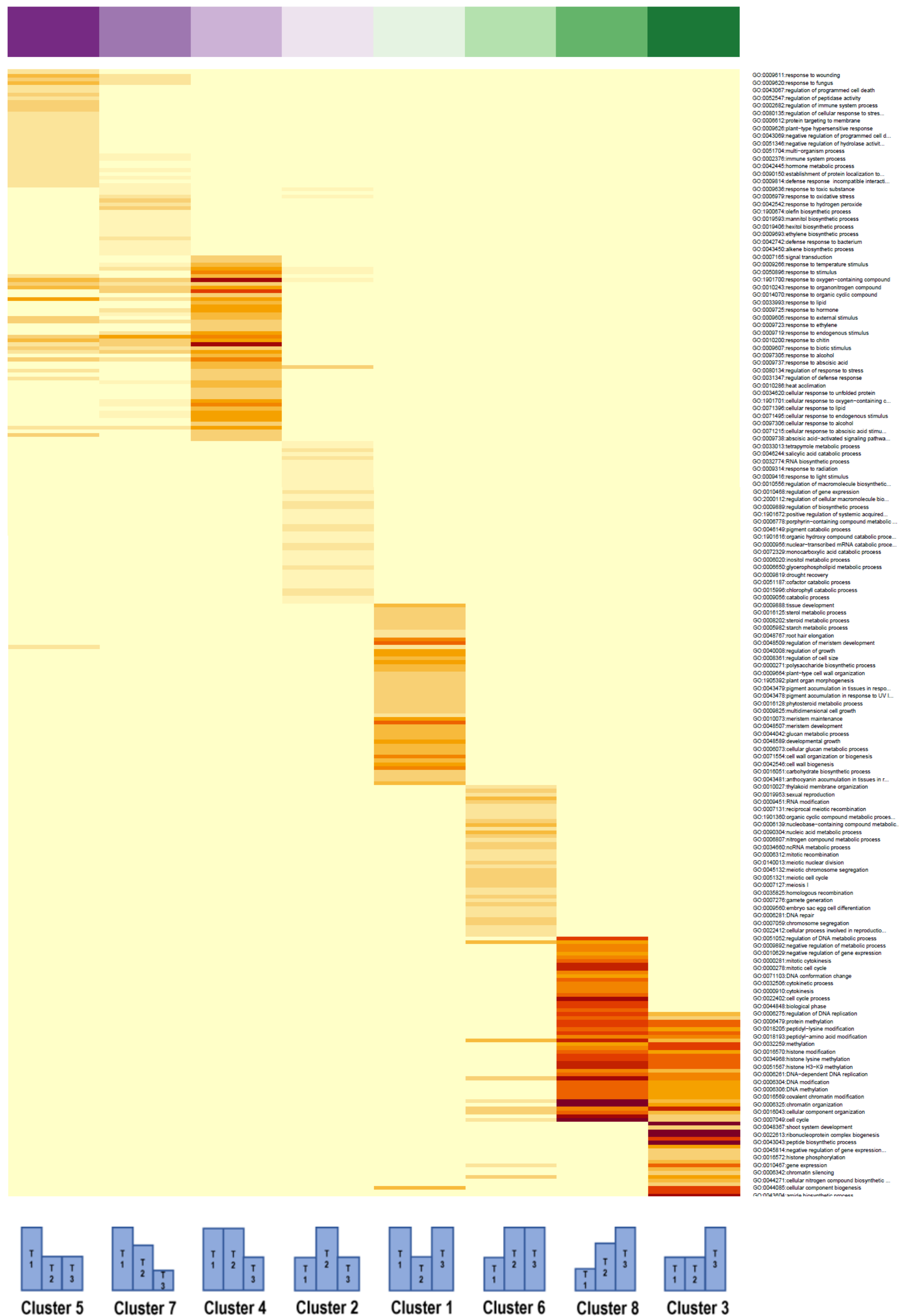


Figure 6. Representation of the most significant GO terms during dormancy progression of Cabernet Sauvignon buds. GO terms derive from enrichment analysis for Biological process (BP) in DEGs of the eight clusters (see paragraph 3.3).

GO:0009810:stilbene metabolic process
GO:0009069:serine family amino acid metabolic proce...
GO:0019748:secondary metabolic process
GO:0009751:response to salicylic acid
GO:0010243:response to organonitrogen compound
GO:0014070:response to organic cyclic compound
GO:0009620:response to fungus
GO:0010200:response to chitin
GO:0031347:regulation of defense response
GO:0006468:protein phosphorylation
GO:0009698:phenylpropanoid metabolic process
GO:0055114:oxidation−reduction process
GO:1900674:olefin biosynthetic process
GO:0009809:lignin biosynthetic process
GO:0002376:immune system process
GO:0042446:hormone biosynthetic process
GO:0006952:defense response
GO:0009805:coumarin biosynthetic process
GO:0008037:cell recognition
GO:0019419:sulfate reduction
GO:0009414:response to water deprivation
GO:0009636:response to toxic substance
GO:0006950:response to stress
GO:0000302:response to reactive oxygen species
GO:0006979:response to oxidative stress
GO:0009642:response to light intensity
GO:0042542:response to hydrogen peroxide
GO:0009644:response to high light intensity
GO:0009719:response to endogenous stimulus
GO:0046688:response to copper ion
GO:0046677:response to antibiotic
GO:0001101:response to acid chemical
GO:0009628:response to abiotic stimulus
GO:0009788:negative regulation of abscisic acid−act...
GO:0042538:hyperosmotic salinity response
GO:0050832:defense response to fungus
GO:0032870:cellular response to hormone stimulus
GO:0097306:cellular response to alcohol
GO:0019439:aromatic compound catabolic process
GO:0009738:abscisic acid−activated signaling pathwa...
GO:0006986:response to unfolded protein
GO:0036211:protein modification process
GO:0070647:protein modification by small protein co...
GO:0034655:nucleobase−containing compound catabolic...
GO:0046700:heterocycle catabolic process
GO:0034620:cellular response to unfolded protein
GO:0006464:cellular protein modification process
GO:0044265:cellular macromolecule catabolic process
GO:0009056:catabolic process
GO:0006401:RNA catabolic process
GO:0009416:response to light stimulus
GO:2001141:regulation of RNA biosynthetic process
GO:0010468:regulation of gene expression
GO:0000956:nuclear−transcribed mRNA catabolic proce...
GO:0010228:vegetative to reproductive phase transit...
GO:0048511:rhythmic process
GO:0051252:regulation of RNA metabolic process
GO:0019219:regulation of nucleobase−containing comp...
GO:0051239:regulation of multicellular organismal p...
GO:0019222:regulation of metabolic process
GO:0010556:regulation of macromolecule biosynthetic...
GO:2000112:regulation of cellular macromolecule bio...
GO:0009889:regulation of biosynthetic process
GO:0009585:red or far−red light signaling pathway
GO:0007602:phototransduction
GO:0009648:photoperiodism
GO:0009059:macromolecule biosynthetic process
GO:0007623:circadian rhythm
GO:0071478:cellular response to radiation
GO:0034645:cellular macromolecule biosynthetic proc...
GO:0010025:wax biosynthetic process
GO:0035383:thioester metabolic process
GO:0016126:sterol biosynthetic process
GO:0006694:steroid biosynthetic process
GO:0010015:root morphogenesis
GO:0048364:root development
GO:0048509:regulation of meristem development
GO:0008361:regulation of cell size
GO:0000271:polysaccharide biosynthetic process
GO:0009832:plant−type cell wall biogenesis
GO:0090627:plant epidermal cell differentiation
GO:0016129:phytosteroid biosynthetic process
GO:1901617:organic hydroxy compound biosynthetic pr...
GO:0009825:multidimensional cell growth
GO:0006629:lipid metabolic process
GO:0044042:glucan metabolic process
GO:0060560:developmental growth involved in morphog...
GO:0044264:cellular polysaccharide metabolic proces...
GO:0044262:cellular carbohydrate metabolic process
GO:0071555:cell wall organization
GO:0016049:cell growth
GO:0016051:carbohydrate biosynthetic process
GO:0016132:brassinosteroid biosynthetic process
GO:0006637:acyl−CoA metabolic process
GO:0010027:thylakoid membrane organization
GO:0009451:RNA modification
GO:0034504:protein localization to nucleus
GO:0017038:protein import
GO:0051644:plastid localization
GO:0006913:nucleocytoplasmic transport
GO:0090304:nucleic acid metabolic process
GO:0034470:ncRNA processing
GO:0061024:membrane organization
GO:0051170:import into nucleus
GO:0007276:gamete generation
GO:0072594:establishment of protein localization to...
GO:0051649:establishment of localization in cell
GO:0009553:embryo sac development
GO:0019750:chloroplast localization
GO:0006725:cellular aromatic compound metabolic pro...
GO:0071824:protein−DNA complex subunit organization
GO:0006479:protein methylation
GO:0018205:peptidyl−lysine modification
GO:0034728:nucleosome organization
GO:0009892:negative regulation of metabolic process
GO:0045814:negative regulation of gene expression...
GO:0016571:histone methylation
GO:0016458:gene silencing
GO:0006304:DNA modification
GO:0006306:DNA methylation
GO:0006305:DNA alkylation
GO:0006342:chromatin silencing
GO:0006333:chromatin assembly or disassembly
GO:0051301:cell division
GO:0006364:rRNA processing
GO:0009657:plastid organization
GO:0043414:macromolecule methylation
GO:0034641:cellular nitrogen compound metabolic pro...
GO:0006081:cellular aldehyde metabolic process
GO:0006996:organelle organization
GO:0061647:histone H3−K9 modification
GO:0006261:DNA−dependent DNA replication
GO:0016043:cellular component organization
GO:0006412:translation
GO:0019693:ribose phosphate metabolic process
GO:0006275:regulation of DNA replication
GO:0019684:photosynthesis light reaction
GO:0009853:photorespiration
GO:0043043:peptide biosynthetic process
GO:1901566:organonitrogen compound biosynthetic pro...
GO:0009117:nucleotide metabolic process
GO:0055086:nucleobase−containing small molecule met...
GO:0046490:isopentenyl diphosphate metabolic proces...
GO:0019288:isopentenyl diphosphate biosynthetic pro...
GO:0006091:generation of precursor metabolites and ...
GO:0006270:DNA replication initiation
GO:0071840:cellular component organization or bioge...
GO:0043603:cellular amide metabolic process
GO:0043604:amide biosynthetic process

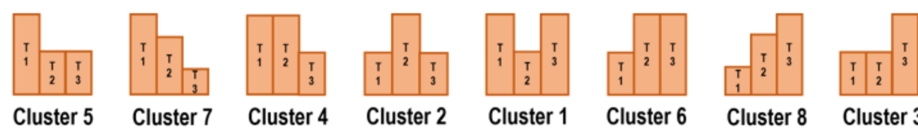


Figure 7. Representation of the most significant GO terms during dormancy progression of Sauvignon Nepis buds. GO terms derive from enrichment analysis for Biological process (BP) in DEGs of the eight clusters (see paragraph 3.3).

3.5 Functional annotation of cultivar-specific DEGs during cold acclimation

Upregulated and downregulated DEGs detected in T1 vs T2 pairwise comparisons were intersected to identify molecular mechianism unique to each cultivar in the cold acclimation phase (Figure 8).

Considering upregulated DEGs in T1 vs T2 in CS and SN, 4114 were shared between the two cultivars, whereas 1087 DEGs were unique to SN and 1794 to CS. Regarding upregulated DEGs in T2 vs T1 DEGs, 2246 DEGs appeared common between CS and SN, while 1173 DEGs were found in SN only and 1059 DEGs in CS exclusively.

BP terms enrichment of up- and downregulated T1 vs T2 DEGs exclusive to each variety was analyzed.

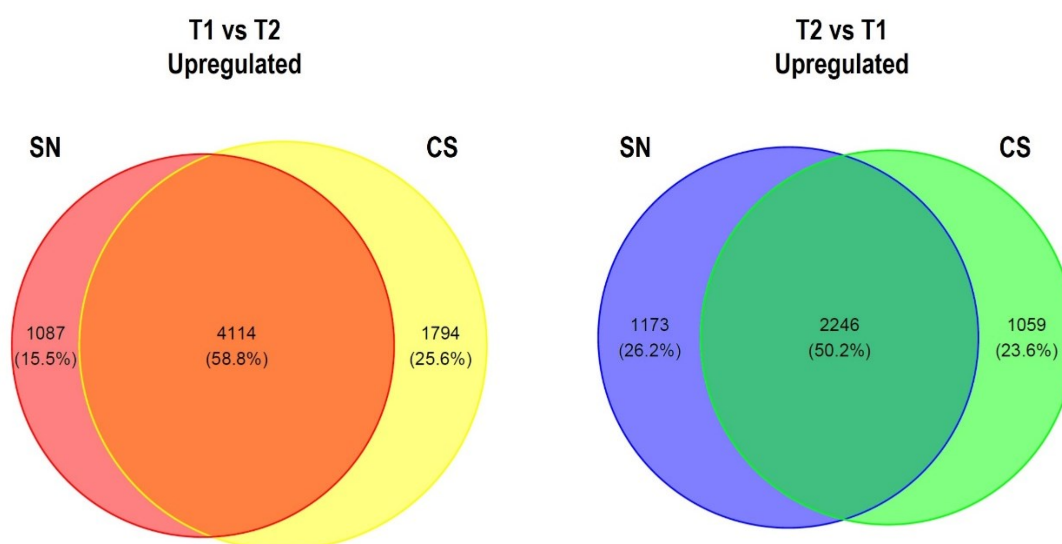


Figure 8. Venn diagrams of shared and differential DEGs between Cabernet Sauvignon and Sauvignon Nepis in T1 vs T2 pairwise comparisons.

BP terms found to be highly relevant in DEGs upregulated in T1 vs T2 exclusive to CS concerned the regulation of protein metabolism ('GO:0042254 ribosome biogenesis', 'GO:0030162 regulation of proteolysis', 'GO:0010466 negative regulation of peptidase activity', 'GO:0006468 protein phosphorylation'), flavonoid metabolism ('GO:0051554 flavonoid metabolic process', 'GO:0051555 flavonol biosynthetic process'), developmental processes ('GO:0009955 adaxial/abaxial pattern

specification', 'GO:0048509 regulation of meristem development') and light responses ('GO:0010218 response to far red light', 'GO:0009637 response to blue light', 'GO:0009765 photosynthesis, light harvesting').

As for upregulated processes in T2 vs T1 CS DEGs, processes related to DNA and organelle organization appeared relevant ('GO:0006260 DNA replication', 'GO:0061647 histone H3-K9 modification', 'GO:0000280 nuclear division', 'GO:0048285 organelle fission'). Cell cycle-related processes ('GO:0000278 mitotic cell cycle', 'GO:0051322 anaphase') and mitochondrial ATP synthesis ('GO:0006120 mitochondrial electron transport, NADH to ubiquinone', 'GO:0022904 respiratory electron transport chain') appeared active. Interestingly, terms related to water stress were also found ('GO:0009819 drought recovery').

A more detailed list of CS-related BP terms can be found in Table A1.

Moving to SN upregulated DEGs in T1 vs T2, lignin and phenylpropanoid metabolism appeared highly active ('GO:0009808 lignin metabolic process', 'GO:0009699 phenylpropanoid biosynthetic process', 'GO:0009804 coumarin metabolic process'). Additionally, lipid metabolism-related process also resulted relevant ('GO:1901570 fatty acid derivative biosynthetic process', 'GO:0006629 lipid metabolic process').

As for upregulated DEGs in T2 vs T1 of SN, gene expression appeared highly regulated ('GO:0010468 regulation of gene expression', 'GO:0016070 RNA metabolic process', 'GO:0006351 transcription, DNA-templated') together with light perception ('GO:0009639 response to red or far red light', 'GO:0009416 response to light stimulus'). Lastly, intracellular transport was also detected as highly relevant ('GO:0017038 protein import', 'GO:0007031 peroxisome organization', 'GO:0006625 protein targeting to peroxisome').

A more detailed list of SN-related BP terms can be found in Table A2.

3.6 Functional annotation of cultivar-specific DEGs during deacclimation

Upregulated and downregulated DEGs detected in T2 vs T3 pairwise comparisons were intersected to identify molecular mechanisms unique to each cultivar in deacclimation phase (Figure 9).

Starting from upregulated DEGs in T2 vs T3 in the comparison between CS and SN, 3193 were common between the two cultivars, whereas 2183 DEGs were unique to SN and 900 to CS. Regarding upregulated DEGs in T3 vs T2, 4755 DEGs appeared shared between CS and SN, while 2085 DEGs were found in SN only and 1325 DEGs in CS exclusively.

BP terms enrichment of up- and downregulated T2 vs T3 DEGs exclusive to each variety was analyzed.

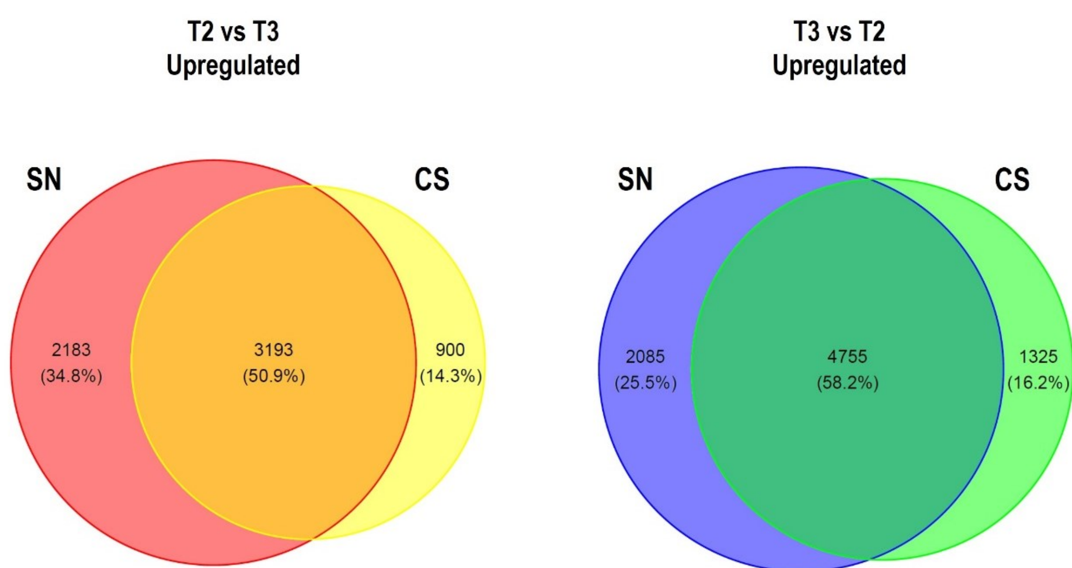


Figure 9. Venn diagrams of shared and differential DEGs between Cabernet Sauvignon and Sauvignon Nepis in T2 vs T3 pairwise comparisons.

The most significant BP terms in CS upregulated DEGs in T2 vs T3 were mostly related to secondary metabolism processes, such as 'GO:1900674 olefin biosynthetic process', 'GO:0009810 stilbene metabolic process', together with 'GO:0009804 coumarin metabolic process' and 'GO:0009809 lignin biosynthetic process'. Biotic stress responses and abscisic acid-mediated signaling also appeared relevant.

As for upregulated DEGs in T3 vs T2 in CS, developmental and reproductive processes appeared to be active ('GO:0007389 pattern specification process', 'GO:0009799 specification of symmetry', 'GO:0010073 meristem maintenance', 'GO:0048827 phyllome development', 'GO:0048440 carpel development'). Responses to abiotic stress were also detected, with the term 'GO:0006952 defense response' being the most significant. Moreover, terms related to transcription regulation

processes and hormonal signaling connected to gibberellins were enriched. Interestingly, biological processes related to transposable elements (TEs) activity were detected.

A more detailed list of CS-related BP terms can be found in Table A3.

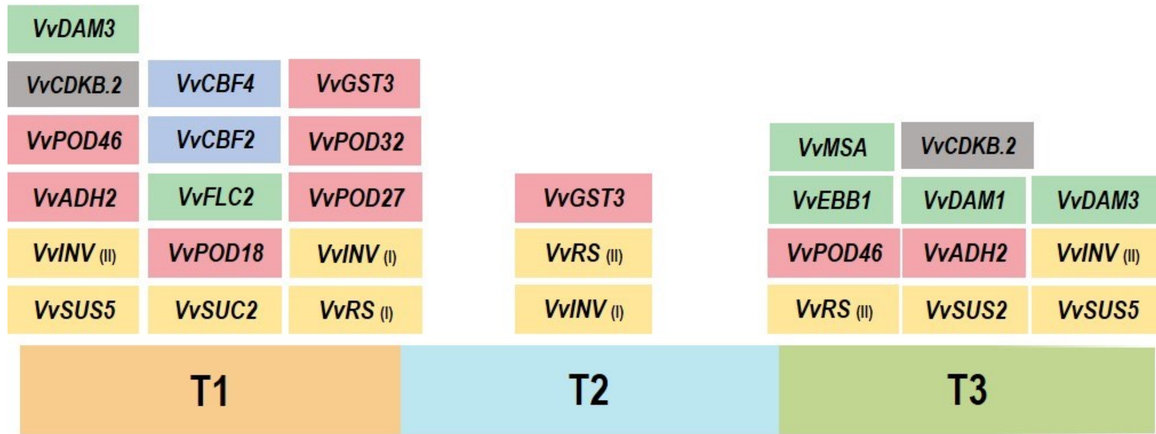
As for SN upregulated DEGs in T2 vs T3, the most significant BP terms found in this context were related to RNA processing and catabolism ('GO:0000956 nuclear-transcribed mRNA catabolic process', 'GO:0010468 regulation of gene expression'). Processes related to energy production were also indicated by terms such as 'GO:0015980 energy derivation by oxidation of organic compounds', 'GO:0045333 cellular respiration' and 'GO:0042775 mitochondrial ATP synthesis coupled electron transport'. Moreover, reproduction-related processes (GO:0010228 vegetative to reproductive phase transition of meristem; GO:0009553 embryo sac development), and light response-related processes (GO:0048573 photoperiodism, flowering; GO:0009416 response to light stimulus) were also detected.

Moving to SN upregulated DEGs in T3 vs T2, the most relevant BP terms appeared involved in energy derivation from glycolysis ('GO:1901135 carbohydrate derivative metabolic process', 'GO:0019682 glyceraldehyde-3-phosphate metabolic process', 'GO:0006090 pyruvate metabolic process'). Moreover, energy derivation processes are also suggested by terms such as 'GO:0046034 ATP metabolic process' and 'GO:0046496 nicotinamide nucleotide metabolic process'. Lastly, photorespiration and photosynthesis-related processes were also detected, as indicated by terms such as 'GO:0015979 photosynthesis', 'GO:0010027 thylakoid membrane organization', 'GO:0009853 photorespiration' and several others.

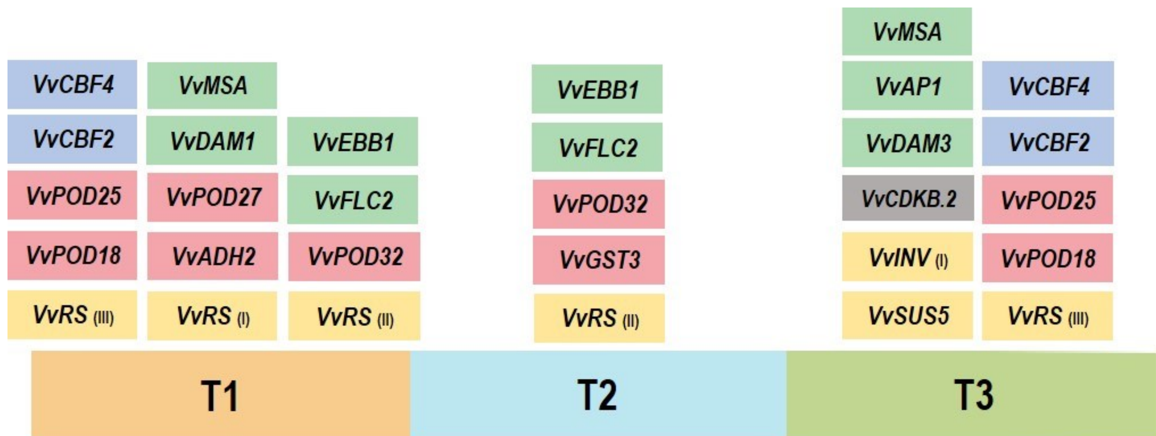
A more detailed list of BP terms derived from SN DEGs can be found in Table A4.

3.7 Differentially expressed genes during dormancy progression

DEGs were searched for specific genes of interest found in recent literature, with particular attention to differentially expressed DEGs between CS and SN (Figure 9).



(**a**)



(**b**)

Figure 10. Differentially expressed DEGs between Cabernet Sauvignon (**a**) and Sauvignon Nepis (**b**) during T1, T2 and T3. Yellow boxes: carbohydrate metabolism-related genes; red boxes: oxidative stress-related genes; blue boxes: cold response-related genes; grey boxes: cell cycle-related genes; green boxes: putative dormancy regulation-related transcription factors. *VvRS* (I) = VIT_217s0000g08960; *VvRS* (II) = VIT_217s0000g09670; *VvRS* (III) = VIT_200s0878g00020; *VvINV* (I) = VIT_205s0077g00510; *VvINV* (II) = VIT_216s0022g00670.

3.7.1 Differentially Expressed DEMETERs during dormancy progression

As previously reported (De Rosa *et al*., 2022a, see Chapter IV), three *DMLs* were detected in the grapevine genome and putatively named *VvDEM1* (VIT_213s0074g00450), *VvDEM2* (VIT_206s0061g01270) and *VvDEM3* (VIT_208s0007g03920). *VvDML* expression appears similar in the two cultivars. In particular, *VvDEM1* and *VvDEM3* were both found to be downregulated in T2 and located in clusters 1 of CS and SN (Figure 5). *VvDEM2* was not detected among DEGs in CS and SN.

3.7.2 DEGs associated with cold-related responses

Several DEGs, related to cold sensing and freezing-induced dehydration response were identified.

Cold response is driven by a signaling cascade regulated by CBF/DREB (*C-repeat Binding Factors/Dehydration Responsive Element Binding*) transcription factors. Four CBFs have been previously described (Xiao *et al*., 2006; Xiao *et al*., 2008); however, *VvCBF1* sequence does not map on the 12X grapevine genome (Vazquez-Hernandez *et al*., 2017) and was thus excluded from this study. In CS buds, two *CBF*s, namely *VvCBF2* (VIT_206s0061g01390) and *VvCBF4* (VIT_216s0100g00380) were both upregulated in T1 and found in cluster 5 (Figure 5a). As for SN, both *VvCBF2* and *VvCBF4* were detected in cluster 1, downregulated at T2. *VvCBF3* (VIT_206s0061g01400) expression was not found among DEGs in both CS and SN.

In regard to cold-related dehydration response, freezing-induced dehydrins *VvDHN1* (VIT_204s0023g02480), *VvDHN2* (VIT_218s0001g00360) and *VvDHN4* (VIT_203s0038g04390) were all found to be upregulated in T2 and located in clusters 2 of both CS and SN (Figure 5).

3.7.3 DEGs associated with carbohydrate metabolism

Starch and soluble sugar composition inside grapevine buds change during dormancy progression (Grant and Dami, 2015). Specific carbohydrate-related transcriptomic variations were recently documented for grapevine buds (Shangguan *et al*., 2020). On these premises, sugar metabolism was analyzed in DEGs data. Starting from shared transcriptomic changes, sucrose synthases such as *VvSUS1* (VIT_204s0079g00230) and *VvSUS4* (VIT_211s0016g00470), and a raffinose synthase (*VvRS*, VIT_219s0015g01350) were found in cluster 1, upregulated at T1 and T3 (Figure 5) in both CS and SN buds. In addition to this, a second *VvRS* (VIT_205s0077g00840), one galactinol synthase (*VvGolS*, VIT_201s0127g00470), *VvSUS3* (VIT_207s0005g00750), one α-amylase (VIT_218s0001g00560), *Sucrose Phosphate Synthase,* (*VvSPS1,* VIT_211s0118g00200), and three β-amylases (VIT_205s0077g00280, VIT_202s0012g00170, VIT_205s0020g01910) were all upregulated at T2 in clusters 2 (Figure 5). Moving to clusters 3 (Figure 5), while one α-amylase (VIT_214s0068g00420) was detected in both varieties, Moreover, *VvSPP*

(*Sucrose Phosphatase*, VIT_208s0032g00840), and one α-amylase (VIT_201s0026g01660) were found to be upregulated in both T1 and T2 in both cultivars in cluster 4. Lastly, a third *VvRS* (VIT_214s0066g00810), three *VvGolS* (VIT_214s0060g00810, VIT_214s0060g00790, VIT_214s0060g00760) and one invertase (VIT_209s0002g02320) were all found to be gradually downregulated from T1 to T3 (clusters 7) (Figure 5)

Moving to differentially expressed DEGs between CS and SN, *VvSUS5* (VIT_217s0053g00700) and one invertase (VIT_216s0022g00670) were found in cluster 1 of CS (Figure 5a) and cluster 3 of SN, were genes upregulated at T3 are grouped (Figure 5b). Conversely, *VvSUS2* was found in cluster 3 in CS (Figure 5a) and cluster 1 in SN (Figure 5b). A fourth *VvRS* (VIT_217s0000g09670) was upregulated at T2 and T3 in CS (cluster 6) (Figure 10a), whereas its upregulation appeared anticipated to T1 and T2 in SN (cluster 4) (Figure 10b). The expression of another *VvRS* (VIT_217s0000g08960) gradually decreased from T1 to T3 in CS (cluster 7) (Figure 10a), while its upregulation was only detected at T1 in SN (cluster 5) (Figure 10b). One invertase (VIT_205s0077g00510) was found in cluster 4 of CS only (Figure 10a), while *Sucrose transporter 2* (*VvSUC2,* VIT_218s0076g00250) was found upregulated at T1 of CS (Figure 10a). One last *VvRS* (VIT_200s0878g00020) was only found in cluster 1 of SN (Figure 10b).

3.7.4 DEGs associated with oxidative stress

Recent research regarding the possibilities of grapevine adaptation to climate change led to the identification of two QTLs connected to budbreak on chromosome 4 and chromosome 19, where four *Glutathione-S-Transferases* (GSTs) genes were detected (Duchêne *et al*., 2012). On this regard, *VvGST4* (VIT_219s0015g02730) was found to be upregulated at T2 (clusters 2) of both CS and SN (Figure 5). *VvGST3* (VIT_219s0015g02610) was instead upregulated in both T1 and T2 of CS (Figure 10a) and located in cluster 2 of SN (Figure 10b). *VvGST1* (VIT_204s0079g00710) and *VvGST2* (VIT_204s0159g00040) were not detected among DEGs in both varieties.

Since H_2O_2 was recently associated with depth of dormancy in grapevine buds (Pérez *et al*., 2021), the expression of specific peroxidases (PODs) of interest (Díaz-Riquelme *et al*., 2012) was searched for in DEGs data. In both CS and SN buds,

peroxidase encoding genes such as *VvPOD2* (VIT_207s0130g00220), *VvPOD17* (VIT_200s1677g00010) and *VvPOD38* (VIT_218s0001g06850) were all downregulated at T2 and located in clusters 1 (Figure 5). *VvPOD30* (VIT_202s0012g00540) was also similarly regulated between varieties, its expression gradually decreasing from T1 to T3 in clusters 7 (Figure 5). Moving to differentially expressed *VvPODs* between CS and SN, *VvPOD18* (VIT_218s0001g01140) was found most expressed in T1 in cluster 5 of CS (Figure 8a) while being downregulated in T2 in SN cluster 1 (Figure 10b). *VvPOD25* (VIT_212s0055g00810) was also found in cluster 5 of SN (Figure 10b), but it was not detected among DEGs for CS. *VvPOD27* (VIT_218s0072g00160) was encountered in CS cluster 7, together with *VvPOD32* (VIT_207s0191g00050) (Figure 10a), and SN cluster 5 (Figure 10b). *VvPOD32* was detected in cluster 4 of SN, downregulated at T3 (Figure 5b). Lastly, *VvPOD46* (VIT_206s0004g01180) was only found in cluster 1 of CS, downregulated at T2 (Figure 10a).

Respiratory stress was indicated to activate dormancy release within buds (Vergara *et al*., 2012). Moreover, a set of HRGs (*Hypoxia Responsive Genes*), was proved to be induced by both hypoxia and dormancy-breaking compound hydrogen cyanamide (H_2CN_2), specifically *VvPDC* (*Pyruvate Decarboxylase*, VIT_208s0217g00100) and *VvADH2* (*Alcohol Dehydrogenase 2*, VIT_204s0044g01120) (Vergara *et al*., 2012). *VvPDC* expression gradually decreased from T1 to T3 in clusters 7 of both CS and SN (Figure 5). *VvADH2* was downregulated in T2 (cluster 1) of CS (Figure 10a) and upregulated in T1 of SN (cluster 5) (Figure 10b).

3.7.5 DEGs associated with cell cycle regulation

Dormancy-breaking compound H_2CN_2 was demonstrated to induce upregulation of two cyclin-dependent kinases, *VvCDKA* (VIT_215s0045g00310) and *VvCDKB.2* (VIT_218s0122g00550), after 48 h post-treatment in grapevine buds. Cyclin *VvCYCA1* (VIT_218s0001g02060) was also upregulated by the same treatment (Vergara *et al*., 2016). On these premises, the expression of these cyclins was looked for in DEGs data. *VvCDKA* was found in clusters 1 of both CS and SN, downregulated at T2 (Figure 5). *VvCDKB.2* was also observed in cluster 1 of CS (Figure 10a), but its

downregulation appeared extended to T1 in SN, leading to its inclusion in cluster 3 (Figure 10b). Lastly, *VvCYCA1* was detected in clusters 3 of both CS and SN (Figure 5).

Dormancy release in buds has been connected to the upregulation of D-type cyclins (*VvCYCDs*), which act at the G1-S-phase transition of the cell cycle (Horvath *et al*., 2003). Five *VvCYCDs* (VIT_207s0129g01100, VIT_203s0091g01060, VIT_218s0001g09920, VIT_218s0001g07220, VIT_203s0180g00040) have been recently associated with dormancy release, with low expression levels during endodormancy and upregulation in spring (Shangguan *et al*., 2020). In both CS and SN, all five *VvCYCDs* were found to be downregulated at T2 and located in clusters 2 (Figure 5).

3.7.6 Differentially expressed transcription factors during dormancy progression

Dormancy-associated MADS-box genes (DAMs), called SVP (*Short Vegetative Phase*) in non-*Rosaceae* deciduous trees, have been associated with dormancy regulation (Bielenberg *et al*., 2008). Several grapevine *SVP*s have been recently described (Min *et al*., 2017; Shangguan *et al*., 2020; Vergara *et al*., 2021). An association with *VvFT* (*Flowering Locus T*, VIT_200s0203g00080) and *VvAP1* (*APETALA 1*, VIT_201s0011g00100) has been suggested (Vergara *et al*., 2016; Vergara *et al*., 2021). On these premises, the expression of known *VvDAMS-SVPs*, *VvFT* and *VvAP1* was explored in DEGs clusters. *VvFT* was not found among DEGs in both CS and SN. *VvDAM2-SVP* (VIT_218s0001g07460) and *VvDAM5-SVP* (VIT_203s0167g00070) were the only genes in the family with similar expression patterns in both CS and SN, found in clusters 2 and clusters 3, respectively (Figure 5). *VvDAM1- SVP* (VIT_200s0313g00070) was detected in cluster 3 of CS (Figure 10a) and upregulated at T1 (cluster 5) in SN (Figure 10b). *VvDAM3-SVP* was downregulated at T2 in CS (cluster 1, figure 10a) and upregulated at T3 in SN (cluster 3) (Figure 10b). Lastly, *VvDAM6-SVP* (VIT_200s0729g00010) and *VvDAM8-SVP* (VIT_215s0024g02000) were not detected among DEGs in both varieties. Lastly, *VvAP1* was found to be upregulated at T3 (cluster 3) of SN (Figure 10b), and not found among DEGs of CS.

The grapevine FLC (*Flowering Locus C*) homolog *VvFLC2* (VIT_214s0068g01800) is a MADS-box transcription factor which was recently proposed to have a role in dormancy regulation (Díaz-Riquelme *et al*., 2012). In detail,

a negative regulation of *VvFT* by *VvFLC* and *SVP-like* genes was hypothesised in *Arabidopsis* (Lee *et al*., 2007). *VvFLC2* expression was higher at T1 (cluster 5) of CS (Figure 10a) and downregulated at T3 (cluster 4) of SN (Figure 10b).

Early Bud-Break 1 (EBB1) is a recently identified AP2/ERF family transcription factor which regulates budbreak in poplar by playing a role in shoot apical meristem reactivation following dormancy (Yordanov *et al*., 2014). One grapevine *EBB1* homolog (VIT_200s0291g00030) has been described and was proposed to positively regulate budbreak (Busov *et al*., 2016). *VvEBB1* expression was detected in DEG data and its expression appeared shifted between CS and SN. In particular, *VvEBB1* expression was highest at T3 (cluster 3) of CS (Figure 10a), whereas it appeared at its lowest at T3 (cluster 4) of SN (Figure 10b).

VvMSA (*Maturation, Stress, ABA,* VIT_218s0072g00380) is the the only identified member of ASR proteins (*ABA-, stress- and ripening-induced*) in grapevine. This transcription factor was hypothesized to connect sugar and ABA signaling (Çakir *et al*., 2003; Saummoneau *et al*., 2012). Recently collected evidence suggests a role for *VvMSA* in the release of bud dormancy in grapevine (De Rosa *et al*., 2022b; see Chapter III). *VvMSA* expression was detected in DEG data and presented different trends in the two cultivars. In detail, *VvMSA* appeared upregulated at T3 in CS (cluster 3) (Figure 10a) and downregulated at T2 compared to the other time-points in SN (cluster 1) (Figure 10b). Lastly, recent research has included transcription factor *VvWRKY3* (VIT_219s0015g01870) in a budbreak-related QTL located on chromosome 19 (Duchêne *et al*., 2012). However, *VvWRKY3* was not found among DEGs in both CS and SN.

3.8 Genomic methylation changes during dormancy progression

Whole Genome Bisulfite Sequencing (WGBS) represents the gold standard of DNA methylation detection. Bisulfite treatment converts unmethylated cytosines in uracil, while methylated cytosines remain intact. The comparison of treated DNA strands with an untreated reference allows the identification of methylated sites in all methylation contexts found in plants, namely CG, CHH and CHG (H = A, T, or G) (Chan *et al*., 2005).

The expression profile of the grapevine *VvDMLs* and other epigenetic factors that appeared to be modulated in buds in transition from T2 to T3 prompted an exploration of the DNA methylation landscape, and a search for possible methylation changes in genes and other targets of epigenetic pathways, in particular transposable elements. Using WGBS, cytosine methylation levels at a genome wide scale were quantified in triplicates of the three time-points for the CS variety, producing high quality information (10X sequencing coverage or higher) for 4.5M cytosines of the CG context (amounting to 38% of the grapevine reference genome), 11M of the CHG context (62%) and 57M of the CHH context (43%) on average across the samples.

A Principal Component Analysis (PCA) of the nine samples based on the overall methylation data showed substantial overlapping of the triplicates except for a T1 replicate (T1-R1), which segregated from the other replicates of the same time-point. To a lower extent and only in the CHG and CHH contexts, a T3 replicate (T3-R3) also appeared separated from the others. Excluding the two outliers from consideration, the PCA revealed a major separation of the T3 samples in the CG context and very minor differentiation between the three time-points in the CHG and CHH contexts (Figure 11).

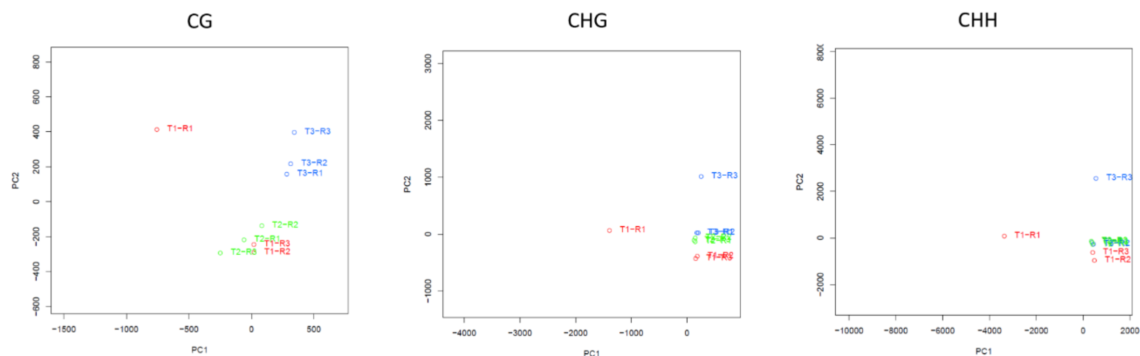


Figure 11. Principal component analysis of genome wide methylation profiles for sequence contexts CG, CHG, and CHH in T1, T2 and T3 of Cabernet Sauvignon buds.

To further investigate this scenario, a meta-analysis was carried out across the gene set and a subset of full-length transposable elements to evaluate the average methylation differences between time-points and within triplicates in these two compartments of the genome. Both compartments were investigated within the actual genomic coordinates of genes and TEs, as well as in their flanking regions, up

to 2 Kb up- and downstream. In both gene and TE meta-analyses, the methylation profiles and the relative CG/CHG/CHH levels yielded by our sequencing data were consistent with the scenarios widely described by the literature on the topic in most plant species (Saze *et al.*, 2012; Bräutigam and Cronk, 2018), supporting the quality of the data. Despite the PCA indication of a distinction between T3 and the previous stages, the gene meta-analysis for the CG context showed high overlapping of methylation levels between samples across the entire regions analysed. Larger variation, albeit very minor, was observed for the CHG and CHH contexts, although the differences between samples could not be ascribed to a clear differentiation between time-points more than to background noise (Figure 12). It should be noticed that the much lower levels of CHG and CHH methylation determine higher uncertainties in their quantification.

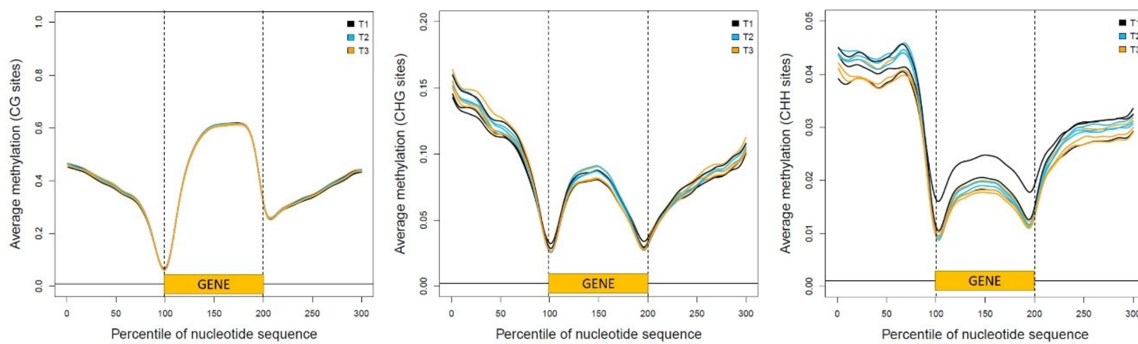


Figure 12. Gene body methylation profiles for sequence contexts CG, CHG, and CHH in T1, T2 and T3 of Cabernet Sauvignon buds.

These results suggest that the transition between the three developmental stages is not accompanied by pivotal genome wide changes in the methylation profiles of genes and their immediate genomic context, and that potential evidence for the methylation differentiation suggested by the PCA should be searched for at the single locus level or at lower scale in following analyses.

Like the gene meta-analysis, average profiles of methylation within TEs did not reveal prominent differences between time-points and rather showed a general consistency in the methylation levels. This analysis was carried out using a subset of 500 DNA transposons, 670 RIL retrotransposons (mainly LINE elements, which in the grapevine genome tend to be integrated in genic introns (Dal Santo *et al.*, 2022)), 889

Copia-like LTR-retrotransposons (RLC, mainly heterochromatic) and 592 Gypsy-like LTR-retrotransposons (RLG, highly heterochromatic). The CHH context in these elements was unique in showing average methylation signals suggestive of a lower amount of methylation at T3 relative to T2, in at least three of the four TE groups, mainly within the DNA and RIL types and partially in the RLC type (Figure 13).

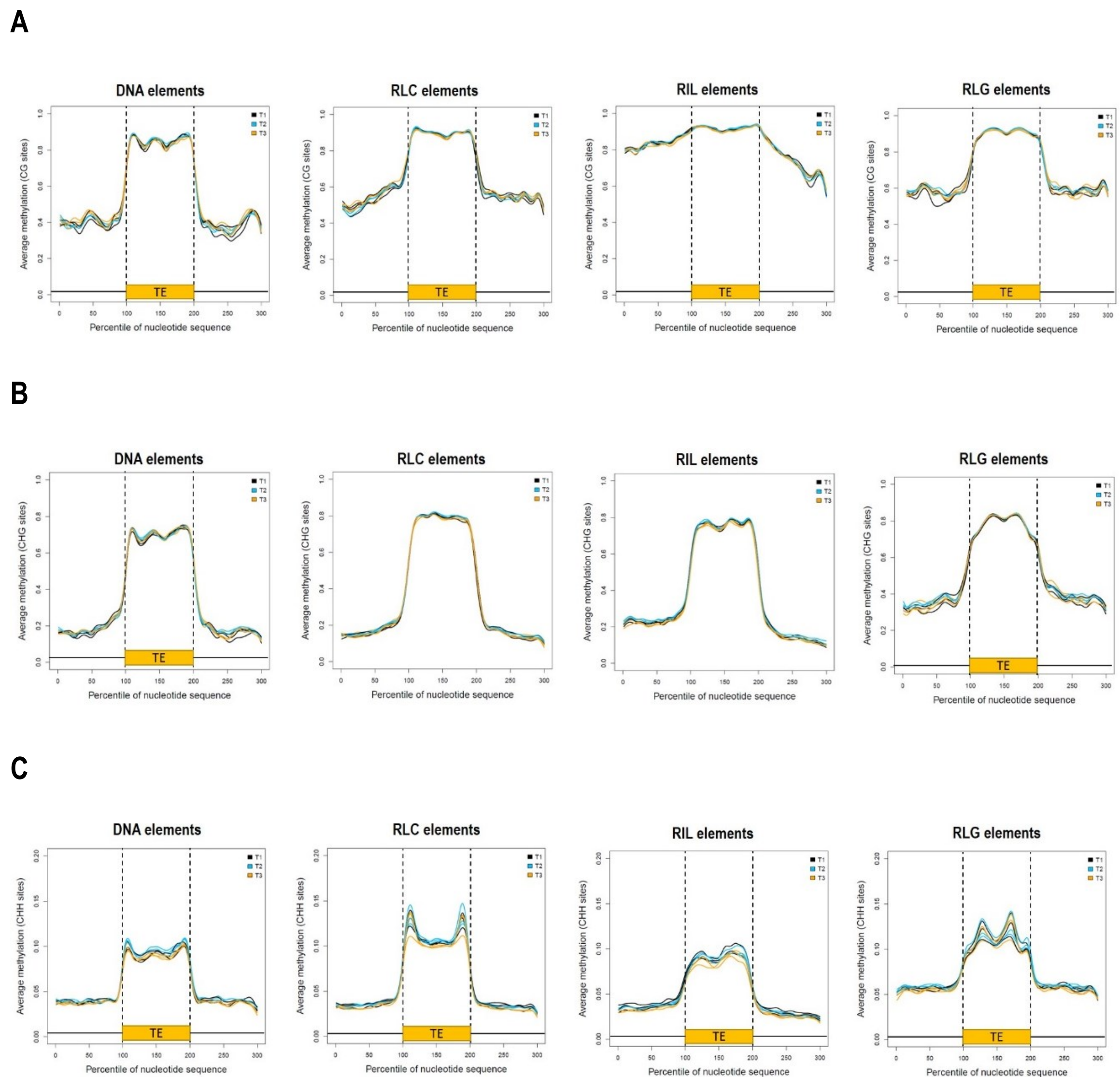


Figure 13. Transposable element methylation profiles for sequence contexts CG (**A**), CHG (**B**), and CHH (**C**) in T1, T2 and T3 of Cabernet Sauvignon buds.

Despite the small methylation differences, the distribution of cytosine methylation in the aggregated triplicates of each time-point showed statistically significant differences (Wilcoxon test, T3 vs T2, pValue 1.406e-13, 1.062e-15, < 2.2e-

16 for DNA, RIL and RLC elements respectively, 0.8952 for RLG). In particular, the group of DNA transposons showed consistently higher mean methylation levels at T2 than in T3. This trend appeared to be brought about by a small number of transposable elements rather than the whole set, most of which did not exhibit differences between time-points at all. The rare elements that showed differential methylation were mainly contributed by the Harbinger and Mutator families and to a lower extent by the hAT family, but never by the CACTA family, despite the similar abundance of elements in the subset used. Further analyses will be required to evaluate the significance of such findings, which in any case do not provide compelling evidence for important methylation changes at genome scale during the developmental transition investigated in this study.

3.9 Locus-specific demethylation during dormancy progression

Locus-specific DNA methylation levels were checked by aligning CS bud DNA reads with the PN40024 12X grapevine reference genome (Jaillon *et al*., 2007). One Kbp windows were searched for at least ≥ 3 adjacent demethylated cytosines. Genes found at a distance of < 1 Kbp from the selected cytocines were searched in DEG data. Three gene targets were found to be upregulated in T3 vs T2: VIT_202s0012g01660, VIT_213s0067g03260 and VIT_218s0117g00200. BLAST tool on Phytozome database (www. phytozome-next.jgi.doe.gov) was used to align the three genes of interest on the *A. thaliana* TAIR 10 genome.

Firstly, VIT_ 213s0067g03260 was found to be a homolog of At3g10870, annotated as *AtMES17* (*Methylesterase 17*). *AtMES17* hydrolyzes MeIAA (*Methyl indol-3-acetate*) into IAA (*Indol-3-acetic acid)*, the active form of auxin (Yang *et al*., 2008). *AtMES17* was found to be expressed at its highest levels in several Arabidopsis tissues, including shoot apex (Yang *et al*., 2008). Secondly, VIT_202s0012g01660 was detected as homolog of At4g25960, annotated as *AtABCB2*. Interestingly, a set of *AtABCBs* (*ATP-binding Cassette Transporters*) was recently associated to auxin transport (Kaneda *et al*., 2011).

Lastly, VIT_218s0117g00200 was found to be related to Arabidopsis At4g38620, annotated as *AtMYB4* (*Transcription Repressor MYB4*). *AtMYB4* was connected to UV-B acclimation in Arabidopsis (Jin *et al*., 2000).

4 DISCUSSION

Although several players have been documented to participate in grapevine dormancy progression, the intricacies of dormancy regulation remain largely unknown. In changing climatic conditions, which put grapevine buds at significant risk of spring frost damage, a deeper knowledge of budbreak control is pivotal. Comparative transcriptomic analyses are useful tools to define key gene regulation pathways and processes involved in plant development and targets for climate adaptation. Since epigenetic mechanisms have also been associated to bud phenology and cold acclimation (Lloret *et al.*, 2018), highlighting the role of epigenetic regulation and modifications is also necessary. In this study, early-budbreak Sauvignon Nepis and late-budbreak Cabernet Sauvignon were compared to shed light on differential timings of molecular processes ascribable to bud phenological advancement towards budburst.

Cold hardiness assessment by DTA suggested a slower reactivity to forcing conditions in Cabernet Sauvignon as compared to Sauvignon Nepis (Figure 2). Specifically, the latter exhibited a faster loss of cold hardiness as compared to Cabernet Sauvignon in the same time span. The different rhythm at which deacclimation progressed in the two cultivars is possibly the result of the recognized features of grapevine hybrids, generally most vulnerable to cold temperature damage from late-winter (Ferguson *et al.*, 2014), in support of our assumption that the two genotypes behave differently in terms of dormancy progression.

Recent evidence based on genome-wide transcriptome and methylome analyses in poplar associated reduction of 5-mCs in targets genetically associated with budbreak to a DEMETER-like dependent demethylation (Conde *et al.*, 2017b). As for the results collected in this study, firstly, *VvDMLs* expression patterns resembled those reported in Chapter IV (De Rosa *et al.*, 2022a), assuring that artificial dormancy simulation and growth forcing conditions do not seem to alter *VvDMLs* expression tendencies compared to open field conditions. DEGs data confirmed these patterns, with *VvDEM1* and *VvDEM3* found to be downregulated at T2 as compared to similar expression levels at T1 and T3 both (Figure 5). *VvDMLs* downregulation at T2 in both cultivars is aligned with the evidence collected in poplar, while no obvious difference

is detectable when it comes to CS and SN comparison. While WGBS did not highlight major genome wide changes in the methylation profiles of genes and their immediate genomic context in CS, the possibility of finer changes of methylation at single locus level is a possibility in all sequence contexts. Moreover, *DMLs* have been found to act on very few gene targets to produce significant effects on the developmental of several plant species. For example, active demethylation of four gene targets by *AtDMLs* was found to be required for pollent tube function in Arabidopsis (Khouider *et al*., 2021). *SlDMLs* were shown to demethylate major fruit ripening regulators in tomato (Liu *et al*., 2015). *AtDMLs* were also found to regulate a subset of defense-related genes in Arabidopsis (Schumann *et al*., 2017).

A preliminary analysis of locus-specific changes in the CG context, which showed the greatest differences between time-points in PCA analysis (Figure 11), highlighted the presence of three putative genes targeted by demethylation. Two of them appear related to *AtMES17* and *AtABCB2*, both presenti connections to auxins signaling (Yang *et al*., 2008; Kaneda *et al*., 2011). Auxins are plant hormones which have been found to influence cell division, elongation and differentiation. Their signaling greatly influences plant development in response to changing environmental conditions (Gomes and Scortecci, 2021). Auxins have been connected to budbreak in grapevine, with several genes connected to their biosynthesis being upregulated by dormancy-breaking compound H_2CN_2 (Noriega and Pérez, 2017).

The third putative demethylated target is a homolog of *AtMYB4*. *AtMYB4* was found to negatively regulate the phenylpropanoid pathway in Arabidopsis (Jin *et al*., 2000), resulting in the reduction of both flavonoids and anthocyanins synthesis. On the other hand, *NtMYB4* was found to positively regulate anthocyanin biosynthesis in tobacco (Luo *et al*., 2020). Flavonoids biosynthesis is activated in response to light exposure to avoid photo-oxidative damage. These molecules can also act as scavengers of reactive oxygen species produced following UV exposure (Ferreyra *et al*., 2021). Flavonoids have been implicated in the protection of newly formed bud tissues from UV damage (Conde *et al*., 2017a). Cuncurrently, H_2O_2 levels have been putatively connected to the breaking of bud dormancy (Beauvieux *et al*., 2018). Evidence of DMLs influence on flavonoid metabolism has been previously reported (Bharti *et al*., 2015; Conde *et al*., 2017a).

A modulation of auxin and flavonoid metabolism could be expected in proximity to budbreak, as is the case of T3 for CS buds. On these premises, further analyses are required to clarify the localization of the demethylated cytosines in the sequence of the putative genes of interest. CHG and CHH contexts should also be searched for demethylated loci. Finally, functional studies will be required to confirm whether *VvDMLs* are involved in the active demethylation of the proposed targets.

In the attempt to describe the biological processes taking place in slow-budburst Cabernet Sauvignon and fast-budburst Sauvignon Nepis, functional annotation was performed on different clusters which grouped genes according to their expression patterns (Figure 5, see Section 3.3). In detail, during the cold acclimation phase of CS, encompassed by clusters 5, 7, 4 and 2, abiotic stress responses appeared the main recurring processes. In particular, the GO term 'oxygen-containing compound' appeared enriched in clusters 5 and 7 and represented the most significantly active biological process in cluster 4 specifically. H_2O_2 concentration has been very recently associated with depth of dormancy in grapevine buds (Pérez *et al*., 2021). The same term was also found in cluster 5, 7 and 4 of SN (Figure 5, Figure 7) although terms related to phenylpropanoid and lignin metabolism appeared more relevant. While not appearing as relevant in the functional annotation performed on each gene cluster, evidence of active lignin metabolism was found among the unique biological processes upregulated at T2 vs T3 in CS (Figure 9). An association between cell wall thickening during endodormancy and the development of cold hardiness has been proposed (Rubio *et al*., 2016). Thicker-walled cells constitute structural ice barriers designated to meristem protection against ice intrusion (Kuprian *et al*., 2014); moreover, cell wall related enzymes, including lignin and phenylpropanoid biosynthesis genes, were recently associated to dormancy induction (Fennell *et al*., 2015). Together, these results suggest differential or even genotype-specific approaches, for cold hardiness acquisition in view of dormancy. An active synthesis of lignin could be associated to ongoing adaptive measures for cold hardiness acquisition in both genotypes, although the activation of a part of lignin metabolism-related genes being at T2 in CS alone suggests a persisting deeper level of dormancy in this cultivar. Moreover, the observed active response to reactive oxygen species (ROS) in CS suggests that endodormancy had already reached deeper levels in this cultivar compared to early-budbreak SN at T1, and possibly maintained it throughout

T2. A delay of dormancy-related processes in SN could be hypothesized due to evolutionary reasons. In fact, wild Vitis species, originating from colder environments, have the necessity to take advantage of favourable environmental cues as long as possible. This was hypothesized in the light of faster deacclimation rates (Ferguson *et al.*, 2014), and is conceivable also for cold acclimation. Differential progression of dormancy-related measures is also supported by the absence of chlorophyll and pigment catabolic processes in SN, as observed in CS (cluster 2).

Differences in the annotated biological processes, in the two cultivars, were also found in clusters 6, 8 and 3, were genes gradually more expressed at T3 are grouped (Figure 5). At this stage, epigenetic regulation mechanisms appeared highly represented (cluster 8 and 3) in CS. Similarly, several epigenetic modifications-related terms were enriched in cluster 3 of SN. In detail, GO terms related to chromatin organization and histone modifications (i.e. 'histone H3-K9 methylation') are highly relevant in both CS and SN. H3-K9 methylation in plants is connected to transposable elements (TEs) silencing to avoid genomic instability (Xu and Jiang, 2020). Consistently, biological processes related to cell cycle and cell proliferation in CS can be found in clusters 8 and 3, while DNA replication is detected in SN cluster 3. This is coherent with TEs methylation being epigenetically transmitted to new DNA molecules during cell division (Fultz *et al.*, 2015). Interestingly, cluster 3 of SN encompassed significant biological processes related to photosynthesis and photorespiration. Recent evidence has documented early activation of cell-cycle related genes, as compared to respiration-related genes, in sprouting grapevine buds exiting endodormancy (Noriega and Pérez, 2017). Variety-specific functional annotation analyses also highlighted that genes upregulated at T3 in CS exclusively are connected to pattern specification processes and development of vegetative and reproductive tissues, while biological processes unique to SN T3 are oriented towards carbohydrate-derivative energy production through glycolysis, photosynthesis and photorespiration (Figure 9). These results agree with the observed differential phenological progression at T3 sampling of CS and SN, when SN buds reached the more advanced BBCH 05 stage (wooly bud) while CS buds appeared still at BBCH 00 (winter bud). Taken together, these findings allow to suggest that early-budbreak cultivars, such as Sauvignon Nepis, start differentiating vegetative and reproductive structures earlier as compared to late-budbreak ones. Since both cultivars were

exposed to identical environmental conditions, differences in their developmental rhythm could be ascribable to diverging transitions from endodormancy to ecodormancy due to varying chilling hours requirements (Anzanello *et al.*, 2018).

Several differences were detected comparing multiple pathways related to cold sensing and acclimation, deacclimation and bud development during dormancy progression. *VvCBF2* and *VvCBF4,* encoding transcription factors involved in low temperature and ABA perception signalling (Wisniewski *et al.*, 2014; Rubio *et al.*, 2019), displayed similar expression patterns but appeared differentially regulated in CS and SN. In detail, in CS the two factors are upregulated in T1 only, while they are similarly expressed in T1 and T3 in SN. *VvCBFs* expression at T1 is in line with previous knowledge; however, in SN it allows to speculate that *VvCBF2* and *VvCBF4* might not only be involved in cold acclimation, but also in deacclimation in a cultivar-specific manner. Concerning carbohydrate metabolism, it is well known that cell osmotic potential in bud tissues is increased, following cold exposure, through soluble sugars accumulation and water reduction to avoid freezing damage. Soluble sugars exert a role as metabolic substrates, signaling molecules and osmoprotectant agents by limiting ice nucleation within the apoplast (Grant and Dami, 2015). In regard to cold tolerance, RFOs (*Raffinose Family Oligosaccharides*) were demonstrated to exhibit the highest correlation with freezing tolerance (Grant *et al.*, 2009). Three *VvRSs* appeared differentially expressed between cultivars (Figure 10). These results support *VvRSs* role in cold-related sugar metabolism and allow to hypothesize a greater inclination of SN to employ measures to reach freezing tolerance. Interestingly, cold-tolerant genotypes that are adapted to very low freezing temperatures were observed to begin accumulating raffinose in their tissues earlier than the cold-sensitive genotypes (Grant *et al.*, 2009). The activation of three *VvRSs* in SN T1 is aligned with this observation.

On the other hand, the expression of four sucrose synthases, which reversibly catalyze sucrose cleavage (Stein and Granot, 2019), has been recently shown to significantly increase in spring compared to grapevine bud dormancy period (Shangguan *et al.*, 2020). In this regard, *VvSUS2* and *VvSUS5* were found to be differentially expressed between cultivars. In particular, the expression of both sucrose synthases was detected at T3 in CS, while only *VvSUS5* was found in SN. This

suggests increased metabolic activity based on sucrose breakdown (or hydrolysis by invertases) as energy source in T3, while sucrose breakdown in T1 of CS could possibly be connected to hexoses accumulation due to increased reactivity to lowering temperatures.

The deletion of several dormancy-associated MADS-box genes (*DAMs*), or SVPs (Wu *et al*., 2017), was first associated to a non-dormant phenotype in *Prunus persica* (Bielenberg *et al*., 2008). Several *SVP*s have been recently described in grapevine (Min *et al*., 2017; Shangguan *et* al., 2020; Vergara *et al*., 2021). Recent evidence suggested *VvDAMs-SVPs* regulation by short-day conditions (Vergara *et al*., 2021). Moreover, an association between *VvDAM3-SVP* (VIT_215s0107g00120), *VvFT* (*Flowering Locus T*) and grapevine endodormancy has been hypothesized (Vergara *et al*., 2021). Concomitant upregulation of cyclins *VvCDKA, VvCDKB.2*, *VvCYCA1*, *VvFT* and *VvAP1* by H_2CN_2 opened the possibility of *VvFT*-mediated regulation of cell cycle genes through *VvAP1* (Vergara *et al*., 2016). Firstly, the results presented in this study depict differential expression of *VvDAM3-SVP* in cvs. CS and SN. In detail, *VvDAM3-SVP* appeared expressed in both T1 and T3 of CS, while being upregulated in T3 only in SN. *VvDAM3-SVP* expression was always concomitant to *VvCDKB.2* upregulation. In SN, *VvAP1* was also induced in the same conditions. Although *VvFT* expression was not detected among DEGs in CS and SN, these results suggest a role for *VvDAM3-SVP*, cyclin *VvCDKB.2*, and possibly for *VvAP1*, in dormancy release processes, even if a distinctive budbreak precocity marker cannot be clearly identified.

VvMSA is thought to represent a point of convergence between sugar and hormone metabolism (Çakir *et al*., 2003). Recent evidence of *VvMSA* expression in grapevine buds suggests a role in the dormancy release process (De Rosa *et al*., 2022b, see Chapter III). DEG data highlight a differential expression of *VvMSA* in the CS and SN, which was found to be downregulated at T2 in the early-budbreak genotype and upregulated at T3 only in the late-budbreak one. A connection between *VvMSA* and sugar metabolism has been proposed (Çakir *et al*., 2003). Coherently, carbohydrate metabolic processes were found to be the most relevant biological processes taking place in cluster 1, which includes genes downregulated at T2. These results suggest a persisting higher metabolic activity in SN at T1, or a more intense activation of cold response-related carbohydrate metabolism, and an early restart of activity at T3.

Moreover, this is supported by the more progressed phenological stage reached by SN at T3.

Lastly, the expression of *VvEBB1* was detected at T3 in CS, and at T1 and T2 in SN. *VvEBB1* is expected to be a positive regulator of budbreak (Busov *et al*., 2016). This hypothesis appears supported by *VvEBB1* expression at T3 in CS, while its anticipated expression in SN introduces the possibility of novel functions in dormancy regulation, or sensing of environmental cues.

5 CONCLUSIONS

In conclusion, the comparison of grapevine buds at transcriptomic and epigenetic level has proved to be a powerful tool to detect evidence of differential metabolic processes taking place during dormancy development. Several differential biological processes have been detected in buds of cultivars characterized by contrasting budbreak timings, namely late-budbreak Cabernet Sauvignon and early-budbreak Sauvignon Nepis. Specifically, proof supporting differential approaches to cold acclimation and dormancy-related processes, or even genotype-specific mechanisms, were cued by several biological processes involved in cold sensing pathways, carbohydrate metabolism, cell wall-related metabolism and oxidative stress responses differentially active between cultivars. On the other hand, early deacclimation in Sauvignon Nepis was supported by active biological processes connected to energy production and photosynthesis. Overall, evidence collected in this study allows to hypothesize that a plethora of developmental and reproductive processes are differentially activated in winter buds of early-budbreak cultivar during dormancy, possibly anticipating late-budbreak one due to an early chilling requirement fulfilment.

Moreover, new insight has been provided regarding the potential participation of specific transcription factors in dormancy release regulation, with possible genotype-specific roles. *VvDAM3-SVP* and *VvEBB1* appear particularly interesting since their expression is significantly different in the two cultivars. *VvDAM3-SVP* was associated with para- to endodormancy transition in grapevine (Vergara *et al*., 2021). While this could be reflected in *VvDAM3-SVP* expression during cold acclimation in

Cabernet Sauvignon, its expression in cold deacclimation in both varieties supports a role in dormancy release as well. *VvEBB1* activity was also recently observed in grapevine buds and connected to budbreak (Busov *et al*., 2016). *VvEBB1* expression in Cabernet Sauvignon is coherent with its expected role, while its shifted upregulation taking place earlier in Sauvignon Nepis needs further inspection.

Future in-depth analyses are also required for WGBS data, since targeted changes of methylation involving specific genes of interest in dormancy regulation could be present even when average whole-genome methylation levels do not appear significantly different. While functional studies are required to corroborate *VvDMLs* connection to the demethylation of the target genes identified in this study, this early evidence allows to speculate the involvement of *VvDML* in grapevine bud dormancy release through the modulation of auxin signaling and flavonoid metabolism.

In conclusion, dormancy release regulation remains an intricate phenomenon operated by a wide range of metabolic processes, possibly tightly connected to differential chilling requirement. This work has contributed to pinpointing key players which need to be further characterized through functional studies.

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♦ CHAPTER VI

The freeze of April 7th, 2021: a case study in Italian Region Friuli Venezia Giulia

INTRODUCTION TO THE STUDY

Spring frost events are well-known as unpredictable occurrences, which is precisely what makes them an insidious issue. As researchers, we were very fortunate to assist to a late frost occurrence on April 7th, 2021, which impacted many cultivars grown in field at the Experimental Farm "A. Servadei" in Udine. To our great interest, early-budbreak hybrid Sauvignon Nepis appeared to show good tolerance to the event despite having reached budburst.

To gain insight on how deacclimated buds react to sudden freezing temperatures, gene expression analysis was performed on buds collected on the day of the spring frost and buds collected in previous weeks. Since by definition budburst is reached when at least 50% of all buds are broken, we also separated spring frost-subjected buds into three pools depending on their phenological phase: BBCH 00 (winter buds), BBCH 05 (wooly buds) and BBCH 09 (green tipped buds). This separation turned out to be fundamental, since differential responses to late frost exposure were seen depending on bud developmental stage. In particular, only BBCH 00 buds appeared competent to respond to the spring frost. In detail, specific genes which showed a decreasing expression trend during deacclimation such as cold tolerance-related dehydrin *VvDHN2* and raffinose synthase *VvRS*, together with abiotic stress response-related *VvNCED1* and *VvEIN3* were all significantly upregulated.

These results allowed us to speculate that BBCH 05 and BBCH 09 buds are no longer competent to respond to sudden freezing temperatures exposure, or that additional or different cold resistance mechanisms may be active at these stages. This work should hopefully inspire future comparative studies involving cultivars with contrasting cold resistance and budbreak precocities, to delineate which pathways are most relevant in determining late frost tolerance.

These results were first presented in a flash talk at the XI International Symposium on Grapevine Physiology and Biotechnology which took place from October 31st to November 4th, 2021 in Stellenbosch (South Africa). A paper is currently under review in *Acta Horticulturae* journal.

Early molecular response of cold-deacclimated grapevine buds to spring frost events[4]

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Abstract

Despite the observed increase of average global surface temperatures, spring frosts are expected to remain a threat to grapevine cultivation in several areas of the world. Due to higher vulnerability in proximity to budbreak, late freezing events can greatly damage bud vegetative and reproductive tissues thus affecting multiple years of production. Therefore, improving our understanding of the mechanisms underlying tolerance to unpredictable freezing events during cold deacclimation is of paramount importance to enhance grapevine's resilience in a changing climate. A spring frost was registered, on April 7th, 2021, in the Italian Region Friuli Venezia Giulia, allowing the field study of the early molecular responses of cold-deacclimated buds in the hybrid Sauvignon Nepis (Sauvignon Blanc × Bianca), showing a good tolerance to this event. Results highlighted differential responses to late frost exposure depending on bud developmental stage, with the BBCH 00 stage (winter bud) being the most reactive. In particular, freezing tolerance-related genes (*VvDHN1/2*) and raffinose synthase encoding gene (*VvRS*) were upregulated in response to freezing shock. *VvNCED1*, involved in abscisic acid biosynthesis, and ethylene receptor *VvEIN3* also appeared involved in this process. This work outlines several molecular pathways activated in cold-deacclimating buds in response to sudden frost occurrence and draws attention to the varying adaptation capabilities in relation to bud phenological stage.

[4] The data presented in this chapter are the object of a conference paper which is currently being reviewed by *Acta Horticulturae* journal.

1 INTRODUCTION

Climate change represents an undeniable danger for fruit crop production all around the world (IPCC, 2021). The ongoing global increase of average surface temperatures is not only at the base of early cold deacclimation due to warmer winters, but also of faster phenological progression rates resulting in early budbreak, flowering and harvest times (van Leeuwen and Darriet, 2016). However, a considerable degree of variability can be ascribed to different genotypes characterized by dissimilar chilling requirements and budburst rates (Londo and Johnson, 2014). Due to higher hydration levels and low supercooling abilities, budbreak is a most critical phase in terms of bud vulnerability to freezing injuries (Fennell, 2004). Late frost occurrences following an interval of vegetative growth, known as spring frosts, can cause relevant damage to bud tissues, affecting multiple production seasons (Carmona *et al*., 2008). Spring frosts are currently expected to remain an enduring threat in several areas worldwide (Augspurger, 2013; Sgubin *et al*., 2018). Variations of cultural practices have been recently tested to delay budbreak, being frost avoidance the most effective strategy for the short term (Palliotti *et al*., 2017). However, breeding effort needs to be considered as an adaptive measure to cope with the future scenarios posed by climate change, and a better understanding of early-responding genes involved in spring frosts response could be helpful in this approach (De Rosa *et al*., 2021).

Cold acclimation and freezing tolerance have been extensively explored in the context of winter frost tolerance in woody perennials, and several key players have been identified. Multiple mechanisms are set in motion by plant tissues to produce modifications that lead to higher cold hardiness levels, such as soluble sugars accumulation (Grant and Dami, 2015; Ershadi *et al*., 2016), water content reduction (Fennell, 2004), and alterations in gene expression and hormonal regulation (Liu and Sherif, 2019). Soluble sugars accumulate in response to cold exposure, with differences ascribable to genotype (Grant and Dami, 2015; Ershadi *et al*., 2016). Soluble sugars act as cryoprotective compounds by decreasing cell osmotic potential, which lowers water freezing point, thus slowing down and preventing ice crystals formation (Tixier *et al*., 2019). Raffinose and raffinose family of oligosaccharides (RFOs) in particular have been observed to correlate strongly with freezing tolerance

(Stushnoff *et al*., 1993). In addition, dehydrins (DHNs) accumulate in response to cold exposure to protect cell components from freezing-induced dehydration damage (Wisniewski *et al*., 2014; Vazquez-Hernandez *et al*., 2021). Four grapevine DHNs have been identified (Yang *et al*., 2012). Hormonal orchestration in the context of dormancy and cold acclimation in woody perennials is highly documented, especially concerning abscisic acid (ABA), a key regulator of bud dormancy in grapevine (Liu and Sherif, 2019). Moreover, recent findings suggest that ABA may play a role in cold hardiness acquisition in grapevine dormant buds (Rubio *et al*., 2019). In addition, ethylene also has a species-dependent role in plant freezing tolerance. Cold stress was shown to enhance ethylene production and the expression of related downstream genes in grapevine (Sun *et al*., 2016). Cold acclimation and dormancy have been more widely explored compared to deacclimation and dormancy release. Re-acclimation following a phase of deacclimation seems to be possible in herbaceous plants (Vyse *et al*., 2019), but is still uncertain for woody perennials (Shin *et al*., 2015). Wild Vitis species, characterized by high tolerance to winter minima, but also greater reactivity to early mild temperatures compared to cultivated grapevines (Londo and Kovaleski, 2019), have been used to produce more freezing tolerant hybrid cultivars such as Sauvignon Nepis, the object of this study. This introduces a paradox for which cultivars characterized by the highest freezing tolerance are also the most vulnerable to spring frost occurrences (Ferguson *et al*., 2014).

Based on these assumptions, this work aims to explore the molecular mechanisms activated in buds of Sauvignon Nepis, at different stages of deacclimation, in response to a natural and sudden late frost occurrence observed in Northern Italy in 2021. This cultivar showed good tolerance to this event and provided a fitting opportunity for these studies.

2 MATERIALS AND METHODS

2.1 Plant material

Early-budbreak Vitis hybrid Sauvignon Nepis (Sauvignon Blanc × Bianca) was selected for this study. Plants were located at the Experimental Farm "A. Servadei" (University of Udine, Northern Italy) and grown in the field. During the 2020/2021 winter season, buds were collected at ~15 days intervals and immediately used for cold hardiness determination or stored at -80°C for subsequent analysis. Buds collected on April 7th 2021 following the spring frost event, which also coincided with the budbreak date (>50% broken buds), were divided into three pools, separately analyzed, based on bud phenological progression as described by the BBCH scale: BBCH 0 (winter bud), BBCH 05 (wooly bud) and BBCH 09 (green tip) (Lorenz *et al*., 1995).

2.2 Cold hardiness monitoring

For each sampling time, 3 biological replicates of 5 buds each were used for cold hardiness determination with differential thermal analysis (DTA) using thermoelectric modules (TEM) and temperature probes placed in a T700BXPRO temperature-controlled freezing chamber (FDM, Rome, Italy). Temperature was quickly lowered to 7°C for 1 hour and subsequently lowered to -25°C at a rate of -2.5°C·h^{-1}. A CR1000 data-logger (Campbell Scientific, Logan, UT, USA) was used for data recording. Temperature and voltage signals were analyzed using RStudio software (https://www.r-project.org/).

2.3 Gene expression analysis

For each sampling time, RNA extraction was performed from 3 biological replicates of 10 buds using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized with QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany), and qRT-PCR was carried out with SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), as described in Sivilotti *et al*., 2017. Primers used to detect gene expression were either found in the literature or designed with Primer3 tool (https://primer3.ut.ee/) (Table 1). Statistical analyses were performed using SigmaPlot 14.0 (https://systatsoftware.com/).

Table 1. List of primers used for qPCR analysis.

Gene target	Primer sequence
VvDHN1	F GTGGGAGAAGGAAGAAAGGGA
	R AGGCAGCTTCTCCTTGATCT
VvDHN2[1]	F AGAAACTGCCAGGACAGCACA
	R CTTCGGTCTTGGGGTGGTATC
VvNCED1	F TTTGTGCACGACGAGAAGAC
	R AGGGAACTCGTGAGGGAAGT
VvEIN3	F TCCACCAGTGAACCAGTCTC
	R AGGTTCCCAGGATTCAAGCT
VvRS	F CTCTCCCGGGGAAATCTGTT
	R GATCTTGGTTTCTCGGCTGC

[1]Yang *et al*., 2012

3 RESULTS

3.1 Cold hardiness determination

DTA analysis allowed monitoring the cold hardiness dynamics of cv. Sauvignon Nepis buds throughout the 2020/2021 winter season. Recorded low temperature exotherms (LTEs), which represent lethal intracellular water freezing events, outline the evolution of bud freezing tolerance (Figure 1). Results indicate that buds' cold acclimation started in the second half of October 2020 (-5.9°C) and reached its maximum level during the second half of February 2021 (-21.9°C). Subsequently deacclimation proceeded rapidly reaching an average minimum level of -8.1°C at the beginning of April in proximity of budbreak, when the late frost occurred. Therefore, buds collected from March 1st onwards were deemed actively deacclimating.

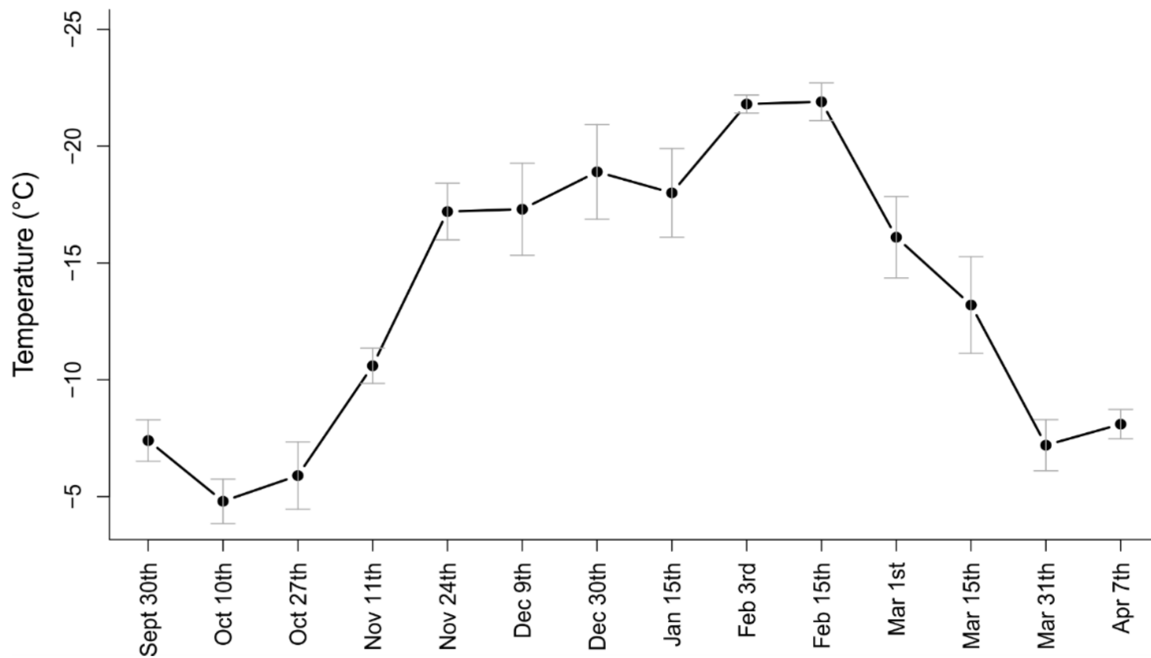

Figure 1. Low-temperature exotherms (LTEs) of cv. Sauvignon Nepis buds. Results are expressed as mean of 3 biological replicates ± standard deviation.

During the spring frost event, which took place in the night between April 6th and 7th 2021, the temperature dropped below 0°C for about 5 hours reaching a minimum of -2.4°C (Figure 2). Since the average LTE recorded on April 7th is lower than the lowest registered temperature during the spring frost event of the same day, buds collected in that date were considered non-lethally damaged and used for gene expression analysis.

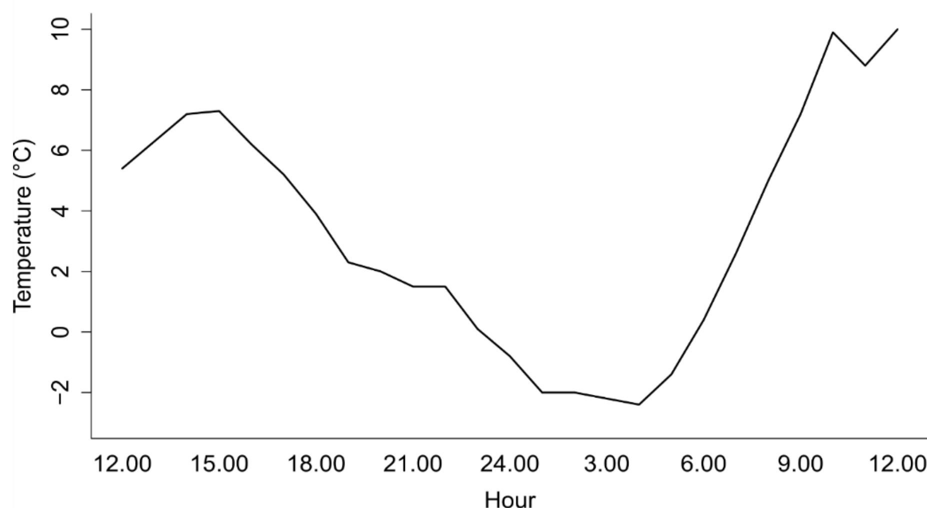

Figure 2. Average hourly temperatures registered between April 6th and 7th 2021 when the spring frost event occurred. Data recorded by the S. Osvaldo (Udine, Italy) weather station managed by ARPA FVG.

3.2 Gene expression

Primer pairs listed in Table 1 were used to assess gene expression variations in response to the late frost occurrence in actively deacclimating buds of cv. Sauvignon Nepis. Among the genes tested in this experiment, only those exhibiting statistically significant differences in their expression pattern after frost were considered (Figure 3).

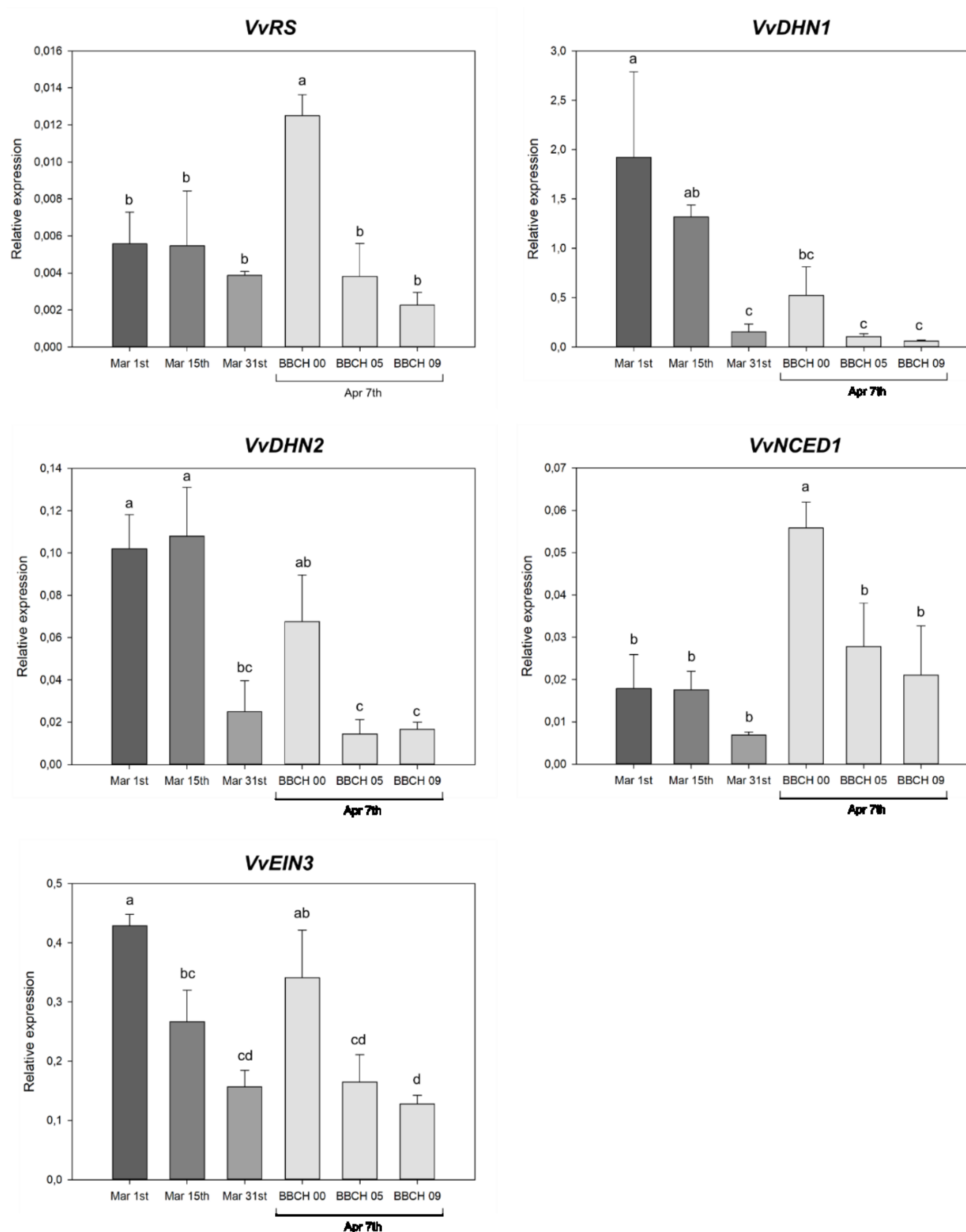

Figure 3. Expression patterns of freezing tolerance- and abiotic stress response-related genes in cv. Sauvignon Nepis buds. *VvRS* = Raffinose synthase; *VvDHN1* = Dehydrin 1; *VvDHN2* = Dehydrin 2, *VvNCED1* = 9-cis-epoxycarotenoid dioxygenase 1; *VvEIN3* = Ethylene-Insensitive 3. Results are expressed as mean of 3 biological replicates ± standard deviation. Statistical analyses were performed using one-way ANOVA and Tukey HSD as *post hoc* test for all pairwise multiple comparison procedures.

Most genes exhibited a significant downregulation before the frost occurrence; *VvRS* (Raffinose synthase) and *VvNCED1* (9-cis-epoxycarotenoi dioxygenase 1) displayed a similar trend, even if not supported by statistical evidence. Most of the genes, and in particular *VvRS* and *VvDHN2* (Dehydrin 2), showed a significant upregulation in the BBCH 00 stage buds, collected on April 7th following the unpredicted frost exposure. *VvDHN1* (Dehydrin 1) also showed an upregulated trend exclusively in winter buds. Similarly, abiotic stress response-related genes *VvNCED1* and *VvEIN3* (Ethylene-Insensitive 3), involved in ABA and ethylene biosynthesis respectively, were also significantly upregulated in the BBCH 00 stage. Interestingly, at the BBCH 05 and BBCH 09 buds' developmental stages, corresponding to "wool stage" and "green shoot tips", all the genes displayed lower expression levels, if compared to the earlier stage (BBCH 00), and similar to those detected before the stress.

4 DISCUSSION

Spring frosts can greatly damage grapevine bud tissue in its most vulnerable phase, negatively influencing vine productivity. Late frost occurrences remain a threat in the present, as demonstrated by the event registered on April 7th 2021 in the Italian Region Friuli Venezia Giulia. Cold deacclimating buds of Sauvignon Nepis appeared to well tolerate the lowest recorded temperature (-2.4°C), thus allowing the study of early molecular responses in this tissue. Results revealed that specific genes, characterized by a clear downregulation trend during deacclimation, were sharply upregulated in conjunction with the sudden frost exposure. In particular, *VvDHN2* expression was rapidly induced in response to the freezing event, and *VvDHN1* also showed an upregulation trend. This can be explained by DHNs putative role as molecular chaperones capable of protecting several cell components from freezing-induced dehydration damage by direct binding, thus preventing protein aggregation and inactivation (Yang *et al*., 2012). The upregulation of *VvRS* is also coherent with the osmoprotectant role associated with soluble sugars accumulation, and RFOs in particular (Grand and Dami, 2015). Cold stress response-related *VvEIN3* and *VvNCED1*, involved in ethylene and ABA biosynthesis respectively (Sun *et al*., 2016;

Rubio *et al.*, 2019) were also upregulated in response to the spring frost. In recent studies, ethylene was hypothesized to positively regulate cold hardiness in *V. amurensis* plantlets subjected to cold stress (Sun *et al.*, 2016) and *VvEIN3* upregulation was detected in response to freeze shock in grapevine leaf tissue (Londo *et al.*, 2018). In addition to this, ABA, coupled with low temperatures, was shown to induce freezing tolerance-related genes in grapevine bud tissue (Rubio *et al.*, 2019). Exogenous ABA application also enhanced bud freezing tolerance in greenhouse-grown grapevines by impacting RFOs biosynthesis and bud water content (Wang *et al.*, 2020).

Interestingly, Sauvignon Nepis buds also showed differential responsiveness depending on their proximity to budbreak. Only buds classifiable as BBCH 00 (winter buds) showed significant reactivity to the spring frost. It can be hypothesized that BBCH 05 and BBCH 09 buds, wooly and green tipped respectively, are no longer competent to respond to sudden freezing temperatures exposure at that stage of phenological advancement. Possibly, additional or different cold resistance mechanisms are active at these stages when the buds appear more and more similar to green tissues.

5 CONCLUSIONS

This work represents a preliminary insight into early molecular responses of actively deacclimating bud tissues to spring frost occurrence. Comparative studies including cultivars with contrasting cold resistance and budbreak precocity could delineate which pathways are the most relevant in determining late frost tolerance. This knowledge could guide future breeding efforts towards the generation of more sustainable varieties in the context of a changing climate.

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♦ CHAPTER VII

Concluding remarks

INSPIRATION FOR THE FUTURE

Dormancy progression and its release are intricate phenomena, involving complex processes such as temperature sensing, cold hardiness acquisition, chilling requirement fulfilment, acclimation and deacclimation dynamics, each one requiring in depth studies to define its contribution in overall dormancy regulation, and to find fitting adaptation targets against the threat of late frost occurrences.

The results presented in this thesis constitute an attempt at clarifying some of the blind spots in this research topic and offer several cues to hopefully inspire further studies (Figure 1). A common recurring theme among chapters refers to carbohydrate metabolism, which appears involved in increased freezing tolerance during dormancy (Chapter III), in deacclimation dynamics (Chapter V), and in rapid response to spring frost occurrences (Chapter VI). Notwithstanding, the role of non-structural carbohydrates as signaling molecules during dormancy progression should be taken into account.

On the other hand, transcriptomic analyses, both at single gene and whole genome level, are powerful tools capable of uncovering entire developmental processes in buds before morphological changes appear (Chapter V). The evidence collected by RNA-Seq analysis shows that winter buds are dynamic entities capable of perceiving internal and external stimuli, thus determining the development of the entire plant. BS-Seq data (Chapter V) uncovered the possibility of fine epigenetic regulation being fundamental in the dormancy release process.

Changing climatic conditions will remain a certainty in the future, and adaptive measures need to be found in step with it. Genetic improvement must be undertaken as a strategy to enhance grapevine’s resilience towards spring frosts. This thesis aims at inspiring focused and directed endeavors for this purpose.

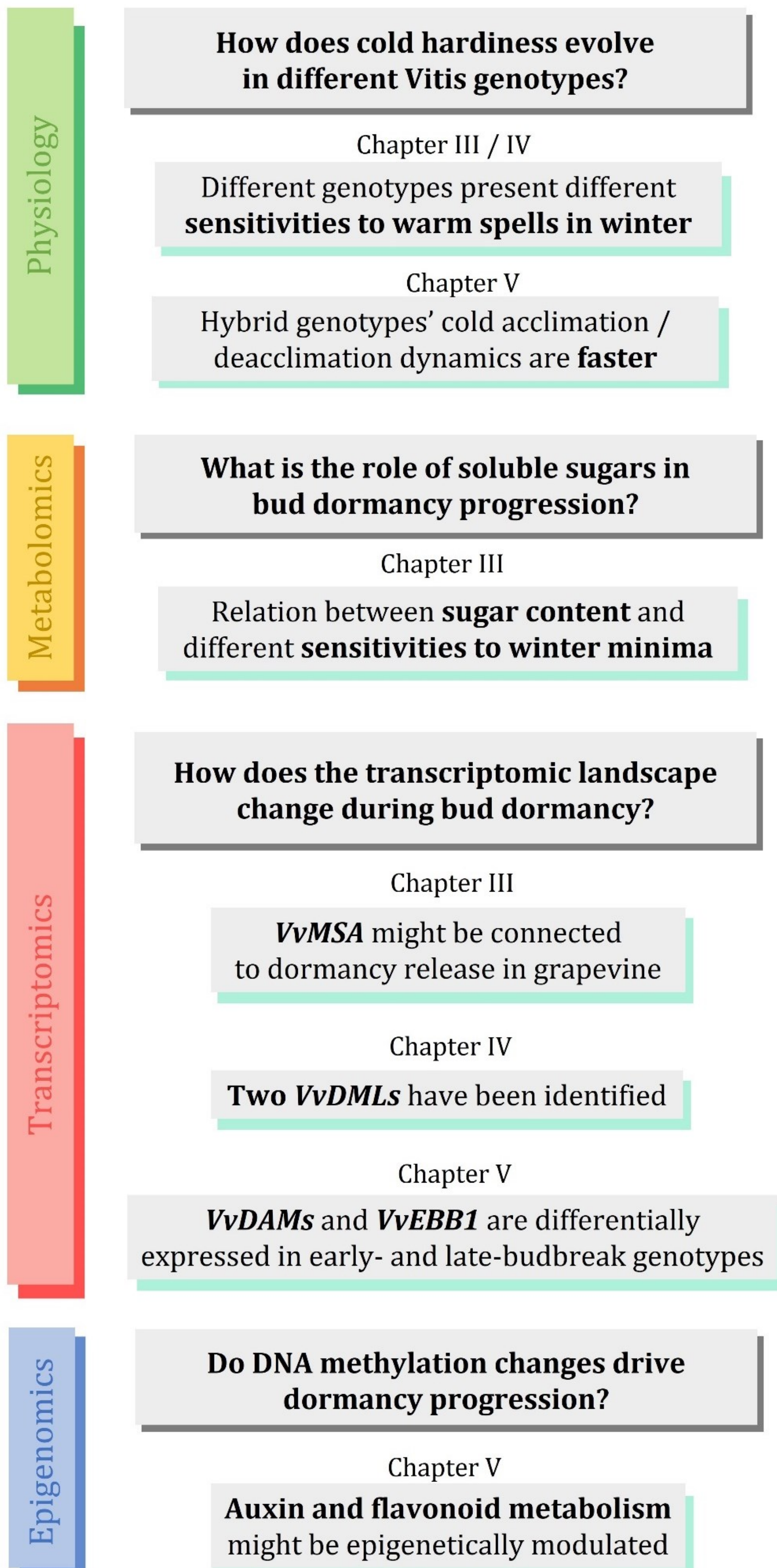

Figure 1. Main results answering the experimental questions posed in Chapter II logic model.

♦ APPENDIX

APPENDIX

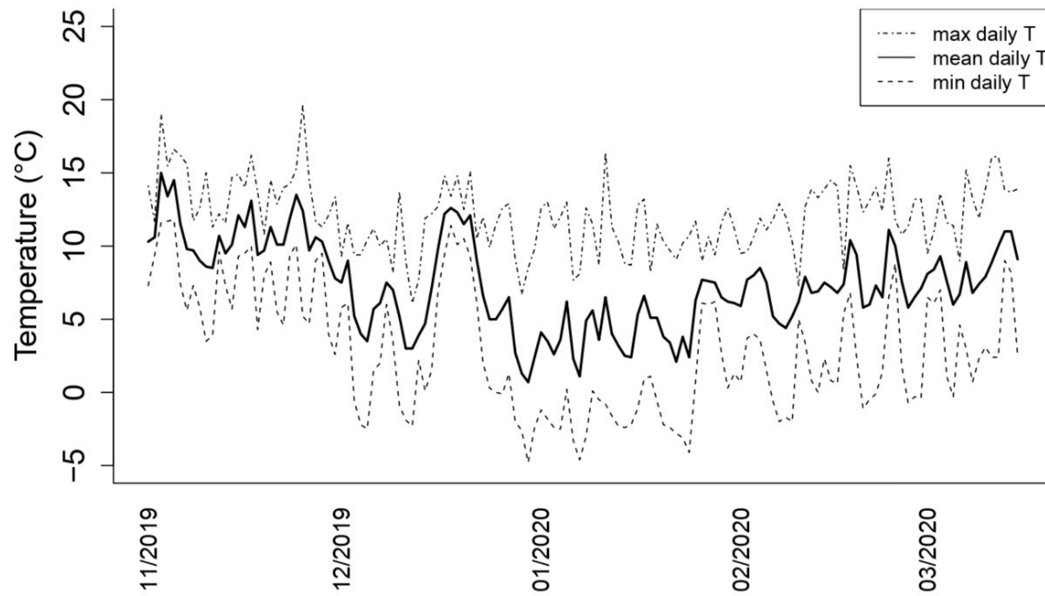

Figure S1. Mean daily temperatures data registered during the 2019/2020 winter season. Data recorded by the S. Osvaldo (Udine, Italy) weather station managed by ARPA FVG.

Table A1. Detailed list of enriched biological processes (BP) terms in up- and downregulated DEGs unique to Cabernet Sauvignon in T1 vs T2 pairwise comparison.

Upregulated DEGs		**Downregulated DEGs**	
GO:0009813	flavonoid biosynthetic process	GO:0000278	mitotic cell cycle
GO:0051552	flavone metabolic process	GO:0051322	anaphase
GO:0051553	flavone biosynthetic process	GO:0006120	mitochondrial electron transport, NADH...
GO:0051554	flavonol metabolic process	GO:1903047	mitotic cell cycle process
GO:0051555	flavonol biosynthetic process	GO:0022904	respiratory electron transport chain
GO:0009812	flavonoid metabolic process	GO:0006743	ubiquinone metabolic process
GO:0006952	defense response	GO:0006744	ubiquinone biosynthetic process
GO:0042254	ribosome biogenesis	GO:0051726	regulation of cell cycle
GO:0022613	ribonucleoprotein complex biogenesis	GO:0008283	cell proliferation
GO:0009955	adaxial/abaxial pattern specification	GO:0042773	ATP synthesis coupled electron transport
GO:0009944	polarity specification of adaxial/abaxia...	GO:0042775	mitochondrial ATP synthesis coupled el...
GO:0010218	response to far red light	GO:0010564	regulation of cell cycle process
GO:0065001	specification of axis polarity	GO:0007049	cell cycle
GO:0009943	adaxial/abaxial axis specification	GO:0022403	cell cycle phase
GO:0009934	regulation of meristem structural organi...	GO:0044848	biological phase
GO:0009765	photosynthesis, light harvesting	GO:0022402	cell cycle process
GO:0009069	serine family amino acid metabolic proce...	GO:1901661	quinone metabolic process
GO:0010466	negative regulation of peptidase activit...	GO:1901663	quinone biosynthetic process
GO:0030162	regulation of proteolysis	GO:0000280	nuclear division
GO:0045861	negative regulation of proteolysis	GO:0006814	sodium ion transport
GO:0052547	regulation of peptidase activity	GO:0042181	ketone biosynthetic process
GO:0015979	photosynthesis	GO:0000911	cytokinesis by cell plate formation
GO:0010114	response to red light	GO:1901616	organic hydroxy compound catabolic…
GO:0009816	defense response to bacterium, incompat...	GO:0032506	cytokinetic process
GO:0048509	regulation of meristem development	GO:1902410	mitotic cytokinetic process
GO:0051346	negative regulation of hydrolase activit...	GO:0000281	mitotic cytokinesis
GO:0014070	response to organic cyclic compound	GO:0006260	DNA replication
GO:0009798	axis specification	GO:0061640	cytoskeleton-dependent cytokinesis
GO:0009637	response to blue light	GO:0019336	phenol-containing compound catabolic…
GO:0007165	signal transduction	GO:0046244	salicylic acid catabolic process
GO:0042549	photosystem II stabilization	GO:0051052	regulation of DNA metabolic process
GO:0023052	signaling	GO:0000279	M phase
GO:0043207	response to external biotic stimulus	GO:0006275	regulation of DNA replication
GO:0051707	response to other organism	GO:0048285	organelle fission
GO:0009607	response to biotic stimulus	GO:0009819	drought recovery
GO:0010216	maintenance of DNA methylation	GO:0000910	cytokinesis
GO:0006468	protein phosphorylation	GO:0007346	regulation of mitotic cell cycle
GO:0009751	response to salicylic acid	GO:0006725	cellular aromatic compound metabolic…
GO:0009624	response to nematode	GO:0006570	tyrosine metabolic process
GO:0007169	transmembrane receptor protein tyrosin…	GO:0000086	G2/M transition of mitotic cell cycle
GO:0043043	peptide biosynthetic process	GO:0044839	cell cycle G2/M phase transition
GO:0044092	negative regulation of molecular functio...	GO:0044770	cell cycle phase transition
GO:0010258	NADH dehydrogenase complex (plastoqu...	GO:0044772	mitotic cell cycle phase transition
GO:0080142	regulation of salicylic acid biosyntheti...	GO:0051301	cell division
GO:0009231	riboflavin biosynthetic process	GO:0009800	cinnamic acid biosynthetic process
GO:0042727	flavin-containing compound biosynthetic ...	GO:0009803	cinnamic acid metabolic process
GO:0009733	response to auxin	GO:0051567	histone H3-K9 methylation
GO:0006412	translation	GO:0061647	histone H3-K9 modification
GO:0019684	photosynthesis, light reaction	GO:0006261	DNA-dependent DNA replication
GO:0040008	regulation of growth	GO:0000278	mitotic cell cycle

Table A2. Detailed list of enriched biological processes (BP) terms in up- and downregulated DEGs unique to Sauvignon Nepis in T1 vs T2 pairwise comparison.

Upregulated DEGs		Downregulated DEGs	
GO:0009808	lignin metabolic process	GO:0010468	regulation of gene expression
GO:0009809	lignin biosynthetic process	GO:0009639	response to red or far red light
GO:0009699	phenylpropanoid biosynthetic process	GO:0009889	regulation of biosynthetic process
GO:0009698	phenylpropanoid metabolic process	GO:0031326	regulation of cellular biosynthetic proc...
GO:0044550	secondary metabolite biosynthetic proces...	GO:0019222	regulation of metabolic process
GO:0009810	stilbene metabolic process	GO:0010556	regulation of macromolecule biosynthetic...
GO:0009811	stilbene biosynthetic process	GO:2000112	regulation of cellular macromolecule bio...
GO:0019748	secondary metabolic process	GO:0010467	gene expression
GO:0009804	coumarin metabolic process	GO:0060255	regulation of macromolecule metabolic p...
GO:0009805	coumarin biosynthetic process	GO:0009416	response to light stimulus
GO:1900673	olefin metabolic process	GO:0080090	regulation of primary metabolic process
GO:1900674	olefin biosynthetic process	GO:0009628	response to abiotic stimulus
GO:0046271	phenylpropanoid catabolic process	GO:0016070	RNA metabolic process
GO:0046274	lignin catabolic process	GO:0006351	transcription, DNA-templated
GO:0010025	wax biosynthetic process	GO:0097659	nucleic acid-templated transcription
GO:0046688	response to copper ion	GO:0032774	RNA biosynthetic process
GO:1901570	fatty acid derivative biosynthetic proce...	GO:0048518	positive regulation of biological proces...
GO:0010166	wax metabolic process	GO:0009314	response to radiation
GO:0055114	oxidation-reduction process	GO:0031323	regulation of cellular metabolic process
GO:0009403	toxin biosynthetic process	GO:0051171	regulation of nitrogen compound metabo...
GO:1901615	organic hydroxy compound metabolic proc...	GO:0007031	peroxisome organization
GO:0006952	defense response	GO:0006355	regulation of transcription, DNA-templat...
GO:0052315	phytoalexin biosynthetic process	GO:1903506	regulation of nucleic acid-templated tra...
GO:1901568	fatty acid derivative metabolic process	GO:2001141	regulation of RNA biosynthetic process
GO:0052314	phytoalexin metabolic process	GO:0048522	positive regulation of cellular process
GO:0006629	lipid metabolic process	GO:0050794	regulation of cellular process
GO:0044255	cellular lipid metabolic process	GO:0072594	establishment of protein localization to...
GO:0030004	cellular monovalent inorganic cation hom...	GO:0065007	biological regulation
GO:1901361	organic cyclic compound catabolic proces...	GO:0017038	protein import
GO:0018948	xylene metabolic process	GO:0050789	regulation of biological process
GO:0018970	toluene metabolic process	GO:0090304	nucleic acid metabolic process
GO:0042184	xylene catabolic process	GO:0019219	regulation of nucleobase-containing com...
GO:0042203	toluene catabolic process	GO:0033365	protein localization to organelle
GO:0072490	toluene-containing compound metabolic pr...	GO:0071478	cellular response to radiation
GO:0072491	toluene-containing compound catabolic pr...	GO:0071482	cellular response to light stimulus
GO:0050000	chromosome localization	GO:0050896	response to stimulus
GO:0019439	aromatic compound catabolic process	GO:0015919	peroxisomal membrane transport
GO:0042335	cuticle development	GO:0016558	protein import into peroxisome matrix
GO:0018879	biphenyl metabolic process	GO:0006625	protein targeting to peroxisome
GO:0006836	neurotransmitter transport	GO:0072662	protein localization to peroxisome
GO:0015807	L-amino acid transport	GO:0072663	establishment of protein localization to...
GO:0019752	carboxylic acid metabolic process	GO:0043574	peroxisomal transport
GO:0032787	monocarboxylic acid metabolic process	GO:0010114	response to red light
GO:0015864	pyrimidine nucleoside transport	GO:0009583	detection of light stimulus
GO:0000038	very long-chain fatty acid metabolic pro...	GO:0051252	regulation of RNA metabolic process
GO:0006558	L-phenylalanine metabolic process	GO:0051649	establishment of localization in cell
GO:1902221	erythrose 4-phosphate/phosphoenolpyruv...	GO:0009584	detection of visible light
GO:0048544	recognition of pollen	GO:0071483	cellular response to blue light
GO:0008037	cell recognition	GO:0044743	protein transmembrane import into intra...
GO:0043447	alkane biosynthetic process	GO:0006357	regulation of transcription by RNA polym...

Table A3. Detailed list of enriched biological processes (BP) terms in up- and downregulated DEGs unique to Cabernet Sauvignon in T2 vs T3 pairwise comparison.

Upregulated DEGs		**Downregulated DEGs**	
GO:1900674	olefin biosynthetic process	GO:0006952	defense response
GO:0009810	stilbene metabolic process	GO:0010073	meristem maintenance
GO:0009811	stilbene biosynthetic process	GO:0009855	determination of bilateral symmetry
GO:1900673	olefin metabolic process	GO:0007389	pattern specification process
GO:0009804	coumarin metabolic process	GO:0009799	specification of symmetry
GO:0009805	coumarin biosynthetic process	GO:0048827	phyllome development
GO:0009809	lignin biosynthetic process	GO:0065001	specification of axis polarity
GO:0009699	phenylpropanoid biosynthetic process	GO:0006468	protein phosphorylation
GO:0042493	response to drug	GO:0048438	floral whorl development
GO:0009808	lignin metabolic process	GO:0048437	floral organ development
GO:0010243	response to organonitrogen compound	GO:0016102	diterpenoid biosynthetic process
GO:0006952	defense response	GO:0009685	gibberellin metabolic process
GO:0071229	cellular response to acid chemical	GO:0009944	polarity specification of adaxial/abaxia...
GO:0010200	response to chitin	GO:0009686	gibberellin biosynthetic process
GO:0009862	systemic acquired resistance, salicylic ...	GO:0048440	carpel development
GO:0009607	response to biotic stimulus	GO:0009943	adaxial/abaxial axis specification
GO:0006950	response to stress	GO:0006355	regulation of transcription, DNA-templat...
GO:0042221	response to chemical	GO:1903506	regulation of nucleic acid-templated tra...
GO:0043207	response to external biotic stimulus	GO:2001141	regulation of RNA biosynthetic process
GO:0051707	response to other organism	GO:0048507	meristem development
GO:0010033	response to organic substance	GO:0006313	transposition, DNA-mediated
GO:0009627	systemic acquired resistance	GO:0009955	adaxial/abaxial pattern specification
GO:0009698	phenylpropanoid metabolic process	GO:0016101	diterpenoid metabolic process
GO:1901700	response to oxygen-containing compound	GO:0048366	leaf development
GO:0009863	salicylic acid mediated signaling pathwa...	GO:0009798	axis specification
GO:0009814	defense response, incompatible interacti...	GO:0009812	flavonoid metabolic process
GO:0002376	immune system process	GO:0009813	flavonoid biosynthetic process
GO:0098542	defense response to other organism	GO:0032196	transposition
GO:1901701	cellular response to oxygen-containing c...	GO:0051552	flavone metabolic process
GO:0071446	cellular response to salicylic acid stim...	GO:0051553	flavone biosynthetic process
GO:0045087	innate immune response	GO:0051554	flavonol metabolic process
GO:0006955	immune response	GO:0051555	flavonol biosynthetic process
GO:0009751	response to salicylic acid	GO:0048467	gynoecium development
GO:0044550	secondary metabolite biosynthetic proces...	GO:0009908	flower development
GO:0031347	regulation of defense response	GO:0009888	tissue development
GO:0071236	cellular response to antibiotic	GO:0009069	serine family amino acid metabolic proce...
GO:0019748	secondary metabolic process	GO:0006351	transcription, DNA-templated
GO:0001101	response to acid chemical	GO:0097659	nucleic acid-templated transcription
GO:0009617	response to bacterium	GO:0032774	RNA biosynthetic process
GO:0009605	response to external stimulus	GO:0090567	reproductive shoot system development
GO:0032870	cellular response to hormone stimulus	GO:0003002	regionalization
GO:0071407	cellular response to organic cyclic comp...	GO:0006520	cellular amino acid metabolic process
GO:0046677	response to antibiotic	GO:0048439	flower morphogenesis
GO:0071495	cellular response to endogenous stimulus	GO:0048532	anatomical structure arrangement
GO:0080134	regulation of response to stress	GO:0048509	regulation of meristem development
GO:0050896	response to stimulus	GO:0009741	response to brassinosteroid
GO:0009738	abscisic acid-activated signaling pathwa...	GO:0009834	plant-type secondary cell wall biogenesi...
GO:0035690	cellular response to drug	GO:0009933	meristem structural organization
GO:1901698	response to nitrogen compound	GO:0010224	response to UV-B
GO:0071310	cellular response to organic substance	GO:0016310	phosphorylation

Table A4. Detailed list of enriched biological processes (BP) terms in up- and downregulated DEGs unique to Sauvignon Nepis in T2 vs T3 pairwise comparison.

Upregulated DEGs		**Downregulated DEGs**	
GO:0000956	nuclear-transcribed mRNA catabolic proce.	GO:0006081	cellular aldehyde metabolic process
GO:0006402	mRNA catabolic process	GO:0019682	glyceraldehyde-3-phosphate metabolic pro...
GO:0006401	RNA catabolic process	GO:0019693	ribose phosphate metabolic process
GO:0016071	mRNA metabolic process	GO:0006091	generation of precursor metabolites and ...
GO:0010468	regulation of gene expression	GO:0019637	organophosphate metabolic process
GO:0006281	DNA repair	GO:0009117	nucleotide metabolic process
GO:0070647	protein modification by small protein co...	GO:0006753	nucleoside phosphate metabolic process
GO:0019222	regulation of metabolic process	GO:0009657	plastid organization
GO:0009892	negative regulation of metabolic process	GO:0015979	photosynthesis
GO:0060255	regulation of macromolecule metabolic pr...	GO:1901135	carbohydrate derivative metabolic proces...
GO:0010605	negative regulation of macromolecule met..	GO:0051156	glucose 6-phosphate metabolic process
GO:0006397	mRNA processing	GO:0006098	pentose-phosphate shunt
GO:0010467	gene expression	GO:0009668	plastid membrane organization
GO:0006351	transcription, DNA-templated	GO:0010027	thylakoid membrane organization
GO:0097659	nucleic acid-templated transcription	GO:0009853	photorespiration
GO:0032774	RNA biosynthetic process	GO:0006739	NADP metabolic process
GO:0010228	vegetative to reproductive phase transit...	GO:0061024	membrane organization
GO:0006355	regulation of transcription, DNA-templat...	GO:0009658	chloroplast organization
GO:1903506	regulation of nucleic acid-templated tra...	GO:0019288	isopentenyl diphosphate biosynthetic pro...
GO:2001141	regulation of RNA biosynthetic process	GO:0043094	cellular metabolic compound salvage
GO:0010629	negative regulation of gene expression	GO:0009240	isopentenyl diphosphate biosynthetic pro...
GO:0031326	regulation of cellular biosynthetic proc...	GO:0046490	isopentenyl diphosphate metabolic proces...
GO:0006310	DNA recombination	GO:0006364	rRNA processing
GO:0090304	nucleic acid metabolic process	GO:0016072	rRNA metabolic process
GO:0006974	cellular response to DNA damage stimulus	GO:0046496	nicotinamide nucleotide metabolic proces...
GO:0010017	red or far-red light signaling pathway	GO:0055086	nucleobase-containing small molecule met...
GO:0071489	cellular response to red or far red ligh...	GO:0019684	photosynthesis, light reaction
GO:0010556	regulation of macromolecule biosynthetic...	GO:0019362	pyridine nucleotide metabolic process
GO:2000112	regulation of cellular macromolecule bio...	GO:0006090	pyruvate metabolic process
GO:0009889	regulation of biosynthetic process	GO:0009150	purine ribonucleotide metabolic process
GO:0016567	protein ubiquitination	GO:0006163	purine nucleotide metabolic process
GO:0016070	RNA metabolic process	GO:0032787	monocarboxylic acid metabolic process
GO:0008284	positive regulation of cell proliferatio...	GO:0006644	phospholipid metabolic process
GO:0000375	RNA splicing, via transesterification re...	GO:0046394	carboxylic acid biosynthetic process
GO:0006302	double-strand break repair	GO:0016053	organic acid biosynthetic process
GO:0048573	photoperiodism, flowering	GO:0072524	pyridine-containing compound metabolic...
GO:0032446	protein modification by small protein co...	GO:0009259	ribonucleotide metabolic process
GO:0008380	RNA splicing	GO:0006733	oxidoreduction coenzyme metabolic proce...
GO:0080090	regulation of primary metabolic process	GO:0010207	photosystem II assembly
GO:0000377	RNA splicing, via transesterification re...	GO:0042254	ribosome biogenesis
GO:0009648	photoperiodism	GO:0044255	cellular lipid metabolic process
GO:0051013	microtubule severing	GO:0046034	ATP metabolic process
GO:0051171	regulation of nitrogen compound metabol...	GO:0008610	lipid biosynthetic process
GO:0090213	regulation of radial pattern formation	GO:0009126	purine nucleoside monophosphate metabo...
GO:0009416	response to light stimulus	GO:0009167	purine ribonucleoside monophosphate me…
GO:0034655	nucleobase-containing compound catabolic	GO:0051186	cofactor metabolic process
GO:0006120	mitochondrial electron transport, NADH t...	GO:0022613	ribonucleoprotein complex biogenesis
GO:0006473	protein acetylation	GO:0009144	purine nucleoside triphosphate metabolic...
GO:0019219	regulation of nucleobase-containing comp...	GO:0009205	purine ribonucleoside triphosphate metab...
GO:0009553	embryo sac development	GO:0072330	monocarboxylic acid biosynthetic process

♦ ACKNOWLEDGMENTS

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Many people have been part of the achievements accomplished in the last three years, and I want to take this opportunity to acknowledge each of them without, hopefully, forgetting anyone.

Starting from my research group, I want to thank my supervisor, Prof. Giannina Vizzotto, for her guidance and support during this work and life experience, and for financing my work.

I want to thank Dr. Rachele Falchi, who flanked me and put her scientific expertise to my disposal, contributing significantly to my growth as a research scientist.

I also want to thank PA Giorgio Comuzzo for his never-ending kindness and availability to manage my plants, take professional pictures of my research subjects, and have a laugh together.

Among the many collaborators with which I've had the pleasure to interact, I want to thank Prof. Alessandro Peressotti, who designed the DTA system I used for two years, and PA Diego Chiabà, who built it and repaired it when it was needed. On this regard, I also want to thank Dr. Fabio Marroni and Dr. Francesco Savian, who both contributed substantially to the improvement of the R code which allowed me to perform peak detection of DTA data.

I want to thank Dr. Emanuele De Paoli, who performed the bioinformatics analysis of RNA-Seq and BS-Seq data in Chapter V. His acumen and meticulousness are commendable, and a model for me.

I want to thank Dr. Erica Moret, who performed HPLC analysis included in Chapter III, and who was very kind to do it as many times as I wished.

Finally, I want to thank PA Moreno Greatti from the Experimental Farm 'A. Servadei' in Udine, who allowed me to perform all the bud sampling for Chapter VI.

While my research collaborators supported my scientific work, many other people supported me emotionally.

I'd like to acknowledge them starting from Dr. Elisa Scarsella, my PhD-mate for all three years. I wasn't always sure we'd be able to say it in the end, but everything did indeed go well after all. A special mention goes to Dr. Tommaso Montanari, who I'd simply define *extra*.

I want to thank my office-mate Dr. Gloria De Mori, who has assisted to many mental breakdowns and was always willing to give precious counsel over two years, and Arianna Lodovici, a partner in laughs and awkwardness, although *disagio* sounds better.

Finally, I want to acknowledge those who supported me outside of my work environment.

I want to thank my family, particularly my mother, my father and my sister, who have heard the words '*Vitis vinifera*' too often but never complained. They have offered me food and *aperitivi* after long work days more times than I can count, and helped me maintain mental serenity even in the most difficult moments.

I want to thank Giulia Cavalsi, my precious best friend who knows me better than most people. I wish we both find the path in life that will bring us the most joy and fulfillment.

Lastly, although he knows very well he is not least, I want to thank Riccardo Braidotti. Go get the world darling, I wish you all the best always.

Grazie a tutti,

Valeria De Rosa

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