UNIVERSIDAD POLITÉCNICA DE MADRID
Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas

Understanding the Genetic Basis of Grapevine Diversity: Implications for Vineyard Resilience in the Face of Climate Change

DOCTORAL THESIS

Submitted for the degree of Doctor by:

Alberto Rodríguez Izquierdo
Master in Fermented Beverages

Madrid, 2024

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Author: Alberto Rodríguez Izquierdo
Doctoral Programme: Doctoral Degree in Biotechnology and Genetic Resources of Plants and Associated Microorganisms
Thesis Supervision:
Dr. Josefina María Bota Salort, Associate Professor of the Department of Biology, University of Balearic Islands (UIB) (Supervisor)

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A mi familia, amigos y sobre todo a Ángela, mi razón de ser.
Rosa, esto va por ti.

In memoriam of Dr. Rosa Arroyo García, PhD director of this thesis

En estas palabras intentaré recoger el profundo cariño y respeto que he tenido, tengo y tendré a la directora principal de la tesis, Dra. Rosa Arroyo García, que falleció el 6 de diciembre de 2023 tras una larga enfermedad.

Me gustaría comenzar por lo que Rosa ha supuesto en mi carrera profesional y personal, ya que sin ella no hubiese conseguido acabar esta tesis. Conocí a Rosa en 2016, tras contactar con ella para hacer unas prácticas en su laboratorio. Desde entonces, siempre continuó contando conmigo para impulsar mi carrera profesional a lo largo de todos estos años. Siempre me apoyó en todo momento, ofreciéndome oportunidades e impulsándome siempre para que consiguiese muchas metas, para muchos, inalcanzables.

Gracias a Rosa conseguí integrarme en el mundo de la investigación sobre la vid, un mundo muy acogedor y agradable, haciéndome sentir como uno más y consiguiendo que viese a esta planta con la pasión y la entrega con la que la vivía ella. Y ella siempre quiso que lo viera así, sumergiéndome en su atmósfera, conociendo a gente maravillosa y viajando a lugares que nunca antes hubiese imaginado viajar. Gracias a ella descubrí que Estados Unidos era un lugar bastante acogedor, pudiendo ver a través de mis ojos una pequeña parte de todo lo que ella vivió en su etapa en la Universidad de California, Davis, y que yo pude experimentar en mis estancias en la Universidad de Kentucky y en la misma Universidad de California, Davis. Una etapa que recordaba con muchísimo cariño en la que vivió muchas experiencias moviéndose a todos lados en bicicleta, logró ser aún más feliz y en donde tuvo a su primera hija. Y creo que esa mirada tan alegre con la que describía todas sus experiencias en Davis (incluidos sus viajes por el Gran Cañón, San Francisco o los burritos del Dos Coyotes, que ella los recordaba como excepcionales y que me recomendaba siempre probar en Davis) era un fiel reflejo de lo que era ella. Una persona optimista, acogedora y que siempre ayudaba a los demás, haciéndote sentir como uno más y que siempre conseguía sacar lo bueno que tenías.

En definitiva, Rosa era una persona que, ante todo, siempre estaba al lado de todos ayudando y aconsejando, aún en sus peores momentos. Una persona que lo daba todo por los demás, y que nunca se la reconocerá lo suficiente todo el esfuerzo que ha hecho durante toda su vida. Es por ello que esta tesis está dedicada a ella, ya que es un reconocimiento a la figura, no sólo de una gran científica, sino también de una madre y una amiga excepcional.

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"Fui a los bosques porque deseaba vivir deliberadamente; para enfrentar sólo los hechos esenciales de la vida, y ver si podía aprender lo que ella tenía que enseñarme; Quise vivir profundamente y desechar todo aquello que no fuera vida, no fuese a descubrir, al llegar la hora de mi muerte, que no había vivido." Walden, Henry David Thoreau, 1854

Resumen

El proceso de domesticación de la vid a lo largo de la Historia ha generado una enorme diversidad de genotipos. Comenzando en la zona asiática y caucásica, tuvo como principal carácter de selección aquellos individuos con hermafroditismo floral, con vistas a asegurar la producción anual de uvas. Además, caracteres como el color de la uva, la forma o la superficie del hollejo han primado a la hora de seleccionar la planta de interés, favoreciendo unos caracteres dependiendo de los usos culinarios de las uvas producidas. Esta selección ha ido asociada a la adaptación de estos genotipos al clima, surgiendo genotipos más tolerantes a estreses abióticos que otros. Además, el avance en genómica de la vid ha evolucionado mucho estos años gracias a las nuevas técnicas de secuenciación masiva (NGS), pudiendo determinar presiones selectivas que han influido a lo largo de la Historia. Además, estas tecnologías se pueden aplicar a diferentes campos de estudio en vid, como pueden ser los estudios sobre la epigenómica de la vid o sobre los cambios en la expresión génica asociados a diferentes condiciones. En la presente tesis se exponen dos capítulos correspondientes a estos campos atendiendo a la domesticación del cultivo y a la exploración de los correspondientes genotipos derivados de este proceso para poder adaptar el viñedo a nuevos escenarios de cambio climático tales como la sequía en el viñedo. Para ello, en estos estudios se utilizan técnicas de secuenciación masiva aplicadas a diferentes objetivos. En el primer caso, se utilizan estas técnicas de secuenciación para explorar la influencia de la epigenómica en la domesticación del cultivo a partir de vides silvestres y cultivadas para encontrar diferencias entre las poblaciones y detectar cambios en la construcción del metiloma. En el segundo capítulo, se aplican estas técnicas de secuenciación masiva para explorar, dentro de la diversidad producida por la domesticación, cuáles son los mecanismos existentes que confieren a algunos genotipos mayor tolerancia a sequía. Se exploran los cambios a nivel fisiológico, metabolómico, hormonal y transcriptómico de dos variedades con distinto grado de tolerancia a sequía bajo tres distintos niveles de estrés hídrico (moderado, intenso y severo): *Callet*, un cultivar local de las Islas Baleares; y *Merlot*, un cultivar internacional procedente de Francia; ambos injertados sobre portainjertos *110 Ricther*. En el primer capítulo, los resultados muestran una clara influencia de la domesticación en las plantas cultivadas por una mayor acumulación de citosinas metiladas en comparación con las variedades silvestres. Mientras que en las plantas silvestres estas metilaciones aparecen en genes relacionados con la respuesta hormonal y la defensa, en plantas cultivadas aparecen genes relacionados con la reducción del estrés oxidativo y la producción de polifenoles. En el segundo capítulo, se demuestra el importante papel de la sensibilidad al ácido abscísico (ABA) en la respuesta de genotipos con mayor tolerancia a sequía como *Callet* a todos los niveles. Los resultados a nivel metabolómico y transcriptómico muestran que, a consecuencia de esta sensibilidad al ABA, *Callet* modula vías de producción de metabolitos secundarios y regula la expresión de genes relacionados con la respuesta a sequía tanto en la parte aérea como en la parte radicular. En cambio, la baja tolerancia a sequía se ve amplificada por una falta de comunicación entre la parte aérea y el portainjertos en *Merlot*, demostrando que esta comunicación en plantas injertadas con *Callet* es más efectiva para adaptar la planta a

condiciones de estrés hídrico. La investigación desarrollada a partir de estos dos capítulos muestra la importancia de estos estudios basados en esta diversidad producida por la domesticación para poder adaptar el viñedo actual a nuevos escenarios producidos por el cambio climático, demostrando su utilidad en posteriores investigaciones.

Abstract

The domestication process of grapevine throughout history has generated a vast diversity of genotypes. Beginning in the Asian and Caucasian regions, the primary selection criterion was individuals with floral hermaphroditism, aimed at ensuring annual grape production. Additionally, traits such as grape color, shape, and skin surface have been prioritized in selecting the desired plant, favoring certain characteristics depending on the culinary uses of the grapes produced. This selection has been associated with the adaptation of these genotypes to climate, resulting in genotypes more tolerant to abiotic stresses than others. Furthermore, advances in grapevine genomics have evolved significantly in recent years thanks to new sequencing techniques (NGS), enabling the determination of selective pressures that have influenced throughout history. Moreover, these technologies can be applied to various unexplored research areas in grapevine, such as studies on grapevine epigenomics or changes in gene expression associated with different conditions. This thesis presents two chapters corresponding to these fields focusing on crop domestication and exploring the derived genotypes from this process to adapt vineyards to new climate change scenarios such as vineyard drought. For this purpose, these studies utilize massive sequencing techniques applied to different objectives. In the first case, these sequencing techniques are used to explore the influence of epigenomics on crop domestication using wild and cultivated vines to identify differences between populations and detect changes in the methylome construction. In the second chapter, these massive sequencing techniques are applied to explore, within the diversity produced by domestication, the mechanisms that confer greater drought tolerance to some genotypes. Changes at physiological, metabolomic, hormonal, and transcriptomic levels of two varieties with different degrees of drought tolerance are explored under three different levels of water stress (mild, high, and extreme): *Callet*, a local cultivar from the Balearic Islands (Spain); and *Merlot*, a commercial cultivar from France; both grafted onto *110 Richter* rootstocks. In the first chapter, the results show a clear influence of domestication on cultivated plants due to a higher accumulation of methylated cytosines compared to wild varieties. While in wild plants, these methylations appear in genes related to hormonal response and defense, in cultivated plants, genes related to oxidative stress reduction and polyphenol production appear. In the second chapter, a significant influence of abscisic acid (ABA) sensitivity is demonstrated in the response of genotypes with greater drought tolerance such as *Callet* at all levels. Metabolomic and transcriptomic results show that, based on this ABA sensitivity, *Callet* modulates secondary metabolite production pathways and regulates gene expression related to drought response in both the aerial and root parts. The adaptation to drought stress in grafted grapevines is also influenced by a lack of communication between the aerial part and the rootstock in *Merlot*, demonstrating that this communication in grafted plants with *Callet* is more effective. The research developed from these two chapters highlights the importance of these studies based on the diversity produced by domestication to adapt current vineyards to new scenarios produced by climate change, showing their powerful utility for use in subsequent research.

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List of Acronyms

Acronym	Meaning
1H-NMR	1H-Nuclear Magnetic Resonance
ABA	Abscisic Acid
AFLP	Amplification Fragments Length Polymorphism
bZIP	Basic region/leucine zipper motif
CGs	Cultivated Grapevines
ChIP	Chromatin Immunoprecipitation
CHS	Chalcone Synthetase
CMCs	Core of Methylated Cytosines
DMCs	Differentially Methylated Cytosines
DNA	Deoxyribonucleic Acid
DRE/CRT	dehydration-responsive element/C-repeat
DREB2A	DRE-CRT-binding protein 2
ENA	European Nucleotide Database
ERF1	Ethylene Response Factor 1
FAIR	Finding Accessible Interoperabililty Reuse
GBS	Genotyping By Sequencing
GBS	Genome Bisulfite Sequencing
GC-MS	Gas Chromatography coupled with Mass Spectrometry
gDNA	Genomic DNA
Gis	Gibberellins
HIV	Human Immunodeficiency Virus
HPLC-MS	High Pressure Liquid Chromatography-Mass Spectrometry
ITS	Internal Transcriber Spacer
IAA	Indol-Acetic Acid
Jas	Jasmonic Acid
LC-MS	Liquid Chromatography coupled with Mass Espectrometry
lncRNA	Long-intervent/intergenic non coding RNA
LTR	Long Terminal Repeats
miRNA	Micro RNA
Mha	Mega hectarea
MYBs	Myeloblastosis
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
PN	Pinot Noir
PP2C	PROTEIN PHOSPHATASE 2C (PP2C)
PYL	PYRABACTIN RESISTANCE 1-Lyke
qPCR	Quantitative PCR
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNA-seq	RNA sequencing

ROS	Reactive Oxygen Species
RRBS	Reduced Representation Bisulfite Sequencing
RT	Reverse transcriptase
SA	Salicilic Acid
SMRT	Single Molecule-Real Time sequencing
SNPs	Single Nucleotide Polymorphisms
SnRK2	SNF1-RELATED PROTEIN KINASE 2 (SnRK2)
ssp.	Subspecie
SSRs	Simple Sequence Repeats
TE	Transposable Elements
TF	Transcription Factor
UPLC-MS	Ultra-high Pressure Liquid Chromatography-Mass Spectrometry
V. vinífera	*Vitis vinífera*
WGBS	Whole Genome Bisulfite Sequencing
WGS	Whole Genome Sequencing
WUE	Water Use Efficiency

General introduction

What is grapevine?

With a total surface near to 7.3 Mha (International Organisation of Vine and Wine Intergovernmental Organisation, 2022), the grapevine (*Vitis vinifera* L.) is one of the most extended crops in the world. The huge relevance of this crop into the society englobes different aspects, from economical level (generating an average of 40 billion euros in 2022 only in Europe) (International Organisation of Vine and Wine Intergovernmental Organisation, 2022), to the social habits, culture and the design of the landscape in wine regions (**Figure 1**). *V. vinifera* is widely cultivated for the production of grapes used in winemaking. The fruit contains sugars, acids, and other compounds that, when fermented, result in the creation of wine. The grapevine has been selectively bred over centuries, giving rise to numerous cultivars with different flavors, aromas, and characteristics. Apart from wine production, grapes from *Vitis vinifera* are also consumed fresh, dried as raisins, or used to make grape juice. The cultivation of grapevines has become a significant agricultural industry, with vineyards established in various parts of the world. Grapes are known for their versatility, not only as a source of beverages but also as a key ingredient in various culinary dishes. Additionally, the cultural and social aspects of winemaking have contributed to the grapevine's symbolic significance in many societies. The plant's ability to adapt to diverse climates has allowed it to become a global symbol of viticulture and winemaking traditions.

Figure 1: Landscape of a traditional wine region and the integration of vineyards in there. Image from website Ruta del Vino de La Mancha.

From a botanical perspective, the grapevine is a woody perennial liana that belongs to the Vitaceae family. More concretely, the representative genus corresponding to grapevine is the *Vitis* genus into the Vitaceae family. There are many species that correspond to the *Vitis* genus, such as *V. riparia* or *V. berlandieri*, but the most relevant is the *V. vinifera*. Morphologically, *V. vinifera* has distinctive lobed leaves with a long petiole and tends to form long and flexible branches that can climb and attach to structures or other plants thanks to the generation of tendrils (**Figure 2**). Principally there are apical meristems that can develop the principal branches, and lateral shoots coming from lateral meristems. Its greenish flowers develop into clusters of berries called grapes, which come in a variety of colors, including green, red, and purple, depending on the grape variety (in **Figure 2** the representative draw guides the description, where it appears the lobed leaves, the flexible branches, the tendrils to climb and other structures like the cluster flowers). The internode distance, in combination of other parameters like the shape of lobed leaves (see different examples of shapes in **Figure 3**) the presence of trichomes in the back of leaves or berry colors, stablish a characterization of the genotypes and stablish differences between cultivars, whose corresponding discipline is the Ampelography (Edward Hellman, 2007).

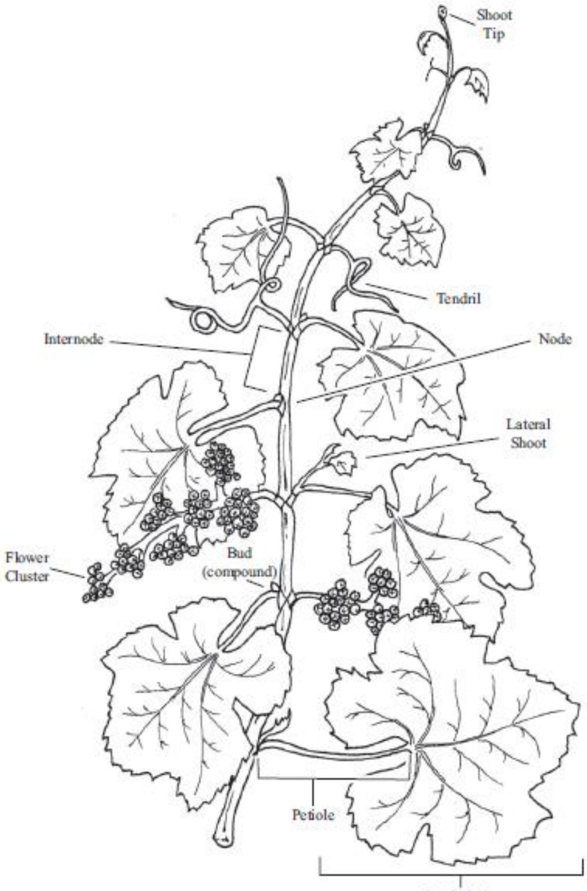

Figure 2: Description of grapevine parts containing the lobed leaves, the flower clusters, tendrils and internodes (available at (Edward Hellman, 2007))

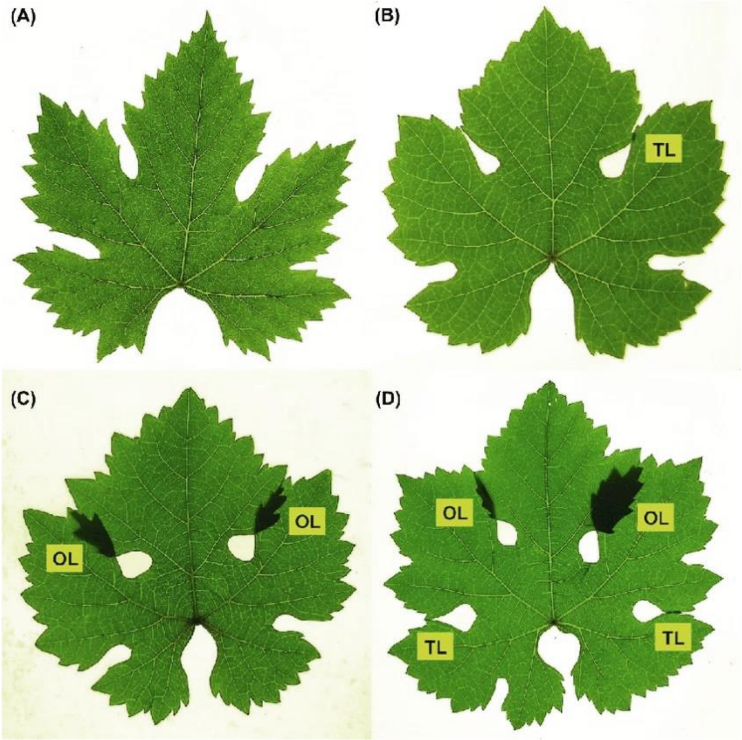

Figure 3: (A) Simple leaf. (B) Leaf with touching lobes [TL]. (C) Leaf with overlapping lobes [OL]. (D) Leaf with both touching and overlapping lobes [TL, OL]. Automatic threshold and contouring methods can be applied only to (A). Image extracted from Diaz, 2017

The annual growth cycle of the grapevine depends on the temperature and the time of light exposure, determining a dormancy period (from November to March in the Northern hemisphere) and a reproductive period (from March to October in the Northern hemisphere), wherein the grapevine start the generation of shoots, blooming and fruit ripening (Goldamer, 2018; Zhu et al., 2020). During this cycle, several changes are involved in the grape ripening, but the most important changes are the "veraison" and the maturity time (**Figure 4 Figure 5** and **Figure 6**). While the "veraison" stage in grapes are determined by the change of colours of the 50% of the grapes in a cluster, the maturity process is involved in the conversion of fruit acids like tartaric and malic acids in sugars, terpenoid and phenolic compounds and the aromatic profile of the berries.

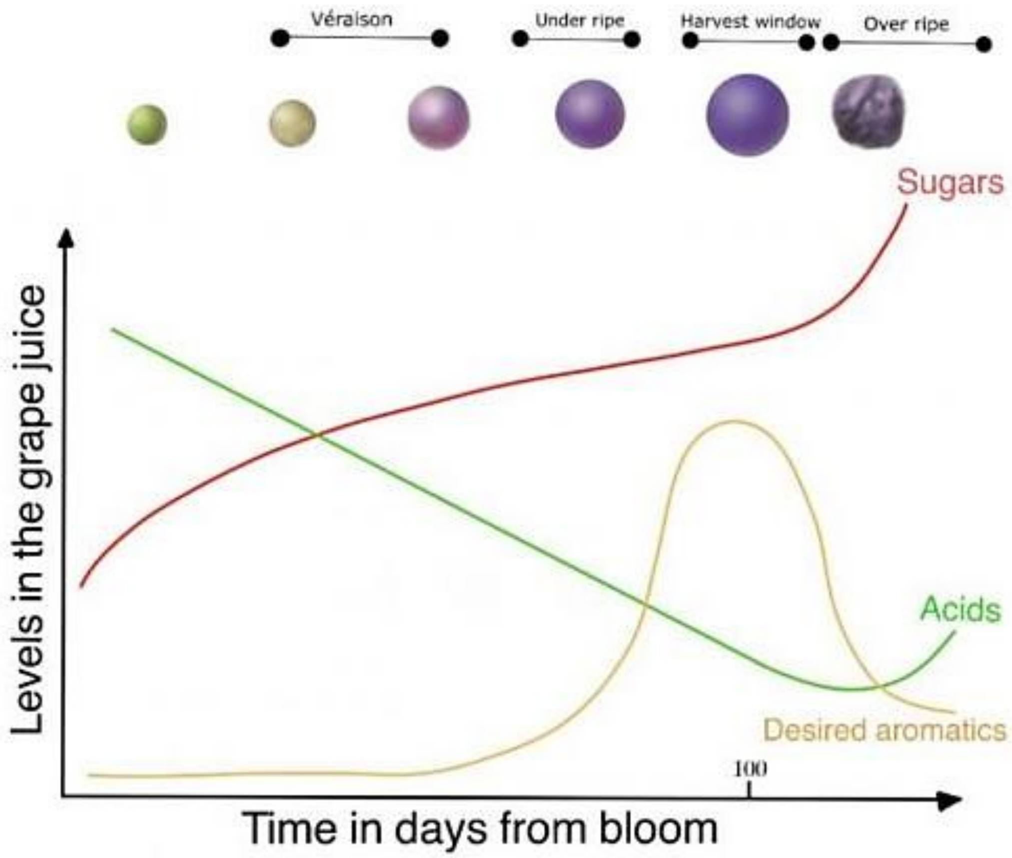

Figure 4: Development of sugars, acids, aromatics, and berry color and eight during ripening. In this graphic, the evolution of grape maturity is developed across the time, and it shows the decrease of grape acids such as malic or tartaric acid by the increase of sugars and the flavor production. Image from Sommelier business

Those events are related to the production of anthocyanidins and polyphenols in the grapes and stablish the possible harvest date to obtain the best quality of grapes to winemaking or table grapes (Goldamer, 2018; Zhu et al., 2020) (**Figure 4** and **Figure 6**). However, the timing grapes depends on the cultivar, and not all the cultivars can ripe grapes at the same time, affecting the harvest management (Goldamer, 2018; Zhu et al., 2020). The timing grapes is also influenced by the latitude of the vineyard, becoming more acid grapes in high latitudes than in low latitudes near to the Equator (Edward Hellman, 2007; Goldamer, 2018; Zhu et al., 2020). Nowadays, the different timing of each cultivar is also affected by the effects of climate change, producing a big impact on the grape quality by an early ripeness with a high quantity of sugar in berries, causing a decrease of the acidity in wines and an increase of alcohol produced after the fermentation process (Goldamer, 2018; Lucchetta et al., 2019; Rogiers et al., 2022; Zhu et al., 2020). Indeed, the planning of harvest is also affected by this effect in vineyard, bringing forward the harvest date to summer and risking the workers' health in vineyard (Goldamer, 2018).

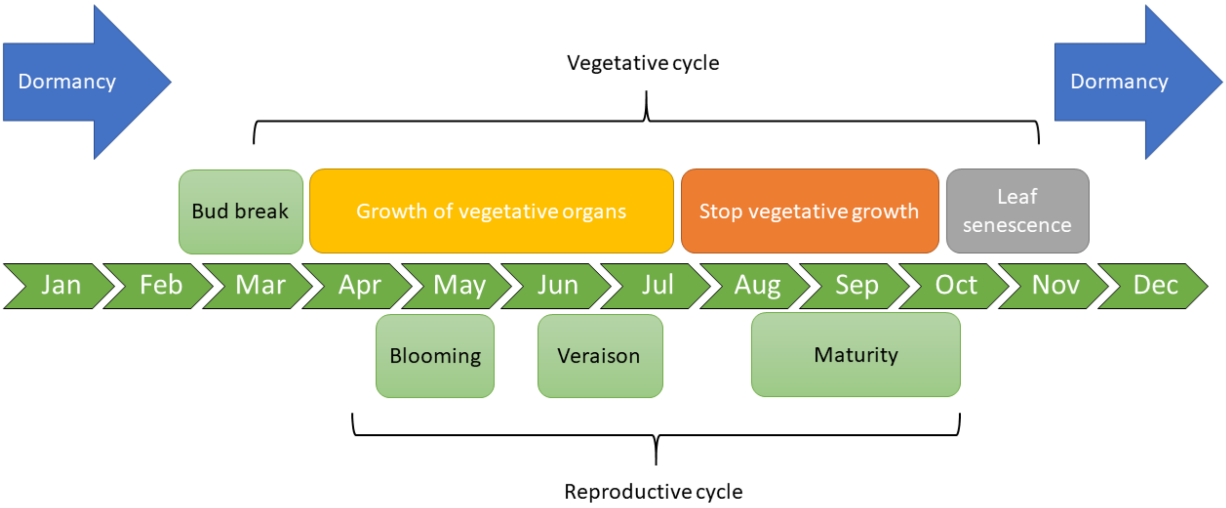

Figure 5: Life cycle of grapevine in a year. The diagram represents all the changes produced in a plant during the year, from brake bud to the leaf senescence and the dormancy period including all the reproductive changes.

Figure 6: Illustrative steps of the grapevine cycle: At left, the budbreak in grapevine. At the center of the image, the veraison stage. At right, the mature berries in a cluster meaning the end of the reproductive season and the start of the harvest. All the images are from stock.

Although the grapes are largely produced in the vineyards along the world, the sexual reproduction using grape seeds is unusual, and a few number of grains are fertile (Denham et al., 2020; Dong et al., 2023; McKey et al., 2010; Schön et al., 2009; Terral et al., 2010). As in many other cultivated perennial crops, the main reproduction system for grapevine in vineyard is the clonal propagation to ensure the genetic stability in the vineyard and the homogeneity of the grape production (Gaut et al., 2015; Schön et al., 2009). Usually, the common method for the vegetative reproduction in grapevine is the use of cuttings (**Figure 7 a)**), but there are other majority methods like layering (**Figure 7 b)**) and grafting (**Figure 7 c)**) that appears in the grapevine reproduction (Goldamer, 2018; Waite et al., 2015).

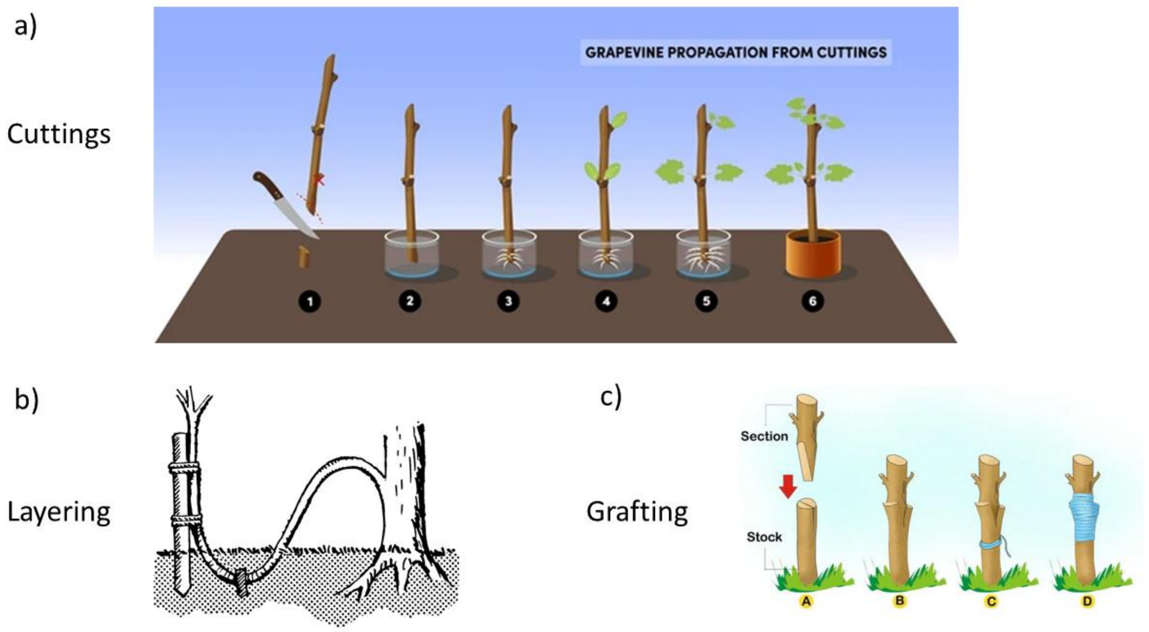

Figure 7: Different ways to vegetative propagate the grapevines: a) Clonal propagation by cuttings, where the dormant cuttings are submitted to root and then it becomes a new clone to cultivate; b) Clonal propagation by layering, where a shoot is covered by soil to root (in this case, there are covered at soil level, but in some cases the layering can be made using a plastic bag with soil in the aerial part), and then it produces a new individual; and c) Grafting, that consists in the use of a rootstock from other grapevine plant to graft the cultivar of interest using a dormant cutting with one or two buds. Images a) and c) from stock and b) from Tablas Creek Vineyard blog.

The vegetative reproduction traits in vineyard described below are followed by the genetic characteristics of the grapevine (Denham et al., 2020; Terral et al., 2010; Tesfamicael et al., 2020). At genetic level, the *Vitis* genus is a diploid plant whose genetic material is distributed in 19 chromosomes and the entire genome is mostly heterozygous (Badouin et al., 2020; Canaguier et al., 2017; Cochetel et al., 2021; Dong et al., 2023; Lodhi & Reisch, 1995; Minio, Cochetel, Massonnet, et al., 2022; The French–Italian Public Consortium for Grapevine Genome Characterization, 2007; Velt et al., 2023). Referring to the cultivated grapevine *V. vinifera*, it exists two principal subspecies classification depending on the sexual dimorphism of the plant, producing the wild grapevine (*V. vinifera* L. ssp. *sylvestris*, with a dioic biotype), and the cultivated grapevine (*V. vinifera* L. ssp. *vinifera*, with a monoic/female biotype) subspecies (see **Figure 8** in next section). More concretely, the taxonomic classification of both subspecies is defined in (Maul, 2003):

Subkingdom: Spermatophyta
  Superdivision: Angiospermae
    Class: Dicotyledonae
      Subclass: Rosidae
        Order: Rhamnales
          Family: Vitaceae
            Genus: *Vitis*
              Subgenus: *Euvitis*
                Specie: *Vitis vinifera*
                  Subspecie: *Vitis vinifera* ssp *vinifera*
                  Vitis vinifera ssp *sylvestris*

History of cultivated grapevines

Due to the significant role of the grapevine in society, the scientific community is dedicated to unraveling the evolutionary ties between grapevines and human societies, as well as exploring the origins of their societal use, a process known as domestication. Beyond the primary applications of this knowledge in enhancing grapevine plant breeding, delving into the origins of the present-day grapevine is crucial for comprehending the mechanisms of artificial selection and the underlying reasons behind it.

The domestication of *Vitis vinifera* L. is intricately linked to the evolution of societies throughout history, influenced by their dietary preferences and behaviors (Dong et al., 2023; McGovern, Fleming, et al., 1996; McGovern, Glusker, et al., 1996). Similar to many plants commonly used as human food sources, the grapevine underwent human selection to align with societal food preferences (Dong et al., 2023; McGovern, Fleming, et al., 1996; McGovern, Glusker, et al., 1996). Notably, the initiation of the domestication process in grapevines was closely linked to ensuring food safety and securing grape production for future years.

Certainly, there is compelling evidence supporting the historical significance of the grapevine in social culture. Traces of tartrate, a key compound in wine derived from tartaric acid present in grapes, have been discovered in containers dating back 6000 years to the Neolithic age. These findings underscore the importance of grapevines in ancient social practices (McGovern, Glusker, et al., 1996), and help date the origins of grapevine use in wine production to around this era (McGovern, Glusker, et al., 1996).

At morphological level, this domestication process acted on the flower shape of grapevine, started with the selective cultivation of female and hermaphrodite flowers (in order to ensure the grape production), leading to a significant phylogenetic separation between cultivated grapevines (*Vitis vinifera* ssp *sativa*) and wild grapevines (*Vitis vinifera* ssp *sylvestris*) (**Figure 8**) (Arroyo-García et al., 2002; Arroyo-Garcia et al., 2006; Arroyo-García et al., 2016; Dong et al., 2023). The breeding efforts initially focused on the selection of sexual characteristics, transitioning from dioecious individuals (*Vitis vinifera* ssp. *sylvestris*) to hermaphrodite flowers (*Vitis vinifera* ssp. *vinifera*). This trend persisted throughout history, with ongoing selection for other desirable traits (Arroyo-Garcia et al., 2006; Terral et al., 2010).

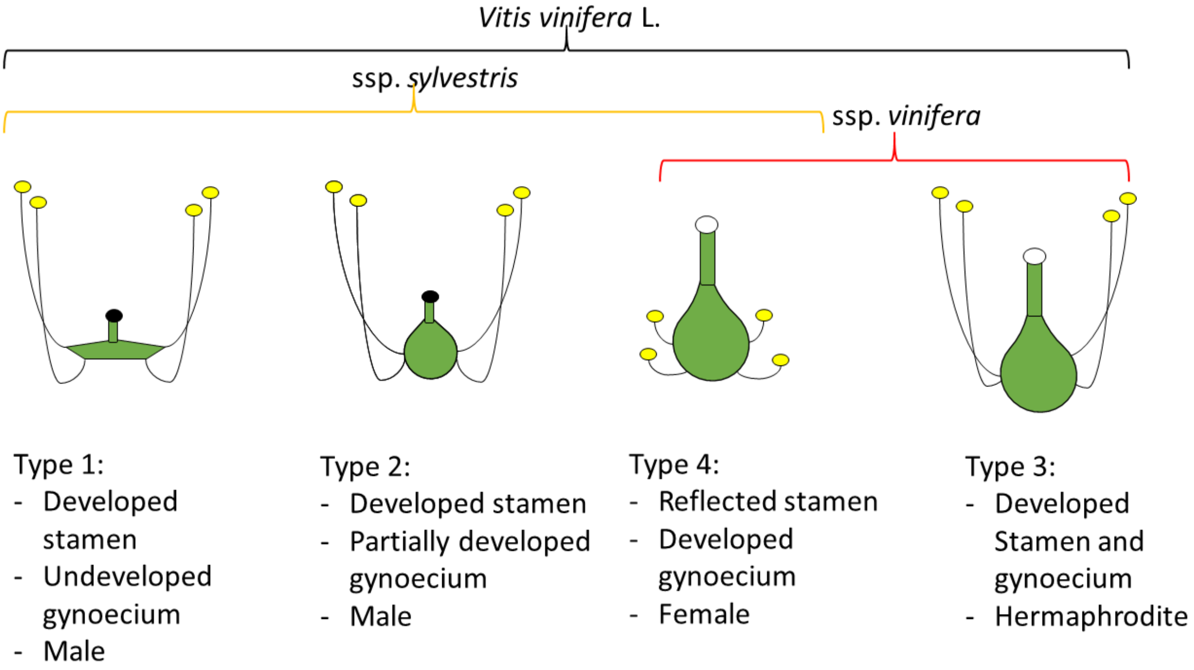

Figure 8: Type of flowers in *Vitis vinifera* L. Type 1 and 2 are commonly considered as males, with a non-development (Type 1) or partial development of the gynoecium (Type 2) and a correct development of the stamens, whereas the Type 4 is commonly considered as female because the low development of the stamens. The Type 3 is the hermaphrodite flower in cultivated grapevines because the development of stamens and gynoecium.

Furthermore, archaeological and morphological findings are corroborated by additional genetic and evolutionary studies, which not only confirm the early initiation of the domestication process in grapevines but also provide insights into its progression throughout human History (Arroyo-Garcia et al., 2006; Margaryan et al., 2021; McGovern, Fleming, et al., 1996; McGovern, Glusker, et al., 1996). The genetic evidences based on SSRs markers on genomic and chloroplast DNA (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2002, 2016; Magris et al., 2021; Margaryan et al., 2021) increased the knowledge about the grapevine phylogeny and the differentiation between the wild and cultivated grapevines during the domestication process in grapevine. Additionally, studies using wild and cultivated grapevines along all the Mediterranean and the Caucasian Area allowed the scientific community to stablish a preliminary date of the domestication (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2016; De Lorenzis et al., 2015; Margaryan et al., 2021). Those studies also confirmed the dependence of the selected traits in grapevine depending on the cultural uses of the grapes, unveiling that multiple origins of cultivated grapevines would be happened depending on the food preferences of the society (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2016).

Thanks to the advances in genomic research using Next Generation Sequencing techniques (NGS) in the last years and the previous genetic information from wild and cultivated grapevines, the research in grapevine genomics unveiled the importance of the domestication process in grapevine and the hypothetical origin of grapevine domestication (Dong et al., 2023; Gaut et al., 2015; Liang et al., 2019). The evidences revealed two different origins of domestication in grapevine, starting in the Caucasian and the Mediterranean area 11000 years ago, producing different genotypes of grapevine that contained specific grape profiles and characteristics, directly related to the food preferences of the societies that were located in these centres of domestication, separating among wine or table grapes (in **Figure 9**, it appears two different domestication events related to the use of berries in the society, defining an origin related to the use of grapes for winemaking (CG2) or for table grape (CG1)). From the whole characters of interest, characteristics like sugar content of berries, shape or colour defined the grapevine phenotype and caused the two domestication events (Dong et al., 2023).

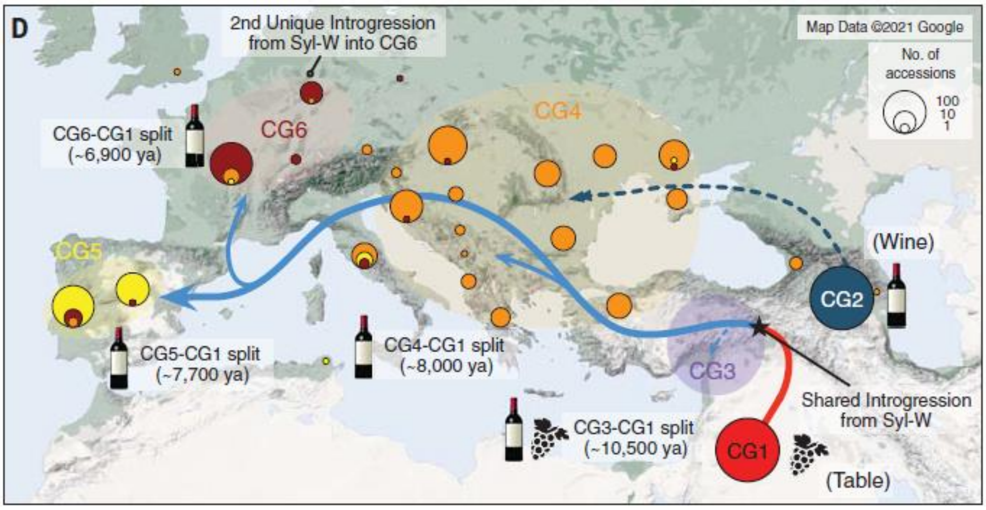

Figure 9: Origination of V. vinifera groups (CG3 to CG6) by the end of the Neolithic. Geographic distributions of CG groups are shown by colored circles. Original image from Dong et al., 2019.

Nonetheless, the human selection of grapevines showing desired traits of interest would affect negatively to other features, for example, in the alleviation of biotic and abiotic stresses (Bota et al., 2001, 2016; Carrasco et al., 2015, 2022; Cramer et al., 2007; Dal Santo et al., 2016a; Kar, 2011). Respect from the effects on abiotic stresses, the human selection would have an influence on the tolerance to some abiotic stresses in cultivated grapevines such as water deprivation or high salt content in soils, enhancing the domestication of grapevines with determinate tolerance to the environmental stresses than others with less tolerance along the History, depending on the climate of the region. Studies about drought (Bota et al., 2001, 2016; Dal Santo et al., 2016; Deloire et al., 2004) and salinity (Carrasco et al., 2022) revealed the fit of some genotypes under water stress conditions and high salt content in soils. For example, the cultivated grapevines whose origin is located in Mediterranean areas (with climate with low precipitations and high temperatures in summer) showed better tolerance to drought than other genotypes whose original place is more related to continental climates (with high water availability and template temperatures in summer) (Bota et al., 2001, 2016; Deloire et al., 2004), in terms of the control of stomatal conductance or the reduction of photosynthetic rate in leaves and organ size to avoid water losses in the plant. Regarding salt stress, some genotypes tolerant to high salt content in soils showed different strategies at transcriptomic and physiological levels in order to face the stress in the plant (Carrasco et al., 2022).

Apart from the abiotic stresses, the domestication process in *V. vinifera* ssp. *vinifera* could affects the evolution of some gene locus related to the resistance to diseases such as mildew (powdery mildew, produced by *Erysipa necator*; downy mildew, produced by *Uncinula necator*), that caused several damages in Europe in the XIXth century because of the pathogen importation from America (Amrine et al., 2015; Cochetel et al., 2021; Riaz et al., 2011, 2013). The evidences suggested to the scientific community that the resistance could disappear along the domestication process in Europe, enhancing the spreading out the parasite in other wild *Vitis* species like *Vitis rotundifolia* (*Muscadinia rotundifolia*) or *Vitis romanettii* in America and Asia (Cochetel et al., 2021; Riaz et al., 2013), and enhancing the selection of gene locus related to the resistance to mildew, like *REN1* and *RUN1* in the *Vitis* of these territories because of the coexistence between American and Asian grapevines and the pathogens (Amrine et al., 2015; Riaz et al., 2013). These evidences, based principally in gene expression data and SSRs markers, defined a possible introgression events detected between cultivated grapevines (*Vitis vinifera* ssp *sativa*) and wild grapevines (*Vitis vinifera* ssp. *sylvestris*), that could support the heterozygosity in origin and the possible loss of this character by human selection of grapevines (Amrine et al., 2015; Cochetel et al., 2021; Riaz et al., 2011, 2013, 2018, 2020).

The advances in the genetic research on grapevine

The richness of the diversity of cultivars in the world is very interesting to explore the genetic mechanisms involved in the grapevine plant breeding and how the domestication process acted on the genome modelling of grapevine. Therefore, the advances in the genetic analysis and technology associated to this changed the basis of the knowledge about the domestication process and improve the plant breeding. The progress of knowledge using genetic techniques like Amplified Fragments Length Polymorphism (AFLP) or Restriction Fragments Length Polymorphism (RFLP) improved the understanding on the phylogeny and the classification of plants depending on the length variability across polymorphic allelic sites (Cervera et al., 1998; Ergül et al., 2006; M. López et al., 2009). In this way, it is remarkable the use of chloroplast Single Sequence Repeats (SSRs) technique in grapevine (Vieira et al., 2016), a genetic technique based on the length and sequence measure of the product of amplification by Polymerase Chain Reaction (PCR) performed on hypervariable regions of chloroplastic DNA (**Figure 10**), allowed the scientific community to understand the mechanisms of domestication in grapevine (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2016; Riaz et al., 2013, 2018). One of the most relevant insights in the research about domestication of grapevine, from the SSRs techniques, came from Arroyo-Garcia et al., 2006; Arroyo-García et al., 2002, that the use of this tool revealed two possible origins of domestication in grapevine, by genotyping cultivated and wild grapevines using chloroplast DNA hypervariable regions. Moreover, SSRs analysis was very useful to improve the knowledge about the genotypic differences between wild and cultivated grapevines in Riaz et al., 2018 or Margaryan et al., 2021 on the whole Mediterranean and Asian grapevine germplasm, studying how the domestication process designed the Mediterranean grapevine genome and exploring along the germplasm bank which genotypes were more susceptible to contain possible resistances to diseases or tolerance to drought or salinity stress (Arroyo-García et al., 2016; Riaz et al., 2013, 2020).

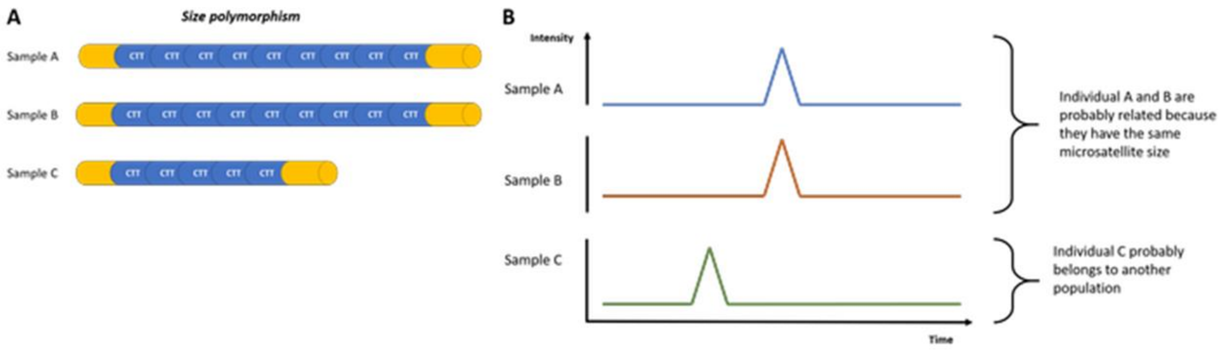

Figure 10: Structure of SSRs studies and basis: Figure A: Representation of the different types of microsatellite polymorphism; and Figure B: Analysis of size polymorphism by capillary electrophoresis. Once amplified by PCR using a labelled primer, the microsatellites of the 3 individuals A, B and C are separated by capillary electrophoresis and their electrophoresis profiles are compared. Image partially modified from Biomnigene website.

Apart from SSRs, the advances in grapevine research was greatly impacted by the sequencing of the first reference genome of grapevine in 2007 (The French–Italian Public Consortium for Grapevine Genome Characterization, 2007) using the homozygous genotype Pinot Noir (PN) 40024 and sequencing by Primary Generation of Sequencing (Sanger) methods, shedding light to the study of genomic differences on grapevine (identifying genomics as the study of all the complete DNA sequences) (**Figure 11**). The production of this reference enabled the use of this genome for the exploration of Single Nucleotide Polymorphism (SNPs) markers along the whole grapevine genome, identifying variants across the individuals in determinate regions (De Lorenzis et al., 2015; Marrano et al., 2017; Riaz et al., 2018).

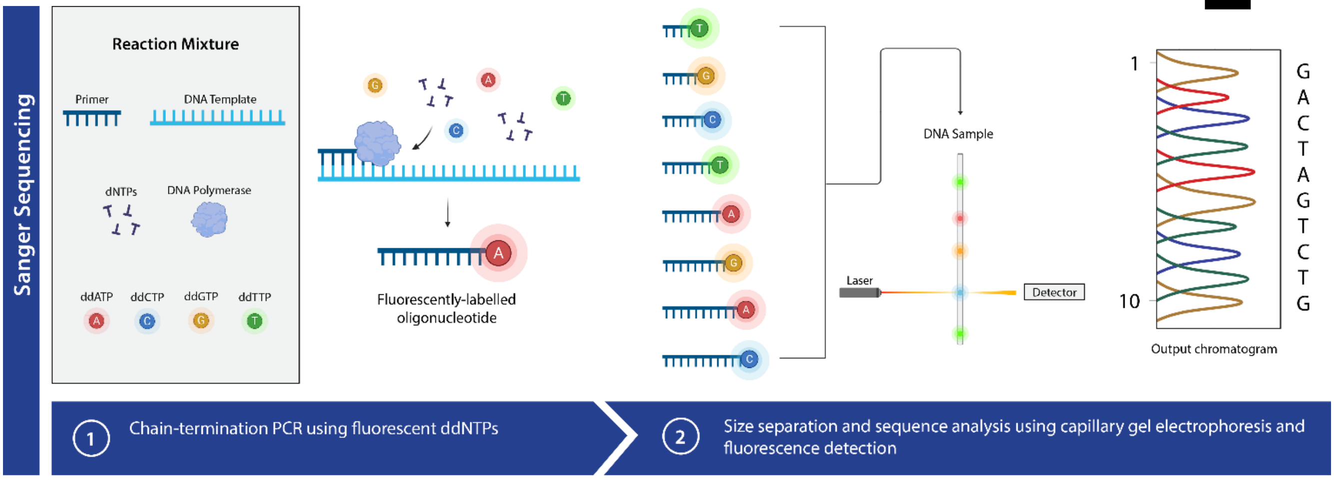

Figure 11: Workflow of Sanger Sequencing method (first generation): 1) PCR chain termination with fluorescent dideoxynucleotides, 2) capillary electrophoresis with detection of fluorescence emission to determine the DNA sequence. Image from AAT Bioquest.

Background information

The first DNA sequencing occurred in 1977, with Maxam-Gilbert and Sanger-Coulson developing a method based on chain termination using dideoxynucleotides to halt DNA synthesis during a PCR reaction. In this method, the dideoxynucleotides were initially labeled with radioactivity, and four distinct reactions were conducted based on the specific dideoxynucleotide (A, T, C, G). Over time, the use of radioactivity was replaced by employing various fluorescent labels for dideoxynucleotides, allowing the combination of all nucleotides in a single reaction. Presently, the Sanger method remains highly accurate, forming the basis for subsequent sequencing methodologies.

The study of somatic variants across a large region of the genome by the study of the SNPs improved the detection of possible mutations that were very difficult to detect using SSRs markers because their specificity of only small repeated regions (Malenica et al., 2011; Vieira et al., 2016).

Following the utilization of SSRs, the knowledge landscape in grapevine research was expanded through the integration of Next Generation Sequencing (NGS) techniques, such as Illumina. The impact of NGS on grapevine research is evident in its influence on understanding different gene expressions under varying conditions (Carrasco et al., 2022; Dal Santo et al., 2016), unraveling gene constitutions and alternative splicing in specific genes of interest (Ding et al., 2020) and exploring the evolution of cultivated grapevines throughout history (De Lorenzis et al., 2015; Dong et al., 2023; Liang et al., 2019; Marrano et al., 2017; Riaz et al., 2018). Techniques like Whole Genome Sequencing (WGS) or RNA-sequencing (RNA-seq) are increasing the knowledge about further research lines in grapevine (Carrasco et al., 2022; Cochetel et al., 2017; Dal Santo et al., 2016). For example, recently, the WGS using second generation of sequencing techniques like Illumina technology (characterized by massive sequencing of small fragments (Sequencing By Synthesis or SBS, schema in **Figure 12**)) allowed the researchers to define the dual domestication of the grapevines by SNPs detection (Dong et al., 2023) from 3525 sequenced genotypes for cultivated and wild grapevines identified across the world. Furthermore, the use of this methodology in gene expression studies increases the richness of the analysis and improve the study of transcriptomic responses to abiotic and biotic stresses in grapevine (Askri et al., 2012; Carrasco et al., 2022; Cochetel et al., 2017; Dal Santo et al., 2016; Gautier, Cochetel, et al., 2020). Beyond gene expression, NGS technology finds additional applications, such as exploring microbial communities around grapevines or modifying DNA strands for the detection of epigenetic modifications in grapevines, including the identification of methylated cytosines (Ahmed et al., 2018; Belda et al., 2017; Darriaut et al., 2022; Fortes & Gallusci, 2017; Gawehns et al., 2022; Hily et al., 2018, 2018; Salvetti et al., 2016; van Gurp et al., 2016).

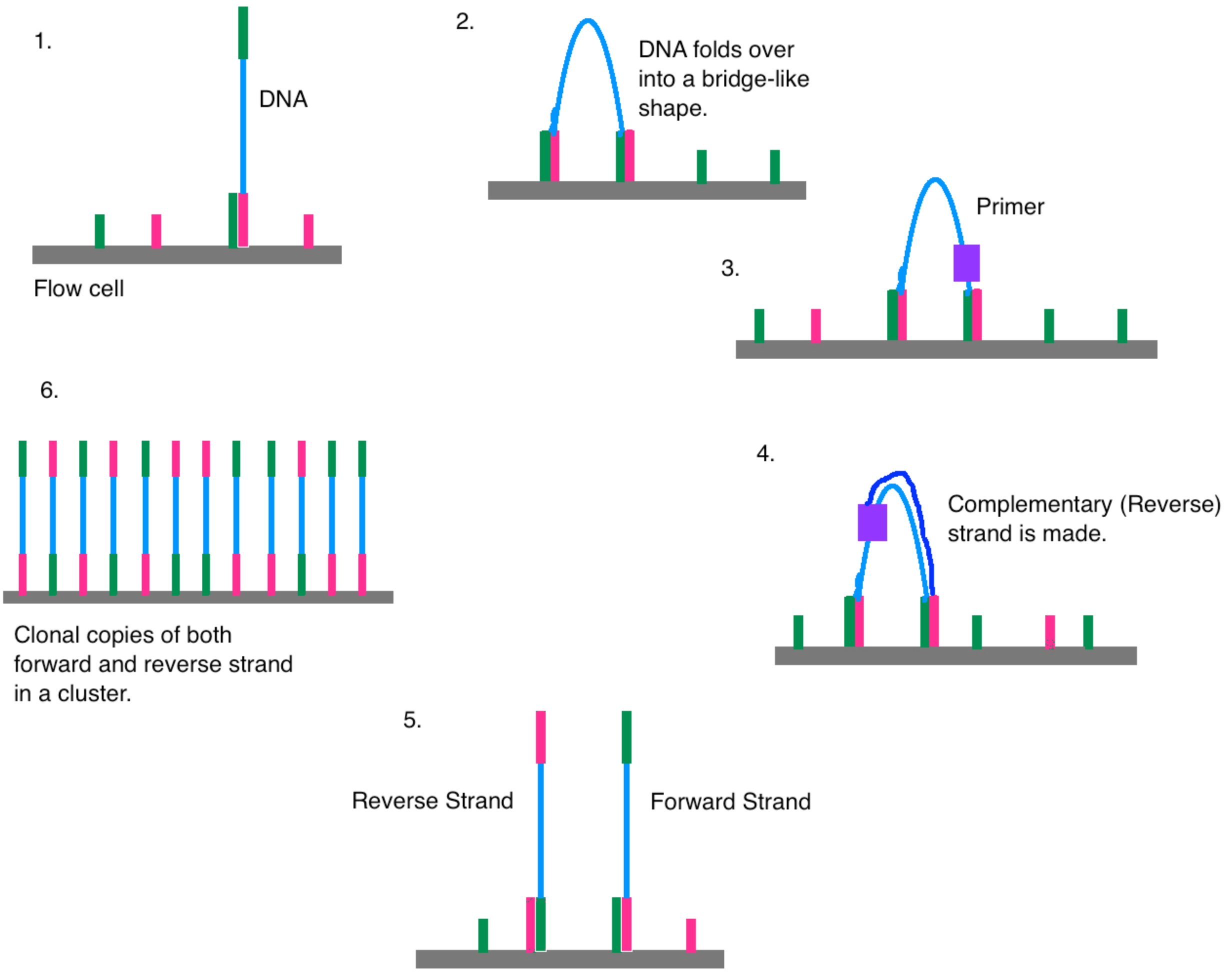

Figure 12: Next-Generation Sequencing Chemistry Overview – Illumina NGS: (1) Forward fragment hybridization using complementary index barcodes, (2) A bridge is made by the hybridization of the reverse index barcodes, (3) PCR chain reaction with fluorescent nucleotides, (4) synthesis of complementary strand, (5) division of forward and reverse strand, and 6) analysis of clonal copies of the fragment for forward and reverse strands. Image from Wikipedia.

Did you know…?

Sequencing by Synthesis (SBS) method developed by Illumina is based on the Sanger methodology (first sequencing generation). The advances in the sequencing methodology are: 1) the addition of known index to identify the sample using barcodes into the sequence to analyze and 2) the hybridization of those samples with the barcode to a complementary matrix to realize the PCR with fluorophores. The subsequent massive reading of fluorescent dideoxynucleotides produces data that can be analyzed to determine the DNA sequence of the fragment. While the accuracy of individual measures may be lower than in the first generation of Sanger technology, the high-throughput nature of massive sequencing and the repetition of fragments (referred to as depth) compensate for this limitation. This depth of sequencing enhances the ability to establish the actual DNA sequence with high accuracy.

In the current landscape of sequencing methods, the application of technologies stemming from the third generation of sequencing has significantly enhanced genomic research in grapevines. Sequencing methodologies based on Single Molecule Real Time (SMRT) techniques, such as Nanopore or PacBio, have proven particularly impactful. These methods offer improved resolution for terminal sequences, including regions like telomeres (Cuber et al., 2023; Rayamajhi et al., 2022). This advancement enables a more detailed and accurate examination of the grapevine genome, contributing to a deeper understanding of its structure and function (Cuber et al., 2023; Rayamajhi et al., 2022).

The most sequencing method utilized in grapevine is PacBio (characterized by the sequencing of long reads in real time), which generated several outcomes in the grapevine genomic research by the generation of genome drafts for other grapevine genomes and the improvement of the initial reference genome of grapevine (PN 40024) (Canaguier et al., 2017; Cochetel et al., 2021; Massonnet et al., 2020; Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022; Minio et al., 2019; Velt et al., 2023).

The PacBio Hi-Fi sequencing technology allowed the grapevine community to remark the relevance of the heterozygosity of the grapevine, unveiling the unknown heterozygosity of the reference genome PN, and stablishing a new paradigm in the genomic research in grapevine (Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022; Velt et al., 2023). The long reads characterizing this SMRT sequencing used by PacBio enhance the gap reduction in genome assemblies (**Figure 13**) and increases the accuracy of the draft model, and stablishing possible heterozygous regions to create haplotypes (Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022; Minio et al., 2019).

Did you know...?

PacBio and Nanopore sequencing technologies are revolutionizing sequencing practices. PacBio's approach relies on fluorophores to monitor fragment sequences (depicted in **Figure 13**), while Nanopore Sequencing Technology detects specific voltage changes caused by the passage of DNA nucleotide strands through a pore. The distinctions between these methodologies are associated with accuracy, with PacBio proving more accurate than Nanopore in long reads, particularly in complex regions like Long Terminal Repeats (LTRs). This accuracy is crucial for a more in-depth exploration of grapevine evolution through the analysis of heterochromatin changes.

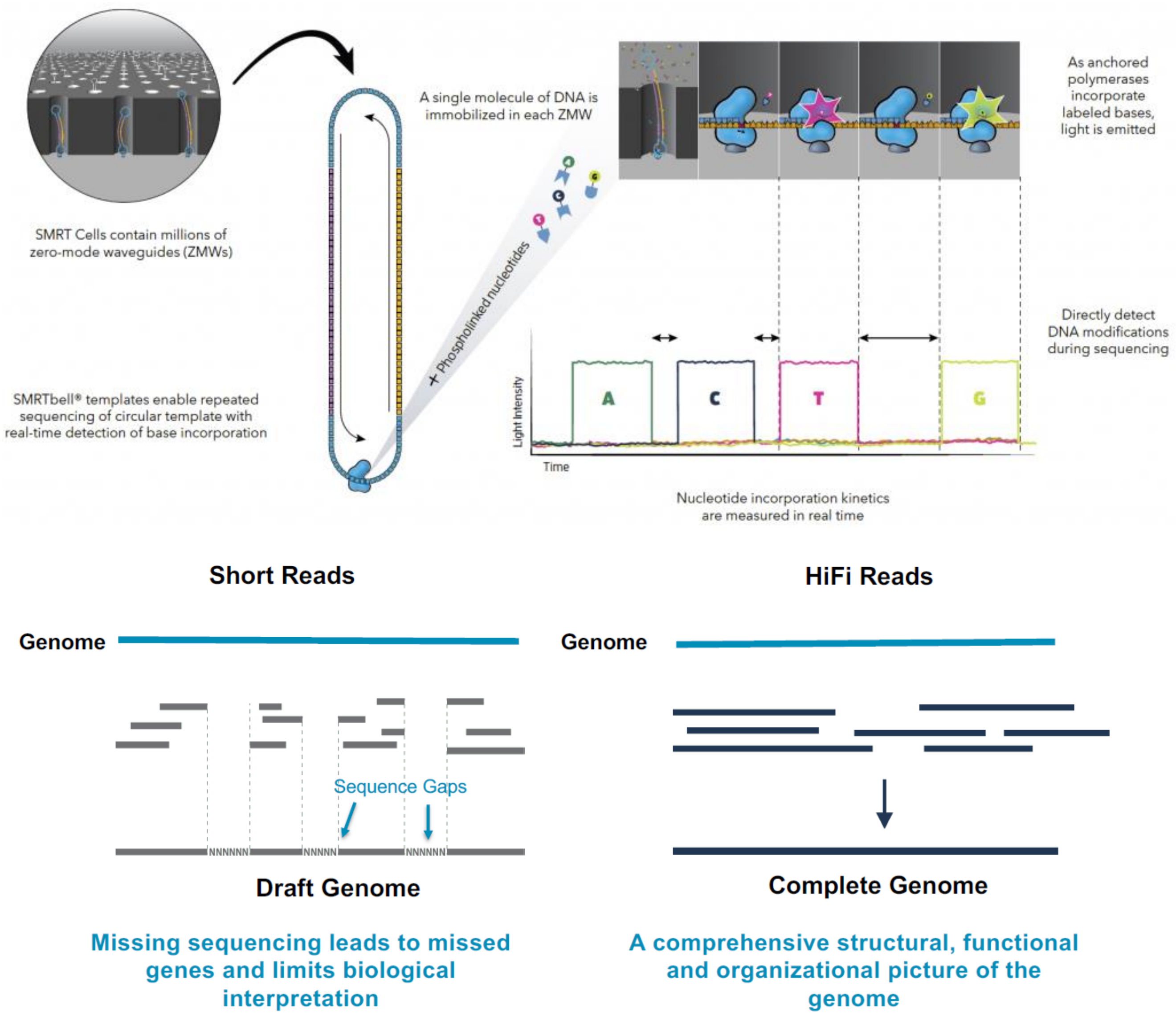

Figure 13: At the top, diagram for SMRT PacBio Sequencing and at the bottom, the comparison between the use of Short Reads coming from Illumina Sequencing with the use of PacBio reads from SMRT sequencing. At the top, the figure describes the PacBio Sequencing method, based on the use of a single molecule of DNA immobilized in a hole which contains a polymerase enzyme. In the DNA replication, PacBio technology measures the incorporation of fluorescent nucleotides, each one with a different fluorophore depending on the nitrogen basis, building an accurate sequence measured in real time. At the bottom, it appears a comparison with short read sequencing methods like illumine to build a draft genome, in comparison with the use of long reads coming from PacBio. Figure from PacBio Biosciences Introduction.

The creation of novel genome drafts for widely cultivated varieties is offering a fresh perspective beyond relying solely on the reference genome for genomic experiments (Cantu & Walker, 2019; Dong et al., 2023; Girollet et al., 2019; Massonnet et al., 2020; Minio, Cochetel, Vondras, et al., 2022). Generating genomic drafts using third-generation methodologies like PacBio, along with complementary sequencing approaches from the second generation such as Illumina, can unveil potential gene modifications, unearth new gene models not present in the reference genome or detect several methylated regions that are presents or absents in each cultivar (Azevedo et al., 2022; Badouin et al., 2020; Cochetel et al., 2017; Fortes & Gallusci, 2017; Gawehns et al., 2022; Girollet et al., 2019; Minio et al., 2019; Trucchi et al., 2016; van Gurp et al., 2016; Velt et al., 2023).

Influence of the epigenetic modifications in grapevine

What is epigenetics? Identification and quantification of DNA methylation

Epigenetics is a fascinating field within molecular biology that explores heritable changes in gene expression that do not involve alterations to the underlying DNA sequence. It goes through the dynamic and intricate regulatory mechanisms that influence how genes are activated or silenced, impacting cellular functions and development. Epigenetic modifications, such as DNA methylation and histone modifications, serve as crucial molecular tags that orchestrate gene activity, playing a pivotal role in processes ranging from embryonic development to responses to environmental cues (Guarino et al., 2015; Y. Li, 2021; Raj et al., 2011; Trucchi et al., 2016). Unraveling the complexities of epigenetics provides valuable insights into the interplay between genetics and the environment, shedding light on the mechanisms that shape an organism's phenotype and contribute to diverse biological phenomena, including diseases and cellular differentiation (Guarino et al., 2015; Raj et al., 2011).

Epigenetics involves different methodologies to understand the plasticity of the phenotype, but the most relevant techniques at molecular level are the DNA-protein interactions and the methylation of DNA (Bräutigam & Cronk, 2018; Dey et al., 2012; Rauluseviciute et al., 2019). The study of DNA-protein interactions englobes several methodologies based on the nature of the protein and the DNA sequence. Techniques like EMSA (Electrophoretic Mobility Shift Assay) – Western Blot are often used in laboratory to study the interaction of some protein of interest and DNA sequences (Dey et al., 2012). In EMSA assays, the different mobility of the proteins in a denaturing gel electrophoresis determines the DNA-protein bindings (Dey et al., 2012). However, due to the possible artifacts and the DNA-protein binding sites that can block the union site with the specific antibody of the targeted protein, these techniques are not as well accurate as the new developed techniques based on ChIP (Chromatin Immunoprecipitation), such as ChIP-seq (Dey et al., 2012). This technique allows a real-time DNA-protein binding by a principal immunoprecipitation followed by a sequencing of the binding DNA fragments using NGS techniques like Illumina (Dey et al., 2012). The study of the interaction between the chromatin and DNA is very interesting and it implies several modulations related to regulatory proteins like TF (Transcription Factors) that could affects the individual to be more resilient and generate different environmental cues (Cantu & Walker, 2019; Guarino et al., 2015; Raj et al., 2011; Sims et al., 2003). In spite of the quantity of information, there is a small image of the epigenetics landscape, and the cytosine methylation in the DNA sequences could show more resolution in epigenetic studies.

In epigenetics, different modifications in DNA can be presents in order to enhance the plasticity in the organisms. One of the most relevant modification is the methylation of cytosines, gain relevance in the modulation of the gene expression by silencing or activating the transcription, but also it has an important role for silencing SNPs or transposable elements in the organisms (Bräutigam & Cronk, 2018; Y. Li, 2021; Muyle et al., 2022). In plants, three different contexts of methylated cytosines are presents: CG, CHG and CHH (where H= A, T, C) (Muyle et al., 2022). Depending on the targeted position of the methylated cytosines and the context, the methylated cytosines can produce different effects. For example, the cytosine methylation around the gene-body can silence a gene or maybe enhance the alternative splicing. The consequences are directly related to the environmental conditions, enhancing the plant resilience (Bräutigam & Cronk, 2018; Muyle et al., 2022; Vondras et al., 2019). However, the cytosine methylation in non-coding regions could be related to silencing possible mutations related to transposable elements or virus infections and their integration into the plant genome (Baduel & Colot, 2021; Carrier et al., 2012; Ramakrishnan et al., 2021). Apart from the position of the methylated cytosines, different functions are related to the type of cytosine methylation context (Bräutigam & Cronk, 2018; Muyle et al., 2022). While CHG and CHH contexts are linked to a reduction of gene expression or alternate splicing by the methylation of introns located in the Gene Body Methylation (GbM), CG is more related to the methylation of promoters and exons in the GbM to silence genes (Muyle et al., 2022). Besides the different effects on position and context in the genome, those methylated cytosines can be transmitted to the offspring, creating a epigenetic fingerprint by epigenetic marks into the genome (Guarino et al., 2015; Muyle et al., 2022; Raj et al., 2011; Schmitz & Ecker, 2012).

In order to identify and quantify the methylation of DNA, there are different strategies depending on the nature of the experiment that can be used for, dividing the diversity of procedures in non-NGS and NGS based approaches (Rauluseviciute et al., 2019). Apart from the immunoassays to detect the global paradigm of methylated cytosines in the DNA, thanks to the NGS techniques the development of new techniques increased the resolution of the epigenetic research (Rauluseviciute et al., 2019). The use of sensitive restriction enzymes to methylation (when a cytosine is methylated, the enzyme do not realize the restriction on the targeted sequence) is a common procedure to obtain information about methylated cytosines depending on the sequence of the targeted restriction enzyme (Rauluseviciute et al., 2019). The sequencing of the resulted fragments is compared with the known sequences and the non-methylated cytosines are detected by length exclusion (Rauluseviciute et al., 2019). Other strategy is based on the immunoprecipitation of fragments with methylated cytosines using methylcytosine-specific antibodies and a posterior sequencing of the selected fragments (Rauluseviciute et al., 2019).

However, the most effective method to study the DNA methylation is the DNA bisulfite conversion and sequencing, where the non-methylated cytosines are susceptible to be attacked by the conversion agent (generally sodium bisulfite) and converted to uracil by deamination (**Figure 14**), leaving the methylated cytosines intact (Gawehns et al., 2022; Rauluseviciute et al., 2019; van Gurp et al., 2016). After the conversion, the uracil modified nucleotides can be replicated as thymine by PCR amplification, and the sequence can be compared with the untreated sequences or the reference genome to identify the positions of the methylated cytosines (Gawehns et al., 2022; Rauluseviciute et al., 2019; van Gurp et al., 2016).

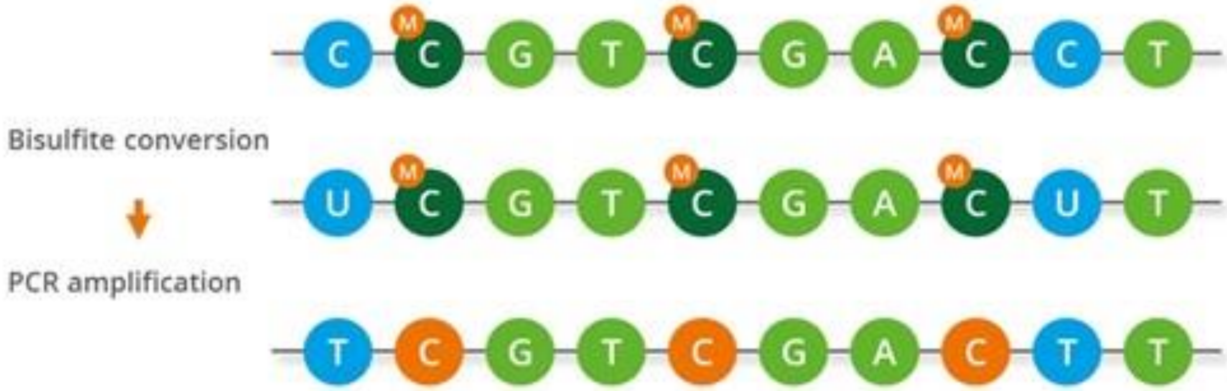

Figure 14: Bisulfite conversion of DNA sequence with methylated cytosines (in dark green) and non-methylated cytosines (in blue). After a PCR amplification, the non-methylated cytosines are converted to timines, while the methylated cytosines stay as cytosines. Figure provided by Eurofins.

The use of the bisulfite sequencing is a good tool to know the positions of the methylated cytosines comparing the methylated positions with the reference genome. Nonetheless, it is difficult to use in large genomes like the plant genomes (>100 Mbp). Limitations like the large genomes or the low resolution of the reference genome of some species can affect seriously the universalization of the technique and the application in experiments based on plant epigenomic studies (Gawehns et al., 2022; van Gurp et al., 2016). The use of the Whole Genome Bisulfite Sequencing (WGBS) is very useful when the coverage of the sequencing is large enough, for example in small-medium genomes (Trucchi et al., 2016). For large genomes, like the grapevine genome (~500 Mbp), some methodologies can help to supply these bias by a reduced representation of the sequenced genome

(Gawehns et al., 2022; van Gurp et al., 2016; Velt et al., 2023). The Reduced Representation Bisulfite Sequencing (RRBS) is based on the sequencing of precise fragments located on the genome that are restricted by specific restriction enzymes whose restriction produces a large number of theoretical fragments in a desired range of fragment size (Gawehns et al., 2022; van Gurp et al., 2016) (**Figure 15**). The resulted fragments can be easily compared between different populations in order to identify methylations in similar regions, with or without a reference genome, becoming very interesting to explore possible changes in methylation across different grapevine populations (Gawehns et al., 2022; Trucchi et al., 2016; van Gurp et al., 2016).

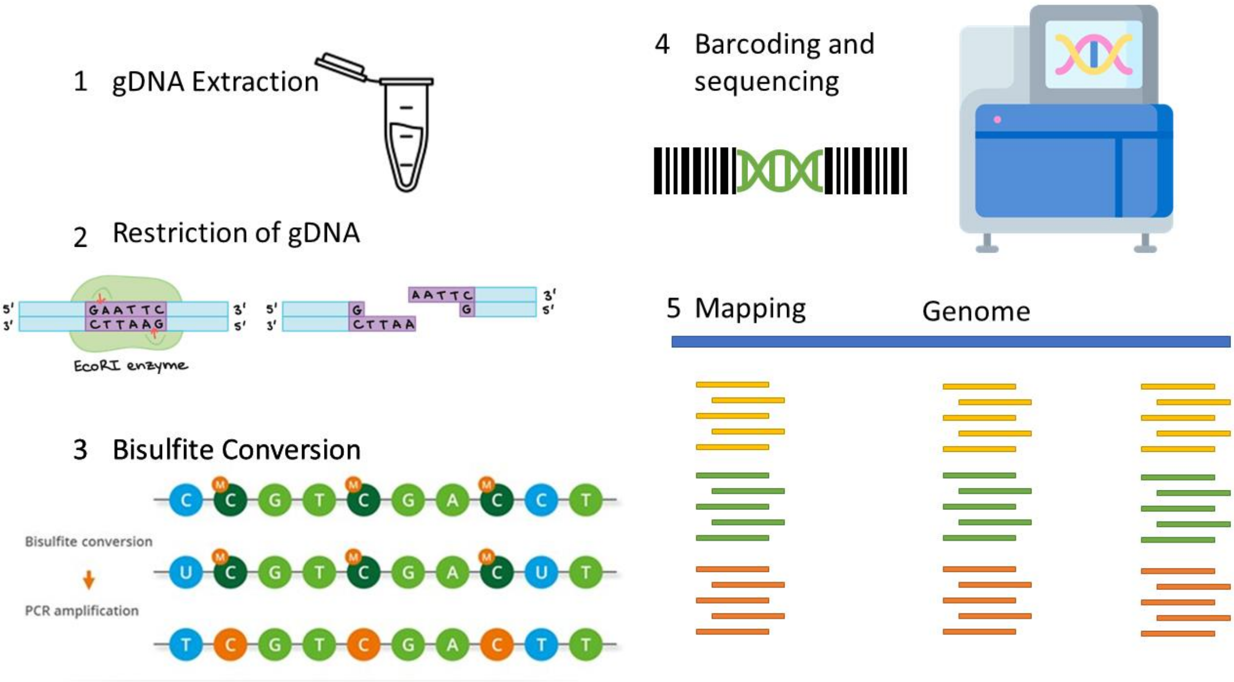

Figure 15: Workflow for Reduced Representation Bisulfite Sequencing (RRBS): 1: Extraction of genomic DNA (gDNA) from tissue or cell sample; 2: Restriction of gDNA using a specific Restriction Enzyme like EcoRI; 3: Bisulfite conversion of non-methylated cytosines; 4: Barcoding and sequencing of fragments; 5: Mapping using a reference genome or a de novo assembly from non-treated sequences to compare the methylation with other samples (in yellow, green and red). Images from stock and Eurofins.

Influence of the epigenetics in the domestication of grapevine

Coming from the importance of the epigenetic changes described below, the epigenomics represents a frontier in genomics that investigates the comprehensive and dynamic landscape of epigenetic modifications across the entire genome. In fact, there is a genomic discipline that research the whole epigenetic modifications in the genome, in order to define an epigenetic profile under a determinate situation. It involves the systematic study of modifications such as DNA methylation, histone modifications, and non-coding RNAs, providing a holistic understanding of how these intricate epigenetic patterns orchestrate gene regulation (Fortes & Gallusci, 2017; Guarino et al., 2015; Schmitz & Ecker, 2012). By mapping these epigenetic marks on a genome-wide scale, epigenomics offers insights into the regulation of cellular processes, developmental trajectories, and responses to external stimuli (Fortes & Gallusci, 2017; Guarino et al., 2015; Muyle et al., 2022; Schmitz & Ecker, 2012). This field plays a crucial role in unraveling the complexity of epigenetic control, contributing to our comprehension of various biological phenomena, such as the influence of reproductive traits into the grapevine genome and evolution (Tesfamicael et al., 2020; Q. Wang et al., 2021; Xu et al., 2020).

Reproductive traits play a significant role in the epigenomics of grapevines. Throughout grapevine domestication, certain plant characteristics underwent modifications from their wild progenitor, while others became diluted. This phenomenon is referred to as the "domestication syndrome," encompassing a distinct set of phenotypic traits associated with genetic changes as an organism transitions from a wild progenitor form to a domesticated one (Bouby et al., 2013; Smith, 2014). In the context of grapevines, the various modes of reproduction can impact the preservation of desired traits in the vineyard (Denham et al., 2020; McKey et al., 2010). Despite the existence of sexual reproduction in grapevines, occasionally utilized for propagating cultivars or developing new varieties through plant breeding, clonal propagation techniques dominate the way of reproduction in vineyard. These techniques ensure the preservation of desirable traits in the cultivar, as the offspring is genetically identical to the parental plant. This stands in contrast to sexual propagation, where some of the sought-after characteristics may disappear or dilute through the recombination process (Denham et al., 2020; McKey et al., 2010; Schön et al., 2009).

In spite of the advantages of vegetative propagation in grapevine, the use of vegetative clones increases the cumulation of several mutations through the inheritance (Denham et al., 2020; McKey et al., 2010). These mutations are closely linked to DNA methylation in the affected regions, underscoring the significance of epigenetics in the regulation of gene expression. Epigenetic mechanisms, particularly DNA methylation, play a pivotal role in silencing genomic regions affected by these mutations (Consuegra & Rodríguez López, 2016; Denham et al., 2020; Fortes & Gallusci, 2017; McKey et al., 2010; Schön et al., 2009). These mutations are reported to affect other characters not interesting for the domestication process, like the potential infertility of the seeds in this plant (Denham et al., 2020; McKey et al., 2010; Schön et al., 2009). The infertility of the seeds is directly caused by the domestication syndrome in vegetative propagated crops, that causes changes in plants at reproductive level, like the reduction of fertility in seeds, the self-fertilization of the flowers or the flower asynchrony (Barrett, 2015; Denham et al., 2020; McKey et al., 2010), compared with other related-traits in plants commonly propagated with sexual reproduction, with synchronic flowers, fertile seeds and pollinator-dependent fecundation. Therefore, the vegetatively propagated plants show a “sexual depression” of further genes related to the flowering and gamete formation, enhancing the development of the vegetative structure (Barrett, 2015; Denham et al., 2020; Massonnet et al., 2020; McKey et al., 2010; Schön et al., 2009). For example, in enset (*Ensete ventricosum* (Welw.) Cheesman), a type of banana tree from Ethiopia that is vegetatively propagated, the accumulation of mutations in genes related to flowering could contribute to the domestication process, enhancing the vegetative growth in detriment of the sexual reproduction (Tesfamicael et al., 2020). In grapevine, this effect is also identified in the flowering formation directly linked to the domestication process in grapevine, affecting genes related to the flowering and sexual dimorphism in wild grapes that could originate the cultivated ones (Dong et al., 2023; Massonnet et al., 2020), originating a male partially fertile (male type 2 from **Figure 8**). This effect becomes interesting because the principal characteristic to make a classification in wild grapevines (*Vitis vinifera* L. ssp. *sylvestris*) and the cultivated grapevines (*Vitis vinifera* L. ssp. *vinifera*) is based on the morphology of the flowers and their sex (Arroyo-García et al., 2002; Arroyo-Garcia et al., 2006; Arroyo-García et al., 2016; Dong et al., 2023; Massonnet et al., 2020; McGovern, Fleming, et al., 1996; Riaz et al., 2018).

Apart from the direct effects on the sexual reproduction in the plants, there are other characters accompanying the domestication syndrome that are also related to the loss of versatility in environmental adaptation to different biotic and abiotic stresses (Carrasco et al., 2022; Denham et al., 2020; Jiang et al., 2017; Tan et al., 2023). The epigenetic regulation can be also altered by the cumulus of methylation in regions with a high frequency of mutations that contains genes related to the adaptation to biotic or abiotic stresses (Barrett, 2015; Denham et al., 2020; McKey et al., 2010; Tan et al., 2023; Tesfamicael et al., 2020). Although the studies like Guarino et al., 2015 indicated the powerful tool of the epigenetics in the improvement of plants like *Populus alba* L. in response to drought stress, this resilience becomes restricted when the environmental conditions change. Further studies like Guarino et al., 2015; Raj et al., 2011; Shuai et al., 2013 unveiled the importance of the epigenetic memory in the environmental adaptation to abiotic stresses like drought in different crops like maize (Begum et al., 2019; Q. Wang et al., 2021; Xu et al., 2020). Other genetic actors that interacts in the shape of the grapevine and the epigenome modelling is the transposable elements (TE) like the Long Terminal Repeats (LTR), where there are associated with a high methylation rate in order to silence the affected region where it is inserted (Baduel & Colot, 2021; Carrier et al., 2012). For example, the presence of LTR in grapevine domestication affected the color in grapes in the cultivar Tempranillo Tinto, generating the white grapes in Tempranillo Blanco (Carcamo et al., 2010).

Epigenetic memory in plants

Epigenetic memory in plants refers to the ability of plants to retain and transmit epigenetic information across generations without changes in the DNA sequence. This phenomenon is crucial for adapting to changing environmental conditions and allows plants to retain molecular memories of previous experiences. Epigenetic mechanisms such as DNA methylation, histone modifications, and RNA interference play a vital role in the establishment of this epigenetic memory. For instance, exposure to environmental stresses, such as drought or pathogens, can induce epigenetic changes that enhance plant resistance and are passed on to future generations. Understanding epigenetic memory in plants not only sheds light on the molecular mechanisms underpinning phenotypic plasticity but also presents opportunities to enhance crop adaptability and productivity in response to changing environmental challenges.

Actual grapevine diversity

The domestication process in grapevine englobing genomic and epigenomic changes, from the start of the process to nowadays, generated a huge amount of different cultivars with specific features along the History, with near of 10000 different cultivars (International Organisation of Vine and Wine Intergovernmental Organisation, 2017). Despite the elevated number of existent cultivars coming from the domestication process, the incidence of the human activity and economy in grapevines produced some weakness in vineyards that produced an important damage in Europe. The most important event on vineyard caused by the human activity was the introduction of a foreigner pest from America called Phylloxera into the European vineyards in the XIXth century that causes several losses by grapevine defoliation (Tello et al., 2019; Tello & Forneck, 2019). Since the discover of America, the aim to diversify the offer of agronomical products in Europe and America produced that some plants originally from both continents were cultivated in them. In the case of the grapevine, further cultivars were exported to America in order to produce table grapes and wine in America (Mostly other *Vitis* species) at the same level of Europe from European cultivated grapevines (*V. vinifera*), and vice versa. The exchange of cultivars in Europe led to increased utilization of American grapevines. However, this importation introduced an associated root parasite that also affects the leaves, causing defoliation in European grapevines (complete life cycle of insect in **Figure 16**). The presence of Phylloxera resulted in significant damage in Europe due to the susceptibility of European cultivars to this parasite infection. This impact has been documented in various studies (Cochetel et al., 2017; Cookson & Ollat, 2013; Darriaut et al., 2022; Gautier, Cochetel, et al., 2020; L. Zhang et al., 2016). Due to the absence of the Phylloxera parasite in Europe and the European grapevine's susceptibility to it caused by the absence of coexistence among them, the introduction of this pest to vineyards resulted in a catastrophic impact. The infection of the Phylloxera parasite in the roots led to a significant number of plant deaths and a substantial decrease in the diversity of European cultivars (Tello et al., 2019).

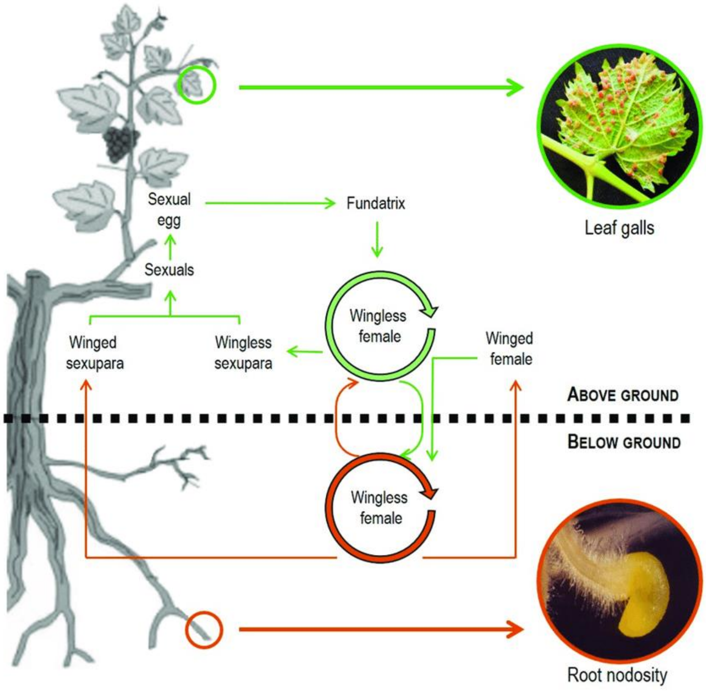

Figure 16: Phylloxera cycle schema in grapevine. In this graphic, the description details the root infection by this insect producing the root nodosity in plants, followed by a colonization of leaves producing leaf galls. Original figure from Tello and Forneck, 2019.

In spite of the incidence of the Phylloxera in the European grapevines and the reduction of the catalog of available cultivars, a large number of genotypes were preserved. The large genetic diversity of cultivars were selected and bred along the History depending on the food preferences of the host society (Dong et al., 2023; McGovern, Fleming, et al., 1996; McGovern, Glusker, et al., 1996). However, the total surface for each cultivar is unequal, becoming much higher in commercial grapevines than in local genotypes, giving the grape and wine market a homogeneity in grapes and excluding exotic local cultivars (International Organisation of Vine and Wine Intergovernmental Organisation, 2017). Studies like Bota et al., 2016 suggested the wine and vine community to explore new alternatives of grapevine genotypes, comparing the possibility to adapt the vineyard management by using local cultivars in front of the highly distributed commercial cultivars that are actually used for. The research about the large diversity of grapevine could solve some problems related to all the mentioned problems due to the domestication and the human selection in grapevine, and could also enhance the plant breeding in grapevine by crossing genotypes with determinate features of interest. Furthermore, exploiting and exploring the existing genotype diversity is presented as an interesting option to solve the threats of climate change (Bota et al., 2022).

Drought Tolerance in Grapevine

Physiological dynamic responses and genotype differences

The climate change is one of the most pressing global issues for the society in the XXIth century, and it is directly caused by humans. Factors such as intensive farming and monocultures, water wastage, the use of pesticides, and reliance on fossil fuels contribute to an environmental impact, resulting in a rise in global temperatures, the extinction of vulnerable species, compromised food security, increased soil salinity, and diminished water availability, among other significant effects (Duchenne-Moutien & Neetoo, 2021; Tirado et al., 2010). One of the most impactful consequences affecting crops is drought and reduced rainfall rates. The decline in water availability for crops can lead to substantial harm, causing losses in fruit production and diminishing global food security (Duchenne-Moutien & Neetoo, 2021; Tirado et al., 2010). Extreme climate events, including prolonged droughts, irregular rainfall, or shifts in monsoon patterns, can profoundly impact vineyard stability, leading to a significant reduction in grape quality and production (Duchenne-Moutien & Neetoo, 2021; Tirado et al., 2010).

More specifically, water availability poses a significant challenge in vineyards, with a considerable portion of the world being impacted by this effect of climate change. This issue is particularly pronounced in winemaking regions such as the Mediterranean area, America, South Africa, and Asia (**Figure 17**) (Duchenne-Moutien & Neetoo, 2021; Hannah et al., 2013; Tirado et al., 2010).

Drought can have deep effects on climate, contributing to a range of climatic challenges. One notable impact is the alteration of atmospheric conditions, where reduced soil moisture levels lead to decreased evaporation and transpiration. This diminishes the transfer of water vapor into the atmosphere, affecting cloud formation and precipitation patterns. As a result, drought can contribute to extended periods of low rainfall and altered weather patterns, exacerbating aridity in affected regions. Additionally, the increased land surface temperature associated with reduced evaporation can contribute to the intensification of heatwaves. Drought conditions often lead to changes in atmospheric circulation, influencing the distribution of high and low-pressure systems. These alterations can further disrupt normal weather patterns, contributing to the persistence of dry conditions and affecting regional climates. Beyond these direct atmospheric effects, drought can also impact ecosystems and land surfaces, leading to changes in vegetation, soil composition, and water availability. These changes, in turn, create a feedback loop that amplifies the overall climatic impact of drought, further emphasizing the interconnected nature of drought and climate dynamics.

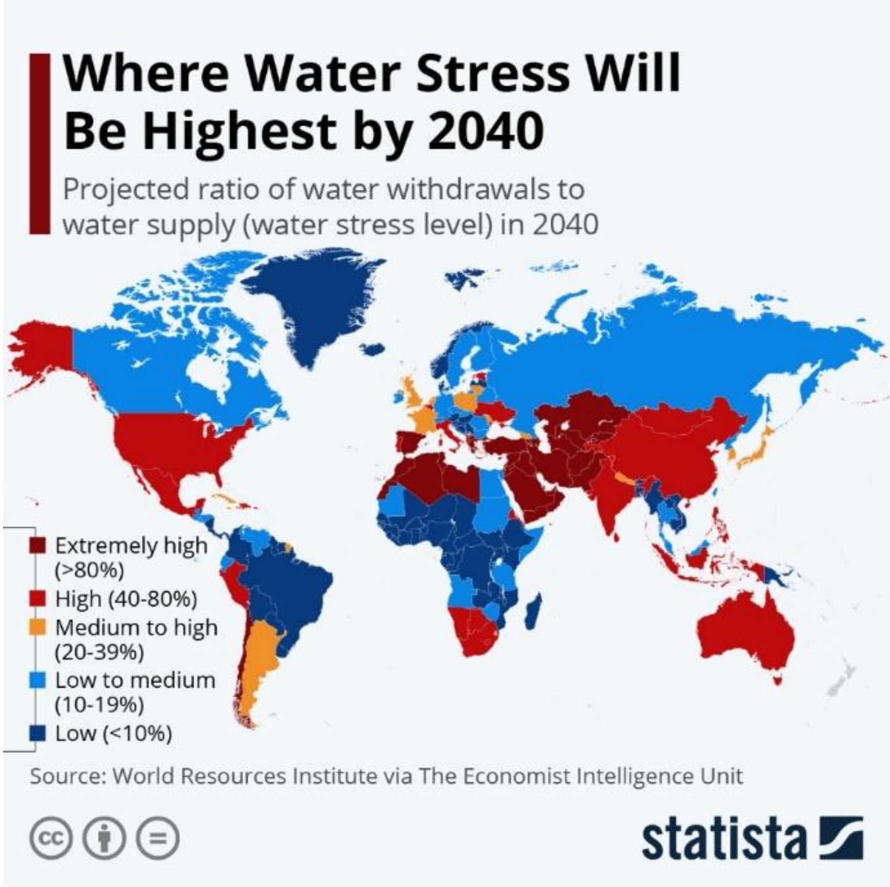

Figure 17: Map of the probably drought stress effects caused by climate change in 2040. Original figure from: World Resources Institute via the Economist Intelligence Unit

The drought causes in the whole crops changes at morphological and physiological level related to the early maturity of the flowers and fruits, a general reduction of the leaf surface in order to avoid possible losses of water by evapotranspiration, the stomatal closure in leaves, the reduction of photosynthesis rate or the reduction of growth (Seleiman et al., 2021). The effects caused by water scarcity are also amplified by the use of large monocultures and predominant cultivars in vineyards, that remarks the vulnerability of this crop for those water requirements and their sensitivity to drought (Bota et al., 2016; Giffard et al., 2022; Pou et al., 2023).

As a principal consequence, the critical reduction in fruit quality and quantity caused by the climate change, and therefore those problems are worrying the viticulturist and winemaking world. Nonetheless, the enormous diversity in grapevine has shown that not all the cultivars are equally sensitive to drought (Bota et al., 2001, 2016). The increase of knowledge about the tolerance to drought of different cultivars could be interesting for adapting the vineyard to new scenarios caused by climate change, almost in regions with general low precipitations and an arid climate like the Mediterranean Area (Bota et al., 2022). Parameters like photosynthesis, stomatal conductance or Water Use Efficiency (WUE) can be used to stablish a classification of the genotypic diversity in field studies depending on their drought tolerance (Bota et al., 2001; Soar et al., 2006; Tomás et al., 2012, 2014). Therefore, the resilience of the vineyard to adverse conditions caused by climate change could reside into the large number of available cultivars originated along the process of domestication.

To comprehend the underlying mechanisms contributing to the drought tolerance in grapevines, different factors play a crucial role in mitigating the damages caused by drought. These factors can be categorized based on their nature and their overarching effects on grapevines. This includes physiological, metabolic, hormonal, or transcriptomic factors, all of which collectively contribute to the general stress response in grapevines (Bota et al., 2022). However, some of these factors go beyond merely participating in the general stress response; they actively induce specific changes that confer a comprehensive and adaptive response to stress in the plant (Bota et al., 2001, 2016; Dal Santo et al., 2016; Lovisolo et al., 2010; Murcia et al., 2017). One of the most important interactors in the response to drought in plants is the signalling by hormones. In plants, the Abscisic Acid (ABA) is one of the most important pivotal player in the response to drought stress (L. Li et al., 2021; Murcia et al., 2017; Rossdeutsch et al., 2016; Villalobos-González et al., 2016). Thanks to the versatility of this hormone, the principal effects in the plants are the stomatal closure and the reduction of photosynthetic rates focusing on the preservation of the energy into the plant. At morphological level, ABA is the principal hormone involved in the leaf senescence, the reduction of growth in shoot, the increase of root elongation or the pruning of fruits (Apel & Hirt, 2004; Cramer et al., 2007; Peleg & Blumwald, 2011; Tillett et al., 2011).

ABA as key to understand the resilience of the grapevine in vineyard: History of ABA along the evolution and mechanisms involved in the response to drought

Since the evolution of plants from algae to the angiosperms, the hormone ABA and its mediated response is key to adapt the plant to water deprivation and to understand the colonization of land by ancestral plants, conferring desiccation tolerance for ancestral aquatic organisms (Y. Sun et al., 2020). In plants, there are two different mechanisms linked to the tolerance to abiotic stresses, the ABA-independent pathway and the ABA-dependent pathway, both in cross talk between them (Yoshida et al., 2014). In ABA-independent pathway, the signal is mediated by an osmotic change that involves a few number of participants, such as the dehydration-responsive element/C-repeat (DRE/CRT) and DRE-CRT-binding protein 2 (DREB2A), proteins related to the induction of stress-responsive genes (**Figure 18**) (Yoshida et al., 2014). This pathway is more oriented to regulate the osmotic changes caused by drought (Budak et al., 2013). Otherwise, the ABA-dependent pathway involves several pathways and proteins, and some of the interactors involved in this cascade arde negative regulators of the ABA-independent pathway (especially those from AREB/ABFs group), improving the response in the plant (Muhammad Aslam et al., 2022; Yoshida et al., 2014). Apart from the transcription factor DREB family, there are other transcription factors and gene families involved in the ABA-mediated response to drought, such as the MYB (myeloblastosis) family, the NCED (9-cis-epoxycarotenoid dioxygenases), the RD family (RD20, RD22, RD29, e. g.) (Response to Dehydration), bZIP (Basic region/leucine zipper motif) or WRKYs families (Khoso et al., 2022; Matus et al., 2014; Muhammad Aslam et al., 2022; Tu et al., 2018).

The signal transduction pathway of the hormone ABA in ABA-dependent response is conserved along the evolution, consisting in a PYRABACTIN RESISTANCE 1-Lyke (PYL)-PROTEIN PHOSPHATASE 2C (PP2C) and SNF1-RELATED PROTEIN KINASE 2 (SnRK2) (Y. Sun et al., 2020). PYL-like proteins, as a receptors, are well conserved along the evolution, and the principal action of this proteins are the reception of ABA and the posterior inhibition of PP2C (Y. Sun et al., 2020).

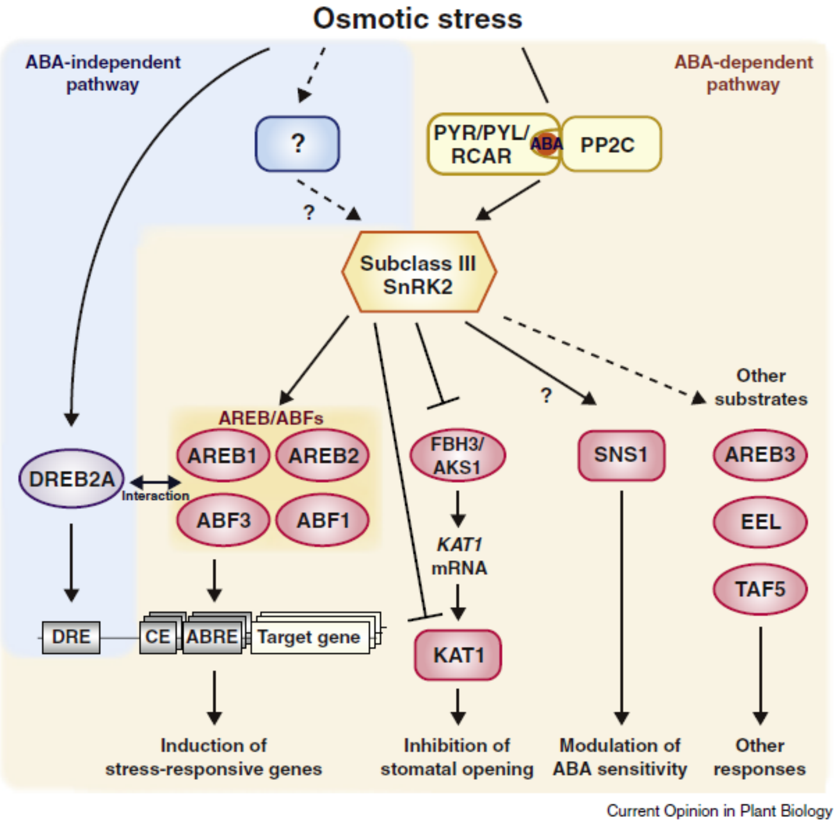

Figure 18: ABA-dependent signaling pathway and cross talk with ABA-independent signaling in response to osmotic stress. From Y. Sun et al., 2020.

More concretely, in the ABA signal transduction pathway, ABA binds to PYL, that allow the receptors to inhibit the PP2C that represses ABA signalling (Y. Sun et al., 2020). The formation of PYL-ABA-PP2C complex releases SnRK2 from the inhibitory complex with PP2C, initiating phosphorylation of transcription factors and ion channels, involved in ABA output responses (**Figure 18** and **Figure 19**) (Y. Sun et al., 2020). From this basis of signal transduction, several modifications along the evolution was identified, such as the inclusion of different subclasses like subclass III SnRK2 kinases in the pathway, anion channel SLAC1 or some transcription factors like AREB/ABFs as substrate of SnRK2 from algae belonging to the *Zygnematophyceae* class like *Zignema circumcarinatum* to angiosperms like *Arabidopsis thaliana* (Muhammad Aslam et al., 2022; Y. Sun et al., 2020; Yoshida et al., 2014). In algae, the ABA plays a crucial role in salt tolerance, that could confer the ability to colonize the land easily. In early divergent land plants, like bryophytes, lycophytes or ferns, the ABA is key to adapt the plant to the desiccation caused by the land colonization thanks to the appearance of stomata (a principal organ whose function in to regulate the evapotranspiration by closing or opening the stomatal closure in plants) and its sensitivity to the ABA hormone (Y. Sun et al., 2020).

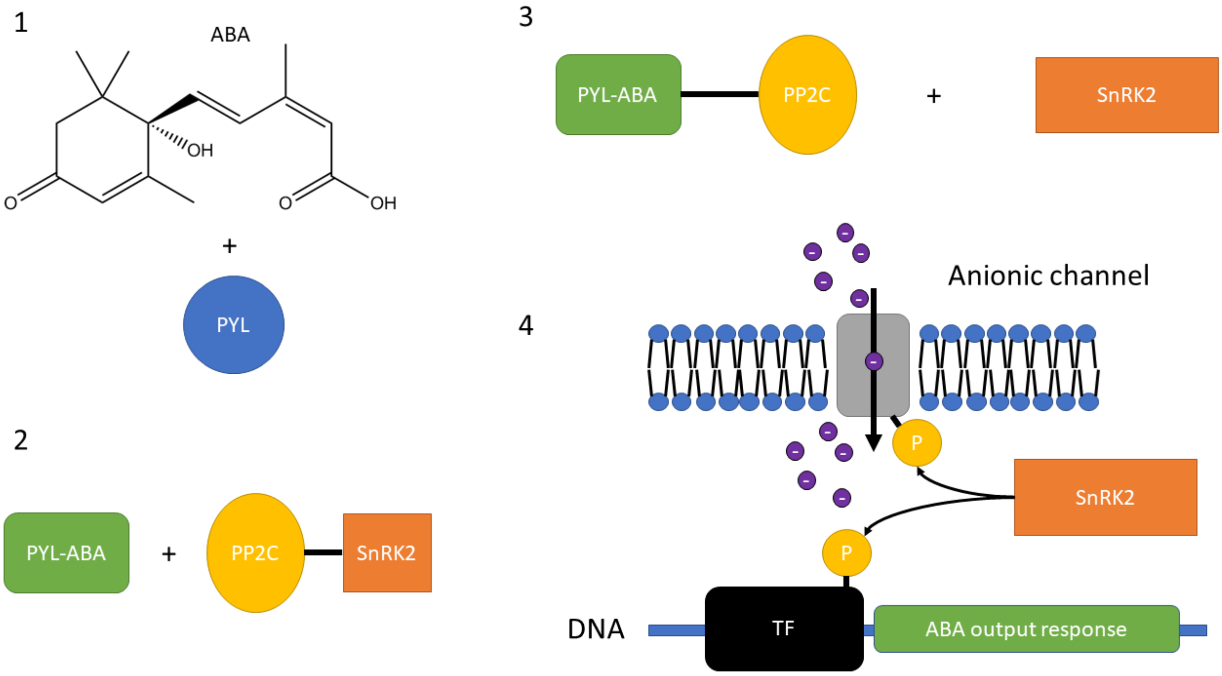

Figure 19: Basic signal transduction pathway for ABA-dependent pathway. 1) Binding PYL-ABA; 2) Formation PYL-ABA-SnRK2 complex; 3) Release SnRK2 from PP2C inhibitory complex; 4) Phosphorilation of ion channels and Transcription Factors for the response.

Indeed, there are several pathways involved in the ABA signal that are important in the reduction of oxidative stress in plants and maintain the cell turgor (Muhammad Aslam et al., 2022; Murcia et al., 2017; Rossdeutsch et al., 2016; Villalobos-González et al., 2016). Apart from the direct mediated response by ABA described in *Arabidopsis thaliana* and grapevine, other phytohormones like auxins, ethylene, Gibberellins (GIs), Jasmonic Acid (JAs), Salicilic Acid (SA) or cytokinin (CK) play an important role in the response to drought, modulating pathways to interact in the response to stress (Corso et al., 2015; Muhammad Aslam et al., 2022; Peleg & Blumwald, 2011). In the case of auxins and GIs, the production of ABA is inversely related to the accumulation and production of Auxins and GIs, becoming antagonists (Kohli et al., 2013; Muhammad Aslam et al., 2022; Peleg & Blumwald, 2011). Conversely, in the case of the JAs, there are a direct relationship between the production ABA and the JAs concentration (Muhammad Aslam et al., 2022; Peleg & Blumwald, 2011). In the case of ethylene, JA and ethylene induced the expression of ERF1 (Ethylene Response Factor 1), that encourages drought tolerance and increases the accumulation of ABA levels, contributing to stress resistance (**Figure 18**) (Muhammad Aslam et al., 2022; Peleg & Blumwald, 2011). Furthermore, cross talk between SA and ABA enhances the response to dehydration, as same as CK, contributing to the cell homeostasis (Muhammad Aslam et al., 2022; Peleg & Blumwald, 2011).

The harmony of the hormone signals under water-limiting conditions have concerted actions through fine-tuned cross talk between the hormones and other possible secondary messengers, like the ROS (Apel & Hirt, 2004; Kar, 2011; Kohli et al., 2013; Muhammad Aslam et al., 2022). Directly related to the effects caused by drought stress at physiological level by the ABA mediation and other hormones, several metabolic pathways are affected in order to alleviate the drought stress in the plant (L. Li et al., 2021; Murcia et al., 2017; Villalobos-González et al., 2016). At metabolic level, the changes in the production of primary metabolites can help to detect the adaptations to face the drought into the plant, such as the concentration of myo-inositol, an osmolyte directly related to maintain the cell turgor and preserve the water into the cell, or the citrate, directly involved in the anthocyanidin synthesis to reduce the damages caused by drought stress (Boeckx et al., 2015; J. Kim et al., 2006; L. Li et al., 2021; Murcia et al., 2017; Villalobos-González et al., 2016).

Therefore, the described ABA-mediated response is addressed by several pathways triggering the modulation of gene expression in the organism (Askri et al., 2012; Corso et al., 2015; Dal Santo et al., 2016; Muhammad Aslam et al., 2022; Murcia et al., 2017; Rossdeutsch et al., 2016; Yoshida et al., 2014). Focusing on grapevine, at transcriptomic level, several genes related to the production of the described metabolites are guided by the ABA signal in grapevine (Corso et al., 2015; Dal Santo et al., 2016). Apart of the cited general genes and TF described before, one of the most representative gene families described in the literature and involved in the response to ABA stress is the BURP family (a part of RD22 TFs, mentioned before), directly related to the response to ABA pulses in grapevine (Matus et al., 2014; Muhammad Aslam et al., 2022; H. Sun et al., 2019). It is notably the role of the expression from other families like the MYBs TF genes, that are presents in the coordination of the adaptation by the flavonoid and polyphenol synthesis for reducing the effects of ROS into the cell (Carrasco et al., 2015; Czemmel et al., 2009; Matus et al., 2008; Orduña et al., 2022). According to the literature, these families are important actors in the response to drought, but there are not the only one. Genes involved in the flavonoid and phenylpropanoid modifications belonging to Chalcone Synthetase (CHS) or the lignin synthesis for root lignification are also related in the response to drought stress (Fujita et al., 2006; Yıldırım et al., 2018).

As a main result of the modulation at genetic and hormonal level in grapevine caused by drought and ABA, the response to ABA affects the grapevine plant physiology, modulating key parameters such as hydraulic conductance, stomatal closure or photosynthesis rate (Coupel-Ledru et al., 2017; Gambetta et al., 2020; Martorell et al., 2015). Depending on the cultivar, the hydric conductance for soil water intake could be affected by the ABA production, reducing the water flux into the plant to preserve the water content into the plant (Coupel-Ledru et al., 2017; Martorell et al., 2015). The low water availability also enhances the stomatal closure in leaves, to avoid evapotranspiration, by ABA mediation, but also decreases in leaves the photosynthesis rate, in order to avoid the generation of ROS into the leaves (Castellarin et al., 2007). Thanks to the measures of those parameters, and depending on the stomatal behaviour, under water deficit stress, the grapevines can be classified as near-isohydric (“pessimistic”) if the genotype have a tight stomatal control, closing stomata at incipient stages of water deficit or anisohydric (“optimistic”) if the stomatal control is not as tight (Schultz, 2003; Soar et al., 2006).

Therefore, with a general vision of the effects of the water scarcity in grapevine, the existence of some cultivars more tolerant to drought than others become very interesting to understand what are the changes at all the described levels that confers that tolerance. Thanks to Bota et al., 2016, there are further information about the near-isohydric genotypes coming from Spain, classifying some local and commercial grapevines by their tolerance to drought at physiological and morphological level. The knowledge about the hydric behavior of cultivars like *Merlot* (classified as anisohydric) and cultivars like *Callet* (classified as near-isohydric) (Bota et al., 2001, 2016; Flexas et al., 2002) could help to understand the different mechanisms involved in the tolerance to drought for near-isohydric cultivars and to enhance the resilience of the vineyard. However, physiological differences only describe superficially the adaptation to drought stress in near-isohydric and anisohydric cultivars. Stomatal behaviour, photosynthetic activity or plant water status only describe the consequences of the adaptation, and it is crucial to go deeper into the molecular mechanisms involved in the response to drought stress in grapevine. For that reason, a good approach to unveil the molecular mechanisms involved in the different tolerance to drought in vineyard could be the use of omics techniques (genomics, transcriptomics, epigenomics, metabolomics, e. g.) (Dai & Shen, 2022; Fabres et al., 2017; Hasin et al., 2017; Jiang et al., 2017; Xu et al., 2020). Omics analysis, in combination with the physiological state of the plants, could be a good approach to observe the complete landscape of changes for each genotype in front of abiotic stresses, gaining relevance for instance the transcriptomics in the ABA-mediated response (Apel & Hirt, 2004; Carrasco et al., 2015; Cramer et al., 2007; Lovisolo et al., 2010; Matus et al., 2008, 2014; Murcia et al., 2017; Orduña et al., 2022; Villalobos-González et al., 2016).

Apart from the relevance of the appropriate cultivar to face possible drought stress situations, nowadays the use of rootstock in vineyard requires special consideration in order to adapt the vineyard to new environmental conditions like the water scarcity. The use of rootstock in vineyard comes from the Phylloxera pest in the XIXth century as a way to mitigate the impact of the insect infection in vineyards. In Europe, due to the phylloxera incidence, several vineyards were affected by this pest, and the vineyard management needed to implement different techniques to stop the infection, such as the use of insecticides or changes in the management practices. However, the most effective trait to mitigate the Phylloxera in the vineyards was the use of resistant rootstocks to the Phylloxera infection (Benheim et al., 2012; Granett, Omer, et al., 2001; Granett, Walker, et al., 2001). Essentially, the solution was based in grafting European grapevines (generally cuttings with two or three nodes with dormant buds) on shoots of American grapevines (with no buds, only shoot with the radicular part), in order to force the union and generate the hybrid plants (see the method in **Figure 7 c)**) (Cochetel et al., 2017; Cookson & Ollat, 2013; Gautier, Cochetel, et al., 2020; Mudge et al., 2009).

This methodology caused a revolution in grape growers and breeders, and the implementation of non-European grapevines in the European vineyards spreads out this technique to the rest of the world (Benheim et al., 2012; Carrasco et al., 2022; Cochetel et al., 2017; Gautier, Cochetel, et al., 2020; Granett, Omer, et al., 2001; Soar et al., 2006; Zhou-Tsang et al., 2021). Thanks to grafting European cultivars (*V. vinifera*) onto American grapevines (such as *V. riparia, V. berlandieri or V. rupestris*) as rootstocks, the incidence of the infection was dramatically reduced (Benheim et al., 2012; Granett, Omer, et al., 2001; Granett, Walker, et al., 2001). This fact was done to the plague resistance of the American grapevine genus and their the coexistence between parasite and host along the evolution that was absent in the European grapevines (Benheim et al., 2012; Granett, Omer, et al., 2001; Granett, Walker, et al., 2001).

Consequently, the use of American rootstocks in grapevine caused a revolution in grape growers and breeders, and the implementation of non-European grapevines in the European vineyards spreads out this technique to the rest of the world (Benheim et al., 2012; Carrasco et al., 2022; Cochetel et al., 2017; Gautier, Cochetel, et al., 2020; Granett, Omer, et al., 2001; Soar et al., 2006; Zhou-Tsang et al., 2021). Furthermore, the use of rootstocks generates a lot of interest in the winegrowers because the rootstocks was useful for other purposes, such as the increase of vigor in plants or the grapevine adaptation to different stresses (Cochetel et al., 2017; Corso et al., 2015; Gautier, Cochetel, et al., 2020; L. Zhang et al., 2016; Zhou-Tsang et al., 2021). Actually, the use of American grapevines as rootstocks is crucial to the winemaking and table grape industry, and there are still in use. Moreover, the plant breeding of the rootstock plant produced hybrid plants (coming from genetical crosses through American grapevines, but also with *V. vinifera* grapevines such as the commercial rootstocks *110 Richter* (*V. rupestris* x *V. berlandieri*), *101-14 Mgt* (*V. riparia* x *V. rupestris*) or *Kober 5BB* (*V. berlandieri* x *V. riparia*)) (Cochetel et al., 2017; Cookson & Ollat, 2013; Minio, Cochetel, Massonnet, et al., 2022; L. Zhang et al., 2016; Zhou-Tsang et al., 2021).

The selection of the appropriate rootstock for a vineyard is an important factor that depends on the environment, water availability and soil quality, and the choice have a crucial influence in the final grape production and plant survival (Corso & Bonghi, 2014; Zhou-Tsang et al., 2021). For example, there are rootstocks more suitable to use in climates with low precipitations, high temperatures and drought seasons like *110 Richter* than others more adapted to grow under high humidity and precipitations along the year and low temperature conditions like SO4 (Corso & Bonghi, 2014; Zhou-Tsang et al., 2021). The great diversity of rootstocks and their attributes enhances the grapevine adaptation to adverse climates, contributing to ensure the grape production in vineyard.

Then, the appropriate rootstock can ensure the successful of the grape production and increase the benefit in vineyard under adverse conditions, like the reduction of available water in soils. Nonetheless, the rootstock is not the only player in the response, and the communication scion-rootstock must be effective in order to avoid water losses into the plant (L. Zhang et al., 2016). In fact, several signals and events are involved in the response to drought stress into the plant, but one of the most important actors is again the ABA in the root part, starting the response by aquaporin synthesis to improve the water intake in roots or the root lignification to avoid water losses, extending the response to the aerial part (L. Zhang et al., 2016) (**Figure 20**).

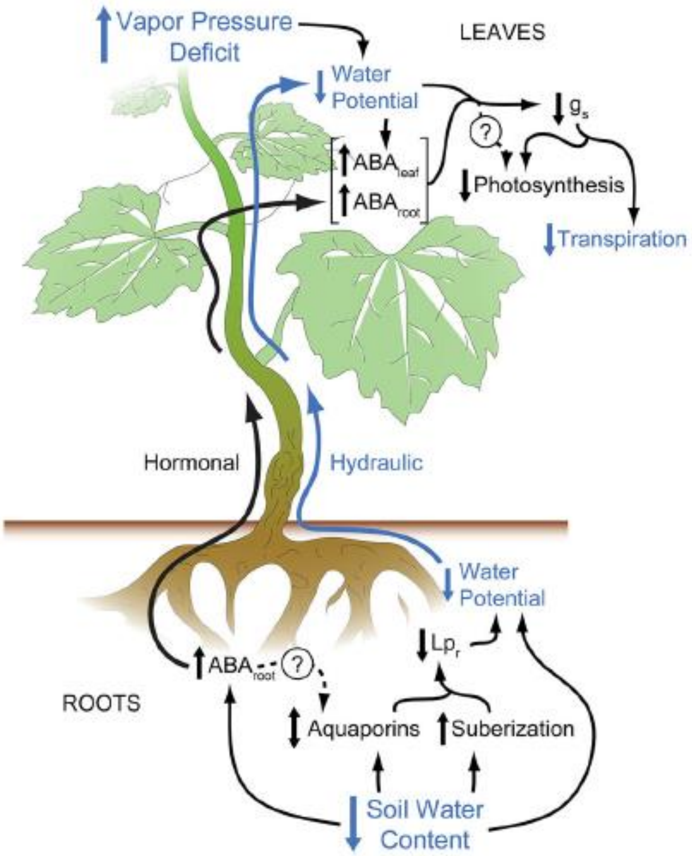

Figure 20: Model of communication from root to shoot in water stress conditions. The information starts in roots and then it is transmitted from roots to shot by hormonal communication and hydraulic changes. Then, the ABA signal increases the ABA production in leaves and roots in order to regulate the stomatal closure and to reduce the photosynthesis rate in leaves. Figure from Zhang et al., 2016.

Genomic and rootstocks, an emerging field…

Despite the crucial role that rootstocks play in vineyards, there has been a notable scarcity of genomic studies focused on these plants, overlooking their significant potential in grape production. While the first genome of *V. vinifera* was published in 2007, the first genome draft for an American grapevine rootstock, specifically *Vitis riparia* Riparia Gloire de Montpellier, was published in 2019 by Girollet et al. in 2019. This pioneering effort primarily utilized third-generation sequencing technology from PacBio. Additionally, Cochetel et al. in 2021 and Minio et al. in 2022 independently produced additional genome drafts. Cochetel et al., 2021 work extended to *Muscadinia rotundifolia*, while Minio et al., 2022 targeted the commercial rootstocks *110 Richter*, *Kober 5BB*, and *101-14 Mgt*. These genomic studies mark important strides in unraveling the genetic makeup of grapevine rootstocks, shedding light on their molecular characteristics. Such insights are crucial for understanding and optimizing their contributions to grape production in vineyards.

Although ABA and hydraulic changes are directly involved in the communication root-shoot, other figurants interplays in the response to abiotic stresses like drought. Changes in gene expression patterns depending on the scion was probed under different situations, evidencing a clear influence of scion-rootstock communication in the response to environmental stresses (Cochetel et al., 2017; Corso & Bonghi, 2014; Gautier, Cochetel, et al., 2020). Those changes in gene expression would be leaded by other intermediaries, modulating several key genetic pathways. In plants like *Arabidopsis thaliana*, it is observed that miRNAs and other polypeptides are messengers in the cross talk between the shoot and the root, together with other hormones signal for regulation (Batool et al., 2018; Curaba et al., 2014; G. Kim et al., 2022; Kohli et al., 2013). At molecular level, miRNAs and polypeptides are transported from root to shoot and from shoot to root in order to modulate the gene expression of key pathways to face the drought stress, and they are also involved in the phytohormone cross talk (involving auxins and CK, Jas and others) (Curaba et al., 2014; G. Kim et al., 2022; Kohli et al., 2013; Takahashi et al., 2020).

The use of omics to explore the global response to drought

Omics is a term used to describe a comprehensive and large-scale study of biological molecules within a cell, organism, or system. It involves analyzing various types of biological data to understand the structure, function, and interactions of the components that make up living organisms (Hasin et al., 2017; Ramalhosa et al., 2011). Some of the principal omics techniques are the genomics (described below in the section "The advances in the genetic research on grapevine"), the epigenomics (described below in the section "Influence of the epigenetic modifications in grapevine"), transcriptomics or metabolomics that will be explained in this section.

The study of transcriptomic changes under water deprivation is key to understand the critical pathways involved in the ABA-mediated response. Since the discovering of PCR, the versatility of the technique enhanced the resolution of the experiments of gene expression thanks to the discovering of the Reverse Transcriptase enzyme or RT in viruses RT-RNA (-) like the HIV (Human Immunodeficiency Virus) (Das & Arnold, 2013; VanGuilder et al., 2008). The RT enzyme can transcribe mRNA into DNA through complementarity, stabilizing the nucleotide strand and enabling DNA polymerase to replicate the information contained in the mRNA fragment (Lowe et al., 2017; VanGuilder et al., 2008). The creation of a stable DNA strand from various mRNA fragments originating from different genes and in varying proportions has allowed the scientific community to quantify gene expression in a cost-effective manner, leading to the development of the Quantitative PCR (qPCR) method (VanGuilder et al., 2008). In spite of the common use of this methodology and its high accuracy, the use of qPCR in gene expression assays is very specific by the measure of only selected genes, that could be a lack in the discovering of new genes involved in the gene expression responses and the cross talk between different interactors (Lowe et al., 2017; VanGuilder et al., 2008). Although the specificity of this technique in gene expression studies, this methodology is still in use, and the use of qPCR is very useful to confirm the results coming from advanced methodologies (NGS) due to its sensitivity and its precision for amplifying candidate genes for representing any transcriptional response. For example, the use of qPCR is very useful to describe gene families involved in the ABA response as mentioned above, such as the BURP family or the MYB family, in different parts of the plant (Matus et al., 2008, 2014). The advances in the study of gene expression in samples under different conditions was targeted to reduce the amount of PCR reactions in order to economize the laboratory times and spaces (Lowe et al., 2017). For that, the microarrays of RNA supplied the needs of this problem. The technique, based on the use of large solid matrix called "chips" with specific DNA probes immobilized in each well to hybridize the complementary mRNA fragments, can be used for exploring differences in genetic pathways, easily detected by fluorescence produced by the hybridization of the fragments with the target molecule (Lowe et al., 2017). The cons of this technique are related to the elevated cost of this methodology, but it produces larger information than qPCR at the same time, allowing the study of gene expression of gene families (Lowe et al., 2017). In grapevine, this methodology is also utilized in gene expression experiments related to the flavonoid and phenylpropanoid pathways for the change of color in berries, for example (Fasoli et al., 2012; Moretto et al., 2016). Nonetheless, due to advancements in sequencing technologies, particularly with second and third-generation sequencing methods, there has been a substantial improvement in the resolution of gene expression studies (Lowe et al., 2017). The conversion of mRNA strands to cDNA has enabled the utilization of technologies such as Illumina to sequence the entire mRNA repertoire present in a sample. This methodology is commonly referred to as RNA-seq. (Lowe et al., 2017). This methodology permits the measure of the abundance of all genes coming from a plant in a specific moment or condition by the massive sequencing of the cDNA fragments. By a posterior alignment to a genome (reference or draft genome), the variations in abundance of genes can be interpreted in global, exploring the behavior of key pathways depending on the condition (**Figure 21**) (Lowe et al., 2017). In grapevine, this methodology is used, for example, to explore the tolerance to nutrient deficiency (Cochetel et al., 2017; Gautier, Cochetel, et al., 2020; L. Zhang et al., 2016) or to unveil the mechanisms involved in drought stress in grapevine (Dal Santo et al., 2016; Tan et al., 2023). Conversely, this methodology is expensive compared with qPCR, and depending on the depth of the represented genes by cDNA, the results could not be enough accurate (Tarazona et al., 2011).

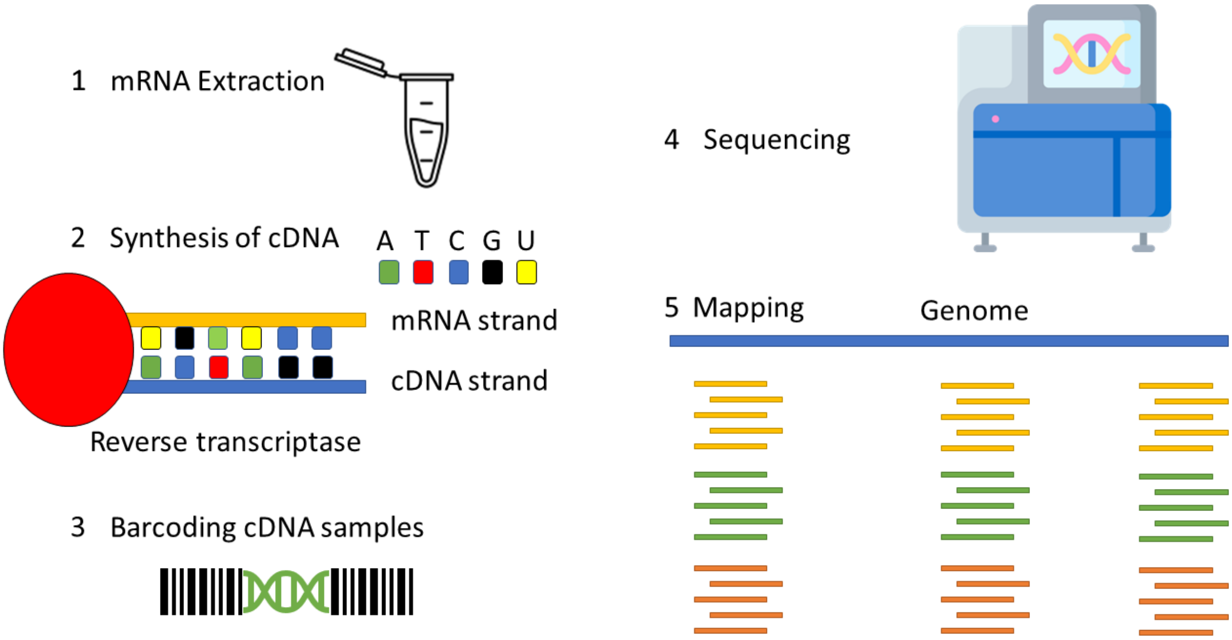

Figure 21: Workflow for RNA-seq analysis: 1) mRNA extraction from cell or tissues; 2) Synthesis of cDNA using reverse transcriptase; 3) Barcoding of cDNA samples; 4) sequencing by Illumina technology; and 5) Mapping sequences with genome to explore the differences between the conditions by abundance of identified fragments.

The measure of key metabolites and hormones is very important, because the relationship between the results of transcriptomic studies and those molecules can generate a global vision of what happens into the plant under determinate conditions or during a period. For that, metabolomics can help to explore the principal mechanisms involved in the response by the measure of metabolites at the same time (Aderemi et al., 2021; Patel et al., 2021). For the characterization of metabolites, two different approaches are presents to understand the metabolic changes in the plant: the characterization of primary or secondary metabolites (Aderemi et al., 2021; Patel et al., 2021). The characterization of primary metabolites is focused on detecting potential changes induced by variations in environmental conditions because it is more sensitive to such changes compared to the study of secondary metabolites, which is more associated with the investigation of the accumulation of protective molecules and their richness in the sample (Aderemi et al., 2021; Patel et al., 2021).

Metabolomics is a part of the functional genomics studies, where the principal objective is to explore the differences between two or more different conditions in order to understand their significances at functional level, relating that with genomic studies such as genomics, transcriptomics or other related fields (Alseekh & Fernie, 2018; Kell & Oliver, 2016). There are many techniques to elaborate a metabolic profile for each sample, since the use of colorimetric techniques to obtain a general image of the sample metabolism

(López-Hidalgo et al., 2021) to molecule separation and characterization techniques like LC-MS (Liquid Chromatography coupled to Mass Spectrometry), GC-MS (Gas Chromatography coupled to Mass spectrometry) or MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight), or spectroscopic techniques like 1H-NMR (1H-Nuclear Magnetic Resonance) (Alseekh & Fernie, 2018; Choi et al., 2004). Despite the low cost of the colorimetric measures and the low quantity of sample required for the study (López-Hidalgo et al., 2021), the resolution of those techniques are limited, and the metabolomics studies are usually more focused in spectroscopic and chromatographic assays (Alseekh & Fernie, 2018). Depending on the nature of the sample and the experimental design, the choice between those principal methodologies are crucial. For primary metabolites with high presence in normal tissues, the most economic and resolutive technique is the 1H-NMR, but the exploration across the large amount of secondary metabolites requires the chromatographic techniques such as LC-MS or GC-MS (Alseekh & Fernie, 2018). The 1H-NMR is a technique that consists in the reaction of a sample submitted to a specific radiofrequency under a strong magnetic field. The basic principles of NMR involve placing a sample in a strong magnetic field, applying radiofrequency pulses to specific nuclei in the sample, and then detecting the signals emitted as the nuclei return to their original alignment or spin (Choi et al., 2004; James, 1998). These signals, in the form of spectra, provide valuable information about the molecular environment and interactions within a substance (**Figure 22**).

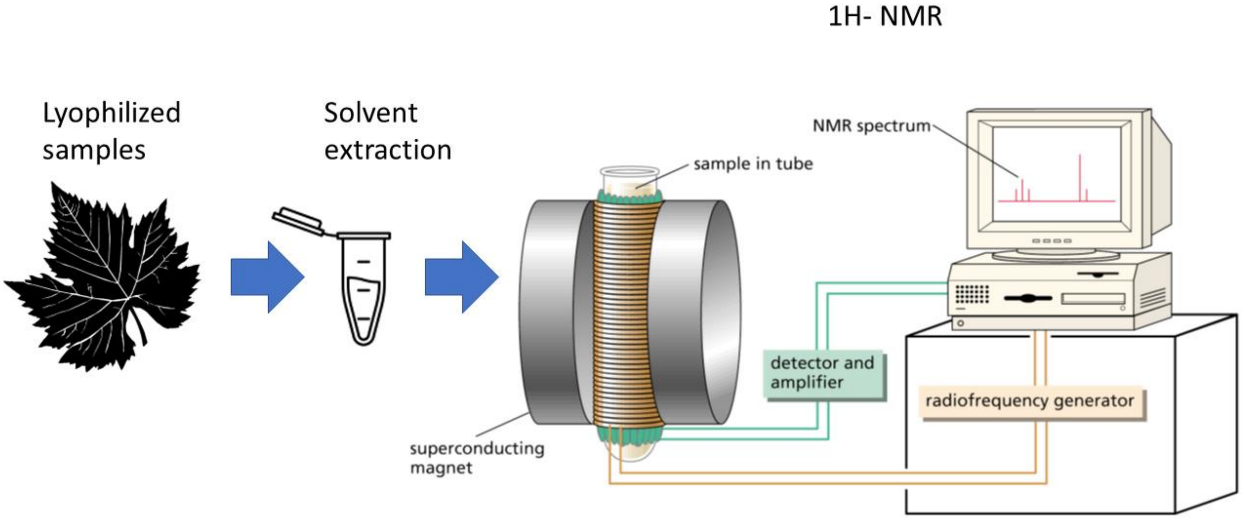

Figure 22: Workflow for metabolomic analysis using 1H-NMR spectroscopy. The basis is the lyophilization of the samples, the solvent extraction and the detection and measurement of absorbance of radiofrequencies under a strong magnetic field. Images from stock and UPV/EHU.

However, this technique is not very useful for the detection of plant hormones. In order to unveil the possible differences through the samples in hormone concentration, the chromatographic methods are the best choice to analyze it. Either LC-MS and GC-MS has a lot of advantages in hormone identification and quantification, but LC-MS is more appropriate thanks to the possibility to detect and quantify more molecules than GC-MS, due to the restriction of the measure of volatile hormones (D. Cao & He, 2023). In LC-MS, a liquid chromatography system is used to separate complex mixtures into individual components based on their chemical properties. The separated compounds are then introduced into a mass spectrometer, where they are ionized and subjected to a magnetic field. The mass spectrometer measures the mass-to-charge ratio of the ions, providing information about the molecular weight, structure and quantification of the hormones (Skoog et al., 2021). Using this methodology, there are several improvements in chromatography, using high pressures in the column to increase the analytical resolution enhancing the separation of particles (High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) or Ultra-high Pressure Liquid Chromatography-Mass Spectrometry (UPLC-MS)), or adding a tandem mass spectrometry (MS/MS) to improve the fragmentation and ionization of the particles (**Figure 23**) (Müller & Munné-Bosch, 2011; Skoog et al., 2021).

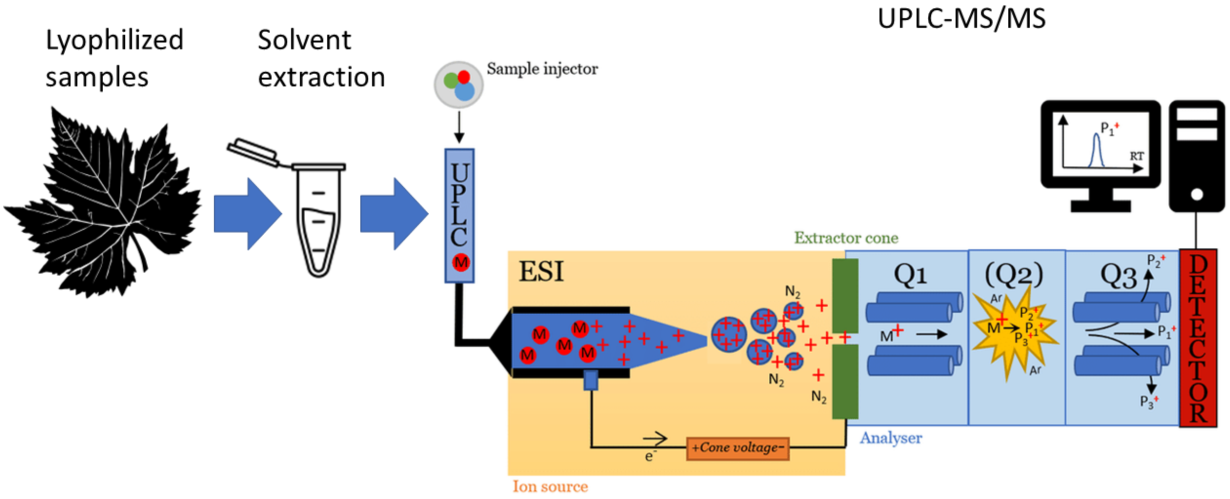

Figure 23: Workflow for hormonal analysis using UPLC-MS/MS. The basis is the lyophilization of the samples, the solvent extraction and the detection and measurement of ionized molecules (ESI) previously separated by liquid chromatography. Figure from University of Sweden.

Main objectives of the thesis

After providing a comprehensive overview of current developments and knowledge in grapevine research and omics approaches, this thesis addresses several unresolved questions. For that reason, omics techniques provide us a battery of tools to observe and answer the questions. In exploring the domestication process of grapevines, genomics studies have emphasized the significance of somatic mutations in the grapevine genotype from its origin of domestication to the present day. However, the it is necessary to explore the crucial role of epigenomics in this process, delving into how methylome modelling has influenced the domestication of grapevines throughout the History, from wild to cultivated grapevines.

Furthermore, several studies evidenced the importance of omics studies in the case of the grapevine adaptation to adverse conditions like drought, but there are not enough omics studies exploring this fact in terms of vineyard conditions, forgetting the importance of the rootstock and the cultivar scion (near-isohydric or anisohydric) in the response to drought stress. The combination of metabolomics, transcriptomics and physiological approaches could show in an integral perspective how the grapevine responds to drought stress depending on the drought tolerance of the genotype and how the scion genotype could affect to the communication scion-rootstock in order to coordinate the drought response in the plant.

The main objectives of this PhD are:

- Explore the differences in methylome modelling between wild and cultivated grapevine accessions (chapter I).
- Identify epialleles that could be associated to phenotypic traits in cultivated grapevines (chapter I).
- Describe the mechanisms that confers the drought tolerance in near-isohydric compared with anisohydric cultivated grapevines (chapter II).
- Study the role of the rootstock in the response to drought stress in the scion/rootstock system using near-isohydric and anisohydric cultivated grapevines (chapter II).
- Determine candidate genes responsible to drought tolerance (chapter II).

The main objectives will be reached along the manuscript in two different chapters (Chapter I and Chapter II).

Diagram of contents

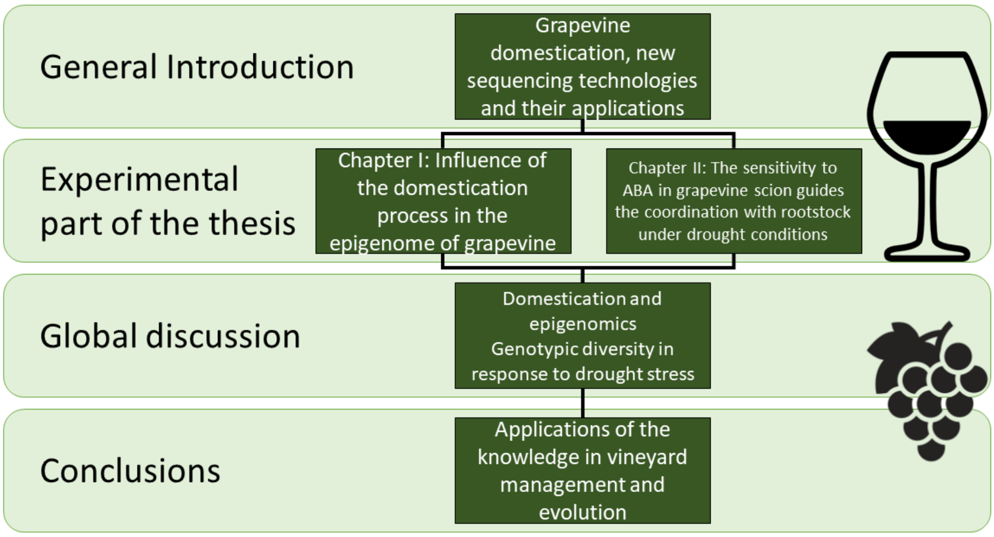

Figure 24: Conceptual design of the thesis with the global aspects of the realized studies.

- Supplementary tables 1, 2 and 3 available in electronic version (see pen-drive or electronic files).

Chapter I: Influence of the domestication process in the epigenome of grapevine

Image generated by IA. Powered by Microsoft Bing Image Creator.

Rodriguez-Izquierdo, Alberto[1]; Carrasco, David[1]; Anand, Lakshay[2]; Magnani, Roberta[2]; Catarecha, Pablo[1]; Arroyo-Garcia, Rosa[1]; Rodriguez Lopez, Carlos M.[2]

[1]Centro de Biotecnología y Genómica de Plantas (CBGP-INIA), CSIC - Universidad Politécnica de Madrid, Campus Montegancedo, Madrid, Spain.

[2]Environmental Epigenetics and Genetics Group (EEGG), Department of Horticulture, College of Agriculture, Food and environment, University of Kentucky, Lexington, KY, USA.

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Abstract

The domestication process in grapevine facilitated the fixation of desired traits. The vegetative propagation of grapevines through cuttings has allowed for easier preservation of these genotypes compared to sexual reproduction. Nonetheless, even with vegetative propagation, different phenotypes often emerge within the same vineyard due to potential genetic somatic mutations in the genome. These mutations, however, are not the sole factors influencing phenotype. Alongside somatic variations, epigenetic variation has been proposed as pivotal player in regulating phenotypic variability acquired during domestication. The emergence of these epialleles might have significantly influenced grapevine domestication over time. This study aims to investigate the impact of the domestication process on the methylation patterns in cultivated grapevines. Reduced-representation bisulphite sequencing was conducted on 18 cultivated and wild accessions. Results revealed that cultivated grapevines exhibited higher methylation levels than their wild counterparts. Differential Methylation Analysis between wild and cultivated grapevines identified a total of 9955 differentially methylated cytosines, of which 78% where hypermethylated in cultivated grapevines. Functional analysis shows that core methylated genes (those consistently methylated in wild and cultivated accessions) are associated to stress response and terpenoid/isoprenoid metabolic processes. While genes presenting differential methylation are associated with proteins targeting to the peroxisome, ethylene regulation, histone modifications, and defense response. Additionally, our findings reveal that environmentally induced DNA methylation patterns are, at least partially, guided by the region of origin of wild grapevine accessions. Collectively, our results shed light on the pivotal roles that epialleles might have played throughout the domestication history of grapevines.

Resumen

El proceso de domesticación en vid facilitó el fijado de caracteres de interés. La propagación vegetativa de vides a través de esquejes ha permitido una fácil conservación de genotipos en comparación con la reproducción sexual. Sin embargo, aún utilizando propagación vegetativa, aparecen diferentes fenotipos en el mismo viñedo debido a la aparición potencial de mutaciones somáticas en el genoma. Estas mutaciones, por el contrario, no son los únicos factores que influencian al fenotipo. Además de estas variaciones somáticas, las variaciones epigenéticas se han propuesto como posibles actores importantes en la variabilidad fenotípica adquirida durante la domesticación. El surgimiento de estos epialelos puede haber influido en la domesticación a lo largo del tiempo. Este estudio se basa en el impacto de la domesticación en los patrones de metilación de vides cultivadas. Para ello, se ha realizado un Reduce-Representation Bisulfite Sequencing sobre 18 accesiones de vides silvestres y cultivadas. Los resultados han revelado que las vides cultivadas contienen mayores niveles de metilación que las vides silvestres. El análisis de metilación diferencial ha identificado un total de 9955 citosinas diferencialmente metiladas, de las cuales 78% se han encontrado hipermetiladas en vides cultivadas. El análisis funcional de genes con posiciones diferencialmente metiladas ha mostrado que el core de genes metilados están asociados a respuesta a estrés y metabolismo de terpenos e isoprenoides, mientras que los genes que presentan metilación diferencial están asociados a marcado de proteínas para el peroxisoma, regulación de etileno, modificación de histonas y respuesta de defensa. Además, las investigaciones han definido patrones de metilación condicionados por la región de origen en vides silvestres. Los resultados, en conjunto, describen el papel importante que los epialelos han jugado durante la etapa de domesticación en vid.

Key-words: domestication, epigenomics, grapevine, epiGBS, wild, cultivated, methylation, epigenetic memory

Introduction

Domestication syndrome is a phenomenon observed in crops. This results in a suite of traits that distinguish cultivated genotypes from their wild progenitors, including changes in morphology, physiology, and phenology that make them more amenable to cultivation. Thanks to the abundance of archaeobotanical, ecological and genetic information available for a handful of economically important seed propagated crops, the domestication syndrome has been well-documented in these species (Denham et al., 2020; Meyer et al., 2012). However, less is known about the domestication trajectories of vegetatively propagated crops (Denham et al., 2020). One of the main advantages of vegetative propagation is that it allows for the preservation of desirable traits from one generation to the next. This is because when a plant is propagated vegetatively, the offspring is genetically identical to the parent plant (Denham et al., 2020; Ku et al., 2020; Raj et al., 2011). This means that desirable traits such as disease resistance, yield, and flavor can be maintained over many generations. This contrasts with sexual reproduction, where traits can be lost or diluted through the process of genetic recombination. The type of propagation used during domestication can result in diametrically opposed domestication syndromes. For example, while the use of vegetative propagation has been shown to negatively affect the capacity for sexual reproduction via the accumulation of mutations in genes associated to flower development, self-fertilization, and seed development, which lead to the production of self-fertilized fruits, flowering asynchrony, and of less viable seeds (Denham et al., 2020; McKey et al., 2010; Tesfamicael et al., 2020), crops domesticated by sexual reproduction, tend to present larger seeds, synchronic flowering and pollinator dependent fertilization (Denham et al., 2020). *Vitis vinifera* is a perennial woody liana belonging to the Vitaceae family. The species is divided into two different forms principally based on the sexuality of the plant and whether it is a cultivated or a wild form. Wild grapevines (*V. vinifera* ssp. *sylvestris*), are commonly dioecious plants (Arroyo-Garcia et al., 2006), and are naturally distributed across Asia and Europe. Cultivated grapevines (*V. vinifera* ssp. *vinifera*) mainly produce hermaphrodites flowers, and are broadly cultivated across the world, both for grape production to be consumed as a fruit, and for winemaking, grape juice or other derived products (Terral et al., 2010).

Although viticulture started at the Paleolithic age as a food source in Europe from wild accessions (Barker, 1997), there are evidences that the use of grapes by humans to produce wine started near to the seventh millennium BC (McGovern, Glusker, et al., 1996), conditioning the domestication process in grapevine by selecting grapevine varieties producing a particular fruit quality and larger berries (Arroyo-Garcia et al., 2006; Terral et al., 2010). It is believed that such selection occurred using vegetative propagation by cuttings to enhance the preservation of phenotypes of interest (Arroyo-Garcia et al., 2006; Levadoux, 1956; Terral et al., 2010), which in turn had a negative effect on the crop tolerance to biotic and abiotic stresses. For example, populations of wild grapevines in North Africa and coastal regions of Northern Spain shown better adaptation to salt stress than cultivated grapevines (Askri et al., 2012; Carrasco et al., 2022), while wild accessions from Germany, Iran and Georgia show higher resistance to mildew infections (Bitsadze et al., 2015; Duan et al., 2015; Riaz et al., 2013; Toffolatti et al., 2016). Moreover, despite the use of vegetative reproduction to maintain a desired genotype, the use of asexual reproduction in grapevine has resulted in novel phenotypes appearing within the same variety (McKey et al., 2010) and same vineyard (This et al., 2006). Such phenotypic variants are frequently found in vegetatively propagated crops and often make up a significant portion of the cultivated varieties. Although a genetic basis is often presumed to be the reason for the noticeable differences in traits observed, epigenetic modifications have also been proposed to play an important role (Fortes & Gallusci, 2017; Noshay & Springer, 2021; Rodríguez López & Wilkinson, 2015; Xie et al., 2017).

Epigenetic modifications are potentially heritable changes in gene expression and function that give rise to a certain phenotype without changes to their underlying DNA sequence (C. -t. Wu & Morris, 2001). The most studied type of epigenetic modification is DNA methylation, defined here as the addition of a methyl group to the carbon 5 of cytosines (Kumar & Mohapatra, 2021). DNA methylation can be transient and can change rapidly during the life span of a cell or organism, or it can be essentially permanent once set early in the development of the embryo. Moreover, recent research has shown that DNA methylation epialleles can be used as an epimutation clock to enable the phylogenetic reconstruction of the recent history of vegetatively propagated plants (Yao et al., 2023), highlighting their heritability and potential contribution to plant diversification.

Several studies suggest that DNA methylation might have played a role in plant domestication. This was first made evident in studies analyzing the effect of polyploidy on DNA methylation in hybrid plant species (Jackson, 2017), including crops such as wheat (Yuan et al., 2020) and cotton (Song et al., 2017). For instance, in hexaploid wheat, the removal of the D sub-genome leads to a genome-wide reduction in DNA methylation. A reduction that is reversed in the resynthesized hexaploid wheat (Yuan et al., 2020). More recently, detailed analysis of DNA methylation in rice (S. Cao et al., 2023) and tomato (Guo et al., 2023) has shown that domesticated cultivars present lower levels of DNA methylation than their wild counterparts. Moreover, multiple studies have shown that differentially methylated regions associated to domestication overlap with genes linked to traits known to be under selection during domestication of soybean (Shen et al., 2018), tomato (Guo et al., 2023), maize (Xu et al., 2020), and cotton (Song et al., 2017). However, our grasp of how these epigenetic modifications might have been utilized or unintentionally modified during the domestication process is currently at an early stage. Particularly, the impact of domestication on DNA methylation is seldom studied in perennial crops (Tan & Rodríguez López, 2023).

In this study, we use reduced representation bisulfite sequencing to characterize and compare the methylome of wild and cultivated grapevine accessions grown under common garden conditions, to explore if the domestication process has affected to methylome modelling in grapevine. We hypothesize that the combination of phenotype selection and vegetative propagation during grapevine’s domestication has resulted in methylomes characteristics of cultivated grapevines which are significantly different from those found in wild accessions (e.g., higher levels of DNA methylation). Moreover, we speculate that the epialleles observed in cultivated accessions could be associated to phenotypic traits traditionally associated to domesticated crops.

Materials and Methods

Experimental design

Single ortets from 10 *V. vinifera* ssp. *vinifera* cultivars (Albillo Mayor, Allaren, Bocalilla, Brujidera, Espadeiro, Graciano, Heben, Jaen, Marfal and Zalema) and 8 *V. vinifera* ssp. *sylvestris* accessions (CA2.9b, CA4.1, CA5.1, H7.8, O1.5, S1.7, SE3.4 and VI3.4) kept in a in vivo grapevine germplasm bank located at IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario, Alcalá de Henares, Madrid, Spain), were used to generated triplicate ramets from dormant wood cuttings. All ortets were generated from material originally collected from different locations in continental Spain (see **Annex 2 Table 1** for more information). Accession unique identifiers (**Annex 2 Table 1**) are denoted by an alphanumeric code e.g. ESP080-BGVCAMXXXX, where XXXX indicates a number unique to each accession. All plants were originally identified by Dr.

Alejandro Benito Barba. Cuttings were collected in winter, January 2021, at dormancy stage, from ortets planted on the same parcel. Cuttings were disinfected with tebuconazole and treated with rooting hormone (indole-butiric acid (IBA) 5 g/L), and then potted in individual containers (1.6 L truncated conic pots with drain sink) filled with potting mix 70% peat / 20% perlite / 10% sand. All propagules were then placed under the same conditions (light 16h 21º C - dark 8 h 16º C) in a single growth chamber, with all the cuttings distributed randomly along the growth chamber. After budbreak, the second and third fully open leaves were collected and immediately snap-frozen using liquid nitrogen and preserve at -80º C until DNA extractions.

DNA extraction and epiGBS protocol

Total DNA (gDNA) extraction from all the samples was performed utilizing the QIAGEN DNEasy Plant Mini Kit, following the instructions provided by the manufacturer, Qiagen N. V., Hilden, Germany. The DNA concentrations for the samples were assessed using the Fragment Analyzer High Sensitivity DNA kit from Agilent. To standardize the sample concentrations, the DNA was adjusted to a uniform concentration of 10 ng/µl. This standardization process ensured uniformity across the samples, enabling consistent and comparable analyses and experiments involving the genomic DNA extracted from the grapevine samples.

The preparation of Reduced Representation Bisulfite Sequencing (RRBS) libraries for all the samples was carried out following the epiGBS2 protocol (Gawehns et al., 2022; van Gurp et al., 2016) by digesting 100 ng of gDNA with restriction enzymes *NsiI* and *Csp6I* (New England Biolabs, UK). Individually barcoded hemimethylated adapters designed for the resulting restriction sites were ligated to the products of these restrictions. Subsequently, the ligated products adapters-DNA strands were amplified via PCR. Each individual library generated from the samples was combined in equimolar amounts, resulting in the creation of two libraries. Then, the built libraries were sequenced using Illumina HiSeq 2500 generating 150bp paired-end reads for each one. The sequencing procedure was conducted by NovoGene USA.

Bioinformatic analysis

All bioinformatics tools included below used their default parameters unless specific parameters are presented. The sequencing library's quality was assessed using FastQC v0.11.8 (Andrews et al., 2012) to ensure data reliability. To tailor the epiGBS workflow (Gawehns et al., 2022; van Gurp et al., 2016; Werner et al., 2020) to our specific dataset, a customized workflow was developed. Initially, demultiplexing was carried out to identify and structure the adapters, facilitating the identification of individual samples (van Gurp et al., 2016). After that, a fastq-filter was applied using Stacks v2.55 (Catchen et al., 2013) to process the demultiplexed sequences. The triplicate sequences from each accession were then merged, and the paired-end sequences were merged using PEAR v0.9.6 (J. Zhang et al., 2014).

For alignment and methylation calling, Bismark v0.23.0 (Krueger & Andrews, 2011) was employed, using the *Vitis vinifera* L. PN40024 v4.1 reference genome. This step allowed for the determination of sequencing depth, coverage, and the identification of methylation differences between the wild and cultivated accessions. To visualize these aspects, ChromoMap R v1.0.0 (Anand & Rodriguez Lopez, 2022) was utilized, providing a comprehensive visualization of the sequencing depth, coverage, and methylation distinctions between the wild and cultivated grapevine accessions. This suite of analyses and visualizations facilitated a detailed understanding of the methylation landscape and differences between the studied grapevine samples.

An analysis of global DNA methylation differences utilizing hierarchical clustering and Principal Component Analysis (PCA) was performed on the cytosine methylation results for all the samples. This analysis was conducted based on the calculated percentage of methylation found in all methylated cytosines present in at least four of the accessions. Following this analysis, the percentage of methylation for each methylated cytosine positions was compared between the cultivated and wild accessions across different methylation contexts (CG, CHG, and CHH, where H = A, T, or C). Statistical significance was assessed using a T-test, after testing for normality in the data using Kolmogorov-Smirnoff Test, considering significant differences between them when the p-value < 0.01. To reduce the effect of genetic mutations on differential methylation data, for a genomic location to be included in the differential methylation analysis, such location must have a cytosine in a minimum of four samples per group and the location must have been sequenced to a minimum coverage of 10X. Additionally, a second more stringent filtering was implemented by identifying all genomic locations containing a SNP using the epiDiverse - SNP pipeline (available at "https://github.com/EpiDiverse/SNP"). Then, all epialleles located in genomic locations containing a SNP were removed from the analysis and hierarchical clustering was performed using all remaining epialleles.

To determine if DNA methylation patterns associated to the geographic origin of wild accessions were present, we performed a comparative analysis following the premises of De Andrés et. al, (2012). For this, the methylation information gathered from wild accessions was filtered for epialleles associated to single nucleotide polymorphism as described above. Both the remaining epialleles and the SNPs identified using epiDiverse - SNP pipeline were used for hierarchical clustering analysis.

Protein coding genes presenting at least one methylated cytosine within 1000 bp of the transcription start site were deemed methylated. The annotated genome PN40024 v4.1 was used to determine the genic location (promoter, intro, exon) of methylated cytosines identified within genes. Genes that exhibited at least one methylated cytosine were considered methylated. Then methylated genes were divided into 6 groups based on the type of methylation observed: 1. Core methylated genes, i.e., genes presenting unchanged methylated cytosines both in wild and cultivated accessions (CMCs); 2. Genes presenting CMCs and hypermethylated dfferentially methylated cytosines (DMCs) in cultivated compared to wild accessions; 3. Genes presenting CMCs and hypomethylated DMCs in cultivated compared to wild accessions; 4. genes presenting CMCs and both hypomethylated and hypermethylated DMCs; 5. Genes presenting hypermethylated DMCs in cultivated compared to wild accessions; and 6. Genes presenting hypomethylated DMCs in cultivated compared to wild accessions. As above, DMCs associate with a SNP were removed from the analysis using the epiDiverse - SNP pipeline.

The Gene Ontology (GO) analysis was performed utilizing the GOstats package (Falcon & Gentleman, 2007) and the rrvgo package (Sayols, 2020) in R environment. This analysis was conducted separately for each of the defined groups, considering all sequenced and identified genes in the reference genome as the gene universe. The genes included in this analysis met the criterion of having at least one read overlapping within a window of 1000 base pairs before and after the 5′ and 3′ untranslated regions (UTRs) respectively. To interpret and visualize the primary GO terms associated with each group, the QuickGO Browser (Binns et al., 2009), based on GO version 2023-09-20, was utilized. The QuickGO Browser provided the means to generate ancestor charts for the predominant GO terms identified within each specific methylation group. These ancestor charts served as visual representations, offering insights into the enriched biological processes, molecular functions, and cellular components related to the genes within each particular methylation category.

Results

Differences in global levels of DNA methylation between wild and domesticated grapevine genotypes

The EpiGBS2 libraries produced a total of 44.5 million reads, averaging approximately 2.5 million reads per sample. The number of reads per sample were different, ranging from 1,106,659 to 8,249,031 reads. Regarding bisulfite conversion efficiency, an average of 90% of unmethylated cytosines were successfully converted to uracils. After de-multiplexing, the average percentage of mappable reads per sample was 49%, with individual samples ranging from 37% to 60%. Consequently, an average genome coverage of 1.5% was achieved, with coverage percentages spanning between 0.7% and 2.6%. This information is detailed in **Table 1**. The distribution of reads across the entire genome was relatively even, as illustrated by the read distribution across chromosome 17 (**Figure 25 A**).

ID	Ssp	Region	Number of Sequencies	Coverage (%)	Number of Usable Reads	Usable reads (%)	Mapping efficiency (%) – *M. rotundifolia* Hap 1	Mapping efficiency (%) – *M. rotundifolia* Hap 2	Aligned Reads (%) - PN
CA2.9b	*sylvestris*	Cádiz	4971432	1.4	2365077	47.6	13.3	13.2	51.7
CA4.1	*sylvestris*	Cádiz	1876093	1.5	817208	43.6	13.6	13.1	47.2
CA5.1	*sylvestris*	Cádiz	3579268	1.6	1630155	45.5	13.7	13.2	51.5
H7.8	*sylvestris*	Huelva	2341797	1.5	1051627	44.9	13.2	12.2	49.6
O1.5	*sylvestris*	Asturias	1106659	1.1	419029	37.9	13.0	12.1	49.6
S1.7	*sylvestris*	Cantabria	1132912	1.1	441593	39.0	14.2	12.8	48.7
SE3.4	*sylvestris*	Sevilla	1400331	1.2	513381	36.7	13.2	12.5	50.6
VI3.4	*sylvestris*	Álava	1210403	1.1	542023	44.8	13.2	12.0	49.6
ALBILLO_MAYOR	*vinifera*	Palencia	1366243	1.6	771275	56.5	13.3	12.8	51.3
ALLAREN	*vinifera*	León	8249031	0.7	4869645	59.0	13.7	13.3	52.4
BOCALILLA	*vinifera*	Madrid	3127912	2.2	1744321	55.8	13.7	13.0	52.5
BRUJIDERA	*vinifera*	Toledo	2047406	1.9	1022147	49.9	13.5	12.4	51.1
ESPADEIRO	*vinifera*	Pontevedra	5445367	2.6	2981903	54.8	14.0	13.5	53.6
GRACIANO	*vinifera*	Navarra	1177539	1.3	609295	51.7	13.6	12.3	50.2
HEBEN	*vinifera*	Guadalajara	1608918	1.7	814404	50.6	13.9	12.8	51.3
JAEN_TINTO	*vinifera*	Granada	1150468	1.3	589110	51.2	13.5	12.5	51.6
MARFAL	*vinifera*	Cáceres	1200311	2.4	727288	60.6	13.6	12.9	50.3
ZALEMA	*vinifera*	Huelva	1540624	1.7	779848	50.6	13.3	12.4	50.2

Table 1: Sequencing stats and coverage results from RRBS protocol, and mapping efficiency using two different genomes, PN (Pinot Noir), and *Muscadinia rotundifolia* genome splitting by haplotypes.

The methylation calling analysis revealed that the CG context exhibited the highest level of cytosine methylation, followed by the CHG and CHH contexts (as depicted in **Figure 25 b)**) in cultivated and wild grapevines. Notably, cultivated varieties (CV) consistently displayed significantly higher levels of DNA methylation compared to wild type accessions (WT) across all sequence contexts (determined using the T-student Test with a p-value < 0.01) as shown in **Figure 25 b)**. The performed Principal Component Analysis (PCA) plots using the percentage of methylation for all sequenced cytosines demonstrated that wild and cultivated accessions formed distinct groups depending on the percentage of cytosine methylation overall. These groups were primarily separated by PC1 across all sequence contexts, as illustrated in **Figure 25 c) to e)**. The observed separation between wild and cultivated accessions was particularly prominent for the CHH context, emphasizing a distinctive methylation pattern between these two subspecies (**Figure 25 e)**).

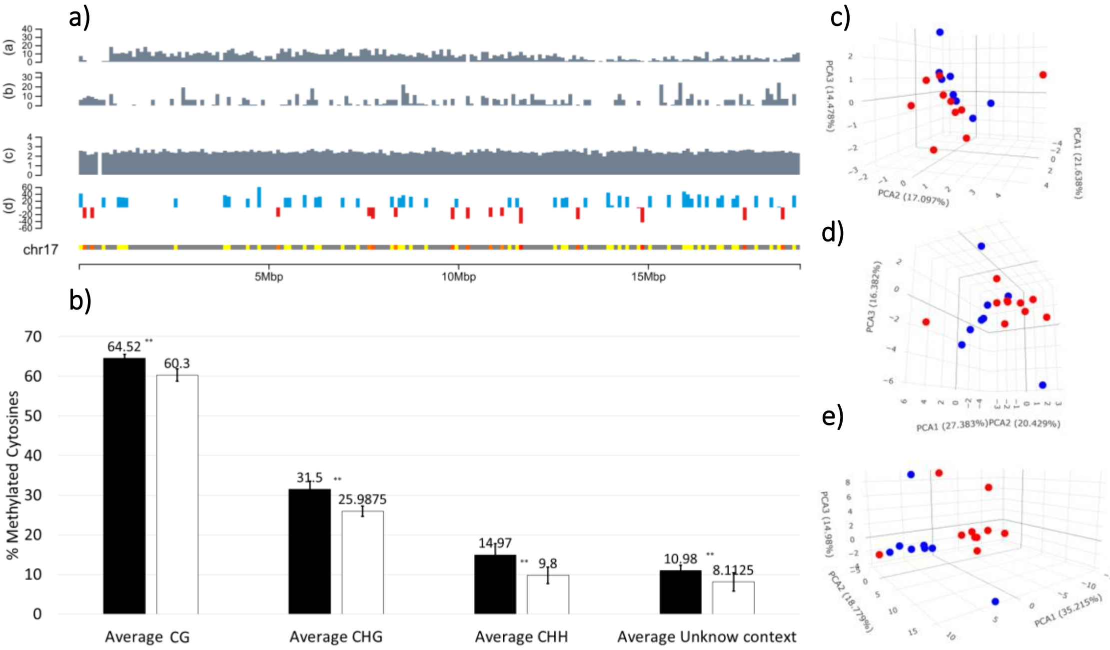

Figure 25: Analysis of differences in global levels of DNA methylation in cultivated and wild V. vinifera accessions.

a) Visualization of genomic and epigenomic information for chromosome 17 of *Vitis vinifera using* 100,000 base pair windows. Vertical bars in panels (a) and (b) show the number of protein coding genes and transposable elements respectively per genomic window. Bars in panel (c) shows average sequencing depth per genomic window (Log 10 of calculated depth for sequenced bases). Panel (d) shows the average fold change in methylation in given window (blue and red bars indicate an average hypermethylated or hypomethylated window in cultivated vs wild accessions. For an interactive version of the figure containing all chromosomes see **Supplementary File 1.** Panels generated using ChromoMap R. **b)** Bars show the average percentage of methylation per sequence context (CG, CHG, CHH, and unknown) in cultivated (*V. vinifera ssp vinifera* (n = 10); black bars), and wild type (*V. vinifera* ssp *sylvestris* (n = 8); white bars) accessions. Error bars indicate the calculated Standard Deviation. ** T-student Test, p-value < 0.01. **c)-e)** Multivariate analysis of percentage of methylation for all individual cytosine sequenced in cultivated and wild *V. vinifera* accessions. Principal Component analysis plots show results for methylation analysis results in the CG (C), CHG (D), and CHH (E) contexts. Blue and red circles represent cultivated and wild accessions respectively. PCs 1 to 3 represent 53, 64, 69% of the total measured variability in CG, CHG, and CHH contexts respectively.

Identification of differentially methylated cytosines (DMCs) associated to domestication

The differential methylation analysis identified a total of 9955 DMCs (Differentially Methylated Regions) between wild and cultivated grapevine accessions, and these regions were evenly distributed across the genome (as depicted in **Figure 26 a)**). Among these, 7793 DMCs exhibited hypermethylation, while 2162 DMCs displayed hypomethylation in cultivated compared to wild grapevine accessions. A notable observation was that the majority of both hypermethylated and hypomethylated DMCs were situated in the CHH context, constituting 77% and 69%, respectively (as shown in **Figure 26 a)**). Regarding their distribution concerning genic features, the DMCs were predominantly found out in intergenic regions (as shown in **Figure 26 b)**). This trend was particularly eye-catching in the CHH context, where 56% of hypermethylated and 60% of hypomethylated DMCs were situated in intergenic regions. The second most striking genic feature presenting DMCs were the introns, where the percentages varies between 24% and 35% in hypermethylated DMCs and between 28% and 32% in hypomethylated DMCs, depending on the methylation context (as illustrated in **Figure 26 b)**).

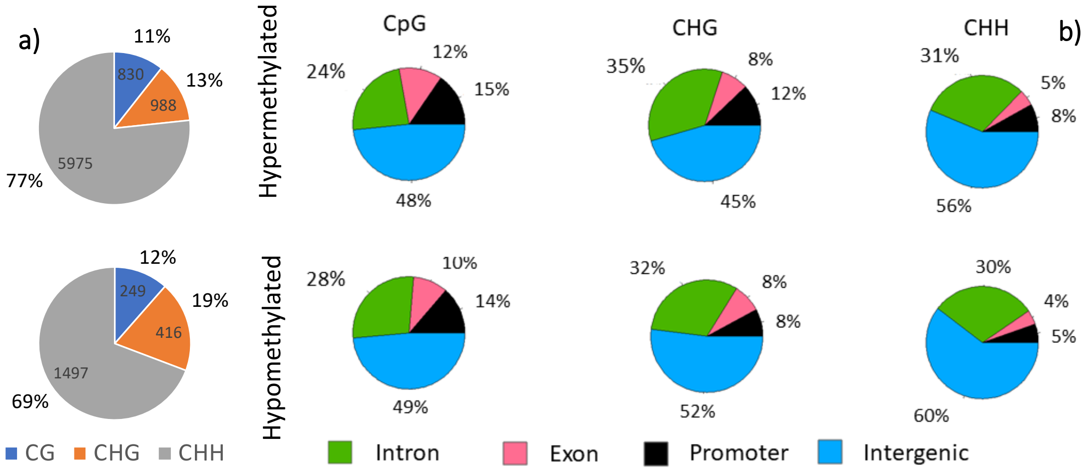

Figure 26: Identification of DMCs associated to grapevine's domestication. Pie charts show **a)** the total number and percentage of hypermethylated (top pie chart) and hypomethylated DMCs identified in cultivated vines compared to wild accessions in each sequence context (CG, CHG and CHH); and **b)** the percentage of DMCs identified per genic feature and sequence context, in cultivated compared to wild type accessions.

Effect of genetic differences to epigenetic differentiation between wild and cultivated accessions

The EpiDiverse-SNP pipeline identified 57,489 SNPs in the 222,711 genomic locations containing methylated cytosines. Of the remaining 165,189 genomic locations containing methylated cytosines, 5869 DMCs were hypermethylated and 1575 DMCs were hypomethylated in cultivated vines compared to wild accessions (i.e., 25% of the original DMCs were associated to a SNP). Hierarchical clustering analysis using all epialleles and only those not associated to SNPs showed no significant clustering differences (**Annex 2 Figure 1**).

Analysis of (epi)genetic signals of provenance in wild type accessions

We then compared wild accessions to determine if a genetic and or epigenetic signal associated to the location from where they were originally collected exist. Hierarchical cluster analysis showed no clear epigenetic signal irrespective of the use of all epialleles sequenced or after removing epialleles associated to a SNP (**Figure 27 a)**). However, when only genetic information was used (i.e., clustering samples using the SNPs identified by the EpiDiverse-SNP pipeline, two separate clusters of wild accessions grouped by their provenance. One cluster contained all three accessions originally collected in the North of the Iberian Peninsula, in oceanic, continental and mountain climatic zones, while the second cluster contained all accession collected from the South of the Iberian Peninsula (Mediterranean climatic zone) (**Figure 27 b)**) (see in **Annex 2 Tables 1 and 2** for metadata associated to each accession).

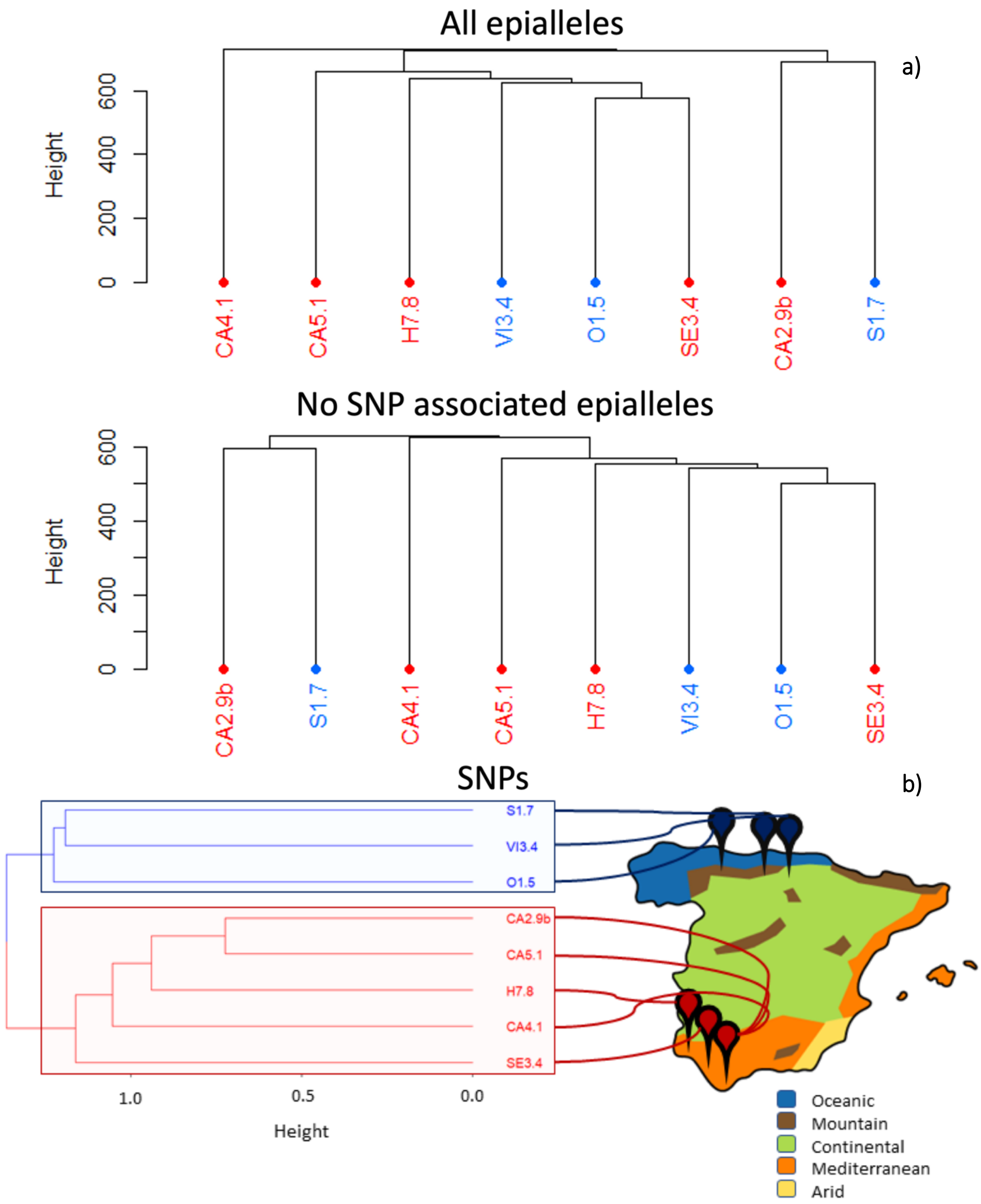

Figure 27: Analysis of genetic (**a)**) and epigenetic (**b)**) differences between wild grapevine accessions grown under a common garden experiment but originally collected from different regions of the Iberian Peninsula. Epigenetic analysis was performed using epialleles not associated to SNPs the epiDiverse-SNP pipeline to remove the effect of underlying genetic variation between wild grapevine populations. Samples highlighted in red, the represented branches correspond to wild accessions belonging to the South of Spain, and in blue, they correspond to wild accessions coming from the North of Spain, placed approximately to the map of the Spanish Climate Zones.

Analysis of domestication associated DMCs within genic features

The epiGBS2 analysis generated reads overlapping with a total of 7174 genes. Among these genes, 2854 (40%) were identified as genes containing at least one methylated cytosine (refer to **Supplementary Table 1 Table 1A**). The distribution of methylated cytosines was primarily observed in introns (66-80%), followed by exons (15-20%), and promoters (4-14%) as depicted in **Figure 28** and detailed in **Supplementary Table 1 Table 1B**. Genes containing methylated cytosines could be further divided into four groups, in order of abundance, 1. Genes presenting methylated cytosines both in wild and cultivated accessions (1883 genes) (core methylated genes (CMCs) hereafter); 2. Genes presenting CMCs and hypermethylated DMCs in cultivated compared to wild accessions (564 genes); 3. Genes presenting CMCs and hypomethylated DMCs in cultivated compared to wild accessions (252 genes); 4. genes presenting CMCs and both hypomethylated and hypermethylated DMCs (116 genes); 5. Genes presenting hypermethylated DMCs in cultivated compared to wild accessions (28 genes); and 6. Genes presenting hypomethylated DMCs in cultivated compared to wild accessions (11 genes).

Functional analysis of genes within each group revealed distinct associations: CMCs were significantly linked to the regulation of cellular response to stress and isoprenoid/terpenoid processes. Hypermethylated genes in cultivated grapevines were mainly associated with processes involved in protein targeting to peroxisomes and histone lysine demethylation, whereas hypermethylated genes in wild grapevines were related to ethylene regulation processes and response to ozone. Additionally, the remaining group (genes exhibiting both hyper and hypomethylated cytosines) presented GO terms related to defense response (**Figure 28** and **Supplementary Table 1 table 1C** for a comprehensive list of GO terms in each group).

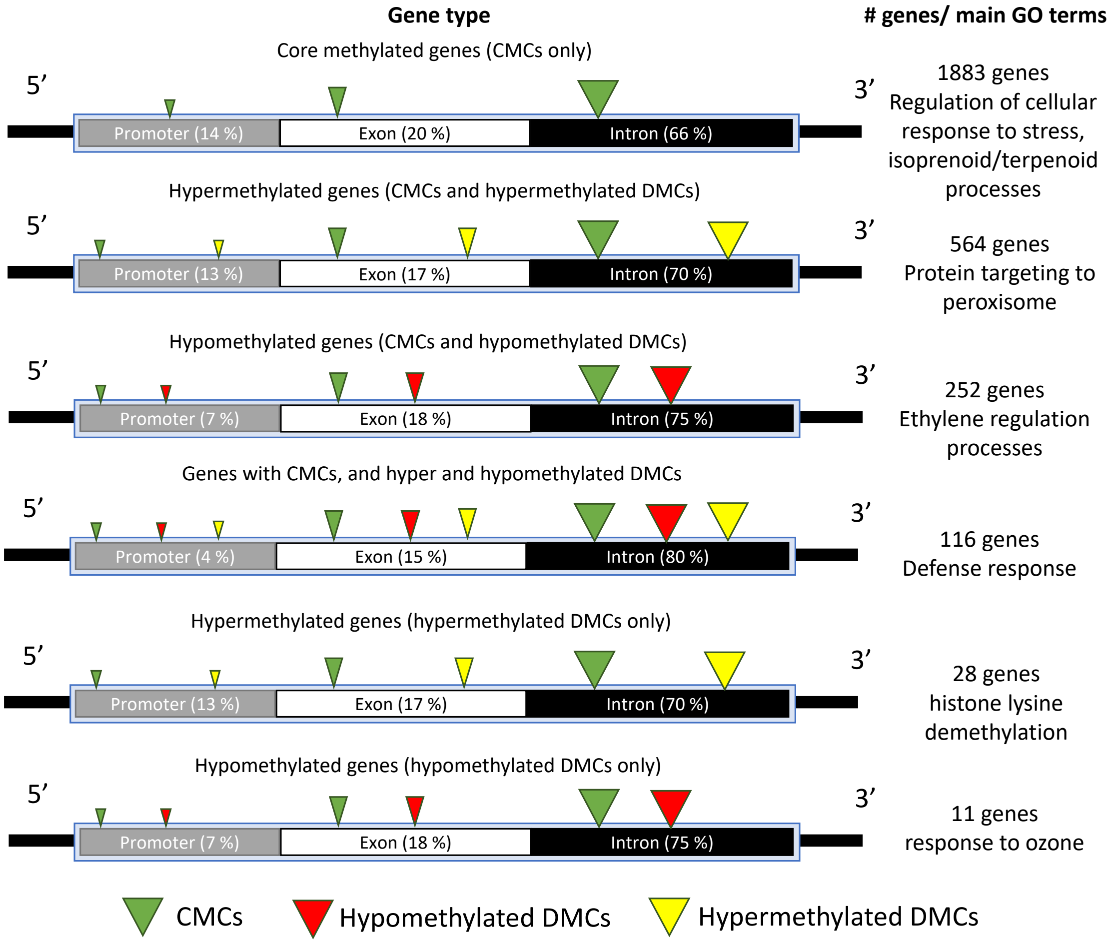

Figure 28: Schematic representation of methylated gene types in wild and cultivated grapevines. Boxes within gene models show the percentage of the total methylated cytosines in each gene group found in each genic context. Arrow heads color and size indicate the type of methylated cytosine found in each gene type (Core methylated cytosines (CMCs); Hypomethylated and hypermethylated cytosines in cultivated vs wild grapevine accessions) and the abundance of that type of methylation within that gene type, respectively. Right panel shows the number of identified genes for each group and their correspondent most representative GO terms.

Discussion

The understanding of the role of epigenetic mechanisms in crop domestication is an area that has generated interest in the last years. While substantial progress has been based in decoding the genetic aspects of grapevine domestication, the comprehension of the influence of epigenetic mechanisms in this process remains relatively limited. Epigenetics, a pivotal regulator in various biological processes across plants and animals, is becoming greatly recognized for its potential role in shaping the adaptation on the phenotypic diversity of domesticated crops. While initial studies have been oriented to the possible involvement of epigenetic changes during the domestication of crops, a comprehensive understanding of how these modifications were utilized, intentionally altered or not, during domestication is still in its early stages. Exploring the potential contribution of epigenetic mechanisms to domestication, independent of genetic changes, could offer new insights into the initial phases of domestication and the challenges faced by our predecessors. Simultaneously, such investigations hold the promise of establishing foundational frameworks that integrate plant adaptation to environmental stress through epigenetic mechanisms. This knowledge could help to develop comprehensive models aimed at cultivating novel resilient crop varieties capable of withstanding environmental stresses, as noted in studies by Bräutigam & Cronk, 2018; Consuegra & Rodríguez López, 2016; Róis et al., 2013; Tan et al., 2023. These models may significantly contribute to the development of resilient and adaptable cultivars crucial for sustaining agricultural productivity in changing environmental conditions.

Epigenetic signal of domestication is independent of genetic variation

Our results show significant epigenetic differences between cultivated and wild grapevine accessions, both at a global level and at genomic feature level. Interestingly, such global differences were maintained after removing all epialleles associated to genetic variability (considered the most abundant type of epigenetic variability (Angers et al., 2020)). Interestingly, the number of DMCs associated to genetic variation in this study (25%), was very similar to the proportion of DMRs associated to soybean domestication that could be explained by local genetic variation (22.4%) (Shen et al., 2018). Since all plants included in this study were grown under the same conditions, this suggests that the remaining epialleles could be considered true epimutations (i.e., not the result of the interaction of the epigenotype with the genotype or with the environment (Angers et al., 2020)). Such differences were particularly evident in the CHH and CG contexts. Such variations indicate that different factors might be influencing the methylation of cytosines in different contexts, potentially outlining diverse selection pressures imposed by domestication for the different sequence context. Conversely, DMCs between wild and cultivated accessions were found evenly distributed across all chromosomes, suggesting that no specific epigenomic region has been under special selection. This could be due to the combination of two factors, the reduced representation methylome sequencing of the approach used in this study, and that DNA methylation might be under weak selection during domestication as previously observed in maize (Xu et al., 2020). A more detailed analysis using whole methylome sequencing is required to validate this hypothesis in grapevine.

Multiples studies have indicated the relationship between geographic origin and genetic differences in grapevine (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2002; Daldoul et al., 2020; De Andrés et al., 2012), which would support the premise that genetic induced epigenetic differences should be observable between grapevines genotypes, independently of how long those accessions have been removed from their original source. However, it is not clear, if environmentally induced epigenetic variability is stable over time. To shed light over that question, we compared the methylome of wild grapevine accessions originally collected in different regions of the Iberian Peninsula, which have been maintained under the same growing conditions over 18 years. Although a clear genetic signal of provenance was observed in the wild accessions included here, no epigenetic differences were found between plants originally collected from populations in the North and South of the Iberian Peninsula.

Grapevine domesticated accessions present higher global levels of DNA methylation

Cultivated accessions consistently exhibit significantly higher global levels of DNA methylation across all sequence contexts and all genomic features. These observations, are in contradiction with previous studies showing that domestication induces a significant decrease in DNA methylation in rice (S. Cao et al., 2023) and tomato (Guo et al., 2023). This is perhaps explained by the differences in the type of propagation used during the domestication of each species (i.e., vegetative vs. sexual propagation), which could have resulted in diametrically opposed domestication epigenetic syndromes (Denham et al., 2020; Tesfamicael et al., 2020). In fact, the historical use of vegetative propagation in cultivated grapevines (McKey et al., 2010; Terral et al., 2010), has been shown to preserve environmentally induced epigenetic variability in vegetatively propagated perennials (Guarino et al., 2015). Having noted this, it's crucial to consider that the observed differences in DNA methylation may not solely result from domestication. Instead, they could stem from the distinct reproductive strategies (hermaphroditic in cultivated accessions versus dioecious in wild accessions) of the plants under study. Irrespective of the driver of the hypermethylation observed in cultivated/clonally propagated accessions, it is also tempting to speculate that the selection for hypermethylation during domestication, might have contributed to the appearance of genetic mutations leading to novel phenotypes, since mutation ratio is higher in methylated cytosines, as previously proposed for clone diversity in grapevines (Vondras et al., 2019).

As seen before (Shen et al., 2018), in our study a large proportion of DMCs between wild and cultivated accessions were found within intergenic regions. Previous work has suggested that intergenic epialleles might be related to the regulation of long intergenic non-coding RNAs (lincRNAs), which are highly prevalent in the intergenic regions of plant genomes and are found to regulate essential biological processes (Chekanova, 2015). It is also possible that the accumulation of methylation in intergenic regions could be related to silencing repeat elements or somatic mutations, which are a major driver of cultivated grapevine genome diversification (Guarino et al., 2015).

Genic regions consistently presented lower levels of DNA methylation in all sequence contexts than intergenic regions, which is a common feature in plant methylomes (see (G. Wang et al., 2020) for an example). Nonetheless, 40% of the genes sequenced here presented methylated cytosines. Of these, 1883 (67%) presented only CMCs, i.e., cytosines which were consistently methylated both in wild and cultivated accessions, while the remaining 33% presented CMCs and or DMCs. Of these, a large majority (73%) presented some form of hypermethylation in cultivated compared to wild accessions.

CMCs and DMCs identified within genes were preferentially found within introns, followed by promoters and exons, irrespective of the sequence context. This positional distribution of methylated cytosines around and within genes revealed different strategies in the methylation of genic features associated to the domestication process. In the context of plant promoters, methylation usually acts to repress gene transcription, thereby controlling the timing and spatial patterns of gene expression throughout development and in response to environmental stimuli (H. Zhang et al., 2018). In introns and exons, DNA methylation plays multifaceted roles. Within introns, it can influence alternative splicing, whereby different mRNA isoforms are generated from a single gene (X. Wang et al., 2016). This can enhance the plant’s adaptive capability by enabling a diverse range of proteins to be produced. In exons, DNA methylation can impact gene-body methylation which is associated with increased gene expression in certain contexts, although the exact mechanism is not fully understood (X. Wang et al., 2016). This intricate interplay between methylation and the genic landscape establishes a regulatory network that finely tunes gene expression and maintains genomic stability, underpinning the complexity and adaptability of plant life.

Domestication is associated with hypomethylation of intergenic regions and hypermethylation of genic features.

Our analysis discovered a substantial number of differentially methylated cytosines (DMCs) within intergenic regions, where wild accessions displayed more prominent hypermethylation compared to cultivated ones, consistent with findings in Shen et al., 2018. However, it's interesting to note that among the genes sequenced, a significant proportion (40%) exhibited methylated cytosines. The majority of these genes (67%) displayed consistent methylation (CMCs) in both wild and cultivated accessions, while the remaining portion exhibited CMCs and/or DMCs.

The distribution of CMCs and DMCs within genes primarily occurred within introns, followed by promoters and exons, irrespective of the sequence context. This methylation pattern in genic features reveals different epigenetic strategies associated with the domestication process. Intergenic epialleles have been previously linked to the regulation of long intergenic non-coding RNAs (lincRNAs), which often exert regulatory control over essential biological processes in plant genomes (Chekanova, 2015). The accumulation of methylation in intergenic regions might also relate to the silencing of repeat elements or somatic mutations, known to drive genomic diversification in cultivated grapevines.

Regarding promoters, methylation typically acts as a repressor of gene transcription, modulating the timing and spatial patterns of gene expression throughout plant development and in response to environmental cues (H. Zhang et al., 2018). In introns, the role of DNA methylation in influencing alternative splicing is notable, where it contributes to generating diverse mRNA isoforms from a single gene, potentially enhancing an adaptive capacity of the plant by producing a wide range of proteins (X. Wang et al., 2016). Within exons, the impact of DNA methylation into the gene-body is linked with increased gene expression in specific contexts, although the exact mechanism remains less understood (Muyle et al., 2022). This intricate interplay between methylation and the genic landscape establishes a regulatory network finely tuning gene expression, promoting genomic stability, and underscoring the complexity and adaptability of plant biology.

Gene specific differential methylation associated to domestication is enriched in response to stress

The analysis of methylated genes revealed substantial variations in DNA methylation levels associated with important agronomic traits during grapevine domestication, notably impacting stress response mechanisms. This aligns with previous research establishing a strong correlation between epigenetic mechanisms, specifically DNA methylation, and the ability of the plants to respond to stress (Konate et al., 2018). Interestingly, while genes displaying differential methylation between wild and cultivated grapevines were fewer in number, they still held significant implications. Hypermethylated genes in cultivated grapevines were linked to crucial cellular processes such as protein targeting to peroxisomes and transport. In contrast, genes hypermethylated in wild grapevines were associated with ethylene regulation processes, responses to ozone and histone lysine demethylation. Ethylene is a pivotal hormone in plants, governing various stress responses and developmental processes (H. Chen et al., 2021), and genes responsive to ethylene have been observed to undergo changes in methylation status under abiotic stress conditions (M.-E. López et al., 2022). Referring to the methylation functions, these functions are vital for maintaining cellular homeostasis and regulating epigenetic modifications, suggesting that the domestication process might have refined or enhanced these functions in cultivated grapevine varieties. Importantly, histone H3-K4 demethylation and DNA hypermethylation have been associated with gene expression repression (Sims et al., 2003). The unique group of genes exhibiting both hypermethylated and hypomethylated cytosines in both wild and cultivated accessions, despite being the smallest group, was associated with defense response mechanisms. This pattern suggests a multifaceted regulatory mechanism and indicates that some genes may have retained functionalities from their wild origins while adapting to new functions in the domesticated environment.

The association of core methylated genes (CMCs) with stress response further emphasizes the idea. The conservation of methylation in CMCs implies that the functions they support are crucial and have remained consistent between wild and cultivated grapevines. This complex and multimodal pattern of methylation during grapevine domestication indicates a sophisticated regulatory network, potentially balancing retained functionalities from wild origins and the acquisition of new functions in cultivated grapevines.

Conclusions

In summary, our research provides compelling evidence that there are significant differences in DNA methylation patterns between wild and cultivated grapevines. These differential methylation patterns between the two types of grapevine accessions offer intriguing insights into the potential origin and roles of DNA methylation in their divergence. The observed prevalence of hypermethylated DMCs in cultivated grapevines across all contexts (CG, CHG, and CHH), underscores our hypothesis that cultivated grapevines accrue more DNA methylation than their wild counterparts. The varied associations of these methylation patterns to vital processes such as stress response, hormone regulation, and defense mechanisms underscore the potential implications of epimutations in shaping the evolutionary and developmental trajectories of domesticated species. Nevertheless, since this study only included hermaphrodite flower producing cultivated accessions, further studies including dioecious cultivated accessions are required to determine if the epigenetic differences identified here are really associated with domestication or to the sexual strategy of the studied plants. Additionally, future studies should analyze complete methylomes and focus on the consequences of methylation changes on gene expression to gain a comprehensive understanding of the role of DNA methylation in grapevine domestication.

Chapter II: The diversity of grapevine as a tool to adapt the grapevine to future climate changes scenarios

Image generated by IA. Powered by Microsoft Bing Image Creator.

Rodriguez-Izquierdo, Alberto[1]; Carrasco, David[1]; Revilla, María Ángeles[2], López-Hidalgo, Cristina[2], Valledor, Luis[2], Bota, Josefina[3] & Arroyo-Garcia, Rosa[1]

[1] Centro de Biotecnología y Genómica de Plantas (CBGP-INIA), CSIC – Universidad Politécnica de Madrid, Campus Montegancedo, Madrid, Spain.

[2] Department of Organisms and Systems Biology, Institute of Biotechnology of Asturias, University of Oviedo, Oviedo, Spain.

[3] Research Group on Plant Biology under Mediterranean Conditions, Departament de Biologia, Universitat de les Illes Balears (UIB) – Agro-Environmental and Water Economics Institute (INAGEA). Carretera de Valldemossa Km 7.5, 07122 Palma, Illes Balears, Spain.

Abstract

The large diversity of grapevine cultivars englobes more tolerant genotypes to drought than others. Widely distributed cultivars, like *Merlot*, are more sensitivity to water deprivation than some local cultivars like *Callet*, more adapted to drought by their Mediterranean origin. Despite their tolerance to drought, the adaptation to drought influenced by grafting in rootstocks like *110 Richter*, is becoming key to face drought in vineyard defining the binomial scion/rootstock. To understand the different response among them, we explored these differences across transcriptomic, metabolic, hormonal and physiologic analysis at three different levels of drought (mild, high and extreme), using *110 Richter* as rootstock in both cultivars. The results revealed the sensitivity to ABA as key for the drought tolerance in the aerial part, guiding the root response accordingly. In this response to ABA, *Callet/110 Richter* develops different gene expression patterns in response to that hormone, addressed to reduce water losses compared to *Merlot*, in aerial and root parts. This modulation in *Callet/110 Richter* involves the regulation of metabolic pathways to increase cell turgor, the reduction of photosynthetic activity, and the production of molecules in response to oxidative stress, such as polyphenols or flavonoids. However, the response to drought in *Merlot/110 Richter* is unspecific in the aerial part and nearly absent in roots, revealing a lack of cross talk. Therefore, the selection of genotypes more ABA-sensitive and their communication with rootstocks is key to manage the vineyard for next scenarios caused by climate change.

Resumen

La gran diversidad de cultivares de vid incluye genotipos más tolerantes a la sequía que otros. Cultivares ampliamente distribuidos, como *Merlot*, son más sensibles a la falta de agua que algunos cultivares locales como *Callet*, más adaptados a la sequía por su origen mediterráneo. A pesar de su tolerancia a la sequía, la adaptación a la sequía influenciada por el injerto en portainjertos como el *110 Richter*, se está volviendo clave para afrontar la sequía en viñedo definiendo el binomio vástago/portainjerto. Para comprender las diferentes respuestas entre ellos, exploramos estas diferencias a través de análisis transcriptómicos, metabólicos, hormonales y fisiológicos en tres niveles diferentes de sequía (leve, moderada y extrema), utilizando *110 Richter* como portainjerto en ambos cultivares. Los resultados revelaron que la sensibilidad al ABA es clave para la tolerancia a la sequía en la parte aérea, guiando en consecuencia la respuesta de las raíces. En esta respuesta al ABA, *Callet/110 Richter* desarrolla diferentes patrones de expresión genética en respuesta a esa hormona, dirigidos a reducir las pérdidas de agua respecto a *Merlot*, en la parte aérea y radicular. Esta modulación en *Callet/110 Richter* implica la regulación de vías metabólicas para aumentar la turgencia celular, la reducción de la actividad fotosintética y la producción de moléculas en respuesta al estrés oxidativo, como polifenoles o flavonoides. Sin embargo, la respuesta a la sequía en *Merlot/110 Richter* es inespecífica en la parte aérea y casi ausente en las raíces, lo que revela una falta de diafonía. Por tanto, la selección de genotipos más sensibles a ABA y su comunicación con los portainjertos es clave para gestionar el viñedo ante próximos escenarios provocados por el cambio climático.

Key-words: drought, ABA, grapevine, RNA-seq, rootstock, scion, cross talk.

CHAPTER II: The diversity of grapevine as a tool to adapt the grapevine to future climate changes scenarios

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most cultivated plant in the world. Due to the large surface of the crop (for example, in Spain, the vineyard extension reach the 950.000 ha), grapevine has a big influence on the economy by the generation of near to 1.000-1.500 Millions of euros per year (approximately the 10% of the value of the total agriculture production) (International Organisation of Vine and Wine Intergovernmental Organisation, 2018) and on the society too, because of the landscape composition, ecosystem services and the different usages of grapevine products. Current climate change scenario is also affecting grapevine, mainly due to the increased length and severity of drought periods, decreasing the grape production and the quality of the berries (Duchêne et al., 2010; Jones and Webb, 2010; Hannah et al., 2013). Nonetheless, not all grapevine genotypes react in the same way, giving us a possible solution to reduce the damages in vineyards by the study of alternative genotypes coming from local cultivars commonly growth at regions with low water availability.

The different responses to drought in grapevines can be categorized as either near-isohydric ("pessimistic") or near-anisohydric ("optimistic"), primarily determined by stomatal behavior and physiological adaptations to water status. Near-isohydric behavior involves conserving current resources and controlling demand for future events, while anisohydric behavior uses available resources with the expectation of future replenishment (Schultz, 2003). Although this classification is somewhat imprecise (Bota et al., 2016), it provides a basic differentiation in grapevine genotypic diversity. Parameters like stomatal conductance and hydric stomatal pressure help establish varying levels of water use efficiency (WUE) based on physiological changes in grapevines (Bota et al., 2016). For example, the globally popular *Merlot* cultivar is considered an anisohydric genotype (Williams and Baeza, 2007; Shellie and Glenn, 2008), as it exhibits less stomatal closure regulation, varying them the WUE respect from other cultivars. In contrast, the local Balearic Islands cultivar *Callet*, known for its interesting wine profile (Pou et al., 2023), is considered near-isohydric due to better control of stomatal closure and water optimization (Bota et al., 2001, 2016; Florez-Sarasa et al., 2020). Drought stress triggers common responses in plants, including morphological and physiological changes to minimize damage. These changes may include reduced aerial growth, decreased evapotranspiration to limit water loss, and lowered photosynthesis rates due to potential damage from Reactive Oxygen Species (ROS) generated during oxidative stress under drought conditions (Apel and Hirt, 2004; Cramer et al., 2007; Kar, 2011; Tillett et al., 2011). However, not all grapevine genotypes respond uniformly to drought stress. Exploring variations in physiological changes within the grapevine genetic pool can enhance grapevine adaptation to climate change effects.

The physiological and morphological changes driven by water deprivation in gravepine are linked to specific metabolism and phytohormone production. Drought alters hormonal rates in response to stress, involving changes in abscisic acid (ABA), jasmonic acid (Jas), auxins or gibberellins (Gas), being an increase in ABA the earliest response of the plant under abiotic stress (Fujita et al., 2006; Peleg and Blumwald, 2011; Corso et al., 2015; Dal Santo et al., 2016). These hormonal changes are related to the regulation of sucrose transport to the phloem or/and the regulation of polyphenol and osmolyte biosynthesis (Murcia et al., 2017). The ensemble of all these responses can help identify differences in metabolite and hormone reactions, aiding the selection of grapevine genotypes better suited to face climate change challenges.

Most of the responses mentioned above are medium and long termed, and consequence of changes in gene expression. Previous transcriptome analyses revealed how grapevine respond to drought at transcriptomic level (Corso et al., 2015; Dal Santo et al., 2016). Further studies showed different strategies and reactions to different biotic and abiotic stresses at transcript level (Dal Santo et al., 2016; Cochetel et al., 2017; Haider et al., 2017; Gautier et al., 2020; Carrasco et al., 2022) which pointed to *BURPs* family, *MYBs* or genes related to the phenylpropanoids pathway, as biomarkers of drought stress in *Vitis vinifera* L. (Matus et al., 2008, 2014; Wu et al., 2014; Dal Santo et al., 2016; Orduña et al., 2022).
The use of specific rootstocks, as it was previously employed for inducing resistance to Phylloxera, may prevent further damages caused by drought periods or increased salinity (Cochetel et al., 2017; Zhou-Tsang et al., 2021; Carrasco et al., 2022). However, a few quantities of studies go through the possible interaction scion-rootstocks under abiotic stresses. Some studies worked on the possible gene expression modification using RNA-seq technology in roots in front of phosphate or nitrogen availability under different scions, revealing possible influences in rootstock by scion (Cochetel et al., 2017; Gautier et al., 2020). Furthermore, the cross talk between rootstock and scion is guided by some factors like hormones or miRNAs (Cookson and Ollat, 2013; Gautier et al., 2020). However, and despite its importance in current climate change scenario, the interaction between the cultivar and rootstock during drought stress at physiological, metabolic and transcriptional levels is unknown.

Consequently, a comprehensive vision of the changes in different cultivars classified as near-isohydric and anisohydric grapevines, considering the potential effects given by the rootstock, would provide us major facilities to select and improve vineyard management by the use of genotypes more tolerant to low water availability. This general point of view needs to know the changes at physiological, metabolic and transcriptomic level, that should contribute to better understand the differences among isohydric and near-isohydric plants. The application of these methodologies allows the study of transcription factors such as MYB or BURP, which are key for drought tolerance and highlight the importance of ABA in the response to water deprivation in grapevine. Furthermore, the exploration through the relationship between scion and rootstock under drought stress is critical. The principal aim of this study is to understand the differences between near-isohydric cultivars like Callet, and near-anisohydric cultivars like Merlot, grafted in rootstocks, at physiological, metabolic, hormonal and transcriptomic levels under drought stress. Indeed, this study also aims to explore their relationship scion/rootstock under water scarcity.

Materials and methods

Plant material, growth conditions and treatments

The experiment was performed on two different years (2020 and 2022), using two different grapevine cultivars: *Callet*, a local cultivar from Balearic Island, characterized as near-isohydric, and *Merlot*, a commercial anisohydric cultivar coming from France. A total of 144 plants (72 plants/year) of two-years old grafted on *110 Richter* rootstocks for each cultivar cited below were used in the performed experiment. These plants (*Callet/110 Richter* and *Merlot/110 Richter*) were growth under greenhouse conditions (16h light - 8h darkness, with temperature intervals of 16-25ºC) using cylindric pots with 21.5 cm diameter and 20.5 cm height, with drain sink and a substrate mix of 70% peat – 30% sand. For each cultivar, the plants were guided in order to have only one shoot. Drought stress was imposed stopping watering. Three different stress levels were defined considering the hydric potential of stem (Ψ_{stem}): Mild (-0.6 MPa $\leq \Psi_{stem} > -0.9$ MPa), High (-0.9 MPa $\leq \Psi_{stem} > -1.4$MPa) and Extreme ($\Psi_{stem} \leq -1.4$ MPa) (Van Leeuwen et al., 2009). Nine plants at each condition and cultivar were sampled and compared to controls (with irrigation, $\Psi_{stem} > -0.6$ MPa). For the determination of each stage, Ψ_{stem} were determined by Scholander chamber (Model 1009, PMS Instruments, Oregon, USA) as in Bota et al. (2016) using the 6-7th last leaf. The 4 last leaves at the end of the branch and a root portion for each year/condition/control were sampled, flash-frozen with liquid nitrogen and stored at -80º C until use (**Figure 29**).

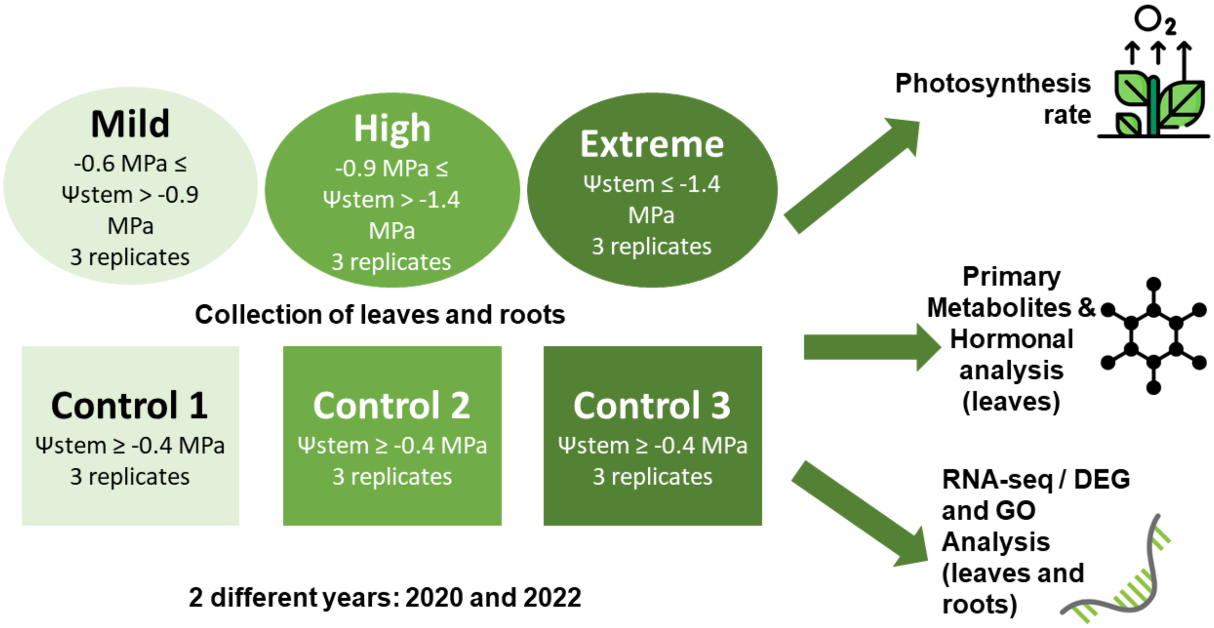

Figure 29: Experimental design on greenhouse conditions at three different drought levels depending on the hydric potential of leaves (Mild: -0.6 MPa ≤ Ψstem > -0.9 MPa; High: -0.9 MPa ≤ Ψstem > -1.4 MPa; Extreme: Ψstem ≤ -1.4 MPa) on two-year study (2020 and 2022).

Physiological Measurements of the plants

Gas exchange measurements, net photosynthetic rate, and stomatal conductance were measured in the 5th leaf from the apex of the shoot using an open gas-exchange system LI-COR 6400 XL (LI-COR Biosciences Inc., USA), under 390 ppm CO_2 concentration and 1000 μE light intensity. The measures were expressed as μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ for the CO_2 fixation units and mol $H_2O \cdot m^{-2} \cdot s^{-}1$ for the stomatal conductance. Provided values averaged the measurements of 5 plants. The intrinsic Water Use Efficiency index (WUEi) was calculated using the ratio between net photosynthesis and the stomatal conductance of the plants and it was expressed as μmol $CO_2 \cdot mol\ H_2O^{-1}$.

Additionally, in order to describe the physiological state for all the plants corresponding to these drought states, a metabolic biomarkers profile was performed in the second year (2022) to explore the differences in response to drought stress in grapevine following the procedure described by (López-Hidalgo et al., 2021) and starting from 10 mg of lyophilized leaves samples.

Primary metabolites and hormonal levels analysis

Regarding to the metabolism study of the response to drought stress in scions *Callet* and *Merlot,* two different analyses were performed. The first analysis was performed on the samples coming from 2020, in order to study the primary metabolites content in *Callet* and *Merlot* leaves. For that, three replicates of leave samples were lyophilized and a primary metabolite analysis was done following the extraction protocol of Choi et al. (2004) coming from 50 mg of dried sample and using 1H-Nuclear Magnetic Resonance (1H-NMR) thanks to the analytical service of CEBAS-CSIC (located at Murcia, Spain). A total of 24 principal metabolites were considered in the primary metabolite analysis (see complete list in **Annex 1 Tables 1 and 2**). Additionally, hormonal levels of 0.03 g of leaves samples coming from the year 2022 from 4 different hormones (salicylic Acid, Indolacetic Acid, Abscisic Acid and Jasmonic Acid) were quantified (**Annex 3 Figure 5**) for each cultivar and drought condition using UPLC/MS using the service for hormonal analysis located at the IBMCP (Insituto de Biología Molecular y Celular de Plantas – UPV/CSIC, Paterna, Valencia, Spain).

Transcriptomic profiles

RNA extraction and RNA-seq performance

RNA extraction from roots and leaves of the two years was performed according to (Reid et al., 2006). Isolated RNA was sequenced at Macrogen Inc., South Korea, using Illumina Hiseq 2000 systems for paired-end sequencing and Strong Specific Libraries, reaching an average of 33 Million of sequences per sample with a length of 150 bp. All the sequencing data were deposited at European Nucleotide Archive (ENA) under BioProject number PRJEB55563 (not available yet).

Differential Expression Genes and Gene Ontology analysis

A Differential Expression Gene (DEG) analysis was developed in order to process all the obtained sequences in RNA-seq using the total of RNA-seq performed on the two years (2020 and 2022). Firstly, a general quality control checking was done on all the samples using FastQC v0.11.8 (Andrews et al., 2012) software and a soft trimming were performed on the samples using Trimmomatic v0.38 (Bolger et al., 2014). After trimming, a second quality checking was done, and a next alignment was performed over all the samples using HISAT2 v2.1.0 (D. Kim et al., 2015) and the reference genome of *Vitis vinifera* L. PN40024 v4.1 INTEGRAPE COST Action (Velt et al., 2023). Once the sequences were aligned, a count of sequences was done using the reference genome annotation of the cited version below and featureCounts tool (Liao et al., 2014) available in Rsubread package v2.4.3, in R software v4.0.3. Finally, the resulted counts were analysed using two different packages of DEG analysis in order to reach a consortium of DEGs from the results of the two different packages (Costa-Silva et al., 2017). For that, we used DESeq2 package v1.30.1 (L. Wang et al., 2010) and NOISeq package v2.34.0 (Tarazona et al., 2011) in the same version of R environment cited below, and only the coincident resulted genes produced by the two packages were be considered. In order to stablish the comparisons, a principal checking of control counts was done comparing all the controls (Control 1, Control 2 and Control 3) to check possible bias among the collected samples among the two years. Because of the non-difference among controls, the analysis was performed using Control 1-Mild, Control 2-High and Control 3-Extreme. For all the comparisons, the DEG analysis were performed on the whole experimental years (2020 and 2022), in order to reduce possible influence of environment and to identify the specific drought response along the transcriptome. All the source code was published at GitHub (available at: https://github.com/alberto-rodriguezizquierdo/RNAseq_analysis). In order to analyse the functional annotation of the resulted genes, only the genes whose $-1 \geq$ log2FoldChange ≥ 1 and p-value < 0.05 were considered as Differentially Expressed Genes. A Gene Ontology Analysis (GOs) was done over all the resulted genes using the package GOStats v2.6 (Falcon & Gentleman, 2007). On that genes, a gene search for possible responsible gene candidates were performed in order to identify possible families involved in. For that purpose, a search using the gene IDs obtained before was performed using the Gene Reference Catalogue of *Vitis vinifera* L. (Navarro-Payá et al., 2022) in order to study the gene expression of each one and comparing with the bibliography. Additionally, from the general counts of each sample, a Principal Component Analysis (PCA) was performed on the overall of the compared conditions, in order to identify the principal variables involved in the differences for each condition. The most important genes according to the loadings representativity on the PCA were annotated manually using the Gramene database (Tello-Ruiz et al., 2022) and finding analogies in model plants. Furthermore, a K-means analysis was performed on the several conditions in leaves and roots for detecting co-expression networks. The functions for each gene cluster was analysed using GOStats v2.6 (Falcon & Gentleman, 2007).

Validation of Differential Expression Gene Analysis results

Seven genes with differential responses to stress were selected to validate differential expression analysis. These genes reflected key elements discovered in this work, combined with other genes with differential expression previously related to drought (*BURP*, *MYBs*, phenylpropanoids). RNAs were retrotranscribed employing qScript XLT cDNA SuperMix (Quanta Bio) and oligonucleotides were designed employing primer-blast (**Table 2**) and the genome of *Vitis vinifera* 12X version 1. Quantitative real-time PCR (qPCR) was performed employing SYBR Green 1 Master and the Roche LightCycler 480 thermocycler. The relative expression of the results was calculated by $2^{-\Delta\Delta Ct}$ and normalized using the referent and constitutive gene β-actin for normalization.

Gene Name	Gene_ID	FW	RV	Tms FW	Tms RV
-	Vitvi04g00442	GGCCCATATGTGTTCCCCAA	GACTCCACCATCCAACCAGG	60	60
-	Vitvi19g01391	CTGTAAGAGAGCGTCGCCTT	TGTGGCGAGAATAGCAGCAA	58.4	58
-	Vitvi06g00149	AAGCCGATTAACGCGAATGC	GAACTGCACCAATCTCCCCA	57.8	57.8
BURP_RD22b	Vitvi11g00340	TGCCCGACCCAAAACCACTGCTTC	GAATAACCCACATCTCCAGCC	60	60.4
MYBs_MYBA1	Vitvi02g01015	TTA TCG CAA GCC TCA GGA CAG	TCC CAG AAG CCC ACA TCA A	57	56.4
MYBs_MYBA2	Vitvi02g01019	GAT GTG GGG TTC TGG GAT AC	AGG GAG TAG AGT ATG AAT GCA	55.2	55.1
FLAV_FLS4	Vitvi02g01473	AAA CCA CCT ACT TAC AGA GC	ACC TAA CCC CAG TGA CAG AC	51.8	56.3

Table 2: Design of primers of the genes involved in the drought stress response and presents in the DEG

Statistical analysis for physiological, metabolic and hormonal data

Statistical analysis was performed in physiological (stomatal conductance, photosynthesis, water potential, WUE), metabolomic and hormonal measures using R environment. In order to perform the analysis, a Kolmogorov-Smirnov normality test was done to check the normality of the data. After that, ANOVA one-way for all the possible comparisons between drought stages and between cultivars was calculated on the whole possible comparatives. A post-hoc analysis was performed using the Tukey HSD (honestly-significant-difference) test on the ANOVA results, considering significant different when p-value < 0.05.

Results

Physiological measurements

Stem water potential indicated no significant differences between *Callet/110 Richter* and *Merlot/110 Richter* cultivars within the same treatment, but significant distinctions emerged among different drought treatments, suggesting consistent water status in both cultivars (**Figure 30 a)**). Physiological measurements revealed significant differences between control and drought stages for both cultivars, demonstrating the common trend of reduced photosynthetic activity and stomatal conductance rates as stress increase (**Figure 30 b) and c)**). Notably, stomatal conductance and photosynthesis exhibited distinct behaviors between cultivars, *Merlot/110 Richter* showing a gradual reduction while *Callet/110 Richter* experienced a substantial decline only when drought stress intensified (high and extreme treatments) (**Figure 30 b) and c)**). Metabolic profiles displayed varying trends in chlorophyll ratio and Free Amino Acid Content (FAA) (**Figure 30 d) and e)**) between the cultivars at each drought stage, but these differences lacked statistical significance across treatments, except in *Merlot* where the FAA increased in extreme drought achieving *Callet* values (**Figure 30 e)**).

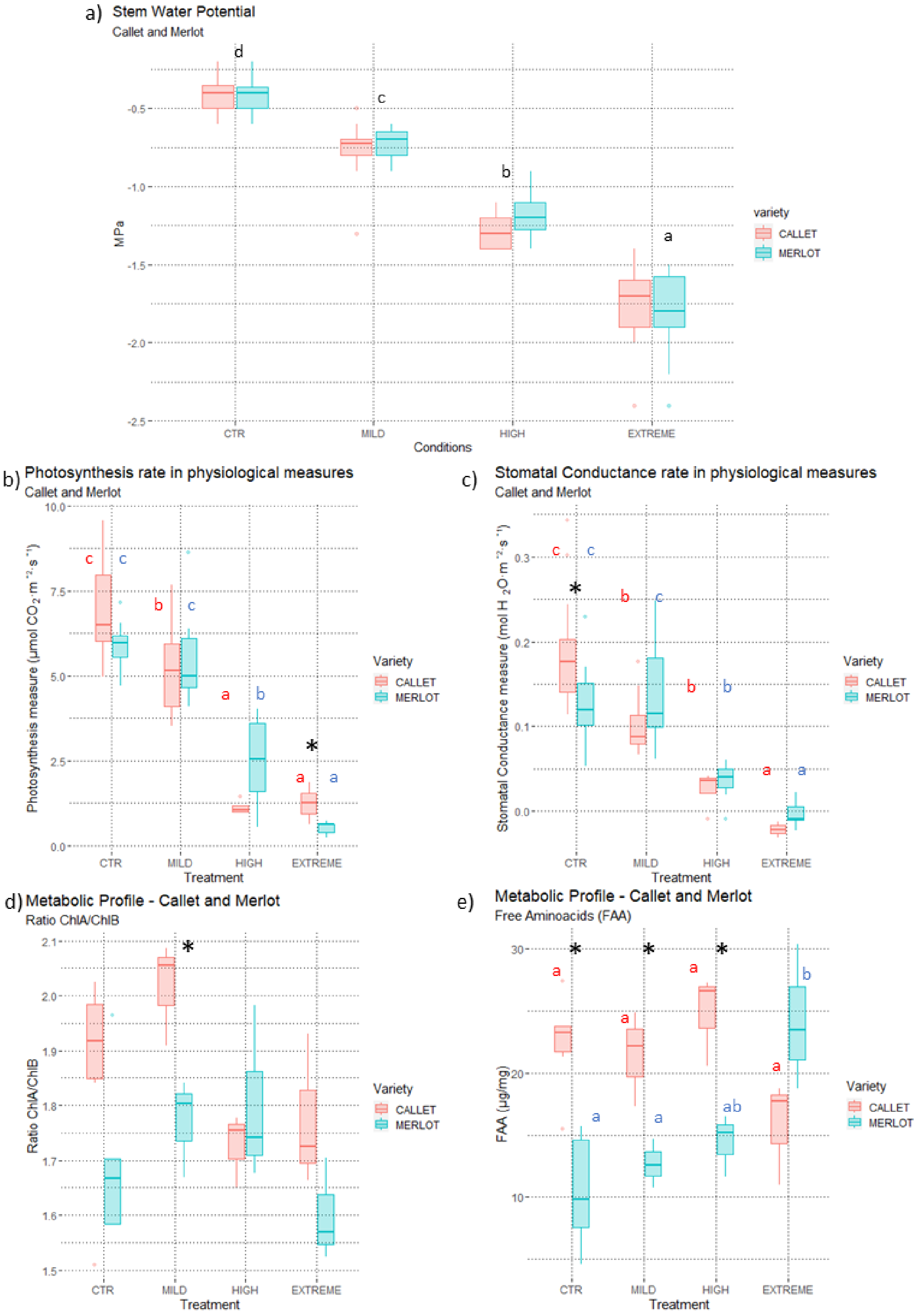

Figure 30: Physiological state of the plants. Boxplot of physiological state measures of the plants describing: a) Stem Water Potential expressed in MPa, b) photosynthetic activity (expressed as µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), c) stomatal conductance (expressed as mol $H_2O \cdot m^{-2} \cdot s^{-1}$), d) Metabolic profile of Chl A/Chl B ratio expressed in units, and e) Metabolic Profile of Free Amino Acid measures expressed in µg/mg dry leaf tissue; in Mild, High and Extreme conditions on two-year study (2020 and 2022) for *Callet/110 Richter* (in red) and *Merlot/110 Richter* (in blue). *Different letters indicate significant differences (p-value < 0.05) for comparisons between treatments in *Callet/110 Richter* (in red) and *Merlot/110 Richter* (in blue). In black, * indicates significant differences between the cultivars.

Primary Metabolite analysis

The results at primary metabolites level revealed further differences between *Callet/110 Richter* and *Merlot/110 Richter*. From the total of 24 principal metabolites considered, we found out that some metabolites presented different concentration in the different drought conditions (**Figure 31**). Significant differences and trends were found among water deficit levels in metabolites directly related to drought stress response as Citrate, GABA, Glutamine, Pyruvate, Valine or Myo-inositol, among others (for further details about all the metabolomic measures, see **Annex 1 Tables 1 and 2**).

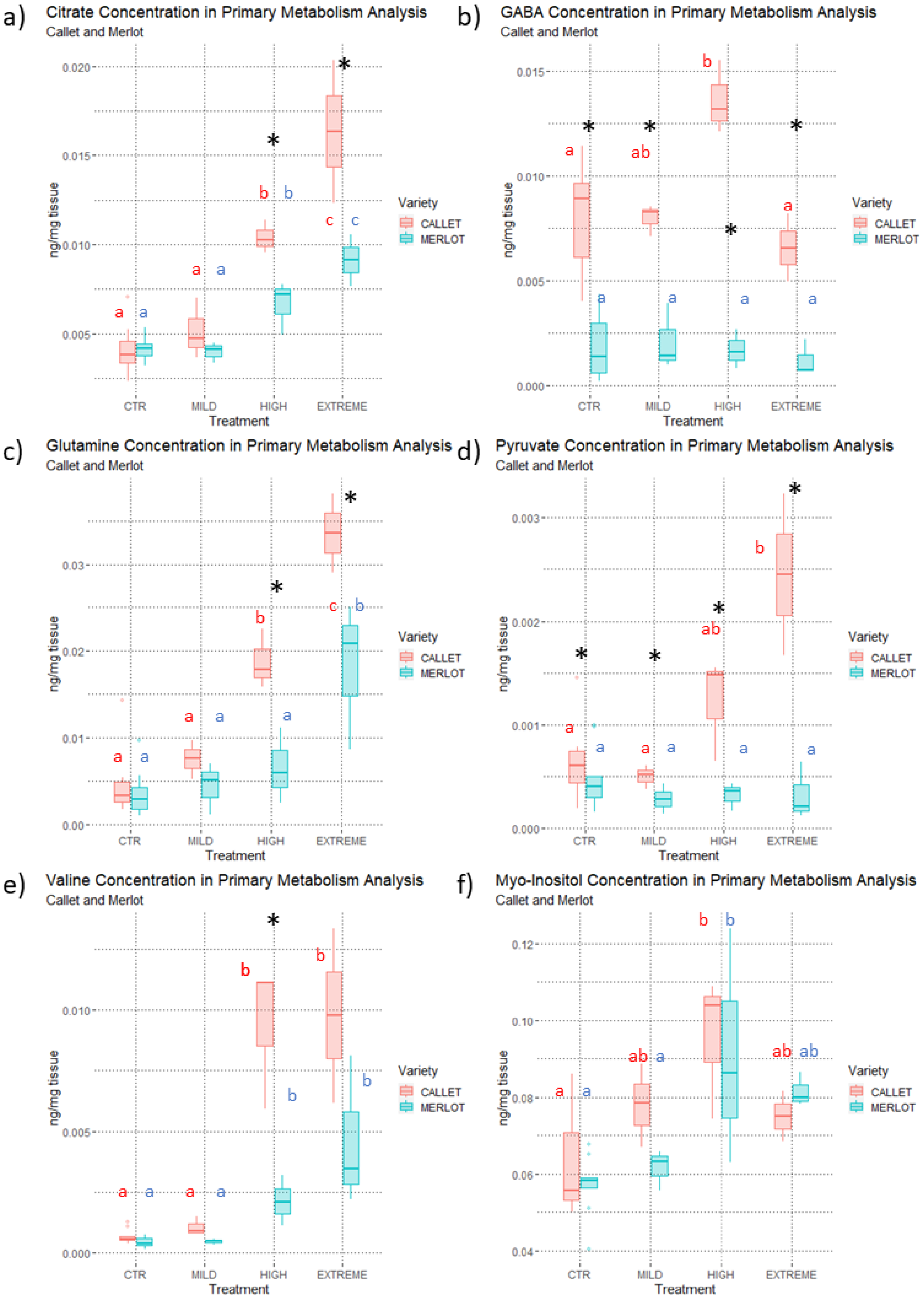

Figure 31: Boxplot of metabolomic results for key primary metabolites: a) Citrate Concentration (in ng Citrate / mg dry tissue), b) GABA Concentration (in ng GABA / mg dry tissue), c) Glutamine Concentration (in ng Glutamine / mg dry tissue), d) Pyruvate Concentration (in ng Pyruvate / mg dry tissue), e) Valine Concentration (in ng Valine / mg dry tissue) and f) and Myo-Inositol Concentration (in ng Myo-Inositol / mg dry tissue) on Mild, High and Extreme conditions in the year 2020 for *Callet/110 Richter* (in red) and *Merlot/110 Richter* (in blue). * Different letters indicate significant differences (p-value < 0.05) for comparisons between treatments in *Callet/110 Richter* (in red) and *Merlot/110 Richter* (in blue). In black, * indicates significant differences between the cultivars.

Hormonal Analysis

The hormonal analysis showed not significant differences in ABA concentration among the studied cultivars, but, as expected, the results revealed a progressive increase of ABA concentration proportional to the applied drought intensity (Mild, High and Extreme) (**Figure 32**). Furthermore, the analysis performed on the other hormones unveiled different patterns depending on the nature of the molecule. A decrease in concentration of Indolacetic Acid (IAA) was described on the hormonal data, caused by drought (**Annex 3 figure 5 a)**), as well as Jasmonic Acid (JA) levels (**Annex 3 figure 5 b)**), defining a pattern across between the two cultivars in response to drought stress. However, not significant differences were identified between *Callet/110 Richter* and *Merlot/110 Richter* on the whole hormonal analysis, including the Salicilic Acid (SA) levels too (**Annex 3 figure 5 c)**).

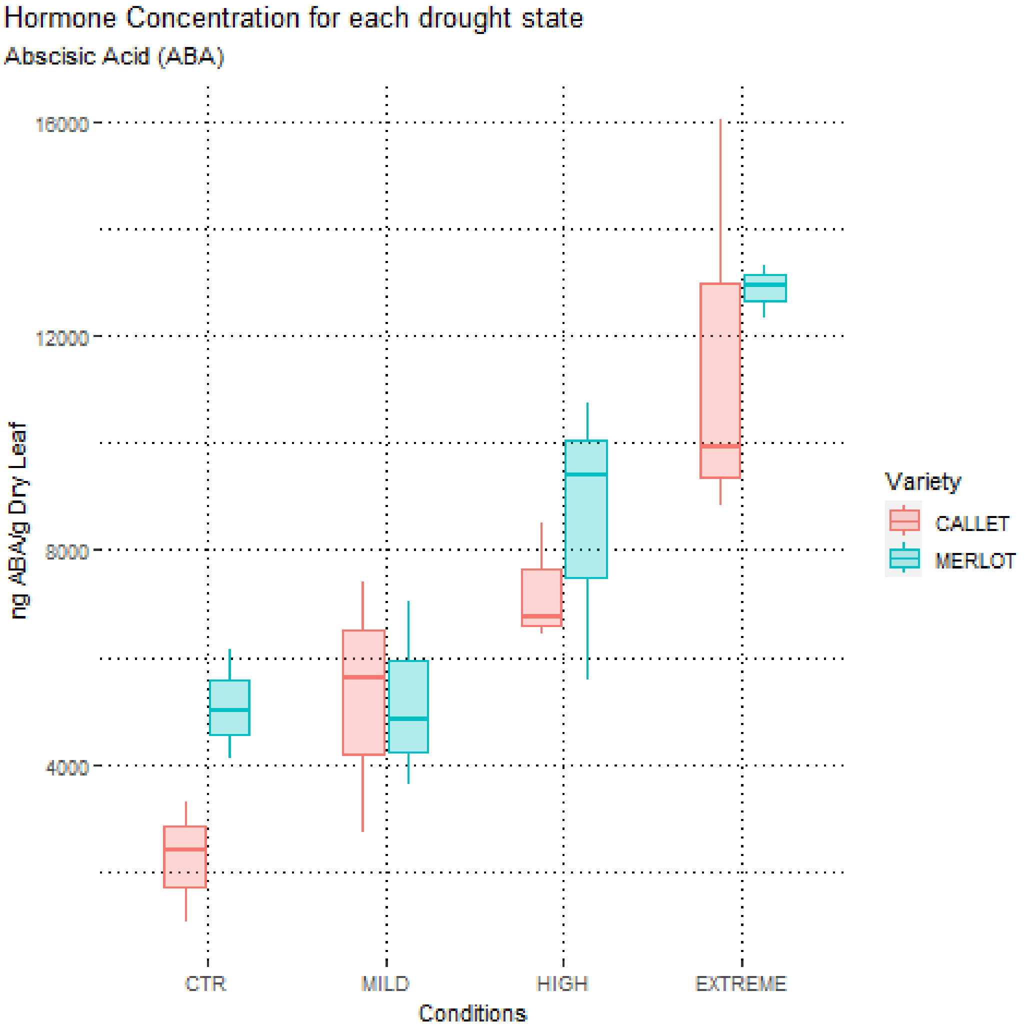

Figure 32: Boxplot of the results obtained at hormonal level for ABA concentration in leaves (expressed in ng ABA / g dry tissue) in 2022 on Mild, High, and Extreme drought conditions for *Callet/110 Richter* (in red) and *Merlot/110 Richter* (in blue). *Significant differences (p-value < 0.05) in black, v indicates significant differences between the cultivars.

Differential Expression Gene (DEG) analysis of RNA-seq data

Differential gene expression analysis of RNA-seq data from the years 2020 and 2022 revealed distinct adaptive strategies in *Callet/110 Richter* and *Merlot/110 Richter* with respect to roots and leaves, depending on drought intensity. Due to the limited number of differentially expressed genes (DEG), the Mild condition was excluded from analysis (all the resulted DEG analysis are available in **Supplementary Table 2** for all the mentioned comparisons).

Venn diagrams were constructed (**Figure 33**), integrating gene expression data for both years, focusing on leaves and roots, considering up-regulated and down-regulated genes derived from the DEG analysis, along with their corresponding Gene Ontology (GO) term summaries (all GO terms are available in **Supplementary Table 2 table 9**). This representation provides new insights into how these different genotypes respond to drought stress at various stages, highlighting a more prominent stress response in *Callet/110 Richter* compared to *Merlot* and indicating distinct patterns between the two cultivars. In broad terms, *Callet/110 Richter* upregulated a higher proportion of genes in both leaves and roots (leaves: 60% upregulated, 39% downregulated; roots: 60% upregulated, 39% downregulated among the identified DEG) compared to *Merlot*. This caused an increase in the percentage of upregulated genes for *Merlot* (leaves: 12% upregulated, 5% downregulated; roots: <0.1% downregulated, 5% upregulated among the identified DEG in leaves and roots) under drought stress (detailed results in **Table 3**).

Cultivar	Organ	Number of Upregulated Genes	Number of Downregulated genes	Percentage Upregulated over the total DEG	Percentage Downregulated over the Upregulated and Downregulated DEG	Percentage Upregulated genes over the Upregulated genes	Percentage Downregulated genes over the Downregulated genes	Number of detected Isoforms
Callet	Leaves	2645	1726	59.91	39.09	99.18	98.74	14
	Roots	995	656	59.40	39.16	100	96.47	
Merlot	Leaves	227	548	5.14	12.41	8.51	31.35	1
	Roots	1	262	0.06	15.64	0.10	38.53	

Table 3: Description of the DEG global results for upregulated and downregulated genes in leaves and roots.

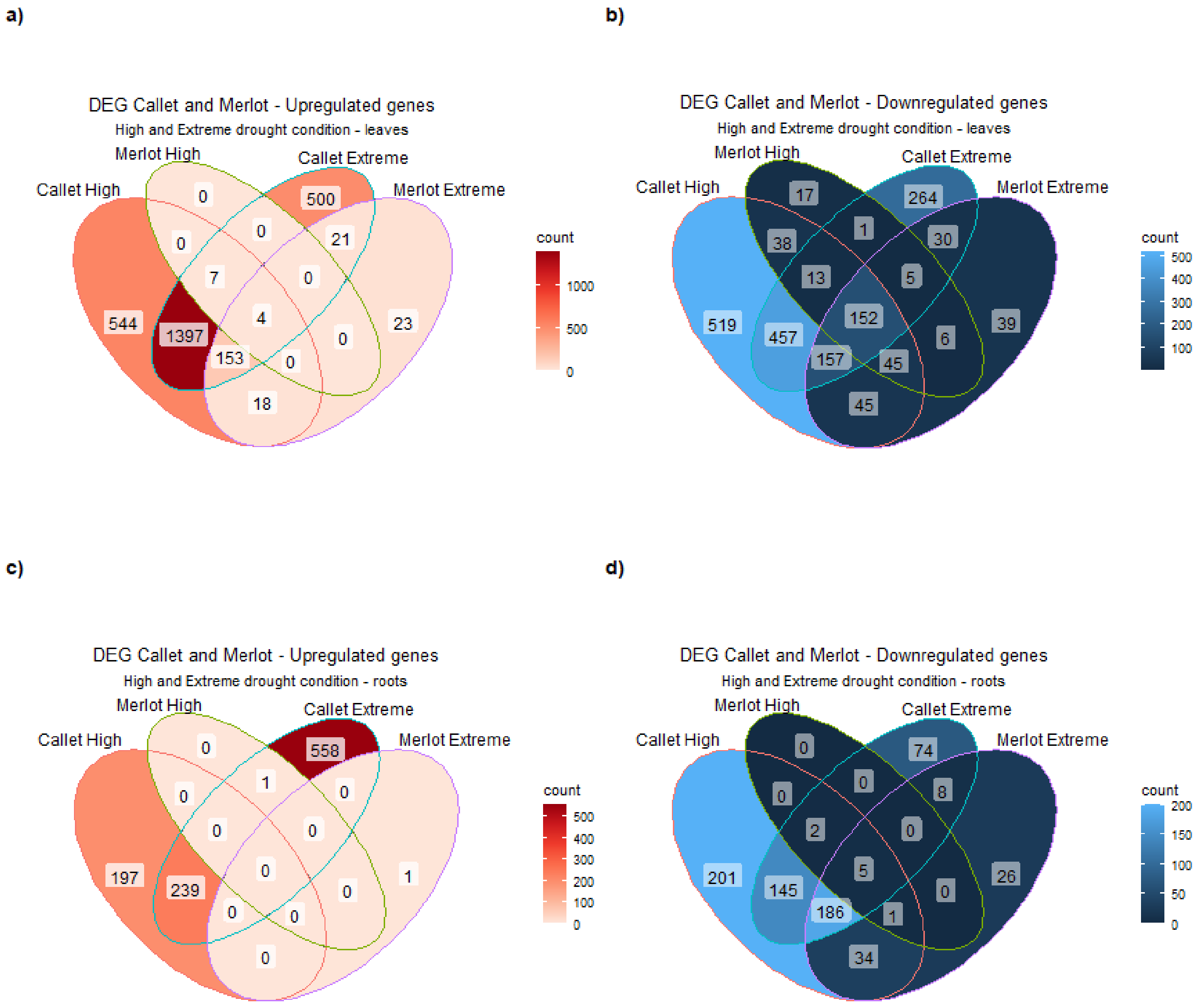

Figure 33: Venn diagram for coincident genes coming from Differentially Expressed Genes analysis results performed on the two years data (2020 and 2022). There are separated at up-regulated (in white-black) and down-regulated level (in red), for a) *Callet/110 Richter* and *Merlot/110 Richter* upregulated genes in leaves, b) *Callet/110 Richter* and *Merlot/110 Richter* downregulated genes in leaves, c) *Callet/110 Richter* and *Merlot/110 Richter* upregulated genes in roots, and d) *Callet/110 Richter* and *Merlot/110 Richter* downregulated genes in roots.

The core set of DEG for each drought stage in *Callet/110 Richter* and *Merlot/110 Richter* exhibited common functions related to stress, governing genes associated with reactive oxygen species (ROS) and defense responses in leaves. However, a limited number of shared DEG were observed (156 genes in leaves and 5 genes in roots), underscoring further distinctions in gene expression regulation between the two cultivars, particularly in the rootstock component. These differences persisted as drought severity increased at each stage. In the High drought state, *Callet/110 Richter* upregulated a substantial number of genes related to hormone response and photosynthesis-related genes (2292 genes) and downregulated other genes related to light stimulus, or growth, while *Merlot/110 Richter* displayed downregulation of genes (277 genes) associated with regulation of cellular processes and growth in leaves (**Figure 33 a) and b)**). In roots, *Callet/110 Richter* upregulated genes associated with growth, metabolic processes and ion homeostasis (436 genes) and downregulated genes linked to the regulation of gene expression and biosynthesis of components, whereas *Merlot/110 Richter* showed minimal response (only 8 genes) (**Figure 33 c) and d)**). As the drought reached the Extreme stage, the gene expression patterns of upregulation and downregulation resembled those observed in the High drought conditions for each cultivar. In leaves, *Callet/110 Richter* exhibited upregulation of genes (2083 genes) associated with photosynthesis and response to abiotic stimulus, while downregulating genes (1079 genes) related to functions like cell communication, catabolic processes, and regulation of gene silencing by miRNAs and protein ubiquitination (**Figure 33 a) and b)**). However, *Merlot/110 Richter* responded differently in leaves, upregulating genes (277 genes) related to photosynthesis and response to radiation and downregulation of genes (548 genes) linked to catabolic processes. In the roots of *Callet/110 Richter*, the response involved upregulation of ion transport, growth, along with downregulation of other metabolic processes like the regulation of gene expression or genes related to macromolecule synthesis (**Figure 33c) and d)**). In contrast, *110 Richter* rootstocks grafted with *Merlot* did not exhibit any specific regulated functions (**Figure 33c) and d)**) (all the GOs related to these stages are available in **Supplementary Table 2 Table 9 and Annex 4 figures 1 to 3**).

Indeed, the Principal Component Analysis performed on the same comparisons were complementary to the DEG. The most representative loadings coming from the Principal Component Analysis performed on the overall of the samples comparing controls with different drought stresses revealed key genes related to the response to ABA (i.e., Vitvi01g00556, Vitvi06g01696, Vitvi08g02226), the response to water deprivation or response to light stimulus in the whole comparisons (**Annex 1 Table 3, Figure 34 and Figure 35** and **Supplementary Table 3 tables 1 to 12**), reaching a separation between the different drought stages in leaves of *Callet/110 Richter* ***Figure 34*** and *Merlot/110 Richter* (**Figure 34**). In roots (**Figure 35**), the irrigated plants showed similar distribution along the PCs with no differences between *Callet* and *Merlot* cultivars at gene expression level. Nonetheless, the distribution changed when the drought stress increased, separating between grafted plants with *Callet* and *Merlot*. Furthermore, the differences are more pronounciated in *Callet/110 Richter* rootstock (**Figure 35**) than in *Merlot/110 Richter*, evidencing a clear difference in the response to drought stress. The co-expression gene analysis on the whole samples revealed different groups of genes with similar expression pattern depending on the drought condition, containing genes related to the stress detoxification and response to ABA in leaves and roots (**Annex 4 figures 4 to 7**, genes by cluster for each condition in **Supplementary Table 3 Table 14** and principal functions for each cluster in **Supplementary Table 3 Table 15**).

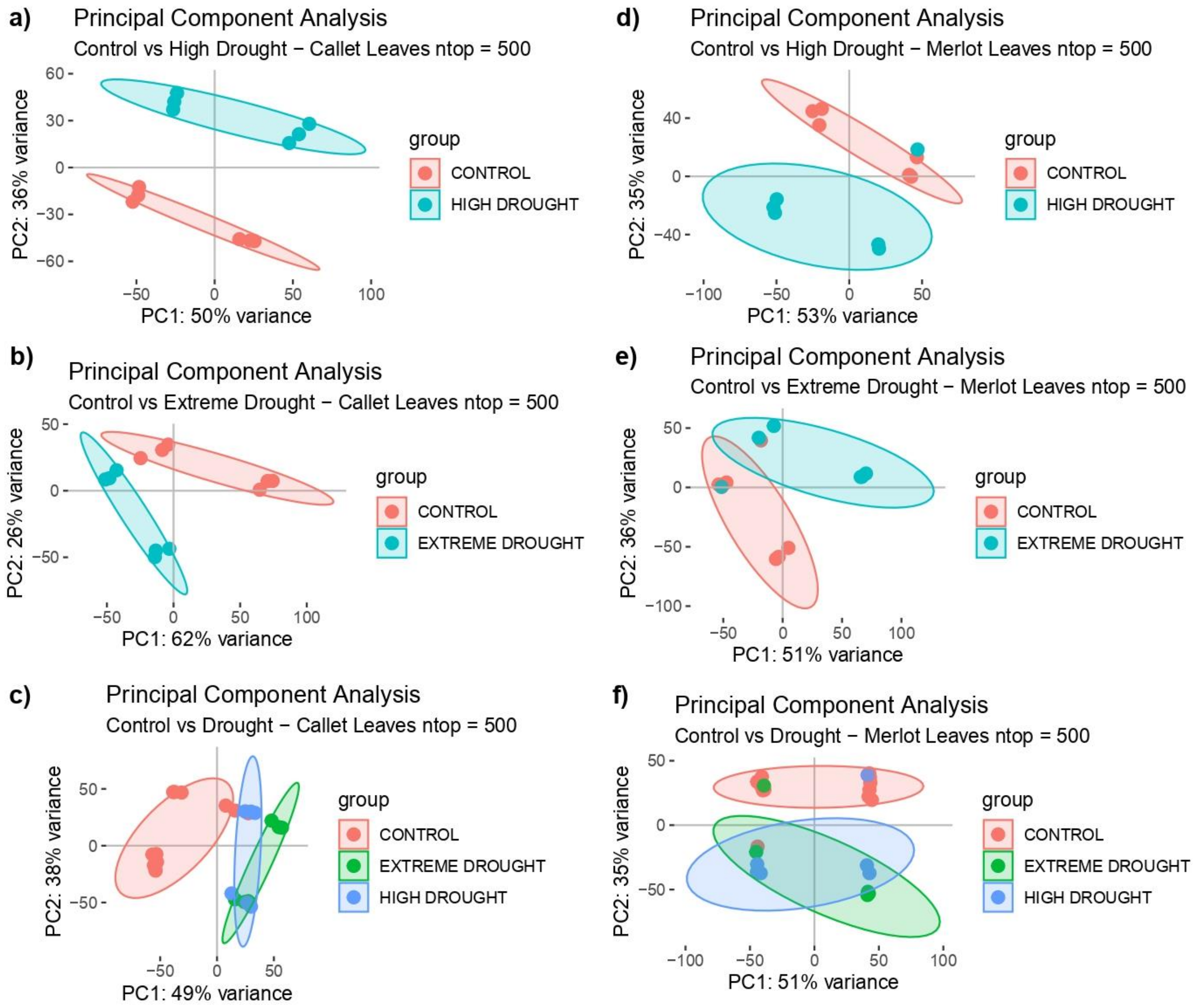

Figure 34: PCA scores for RNA-seq data on leaves of *Callet/110 Richter* and *Merlot/110 Richter* under different drought stages in 2020 and 2022. a) *Callet* Control in red and High drought in light blue; b) *Callet* Control in red and Extreme drought in blue; c) *Callet* control in red, High drought in blue and Extreme drought in green; d) *Merlot* Control in red and High drought in blue; e) *Merlot* Control in red and Extreme drought in blue; and f) *Merlot* Control in red, High drought in blue and Extreme drought in green.

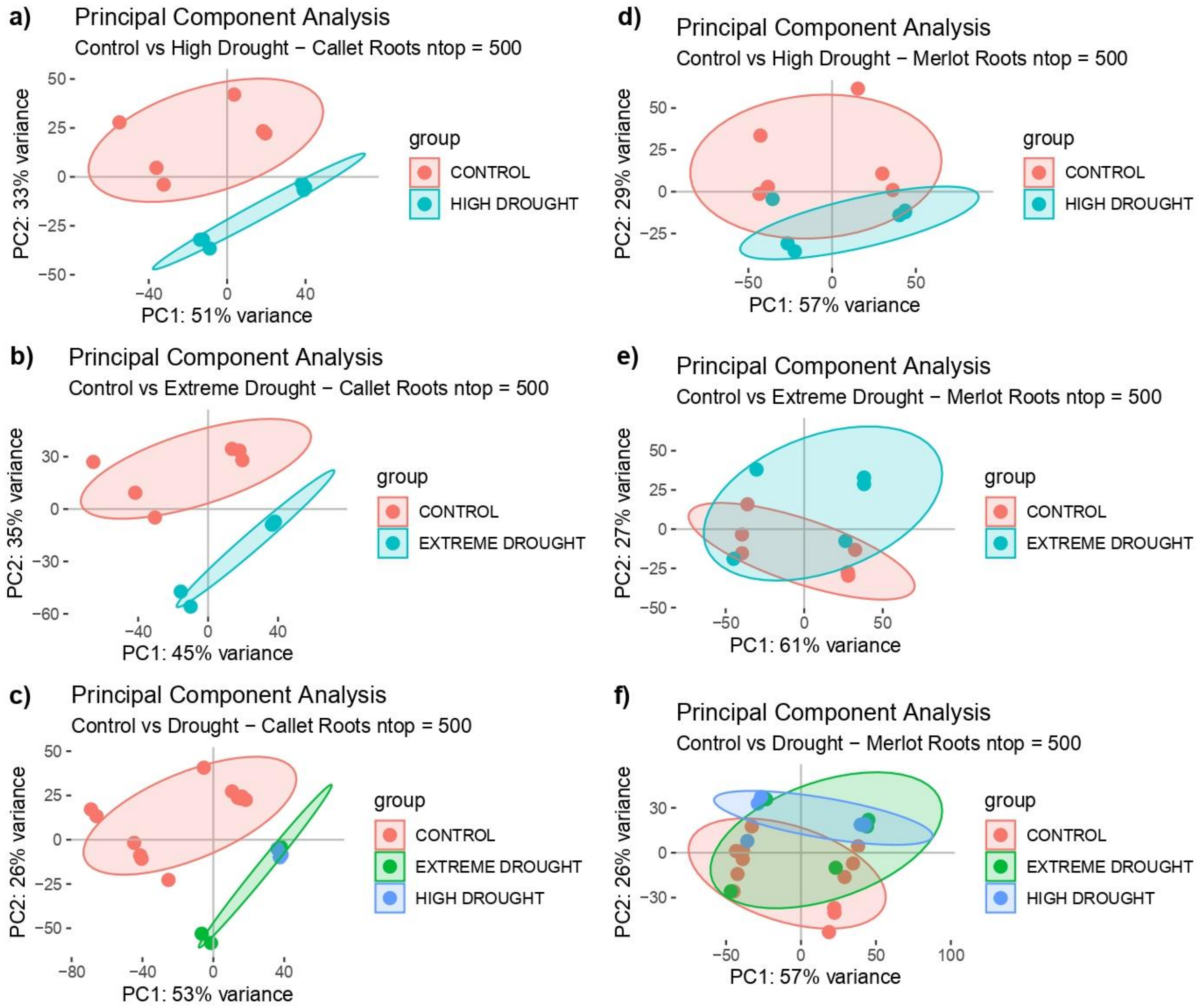

Figure 35: PCA scores for RNA-seq data on roots of *Callet/110 Richter* and *Merlot/110 Richter* under different drought stages in 2020 and 2022. a) *Callet* Control in red and High drought in light blue; b) *Callet* Control in red and Extreme drought in blue; c) Callet control in red, High drought in blue and Extreme drought in green; d) *Merlot* Control in red and High drought in blue; e) *Merlot* Control in red and Extreme drought in blue; and f) *Merlot* Control in red, High drought in blue and Extreme drought in green.

The analysis of co-expressed genes on the whole samples revealed different groups of genes with similar expression pattern depending on the drought condition (**Annex 4 figures 4 to 7**). Despite of the non-specific groups of co-expressed genes because of the large number of genes contained in the cluster, we could detect some groups of co-expressed genes related to the response to stress and detoxification in *Callet/110 Richter* leaves (**Annex 4 figure 4 cluster 6**) that were not represented enough in *Merlot/110 Richter* leaves, with a non-specific co-expressed network of genes (**Annex 4 figure 5 cluster 2**). In roots, the co-expressed gene networks were more unspecific than in leaves, but *Callet/110 Richter* showed co-expressed genes related to the response to stress and plant development (**Annex 4 figure 6 cluster 13**), while *Merlot/110 Richter* showed less specificity in the response because of the large quantity of genes contained in the clusters (**Annex 4 figure 7 cluster 2)** (all GO terms are available in **Supplementary Table 2 table 2**).

Transcriptomics also revealed significant differences among the regulatory families *BURP* (**Figure 36 a)**) and *MYBs* (**Figure 36 b)**). This family responded differentially to the intensity of the stress and cultivars, particularly for genes like *RD22b*, *RD22d*, or *RD22k*, where *Callet/110 Richter* exhibited higher expression levels than *Merlot/110 Richter* in both leaves and roots or, *MYBA1*, and *MYBA2*, mainly in leaf tissue. Moreover, the DEG represented in the **Figure 36a)** and **b)** were identified using Gene Reference Catalogue for *Vitis vinifera* L. (Navarro-Payá et al., 2022). The resulted genes involved in the drought stress response, in that case, were involved in key pathways like stilbene synthesis or cell wall metabolism (see the most important gene families in **Annex 3 Figures 1 to 4**), but one of the most important families related to drought stress described by the literature was found in the gene search analysis (in **Figure 36 a)** and **b)**). In the performed gene selection search on the DEG results on *Callet/110 Richter* and *Merlot/110 Richter*, the gene families like *BURP* (**Figure 36 a)**) or MYBs (**Figure 36 b)**) were found out with differences in gene expression levels among other principal families related to this (in **Annex 3 Figures 1 to 4**). The **Figure 36 a)** showed the differences in gene expression for the *BURP* DEGs identified in each drought stage, revealing a clear difference in gene expression in genes like *RD22b, RD22d* or *RD22k* between *Callet/110 Richter* and *Merlot/110 Richter* in leaves and roots, becoming higher the gene expression in *Callet/110 Richter* than *Merlot/110 Richter*. Furthermore, the gene expression of MYB (**Figure 36 b)**) genes like *MYB15, MYBA1* or *MYBA2* also revealed differences between *Callet/110 Richter* and *Merlot/110 Richter* leaves.

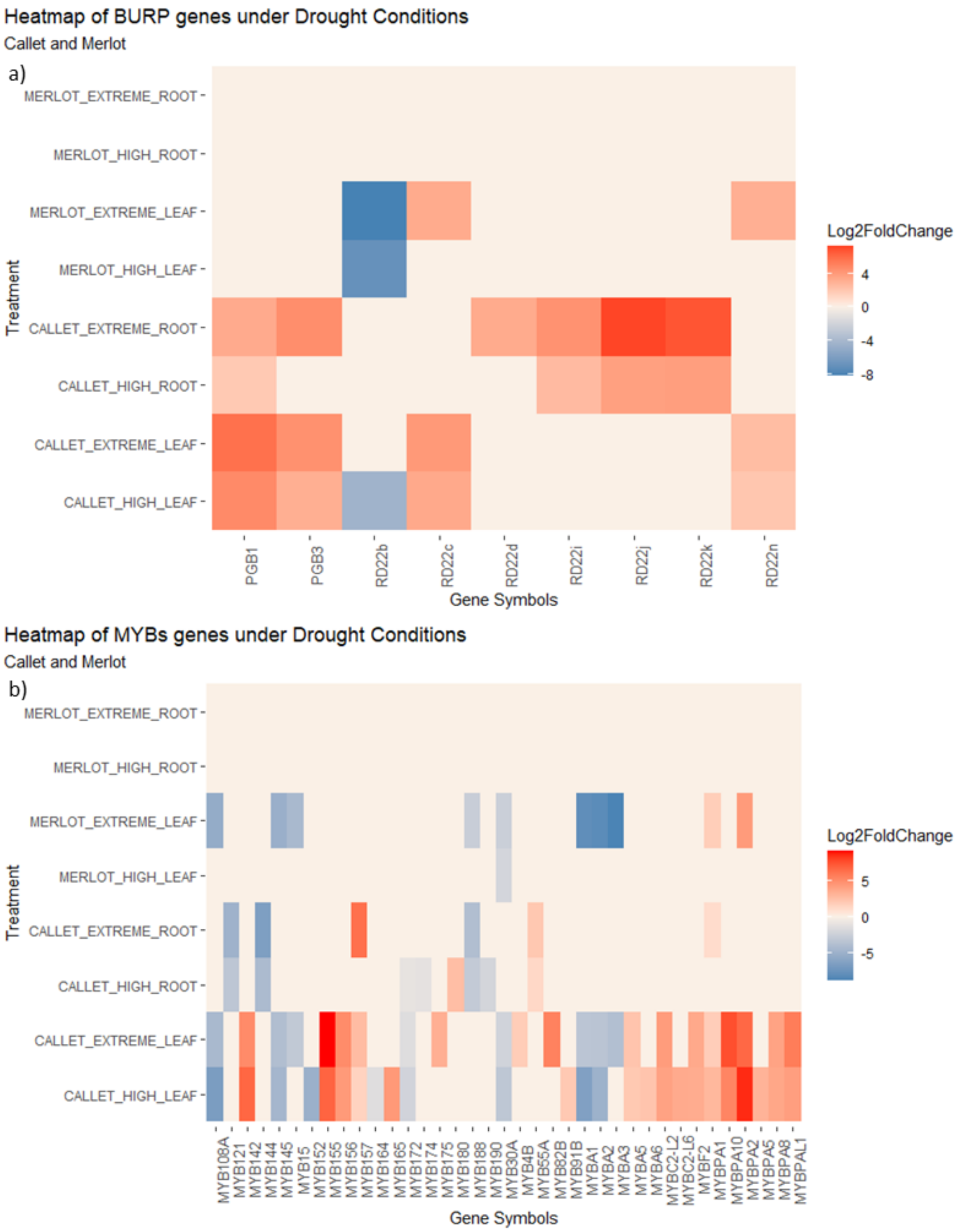

Figure 36: Heatmap of the most represented families in ABA response genes, like a) *BURP* genes and b) *MYBs* genes. Where the Log2foldChange of the downregulated genes are represented in blue scale and the Log2foldChange of the upregulated genes are represented in red scale, on leaves and roots of *Callet/110 Richter* and *Merlot/110 Richter* plants.

Discussion

Drought treatment significantly impacted stomatal conductance and photosynthesis, with effects becoming more pronounced under higher water deficit stress (in both cultivars (**Figure 30 b)** and **c)**). However, results indicate distinct responses to drought between *Callet/110 Richter* and *Merlot/110 Richter*, particularly in these key physiological aspects. Notably, the reduction in carbon fixation rates was more severe in *Callet/110 Richter* under high drought conditions compared to *Merlot/110 Richter*. However, under extreme drought conditions, *Callet/110 Richter* maintained higher photosynthetic rates than *Merlot/110 Richter*, with no differences in stomatal conductance, demonstrating better water use efficiency (WUEi) (**Annex 3 figure 6 a)**). This preservation of carbon assimilation under drought stress, despite stomatal closure regulation, is crucial for survival (Apel & Hirt, 2004; Bota et al., 2001, 2016; Cramer et al., 2007; Tillett et al., 2011).

Although physiological alterations facing drought were notable, metabolite profile analysis also revealed relevant changes between cultivars at different water stress levels (**Annex 3 figure 7**). Variations among Callet/Merlot in photosynthesis-related metabolites supported previous observations explaining the possible differences in photosynthetic rates due to increased tolerance to high illumination in plants (Begum et al., 2019), while the differential free amino acid content suggested additional pathways affected by drought, possibly indicating protein degradation or alterations in phenolic compounds. Changes of quantity of phenolic compounds, involved in reducing oxidative damage, could reflect a differential regulation of metabolic pathways to minimize the impact of drought stress (Król et al., 2014), becoming higher in *Callet/110 Richter* than *Merlot/110 Richter* (**Annex 3 figure 7**). Further analysis of primary metabolites revealed distinct responses to drought stress and differences between genotypes, possibly attributed to variations in Abscisic Acid (ABA) sensitivity. For instance, the different patterns observed in metabolite concentrations such as Citrate, GABA, Glutamine, Pyruvate, and Valine when increasing drought stress (**Figure 31**), suggested different ABA responses between *Merlot/110 Richter* and *Callet/110 Richter* (Huang & Jander, 2017; L. Li et al., 2021; Muhammad Aslam et al., 2022; Murcia et al., 2017).

As expected, the increasing water deficit stress resulted in an increased ABA concentration, in a similar way in leaves of both cultivars (**Figure 32**). Similar concentration coupled with differing responses suggesta different sensitivity of the genotypes to ABA hormone (Dal Santo et al., 2016). This was reflected in different concentrations of other metabolites analyzed as in the case of the myo-inositol, higher in *Callet/110 Richter* than in *Merlot/110 Richter*, that acts as an osmolyte, reducing water loss during drought stress

(Murcia et al., 2017).

All these changes related to physiological functioning in grapevine were also supported by a clear transcriptomic response that allow us to stablish different strategies in response to drought stress. The Principal Component Analysis showed important genes related with the drought tolerance in grapevine, like aquaporins or genes involved in the response to ABA and genes related to the flavonoid biosynthetic process (Baiges et al., 2001; Galmés et al., 2007; Tyerman et al., 2002) (**Annex 1 Table 3**). Furthermore, the co-expression gene clusters also defined different strategies between *Callet/110 Richter* and *Merlot/110 Richter*, attributing to *Callet/110 Richter* more specificity in the response than *Merlot/110 Richter* in terms of plant development and detoxification (**Annex 4 figures 4 to 7 and Supplementary Table 3 Table 15**). According to that results, the DEG analysis presented in figure 33, hightlight different strategy in roots and leaves, with these differences being more pronounced in *Callet/110 Richter* than in *Merlot/110 Richter*. Specifically, *Callet/110 Richter* exibed more upregulated genes than down regulated ones, especially in leaves, unveiling a transcriptomic response to face the water deprivation (Dal Santo et al., 2016). Conversely, *Merlot/110 Richter* exhibits more downregulation than upregulation of genes, indicating a divergent gene expression pattern under drought stress (Dal Santo et al., 2016). In addition, we found out big differences across the drought conditions into the plant cultivars, becoming higher gene modulation in High drought conditions than in Extreme conditions in both tissues. Gene Ontology Analysis, also reveals distinct responses between leaves and roots to alleviate drought stress, suggesting a tissue-specific adaptation strategy in response to drought (Dal Santo et al., 2016; Matus et al., 2014).

In leaves, while *Callet/110 Richter* reacted under High drought condition increasing the expression of genes related to photosynthesis, *Merlot/110 Richter* reacted poorly sharing part of the expressed genes in *Callet/110 Richter*. The upregulation of genes related to the photosynthesis under drought stress is linked with the physiological results because of the molecular function related to these genes. Despite of the critical photosynthesis rate produced by drought, studies like Y. Chen et al. (2016) described an upregulation of genes related to the photosystem II and other related genes in order to reduce the damage in the thylakoid caused by drought stress in *Arabidopsis thaliana*. This damage could be directly related to the critical reduction of photosynthesis and the differences in ChlA and B observed in our results (**Figure 30d)).** It is also important to remark that *Merlot/110 Richter* showed similar gene expression of genes related to photosynthesis and response to radiation only under extreme drought conditions, while *Callet/110 Richter* modulated the gene expression at early drought conditions (Highstress), evidencing the different strategy of each cultivar. However, in roots, *Callet/110 Richter* plants reacted upregulating growth and enhancing metabolic process and ion homeostasis, in front of *Merlot/110 Richter* plants, with much low general transcriptomic response (**Figure 33 b) and d)**).

The differences in gene expression were further supported by the identification of candidate genes associated with drought stress response. Gene expression patterns of families such as *BURP*, *MYB*, and those related to the Flavonoid Pathway, sugar transportation, and lignin (Matus et al., 2008, 2014) differed between the two cultivars, with *Callet/110 Richter* showing coordinated responses in both leaves and roots (**Annex 3 Figures 1 to 4**). The *BURP* family, for example, plays a crucial role in understanding the response of drought-tolerant grapevines to varying degrees of water deficit stress. In *Callet/110 Richter* the variation in gene expression of some genes in leaves was followed by a regulation of gene expression in roots similarly, for example, in genes related to sugar transportation like *VvHT4* (Vitvi16g00479) or *VvHT5* (Vitvi05g00468), related to sugar uptake in extracellular environment in order to reduce the activity into the plant (Medici et al., 2014; Z. Zhang et al., 2019), or in genes related to the flavonoid pathway, like *VvCHS2* (Vitvi14g01449), *VvCHS3* (Vitvi05g01044) and *VvLDOX1* (Vitvi02g00435) previously described as possible related genes in response to drought in grapes and leaves (Castellarin et al., 2007; Matus et al., 2008; Orduña et al., 2022). The importance of the *BURP* family in drought could be essential to understand how to understand drought tolerant grapevines could react to water deficit stress under different stages of water privation, and the results showed similar gene expression patterns in the differentially expressed genes detected, directly related to ABA, like the *VvRD22b* (Vitvi11g00340) downregulation and the *VvRD22c* (Vitvi04g00342) upregulation (**Figure 36**). *RD22* (Response to Dehidration 22) superfamily is involved in drought stress response, encoding structural proteins located in apoplast and cell wall (Bray, 2002; Matus et al., 2014). The expression pattern of these genes, which was not varying during stress, were similar to the observations of Dal Santo et al. (2016), in which near-isohydric genotypes like *Montepulciano* showed more sensitivity to ABA in terms of stomatal closure and upregulation of ABA-related genes than anisohydric genotypes, like *Sangiovese*, that showed low sensitivity to ABA.

The involvement of the *BURP* family genes in the response to abiotic stress in grafted plants presents a new window for exploring their role in scion-rootstock communication. ABA's influence on the scion could potentially enhance responses in the roots, such as root lignification and regulation of root elongation (**Supplementary Table 2 Table 1**). For instance the regulation of genes related to lignin, such as *VvLAC20* (Vitvi13g00117), are crucial for mitigating water loss by increasing impermeability, but reducing root growth (**Figure 36 b) and Annex 3 Figure 2**) (Yıldırım et al., 2018).

Within the *MYB* family, a general overexpression tendency has been observed comparing *Callet/110 Richter* with *Merlot/110 Richter* in leaves and roots (**Figure 36**). Indeed, the DEG analysis showed different regulation in genes like *VvMYB15* (Vitvi05g01733), *VvMYBA1* (Vitvi02g01019) *VvMYBA2* (Vitvi02g01015) or *VvMYBA3* (Vitvi02g01024), that are implicated in stress response and secondary metabolite production (Corso et al., 2015; Dal Santo et al., 2016b; Matus et al., 2008; Orduña et al., 2022). For example, *VvMYB15* is associated with the expression of genes involved in the production of stilbenes or flavonoids, which relieve abiotic stress by regulating genes of the Flavonoid Pathway such as *VvLDOX1* (Vitvi02g00435) or *VvUFGT1* (Vitvi16g00156) (Matus et al., 2008; Orduña et al., 2022), which is consistent with the differential expression between *Callet/110 Richter* and *Merlot/110 Richter* (Figure 38 and Annex 3 Figure 1), supporting previous metabolic observations and probably related to mitigation of oxidative damage.

Moreover, the phenylpropanoid pathway is implicated in the response, with a general upregulation of families involved in drought stress response (Savoi et al., 2016) as occurred in *Callet/110 Richter* (**Annex 3 Figure 3**) . Despite the substantial evidence implicating these gene families, the intricate network among them and their regulation of other unknown genes is likely mediated by transcription factor families like *WRKYs* and *bZIPs*, which are also related to ABA and are regulated in response to drought stress. This regulation is more pronounced in *Callet/110 Richter* than in *Merlot/110 Richter*, particularly in transcription factors like *VvbZIP52* (Vitvi19g00147), which has been reported in rice as a modulator of drought stress response (**Annex 3 Figure 4**) (Liu et al., 2012; Tu et al., 2018; Khoso et al., 2022). The interaction between scion and rootstock also appeared crucial in drought adaptation. When *Merlot/110 Richter* was used as the scion and *110 Richter* as the rootstock, a possible lack of coordination was observed, with lower gene expression levels in leaves and roots compared to *Callet/110 Richter* (**Figure 36** and **Annex 3 figures 1 to 4**). This lack of coordination could be attributed to the cross talk between the cultivar and rootstock, evidenced by differential expression of genes related to miRNA regulation, which was absent in *Merlot/110 Richter*, probably caused by the cross talk between the cultivar *vinifera* and the American grapevine rootstock (Cochetel et al., 2017; Cookson & Ollat, 2013; Gautier, Cochetel, et al., 2020; Gautier, Cookson, et al., 2020; L. Zhang et al., 2016).

This cross talk could be initiated by the increase in ABA production in leaves in response to reduced water availability in roots, subsequently communicating the drought signal to the rest of the binomial plant/scion (Cochetel et al., 2017; Gautier, Cochetel, et al., 2020; Gautier, Cookson, et al., 2020; L. Zhang et al., 2016). The similar ABA concentration in leaves of both cultivars, suggested that transcriptional changes cofered higher sensitivity to ABA in *Callet/110 Richter*, leading to physiological adjustments such as propmt stomatal closure and reduced photosynthesis rates (**Figure 30**). Additionally, it triggered further adaptations related to primary and secondary metabolite production, and root elongation, as evidenced by transcriptomic responses. However, the low sensitivity to ABA in *Merlot/110 Richter* hindered this response, leading to a poor modulation of the gene expression and metabolite analysis, particularly evident in roots under high drought conditions (Dal Santo et al., 2016; Rossdeutsch et al., 2016).

Conclusions

The findings underscore the multifaceted nature of grapevine drought tolerance, highlighting the interaction between genotype and the communication between scion and rootstock. Specifically, our findings suggest that ABA sensitivity played a crucial role in facilitating a more comprehensive response in *Callet* compared to *Merlot*, both grafted with *110 Richter* rootstocks. Notably, the differential responses observed in gene expression and metabolite profiles between *Callet/110 Richter* and *Merlot/110 Richter* underscore the intricate regulatory networks involved in drought response and a differential ABA sensitivity. The impaired communication between scion and rootstock caused by a deficient ABA sensitivity in *Merlot/110 Richter* reduced the capability of the plant to adequately respond to stress, forcing a non-specific response to drought instead of the specialized response exhibited by *Callet/110 Richter*.

Furthermore, the identification of candidate genes associated with drought stress response, such as those in the *BURP* and *MYB* families, sheds light on potential molecular markers for drought tolerance. Understanding rootstock/scion interactions is essential for developing strategies to mitigate the impact of drought on viticulture and ensuring the resilience of grapevine crops in the face of changing environmental conditions, being absolutely necessary to explore new optimal combinations of scions and rootstocks for a better stress tolerance.

General Discussion

The findings of the present thesis showcase the significant impact of advancements in genomics techniques on enhancing our understanding of grapevines. These experiments have contributed a wealth of information to the scientific community, shedding light on the influence of epigenomics on grapevine epigenetic processes and how the evolution of numerous genotypes throughout history has shaped their responses to drought-induced abiotic stresses.

The first chapter, focused on epigenomic sequences from cultivated and wild grapevine genotypes, uncovered distinct epigenetic imprints associated with cultivated and wild grapevines. This discovery suggests that epigenetics played a pivotal role in the domestication process. Notably, the maintenance and growth of these genotypes under the same conditions continued to reflect this epigenetic memory, underscoring its persistence and influence.

In the second chapter, transcriptomic sequences obtained from leaves and roots of *Callet/110 Richter* and *Merlot/110 Richter* plants, highlighted two diverse strategies in responding to drought stress within vineyards. These strategies seemed to involve factors such as sensitivity to abscisic acid (ABA) and the intricate communication between the scion and rootstock.

Both chapters contribute significantly to our comprehension of grapevine biology, emphasizing the nuanced roles of epigenomics in domestication processes and the complex responses of grapevines to environmental stresses like drought detected using transcriptomics and metabolomics. The integration of genomics techniques has opened new avenues for exploring these critical aspects of grapevine biology and may pave the way for targeted improvements in viticulture and agriculture.

Understanding the domestication process in grapevine through the epigenomic perspective

Cultivated grapevines show different methylation pattern than its wild relatives

Following the results generated in Chapter I, the evidences revealed a clear influence of the domestication process in the methylome modelling of grapevine. The high cytosine methylation rates in all the contexts (CG, CHG and CHH) showed in chapter I determined the conservation of methylation across the vegetative propagation along the History (Denham et al., 2020; Tesfamicael et al., 2020; Xie et al., 2017) in cultivated grapevines. The different methylation rate in CHG and CHH between cultivated and wild grapevines is also interesting because the directly relationship between those contexts with the diverse selection pressures caused by the domestication and traits related to the plant reproduction, especially in CHH contexts (Arroyo-Garcia et al., 2006; Dong et al., 2023; McKey et al., 2010). While in cultivated grapevines the predominant method of reproduction is generally the vegetative propagation, the sexual reproduction would be predominant in wild grapevines (Arroyo-Garcia et al., 2006; Dong et al., 2023; McKey et al., 2010), and these methylated cytosines would be preserved across the offspring.

The different methylation rates are also followed by different strategies to build the methylome modelling in wild grapevines across the functional results of the genes containing methylated positions. While in cultivated grapevines, there are some methylated positions related with the reduction of oxidative stress, in wild grapevines, the methylation strategy is more related to the methylation of genes linked with the response to phytohormones like ethylene (H. Chen et al., 2021; Sims et al., 2003), directly response to a defensive strategy to abiotic and biotic stresses (Böttcher et al., 2013). The special methylation in cultivated grapevines on genes related to oxidative stress could be related to the difficulties of some cultivated grapevines to adapt it to environmental stresses. For example, studies like Guarino et al. (2015) and Raj et al. (2011) remarked the important influence of the epigenetic fingerprints to the offspring of poplar in drought stress experiments. Furthermore, Tesfamicael et al. (2020) described the possible cumulus of mutations around the sexual genes in enset, a vegetative propagated crop, that could increase the cytosine methylation around those genes and it could silence the gene expression for those genes (Denham et al., 2020; Konate et al., 2018; McKey et al., 2010; Meyer et al., 2012; Raj et al., 2011; Tan et al., 2023; Tesfamicael et al., 2020; Xie et al., 2017). Indeed, higher methylation of genes related to Histone H3-K4 demethylation can play a great role in the environmental adaptation to grapevine, by gene expression repression of some genes whose product could not be representative for the plant resilience (Sims et al., 2003). Taking account into the influence of the cytosine methylation in the adaptation to different stresses like drought, the epigenetic memory could also influence in the grapevine plant breeding (Fortes & Gallusci, 2017; Konate et al., 2018; Raj et al., 2011; Rodríguez López & Wilkinson, 2015), conditioning the generation of some cultivars with more tolerance to abiotic stresses such as drought (for example, in cultivars like *Callet* or other grapevines with tolerance to drought, referred in Chapter II), versus other less tolerant to water deprivation (for example, genotypes like *Merlot* among others, referred in Chapter II) (Konate et al., 2018; Raj et al., 2011; Rodríguez López & Wilkinson, 2015).

Curiously, in all the hypermethylated genes for each part (hypermethylated for wild accessions and hypermethylated for cultivated accessions), the methylated position of the cytosines were differently distributed across the gene body, revealing possible interferences in gene expression and alternate splicing or a complete gene silencing depending on the methylated cytosine position (Bewick & Schmitz, 2017; Muyle et al., 2022). For example, the identified genes containing hypermethylated cytosines in cultivated grapevines presented slightly differences in the percentage of the presence of methylated cytosines in introns (70%) compared with hypermethylated cytosines founded out in wild accessions, with more presence of methylation in introns (75%), directly related in alternate splicing (Bewick & Schmitz, 2017; Muyle et al., 2022). Conversely, in cultivated accesions, the percentage of methylation in promoters was higher than in wild accessions (14% vs 4%), revealing a possible gene silencing at promoter level (Bewick & Schmitz, 2017; Muyle et al., 2022).

Gene specific differential methylation associated to domestication is enriched in response to stress

Functional analysis of methylated genes showed that genes related to important agronomic traits exhibited significant DNA methylation level variation during grapevine domestication, particularly in terms associated with stress response. Genes with differential methylation in the form of hypermethylation or hypomethylation between wild and cultivated grapevines were less abundant but still significant. The hypomethylated genes in cultivated grapevines were tied to protein targeting to peroxisomes and histone lysine demethylation. These processes are essential for cellular homeostasis and epigenetic regulation, suggesting that the domestication process may have enhanced or refined these functions in cultivated varieties. Interestingly, Histone H3-K4 demethylation, and DNA hypermethylation, have both been associated with gene expression repression (Sims et al., 2003). Moreover, genes hypermethylated in wild grapevines were found to relate to ethylene regulation processes and response to ozone. Ethylene is a critical hormone in plants, mediating various stress responses (H. Chen et al., 2021). In grapevine, ethylene signaling plays a crucial role beyond managing abiotic stress, encompassing various agronomically important traits such as bud dormancy and berry development. For bud dormancy, research has shown that a transient induction of specific ethylene biosynthesis genes may be involved in the regulation of the release of grapevine bud dormancy, indicating a targeted genetic response that mediates dormancy transitions (Shi et al., 2018). Further studies identified potential events following ethylene signaling that are triggered by stimuli promoting bud dormancy release, suggesting a complex network of regulatory mechanisms (Shi et al., 2020). In the context of berry development, interactions between ethylene and auxin have been identified as pivotal in controlling the ripening process of grape berries. This interaction points to a synergistic action between these hormones that is crucial for fine-tuning the developmental processes that lead to optimal fruit maturation (Böttcher et al., 2013). These insights collectively enhance our understanding of ethylene's multifaceted role in grapevine biology, influencing both growth cessation and fruit development. Moreover, recent studies have highlighted the interplay between DNA methylation and ethylene-responsive genes under stress conditions and their relationship with ABA in regulating bud dormancy. For instance, in woodland strawberry, dynamic changes in DNA methylation have been observed in response to stress, affecting genes including those responsive to ethylene, which are crucial for adaptation and survival (M.-E. López et al., 2022). Moreover, research on perennials has demonstrated that ABA plays a significant role in bud dormancy, where changes in DNA methylation patterns might regulate the expression of ethylene-responsive genes critical for dormancy initiation and release (Pan et al., 2021). These findings suggest that methylation changes in ethylene-responsive genes are a key mechanism through which plants modulate developmental and stress-related responses, presenting a fertile area for future research into crop improvement and adaptation strategies.

The unique category of genes that showed both hyper and hypomethylated cytosines in both types of accessions, albeit being the smallest group, was associated with defense response. Intriguingly, core methylated genes (CMCs) were also associated with stress response. This multimodal pattern of methylation during grapevine domestication suggests a complex regulation mechanism and might hint at genes that have retained some functionality from their wild origins, while also adapting new functionalities for the domesticated environment. Also, the conservation of methylation in the core methylated genes could suggest that the functions they support are essential and have remained unchanged between wild and cultivated grapevines.

Concluding remarks in epigenomics: The bias related to the use of RRBS in epigenomic studies guides the resolution of epigenomic studies

One of the highlights of chapter 1 focuses on advancing knowledge of the technique used in this experiment to study the methylome. The Reduced Representation Bisulfite Sequencing (RRBS) has offered relevant results in the grapevine methylome modelling, enabling a good separation and definition of wild and cultivated grapevines methylome modelling strategies. It is showed the efficacy of RRBS methods like epi-GBS (Gawehns et al., 2022; van Gurp et al., 2016) that can help with the elucidation of possible differences between wild and cultivated populations. Although the comparable results with the whole genome sequencing, the reduced representation methods realized in the Chapter I have shown certains limitations in terms of genomic coverage (in average, near to 1.55%) (Gawehns et al., 2022; van Gurp et al., 2016; Wright et al., 2019). In spite of the obtained results on genomic coverage produced by RRBS and the critical implications in the epigenomics research, the low coverage could produce some bias related to the genome definition and it can exclude further differences that could be identified using whole genome sequencing (Wright et al., 2019). Furthermore, although the 1.5% of coverage for the entire genome is low, in a RRBS experiment is high in a genome of 500 Mbp like the grapevine (1.5 Mbp / 200 bp fragment = 7500 sequenced regions), enabling to explore the methylated cytosine positions easily with a good resolution (>10X), in the sequenced regions (Gawehns et al., 2022; van Gurp et al., 2016). Additionally, methods based in Restriction-site Associated DNA sequencing (RADseq) like epiGBS has showed important evidences in the exploration of genetic and epigenetic changes on non-model plants, supporting the use of this methodology (Boukteb et al., 2021; Davey & Blaxter, 2010; Dong et al., 2022; Trucchi et al., 2016). Therefore, the results suggested us that RRBS can be used for general analysis like the comparison between wild and cultivated grapevines (Gawehns et al., 2022; van Gurp et al., 2016; Werner et al., 2020).

Despite the efficacy of the epiGBS method based on RRBS utilized in this experiment, epiGBS could contain some associated problems related to the readable sequenced reads of the experiment and the generated results. Attending to the nature of the non-methylated cytosine modification, the use of bisulfite conversion caused possible damages that could affect the quality of the samples and the quantity of usable reads, as shown in the mapping efficiency percentage, ~40-50% in the present results (Gawehns et al., 2022; van Gurp et al., 2016). According to this problem, studies like Han et al. (2022) revealed the importance of the bisulfite treatment in the percentage of usable reads, evidencing the possible bias related to the methylated cytosine conversion using bisulfite treatment compared with using an enzymatic method like EM-seq (Han et al., 2022), altering the barcoding identification in epiGBS-converted sequences (Gawehns et al., 2022; van Gurp et al., 2016).

Apart from the results in coverage and the use of bisulfite to convert sequences, the combination of two different restriction enzymes (*Csp6I* and *NsiI*) generated comparable and traceable results in terms of coverage and sequence depth. However, the sequencing performance and barcode identification suggested that a huge number of sequences were lost in the process (between 1 to 8 Million of demultiplexed sequences combining three different samples showed in our results compared with < 1M sequences per sample as reported in Gawehns et al. (2022) andvan Gurp et al. (2016)). This fact could be related to possible not reported STAR activity of one of the used enzyme (either *Csp6I* or *NsiI*) (Rauluseviciute et al., 2019; Wright et al., 2019), that could generate inspecific restriction enzymes clouding the barcoding identification (based in the barcoding sequences and a final region to link with DNA ligase to the restricted fragments of interest and becoming key for sequence demultiplexing after sequencing (see schema in **Figure 37**). Because the barcode demultiplexing consists in the identification of specific barcode sequences for each sample and the identification of the restriction site, the STAR activity is a huge problem that could causes several losses of sequences per sample (Catchen et al., 2013; Gawehns et al., 2022, 2022).

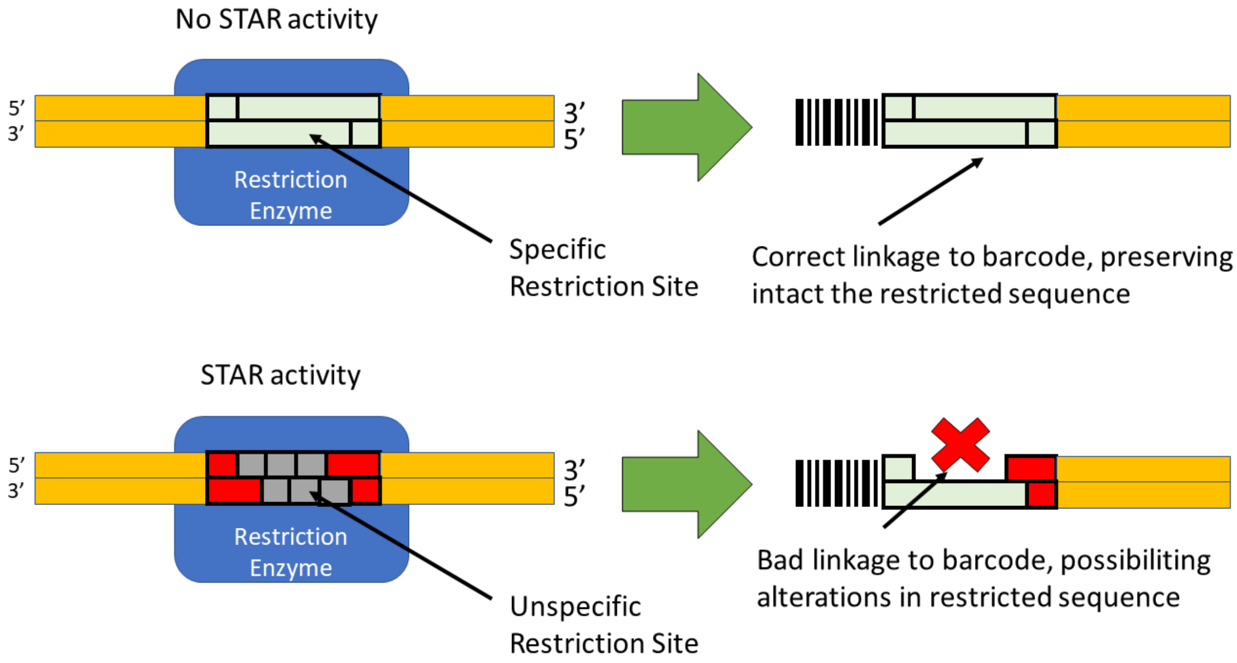

Figure 37: Schematic process of the barcode ligation to DNA sequences using enzyme restriction with and without STAR activity. At top, the current process of barcode ligation after a restriction process is the generation of the restriction site and the posterior ligation preserving intact the restriction site. At bottom, the unspecific fragments generated by STAR activity on restriction enzyme generates bad ligation with the barcodes because of the absence of a part of the restricted site, causing possible substitutions that could reduce dramatically the barcoding identification.

Nonetheless, apart from the results and the possible STAR activity, this method is very useful for other purposes. Due to the combination of two restriction enzymes and the specific primer design to generate the genome fragmentation, it could be also a good tool to sequence regions of interest on a reference genome in order to define the changes in methylation in a known region (Dong et al., 2022; Gawehns et al., 2022; Lepais & Weir, 2014; van Gurp et al., 2016; Werner et al., 2020), guiding the sequencing to these resulted fragments and their related mutations in the resulted fragments. Moreover, despite the sequence length of the generated fragments (~150 bp) that could have a detriment in the definition of Differentially Methylated Regions or DMR by skipping valuable information around the position of methylated cytosines in genic parts (Han et al., 2022; Trucchi et al., 2016), the determination of Differentially Methylated Cytosines positions or DMCs can supply partially this effect by different pattern detection of methylation (Han et al., 2022; Trucchi et al., 2016).

Although the importance of the sequence length and the coverage derived from this experiment, the use of Illumina technology showed an accurate definition (see depth information in **Supplementary File 1** and coverage in **Table 1**) that could be lower using SMRT techniques like Nanopore or PacBio (Cuber et al., 2023; Cui et al., 2020; Rayamajhi et al., 2022). Nonetheless, not only the quality reads and the depth is important, and it is crucial to understand the role of the genome to align the sequences, because the sequences could produce some bias depending on the reference genome to align it (Gawehns et al., 2022; Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022; van Gurp et al., 2016). In this experiment, the use of PN40024 reference genome gave the study a precise image because of the several improvements of the genome along the past years (Canaguier et al., 2017; Velt et al., 2023). But the use of a far variety for this experiment could be accompanied with possible wrong positions and other mobile elements like TE that could affect key positions. This effect is observed in **Table 1**, where the alignment realized using PN40024 was much higher (up to 50%) than other *Vitis* genus, *Muscadinia rotundifolia* (*V. rotundifolia*) (near to 13% in average). Although the RRBS is often utilized with non-model plants with an absence of a reference genome (Lee et al., 2021; Trucchi et al., 2016), the alignment of the methylated positions on a reference genome assessed the information about some genetic functions affected by differences in methylation between two populations like wild and cultivated varieties, such as the response to oxidative stress or the methylome maintenance detected in Chapter I (Lee et al., 2021). The huge differences in this part could be related to different events, depending on the nature of the experiment and the possible divergence between *Vitis* and *Muscadinia* (Han et al., 2022; Tran et al., 2014). On one hand, the conversion of non-methylated cytosines by bisulfite could produce possible bias in the mapping for further software packages like Bismark or BS-seq because the reduction of similarities along the genome and the converted sequences (Chatterjee et al., 2012; Tran et al., 2014). On the other hand, the relevance of the genome to align the sequences could be accompanied with more dissimilarities across the sequences and the genome scaffolds depending on the relationship between the plant subject and the phylogenetic precedence of the genome to align (Chatterjee et al., 2012; Han et al., 2022; Tran et al., 2014). Due to these facts, the use of *Pinot Noir* (belonging to *V. vinifera* ssp. *vinifera*) increased drastically the mapping rate for cultivated and wild grapevines, compared with the use of *M. rotundifolia* as a genome to align.

As mentioned above, despite the cited problems related to the use of a reference genome in this kind of experiments, other of the possible limitations of the use of a reference genome is the skip of important elements like Transposable Elements (TE) that could affects to the positions of methylated cytosines using a reference genome to align two different subspecies (*V. vinifera* ssp. *sylvestris* and *V. vinifera* ssp. *vinifera*) (Baduel & Colot, 2021; Carrier et al., 2012; Ou et al., 2019; Ramakrishnan et al., 2021). Due to the importance of these elements in grapevine along the History and the conformance of characters in vines, there could be related to possible differences in methylation that could not be detected because the utilization of only one reference genome to align the sequences (Chatterjee et al., 2012; Lee et al., 2021; Tran et al., 2014). As indicated in the results (**Table 1**), the bias using a different reference genome could exert a critical influence in the detection of methylated positions, furthermore in RRBS and the reduced coverage of the sequences. This could be related to genomic differences at basic level as a product of recombination, but also with TE, that influences the presence of methylation around them (Baduel & Colot, 2021; Carcamo et al., 2010; Ou et al., 2019; Ramakrishnan et al., 2021). In addition, the possible modifications in genes in the reference genome that are presents in one of the populations could be important to consider in future experiments (Baduel & Colot, 2021; Carrier et al., 2012; Gawehns et al., 2022). The existence of assembled genomes for each genotype of interest could help to improve the accuracy of the methylated cytosines and could also help to detect possible changes at genomic level that could imply the association of methylated regions with that variable elements in wild and cultivated grapevines (Baduel & Colot, 2021; Carcamo et al., 2010; Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022; Ou et al., 2019; Ramakrishnan et al., 2021). Other possible way to increase the resolution of the analysis is to create a general model for each condition (pangenome), that could increase the resolution of the methylated cytosines by the mapping of general regions shared in common (Garcia et al., 2023; W. Li et al., 2022; Morales-Cruz et al., 2021).

The importance of the sensitivity to ABA in grapevines with high tolerance to drought

Across the results showed in Chapter II, a general perspective of the sensitivity to ABA is key to define future perspectives exploring across the genotypic diversity of grapevine, in order to select the appropriate genotype in regions with low precipitations like the Mediterranean Area. At physiological level, as Bota et al., (2001, 2016) showed previously, the existing genetic variability in essential physiological traits as photosynthetic activity, stomatal conductance and WUE under water deprivation could be useful tool for cultivar selection. The different results between *Callet/110 Richter* and *Merlot/110 Richter* cultivars on the changes in physiological paramaters under drought may be drived by different ABA sensitivity (**Figure 38**) (Martorell et al., 2015).

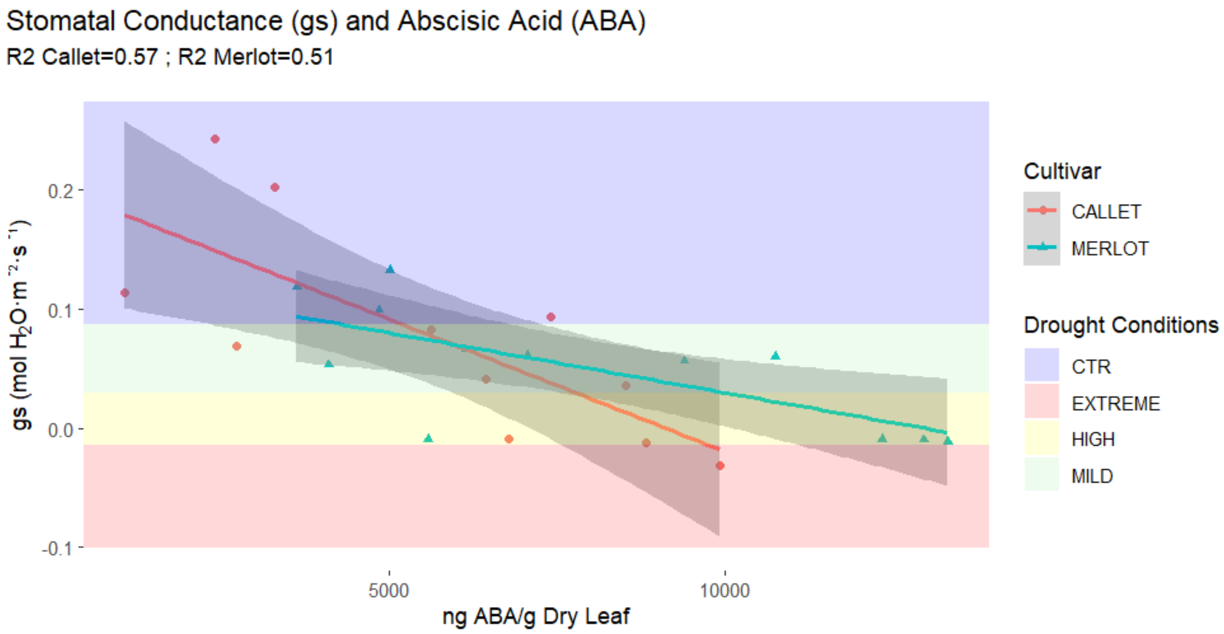

Figure 38: Linear model representing in x-axis the quantity of ABA (ng ABA/g Dry Leaf) and in y-axis the stomatal conductance (mol $H_2O \cdot m^{-2} \cdot s^{-1}$) for *Callet/110 Richter* (in circular red dots and line) and *Merlot/110 Richter* (in triangular blue dots and line) during different drought stages (Irrigated (CTR) in blue; Mild (MILD) in green; High (HIGH) in yellow; and Extreme (EXTREME) in red).

In this way, the low reduction of carbon fixation rate in *Merlot* could imply a high cumulation of ROS that could impact in the plant cell maintenance. The low stomatal regulation (**Figure 38**) could also increase the damages into the cell by possible water loses that could increase the presence of ROS into the cell (Apel & Hirt, 2004; Baranowski et al., 2019; Flexas et al., 2002).

Apart from the results observed at physiological level that could allow the scientific community evidencing different mechanism for plant adaptation to drought, the metabolomic results highlighted some biomarkers that are influenced by the sensitivity to ABA. For example, the study of key primary metabolites like Citrate, directly involved in the anthocyanidin production (Murcia et al., 2017), or other metabolites related to the cell turgor maintenance like myo-inositol in leaves (Murcia et al., 2017) can be practical to characterize the existent cultivars depending on the sensitivity to ABA and, in consequence, to be candidates biomarkers for genotype selection in grapevine adaptation to new climate change scenarios (Dal Santo et al., 2016; Gil et al., 2012; Murcia et al., 2017; Savoi et al., 2016). Nonetheless, the knowledge about isohydric and anisohydric degree in grapevines is unclear, and the combination of those methodologies to characterize these behaviours is not developed in other genotypes to be used as biomarkers.

Therefore, the evidences at physiological and metabolic level needs to be complemented with genetic studies in order to obtain a clear picture of the mechanism to face the water scarcity in grapevine (Duchêne, 2016; Duchêne et al., 2010). As mentioned before, the sensitivity to ABA in grapevine is critical, and all the physiological and metabolical responses are directly mediated by the modulation of gene expression. The transcriptomic results revealed gene families involved in the response to drought in roots and leaves, such as the *BURP* gene family, whose modulation of gene expression is directly related to the presence of ABA (Matus et al., 2014; H. Sun et al., 2019), or the *MYBs* transcription factor genes, directly involved in the synthesis of anthocyanidins and stilbenes, key molecules for cell protection against abiotic stresses (Carrasco et al., 2015; Czemmel et al., 2009; Höll et al., 2013; Matus et al., 2008; Orduña et al., 2022). The intrincate modulation of gene expression by ABA in those families is evidenced in *Callet/110 Richter,* enhancing the drought tolerance by the managing of further pathways related to the alleviation of ROS in cell, that it is not observed in the same way in *Merlot/110 Richter*. By the way, the different gene expression of genes codifying to aquaporins and other less known genes related to ABA responses in *Callet/110 Richter* evidenced the clear influence and sensitivity to ABA to elaborate an appropriate response to drought stress (J.-H. Chen et al., 2012; Coupel-Ledru et al., 2017; Huang & Jander, 2017; Muhammad Aslam et al., 2022; Tu et al., 2018). Interestingly, the identification of differences in gene expression for other gene families related to the carotene metabolism), the *bZIP* TF families or the lignin metabolism reported in other near-isohydric cultivar shed light to possible differences at morphological level too (Dal Santo et al., 2016; Tu et al., 2018). In combination with the differences in gene expression on other gene families involved sugar transportation (Medici et al., 2014; Z. Zhang et al., 2019) or the flavonoids and stilbene pathways (Corso et al., 2015; Orduña et al., 2022; Savoi et al., 2016), a different modulation of gene expression caused by ABA sensitivity in *Callet/110 Richter* could exert an influence indirectly in morphological processes such as root lignification or the maintenance of cell turgor, in order to avoid water losses and to preserve the water into the plant (Murcia et al., 2017; Savoi et al., 2016; Seleiman et al., 2021; Tu et al., 2018). Although the intricate modulation of different pathways is still unclear, the resultingevidence could contribute to build a clear image of the main different mechanisms involved in the drought stress response for near-isohydric cultivars like *Callet/110 Richter*, and help in the detection of possible cultivars with similar characteristics in the huge diversity of grapevine.

The genes related to the sensitivity to ABA gain interest as potential candidates for the study of drought tolerance in the grapevine diversity

The search of candidate genes whose modulation of gene expression is related with the mechanism of near-isohydric cultivars to enhance the adaptation to drought conditions is key to understand the complicate genic network involved in. In addition to the evidences detected below describing the general mechanism to drought tolerance, some cited gene families are described in the literature as possible candidates for determinate the drought tolerance in grapevine (Matus et al., 2008, 2014; Medici et al., 2014; Tu et al., 2018; Z. Zhang et al., 2019). In our results, the gene expression of *BURP* and *MYB* families in grapevine under drought stress conditions was similar to the bibliography, describing similar gene expression patterns involved in the response to ABA and, therefore, the drought tolerance in grapevine (Matus et al., 2008, 2014). According to the literature, a similar gene expression was also found out on families related to Flavonoid Pathway, sugar transportation or lignin, among other gene candidates detected (in **Annex 3 figures 1 to 4**), targeting the response to drought in *Callet/110 Richter* more accurate than in *Merlot/110 Richter*. The results also describes other type of candidate genes involved in the cell turgor or sugar transportation like *VvHT4* or *VvHT5* better modulated in *Callet/110 Richter* than in *Merlot/110 Richter*, that are related to sugar uptake in extracellular environment in order to reduce the activity into the plant (Medici et al., 2014; Z. Zhang et al., 2019), or in genes related to the flavonoid pathway, like *VvCHS2*, *VvCHS3* and *VvLDOX1* previously described as possible related genes in response to drought in grapes and leaves (Castellarin et al., 2007; Matus et al., 2008; Orduña et al., 2022).

Apart from the cited gene families that are susceptible to become candidate genes, the importance of the *BURP* family in drought could be essential to understand how drought tolerant grapevines could react to water deficit stress under different degrees of water privation. The results showed similar gene expression patterns in the differentially expressed genes detected, directly related to ABA, like the *VvRD22b* downregulation and the *VvRD22c* upregulation, almost in *Callet/110 Richter* leaves and roots (Matus et al., 2014). This superfamily, and more concretely the *RD22* (Response to Dehidration 22) characterized genes, are involved in drought stress response, encoding structural proteins located in apoplast and cell wall (Bray, 2002; Matus et al., 2014). However, the gene expression levels for those genes were not different under different drought stress degrees, and this event was also observed into the study conducted by Dal Santo et al. (2016), where these authors reported that near-isohydric genotypes like *Montepulciano* showed more sensitivity to ABA in terms of stomatal closure and upregulation of ABA-related genes than anisohydric genotypes, like Sangiovese, that showed low sensitivity to ABA. The *BURP* family genes were also related to abiotic stress response in grafted plants, becoming interesting as a possible family that could be involved in scion-rootstock communication.

Independently from the influence of ABA on the aerial part, the influence of this phytohormone and its observed modulation in several pathways in the case of *Callet/110 Richter* could enhance the response in roots for other types of functions like root lignification or regulating root elongation in grapevine. For example, the study of candidate genes such as *VvLAC20* in roots could determine the adaptation to drought in roots lignificating roots in order to avoid water losses (Yıldırım et al., 2018). Furthermore, other genes related to the production of secondary metabolites from the *MYBs* gene family like *VvMYB15*, *VvMYBA1*, *VvMYBA2* or *VvMYBA3* also demonstrated in the gene expression analysis their relevance in the drought adaptation for *Callet/110 Richter,* becoming interesting to explore their response on different abiotic stresses on other near-isohydric genotypes (Corso et al., 2015a; Dal Santo et al., 2016; Matus et al., 2008; Orduña et al., 2022). For example, *MYB* genes like *VvMYB15* was also related to gene expression of stilbene or flavonoid production, in order to alleviate some abiotic stresses, by regulation of genes from the Flavonoid Pathway like *VvLDOX1* or *VvUFGT1* or the *STSs* genes, appearing as differentially expressed in *Callet/110 Richter*, but not in *Merlot/110 Richter* (Matus et al., 2008; Orduña et al., 2022). The results in gene expression also remarks the role of the gene expression in genes related with the phenylpropanoids pathway on *Callet/110 Richter*, revealing a general regulation of some families involved in the drought stress response like *C4H* (Savoi et al., 2016). However, although the large evidences of related families, the network among them and their regulation of other unknown genes would be also regulated by some transcription factor families like *WRKYs*, who appeared upregulated in response to drought stress (Khoso et al., 2022), or *bZIPs* gene families, also related to ABA response in grapevine (Tu et al., 2018).

Cross talking between scion and rootstock plays a key role in the adaptation to water scarcity in near-isohydric cultivars

The relevance of the different responses in root depending on the cultivar is key to explore new strategies related with the cross talk between scion and rootstock in grapevine. The evidences coming from the Chapter II unveils the potential effect of the scion on the rootstock, guiding the response to drought stress and leading the general response in the isohydric cultivar. The use of rootstocks in vineyard is largely known, and further traits are associated to each one (Carrasco et al., 2022; Cochetel et al., 2017; Cookson & Ollat, 2013; Gautier, Cochetel, et al., 2020; Gautier, Cookson, et al., 2020; Zhou-Tsang et al., 2021). The use of *110 Richter* rootstock is often linked to arid environments, conferring the vineyard more tolerance to drought soils and increasing the vigour of the plants (Zhou-Tsang et al., 2021). However, the transcriptomic results revealed the potential of the combination scion-rootstock, revealing an exertion from the scion to the rootstock to face the drought stress in the plant, and unveiling the importance of the cross talk between shoot and root to adapt the plant to adverse conditions (see schematic diagram in **Figure 39**) (Cochetel et al., 2017; Cookson & Ollat, 2013; Gautier, Cochetel, et al., 2020; G. Kim et al., 2022).

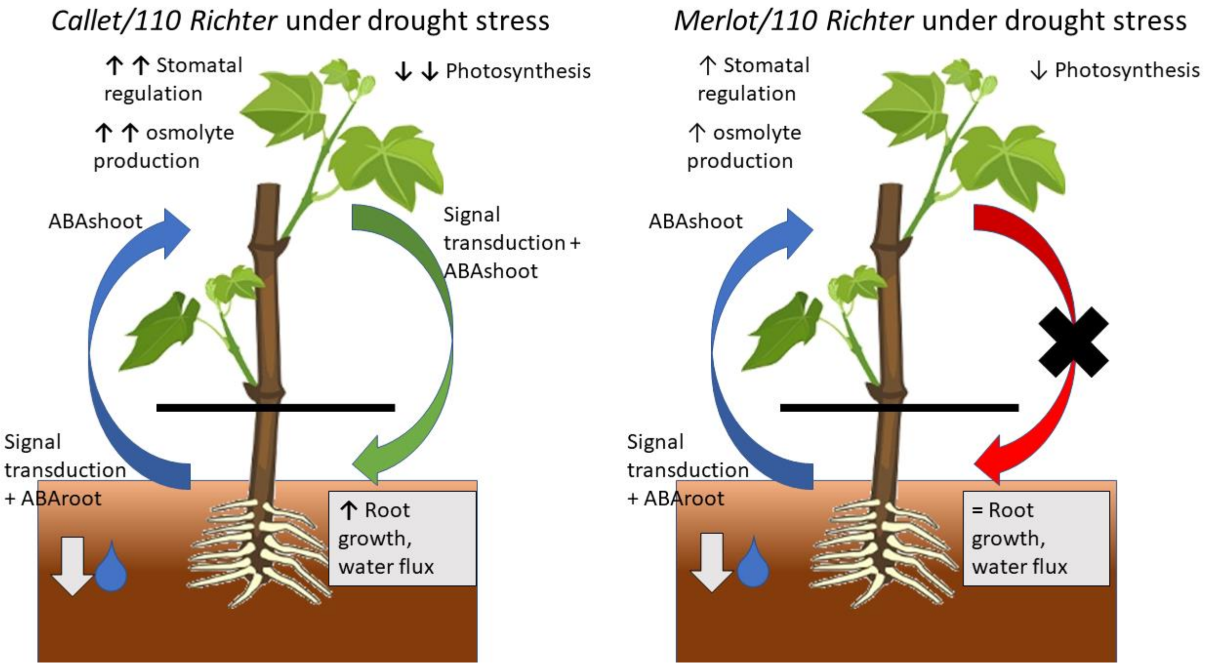

Figure 39: Abstract image for lack cross talk between scion and rootstock depending on the cultivar scion under water scarcity in soil. At left, the schema represents the good communication between scion and rootstock under drought conditions, with an initial signal of ABA in roots that travels to the shoot and then start the response and communicate it to root again. At right, the schema represents a lack of communication between scion and rootstock under drought conditions, with an initial signal of ABA in roots that travels to the shoot but the response is not accurate enough and then, the communication to root is missing.

Several studies on scion/rootstock behavior under different stresses remarked the important role of the scion to guide the transcriptomic reprogramming to face environmental stresses like phosphate or nitrogen limitation in grapevine (Cochetel et al., 2017; Cookson & Ollat, 2013; Gautier, Cochetel, et al., 2020; Guillaumie et al., 2020; Prodhomme et al., 2019). The clear influence on the rootstock is largely manifested in the results showed in Chapter II (**Figure 33 Figure 36** and the complementary **Annex 3 figures 1 to 4**), where the activation of gene expression patterns in *Callet/110 Richter* roots that are absents in *Merlot/110 Richter* roots, revealing different cross talk depending on the cultivar (**Figure 39**).

Furthermore, the identification of differentially expressed genes related to the gene silencing by miRNAs exclusively in *Callet/110 Richter* leaves under Extreme drought stress reveals an important role of the cross talk between scion and rootstock in the adaptation to water deprivation (Curaba et al., 2014; G. Kim et al., 2022). The relevance of the sensitivity to ABA becomes key to understand the pivotal role of that phytohormone in the shoot-rootstock cross talk, allowing the rootstock to respond to drought stress and inducing the modulation of several pathways to face the drought stress (G. Kim et al., 2022).

Due to the similar concentration of this phytohormone in leaves, the response to drought stress would start in roots, sending the same information to the aerial part that should respond consequently to the phytohormonal signal (Albacete et al., 2015; Archer & Fouche, 2017; Cookson & Ollat, 2013; Corso et al., 2015). Then, the different sensitivity to ABA would define the different cross talk responses between scion and rootstocks in spite of the similarities in stem water potential. The differences in gene expression at leaves also reveals the influence of the sensitivity to different metabolite pathways that are also signals to send to the root (Jogawat et al., 2021; Salvi et al., 2021; Yang et al., 2015). The different concentration levels on Gamma Amino Butyric Acid (GABA) between *Callet/110 Richter* and *Merlot/110 Richter*, a primary key metabolite related to the response to stress and involved in the cross talk between different pathways and parts of the plants (L. Li et al., 2021; Sheteiwy et al., 2019), shows the influence of the cross talk between those cultivars. The higher concentration of GABA levels in *Callet/110 Richter* than *Merlot/110 Richter* are also linked with the reduction of starch availability in *Callet/110 Richter* leaves, revealing a clear influence of the cross talk in the different stress response among the cultivars (Jogawat et al., 2021; Salvi et al., 2021; Sheteiwy et al., 2019; Yang et al., 2015). These evidences are also supported by different expression patterns between *Callet/110 Richter* and *Merlot/110 Richter* in gene families related to WRKYs or sugar transportation, that are relevant in the cross talk although the poorly documentation about the specific functions (Jogawat et al., 2021; Salvi et al., 2021; Yang et al., 2015).

Concluding remarks in transcriptomics: Reference or draft genome assembly for RNA-seq alignment in rootstocks?

The use of NGS technique on different drought stress samples across the two different years sequencing the whole transcript universe (RNA-seq) revealed different strategies at transcriptomic level in leaves and roots of isohydric cultivars like *Callet* grafted in *110 Richter* rootstocks. Thanks to the massive sequencing procedures like Illumina (see explained process in **Figure 21**), the resolution of the transcriptomic landscape for stressed grapevines is accurate at genic level. However, the study could be lacked by the small length of the fragments (~150 bp), clouding a possible detection of alternate splicing and latent genic isoforms depending on the drought stages (Cui et al., 2020; Leshkowitz et al., 2022; Rayamajhi et al., 2022; Wright et al., 2019). This effect is observed along the whole comparisons realized in *Callet/110 Richter* and *Merlot/110 Richter*, evidencing a poor abundance in isoforms detected in the DEG analysis (14 isoforms for *Callet/110 Richter* and 1 for *Merlot/110 Richter*). It is important to remark the reduced capacity to detect low expressed sequences related to the gene expression modulation after transcription using this methodology of RNA-seq, such as miRNA or long-intervent/intergenic non coding RNA (lncRNA) that could be interesting for next experiments (Curaba et al., 2014). The effect of miRNA, for example, is observed in the top genes of the signals transmitted by them, but not miRNA presence was detected in RNA-seq analysis due to the low expression of this RNA fragments and the protocol for mRNA extraction (Curaba et al., 2014).

The adequacy of the reference genome represents a significant constraint in RNA-seq analyses, particularly when comparing data across varying drought conditions, particularly in root tissues. As highlighted in the epigenomic chapter (Chapter I), biases can arise from the selection of the genome for alignment (Lee et al., 2021). This emphasizes the importance of using a high-quality and relevant reference genome to ensure accurate mapping and interpretation of RNA-seq data, especially in studies focusing on dynamic environmental conditions like drought stress. The potential biases associated with genome selection underscore the need for careful consideration and validation of reference genomes in order to minimize errors and enhance the reliability of RNA-seq analyses, particularly when investigating responses to specific environmental challenges such as drought (Lee et al., 2021; Wright et al., 2019). While utilizing a suitable genome is advantageous in population and evolutionary studies, as emphasized by studies like Lee et al. (2021) and Wright et al. (2019), the significance of genome selection takes on a slightly different context in Chapter II. In this case, the emphasis lies more on identifying genomes with accurately defined gene models associated with the sequenced transcripts. This becomes crucial for representing variations in transcript abundance between an anisohydric and a near-isohydric genotype in response to drought stress. Therefore, the key consideration shifts towards choosing genomes that precisely define gene structures, ensuring a more accurate portrayal of the differences in gene expression levels under the specific conditions of interest. This nuanced approach acknowledges the importance of gene models in capturing the subtleties of transcriptomic responses to drought stress in distinct plant genotypes (Lee et al., 2021; Wright et al., 2019). Concretely, in the case of the second chapter (Chapter II), the utilized rootstock was *110 Richter*, a hybrid from American *Vitis* grapevines (*110 Richter* comes from a hybridization between *Vitis rupestris* x *Vitis berlandieri*). Consequently, the use of a correct genome to align the transcripts would be very important, and the lineage of *110 Richter* could hinder the development of transcriptomic studies on rootstocks under different abiotic and biotic stresses. Recently, the use of SMRT allows the scientific community to generate complete non-reference models with high quality, uncovered the importance of the haploidization in heterozygous genomes such as *Vitis* genomes. Studies leaded by Dario Cantù, Andrea Minio and Noé Cochetel in (Cochetel et al., 2021; Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022), or Nathalie Ollat in (Girollet et al., 2019) among others provided the scientific community a large catalog of grapevine genomes for *Vitis* species such as *Vitis rupestris, Vitis riparia* or *Muscadinia rotundifolia*. Nonetheless, the generation of transcriptome drafts are not accurate enough compared with the reference genome PN40024, that are curated and evolved along the different versions (Canaguier et al., 2017; The French–Italian Public Consortium for Grapevine Genome Characterization, 2007).

The use of the reference genome in that case is important because of the large gene models defined in successive versions of the genome (Canaguier et al., 2017; Dal Santo et al., 2016; Matus et al., 2008, 2014; Orduña et al., 2022; The French–Italian Public Consortium for Grapevine Genome Characterization, 2007; Velt et al., 2023) that could improve the detection of gene modulation in several metabolic pathways that are not correctly identified in the new genome drafts (Cochetel et al., 2021; Minio, Cochetel, Massonnet, et al., 2022; Minio, Cochetel, Vondras, et al., 2022). For this reason, the use of PN40024 to align and identify the transcriptomic sequences is more appropriated in this kind of analysis. However, the improvements in the definition of several genes by the community can supply this problem by the research about the transcriptomic sequences in rootstocks along the genomic databases like NCBI or EBI in different transcriptomic experiments on rootstock roots under biotic and abiotic stresses (Lim et al., 2021; Sreedasyam et al., 2023; Yokoi et al., 2022). The Finding Accessible Interoperability and

Reuse (FAIR) (Wilkinson et al., 2016) principles of the transcriptomic sequences coming from gene expression studies using NGS techniques and using parts of the rootstocks like leaves or roots can enhance the definition of gene models in the transcriptome and increase the accuracy of future transcriptomic analysis.

Conclusions

Objective 1: Explore the differences in methylome modelling between wild and cultivated grapevine accessions

1. The RRBS techniques combined with Bisulfite-seq are efficient in the study of the methylome modelling in wild and cultivated populations of plants with large genomes.
2. Significant variations in DNA methylation patterns are observed between wild and cultivated grapevines.
3. The differences in methylation profiles between the two grapevine groups provide valuable insights into the possible origins and functions of DNA methylation in their evolutionary divergence.

Objective 2: Identify epialleles that could be associated to phenotypic traits in cultivated grapevines

4. The diverse associations of these methylation patterns with essential processes like alternative splicing, stress response, hormone regulation, and defense mechanisms highlight the significant impact of epimutations in shaping the evolutionary and developmental paths of domesticated species, thereby affecting the crop's plasticity and uniformity.

Objective 3. Describe the mechanisms that confers the drought tolerance in near-isohydric compared with anisohydric cultivated grapevines

5. The sensitivity to ABA is the identified key trait that confers more tolerance to drought and can be used to identify more tolerant genotypes than others derived from the domestication of grapevine.

Objective 4. Determine candidate genes responsible to drought tolerance.

6. Gene families related to the response to ABA stress and response to abiotic stresses such as *BURP* or *MYBs* have demonstrated their potential as candidate genes to identify possible cultivars tolerant to drought based on their sensitivity to ABA.

Objective 5. Study the role of the rootstock in the response to drought stress in the scion/rootstock system using near-isohydric and anisohydric cultivated grapevines.

7. The differential response of cultivars to ABA drives the communication between rootstock and scion. In *Merlot*, a low sensitivity to ABA resulted in a lack of

communication between rootstock and scion, revealing its importance in the cross talk between them under abiotic stresses like water privation.

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Annexes

Annexes

Annex 1: Statistic for Chapter II: Physiological changes, Metabolites and Hormones

Table 1: Tukey honesty test results on ANOVA one-way results by Variety (Callet and Merlot) for each Condition case (Control or CTR, Mild, High or Extreme)

Variety	type_analysis	metabolite	conditions	diff	lwr	upr	p adj
CALLET	ANOVA	Alanine	MILD-CTR	0.00026778	-0.00146722	0.00200277	0.96786227
CALLET	ANOVA	Alanine	HIGH-CTR	0.00162778	-0.00010722	0.00336277	0.06889984
CALLET	ANOVA	Alanine	EXTREME-CTR	0.00118444	-0.00085002	0.00321891	0.35818093
CALLET	ANOVA	Alanine	HIGH-MILD	0.00136	-0.00076493	0.00348493	0.28353238
CALLET	ANOVA	Alanine	EXTREME-MILD	0.00091667	-0.00145907	0.00329241	0.67694581
CALLET	ANOVA	Alanine	EXTREME-HIGH	-0.00044333	-0.00281907	0.00193241	0.94552865
CALLET	ANOVA	Arginine	MILD-CTR	0	0	0	NaN
CALLET	ANOVA	Arginine	HIGH-CTR	0	0	0	NaN
CALLET	ANOVA	Arginine	EXTREME-CTR	0	0	0	NaN
CALLET	ANOVA	Arginine	HIGH-MILD	0	0	0	NaN
CALLET	ANOVA	Arginine	EXTREME-MILD	0	0	0	NaN
CALLET	ANOVA	Arginine	EXTREME-HIGH	0	0	0	NaN
CALLET	ANOVA	Isoleucine	MILD-CTR	0.00015111	-0.00295135	0.00325357	0.99890176
CALLET	ANOVA	Isoleucine	HIGH-CTR	0.00684778	0.00374532	0.00995024	0.00010737
CALLET	ANOVA	Isoleucine	EXTREME-CTR	0.00694278	0.00330482	0.01058074	0.00043803
CALLET	ANOVA	Isoleucine	HIGH-MILD	0.00669667	0.00289694	0.01049639	0.00090233
CALLET	ANOVA	Isoleucine	EXTREME-MILD	0.00679167	0.00254345	0.01103989	0.00208148
CALLET	ANOVA	Isoleucine	EXTREME-HIGH	9.50E-05	-0.00415322	0.00434322	0.99989292
CALLET	ANOVA	Leucine	MILD-CTR	9.78E-05	-0.00203085	0.0022264	0.99907785
CALLET	ANOVA	Leucine	HIGH-CTR	0.00580444	0.00367582	0.00793307	1.17E-05
CALLET	ANOVA	Leucine	EXTREME-CTR	0.00469444	0.00219841	0.00719048	0.00050144
CALLET	ANOVA	Leucine	HIGH-MILD	0.00570667	0.00309964	0.00831369	0.00011667
CALLET	ANOVA	Leucine	EXTREME-MILD	0.00459667	0.00168192	0.00751141	0.00232928
CALLET	ANOVA	Leucine	EXTREME-HIGH	-0.00111	-0.00402474	0.00180474	0.68549003
CALLET	ANOVA	Proline	MILD-CTR	0.00232778	-0.0144604	0.01911595	0.97628124
CALLET	ANOVA	Proline	HIGH-CTR	0.04014778	0.0233596	0.05693595	4.74E-05
CALLET	ANOVA	Proline	EXTREME-CTR	0.02852111	0.00883523	0.04820699	0.0045665
CALLET	ANOVA	Proline	HIGH-MILD	0.03782	0.01725877	0.05838123	0.00061473
CALLET	ANOVA	Proline	EXTREME-MILD	0.02619333	0.00320518	0.04918149	0.02388337
CALLET	ANOVA	Proline	EXTREME-HIGH	-0.01162667	-0.03461482	0.01136149	0.47359051

CALLET	ANOVA	Acetate	MILD-CTR	-3.78E-05	-0.0003905	0.00031495	0.98873373
CALLET	ANOVA	Acetate	HIGH-CTR	0.00058556	0.00023283	0.00093828	0.00151686
CALLET	ANOVA	Acetate	EXTREME-CTR	0.00051889	0.00010528	0.00093249	0.01288368
CALLET	ANOVA	Acetate	HIGH-MILD	0.00062333	0.00019134	0.00105533	0.00471137
CALLET	ANOVA	Acetate	EXTREME-MILD	0.00055667	7.37E-05	0.00103965	0.02226409
CALLET	ANOVA	Acetate	EXTREME-HIGH	-6.67E-05	-0.00054965	0.00041632	0.97658521
CALLET	ANOVA	Ascorbate	MILD-CTR	-0.00460778	-0.02130794	0.01209238	0.84872299
CALLET	ANOVA	Ascorbate	HIGH-CTR	-0.02804111	-0.04474127	-0.01134095	0.00137637
CALLET	ANOVA	Ascorbate	EXTREME-CTR	-0.05504278	-0.07462545	-0.0354601	8.43E-06
CALLET	ANOVA	Ascorbate	HIGH-MILD	-0.02343333	-0.04388677	-0.0029799	0.02309649
CALLET	ANOVA	Ascorbate	EXTREME-MILD	-0.050435	-0.07330264	-0.02756736	0.0001082
CALLET	ANOVA	Ascorbate	EXTREME-HIGH	-0.02700167	-0.04986931	-0.00413403	0.01913968
CALLET	ANOVA	Caffeic.acid	MILD-CTR	-0.02806667	-0.0577165	0.00158317	0.06598394
CALLET	ANOVA	Caffeic.acid	HIGH-CTR	-0.03419	-0.06383983	-0.00454017	0.02219572
CALLET	ANOVA	Caffeic.acid	EXTREME-CTR	-0.04606833	-0.08083584	-0.01130082	0.00882934
CALLET	ANOVA	Caffeic.acid	HIGH-MILD	-0.00612333	-0.04243681	0.03019015	0.95882117
CALLET	ANOVA	Caffeic.acid	EXTREME-MILD	-0.01800167	-0.05860137	0.02259804	0.57799377
CALLET	ANOVA	Caffeic.acid	EXTREME-HIGH	-0.01187833	-0.05247804	0.02872137	0.82559074
CALLET	ANOVA	Lactate	MILD-CTR	0.00038444	-0.00087683	0.00164572	0.80777245
CALLET	ANOVA	Lactate	HIGH-CTR	0.00270778	0.00144651	0.00396905	0.0001415
CALLET	ANOVA	Lactate	EXTREME-CTR	0.00358278	0.0021038	0.00506175	4.15E-05
CALLET	ANOVA	Lactate	HIGH-MILD	0.00232333	0.0007786	0.00386807	0.00341224
CALLET	ANOVA	Lactate	EXTREME-MILD	0.00319833	0.00147127	0.0049254	0.00057792
CALLET	ANOVA	Lactate	EXTREME-HIGH	0.000875	-0.00085207	0.00260207	0.47218383
CALLET	ANOVA	Malate	MILD-CTR	0.03449333	-0.04038124	0.1093679	0.548475
CALLET	ANOVA	Malate	HIGH-CTR	0.15002667	0.0751521	0.22490124	0.00027676
CALLET	ANOVA	Malate	EXTREME-CTR	0.086065	-0.00173321	0.17386321	0.0554263
CALLET	ANOVA	Malate	HIGH-MILD	0.11553333	0.02383109	0.20723558	0.01252094
CALLET	ANOVA	Malate	EXTREME-MILD	0.05157167	-0.05095456	0.15409789	0.47805581
CALLET	ANOVA	Malate	EXTREME-HIGH	-0.06396167	-0.16648789	0.03856456	0.30320741
CALLET	ANOVA	Succinate	MILD-CTR	0.00015556	-0.00119551	0.00150662	0.98610143

CALLET	ANOVA	Succinate	HIGH-CTR	-0.00040111	-0.00175218	0.00094996	0.81938667
CALLET	ANOVA	Succinate	EXTREME-CTR	0.00057722	-0.00100705	0.00216149	0.71324952
CALLET	ANOVA	Succinate	HIGH-MILD	-0.00055667	-0.00221138	0.00109805	0.75897288
CALLET	ANOVA	Succinate	EXTREME-MILD	0.00042167	-0.00142836	0.00227169	0.90689912
CALLET	ANOVA	Succinate	EXTREME-HIGH	0.00097833	-0.00087169	0.00282836	0.43700305
CALLET	ANOVA	Tartrate	MILD-CTR	0.08864556	-0.08241059	0.2597017	0.45365116
CALLET	ANOVA	Tartrate	HIGH-CTR	0.08471222	-0.08634393	0.25576837	0.49074118
CALLET	ANOVA	Tartrate	EXTREME-CTR	0.00676056	-0.19382056	0.20734167	0.99963429
CALLET	ANOVA	Tartrate	HIGH-MILD	-0.00393333	-0.21343347	0.20556681	0.99993659
CALLET	ANOVA	Tartrate	EXTREME-MILD	-0.081885	-0.31611328	0.15234328	0.73764376
CALLET	ANOVA	Tartrate	EXTREME-HIGH	-0.07795167	-0.31217994	0.15627661	0.76472494
CALLET	ANOVA	Fructose	MILD-CTR	0.03357333	-0.01250664	0.07965331	0.19218427
CALLET	ANOVA	Fructose	HIGH-CTR	0.01271333	-0.03336664	0.05879331	0.84874321
CALLET	ANOVA	Fructose	EXTREME-CTR	0.00747	-0.04656356	0.06150356	0.97647992
CALLET	ANOVA	Fructose	HIGH-MILD	-0.02086	-0.07729621	0.03557621	0.70441419
CALLET	ANOVA	Fructose	EXTREME-MILD	-0.02610333	-0.08920094	0.03699427	0.62913522
CALLET	ANOVA	Fructose	EXTREME-HIGH	-0.00524333	-0.06834094	0.05785427	0.99464281
CALLET	ANOVA	Glucose	MILD-CTR	0.08073556	-0.03522649	0.1966976	0.2223962
CALLET	ANOVA	Glucose	HIGH-CTR	0.09677889	-0.01918315	0.21274093	0.11610399
CALLET	ANOVA	Glucose	EXTREME-CTR	0.04017556	-0.09580199	0.1761531	0.8214491
CALLET	ANOVA	Glucose	HIGH-MILD	0.01604333	-0.12598058	0.15806725	0.9868482
CALLET	ANOVA	Glucose	EXTREME-MILD	-0.04056	-0.19934757	0.11822757	0.87517523
CALLET	ANOVA	Glucose	EXTREME-HIGH	-0.05660333	-0.2153909	0.10218423	0.72633322
CALLET	ANOVA	Sucrose	MILD-CTR	0.05939556	-0.04246192	0.16125303	0.35688356
CALLET	ANOVA	Sucrose	HIGH-CTR	0.02619222	-0.07566525	0.12804969	0.87305491
CALLET	ANOVA	Sucrose	EXTREME-CTR	0.00544722	-0.11399125	0.1248857	0.99909734

CALLET	ANOVA	Sucrose	HIGH-MILD	-0.03320333	-0.15795275	0.09154608	0.86160649
CALLET	ANOVA	Sucrose	EXTREME-MILD	-0.05394833	-0.19342242	0.08552575	0.67532027
CALLET	ANOVA	Sucrose	EXTREME-HIGH	-0.020745	-0.16021909	0.11872909	0.97104779
CALLET	ANOVA	Choline	MILD-CTR	-0.00124556	-0.00467515	0.00218404	0.71519845
CALLET	ANOVA	Choline	HIGH-CTR	-0.00163222	-0.00506182	0.00179738	0.52275766
CALLET	ANOVA	Choline	EXTREME-CTR	0.00193778	-0.00208378	0.00595934	0.51285902
CALLET	ANOVA	Choline	HIGH-MILD	-0.00038667	-0.00458705	0.00381372	0.99276201
CALLET	ANOVA	Choline	EXTREME-MILD	0.00318333	-0.00151284	0.0078795	0.24114365
CALLET	ANOVA	Choline	EXTREME-HIGH	0.00357	-0.00112617	0.00826617	0.16600649
CALLET	ANOVA	Methanol	MILD-CTR	0.00168333	-0.00530911	0.00867578	0.89269571
CALLET	ANOVA	Methanol	HIGH-CTR	-0.00483333	-0.01182578	0.00215911	0.22738484
CALLET	ANOVA	Methanol	EXTREME-CTR	0.00693333	-0.00126604	0.0151327	0.1099654
CALLET	ANOVA	Methanol	HIGH-MILD	-0.00651667	-0.01508063	0.00204729	0.165426
CALLET	ANOVA	Methanol	EXTREME-MILD	0.00525	-0.0043248	0.0148248	0.407196
CALLET	ANOVA	Methanol	EXTREME-HIGH	0.01176667	0.00219187	0.02134147	0.01478365
CALLET	ANOVA	Trigonelline	MILD-CTR	0.00025	-0.00033582	0.00083582	0.60661902
CALLET	ANOVA	Trigonelline	HIGH-CTR	0.00054	-4.58E-05	0.00112582	0.07494655
CALLET	ANOVA	Trigonelline	EXTREME-CTR	0.00041	-0.00027693	0.00109693	0.33813827
CALLET	ANOVA	Trigonelline	HIGH-MILD	0.00029	-0.00042747	0.00100747	0.64550275
CALLET	ANOVA	Trigonelline	EXTREME-MILD	0.00016	-0.00064216	0.00096216	0.93471301
CALLET	ANOVA	Trigonelline	EXTREME-HIGH	-0.00013	-0.00093216	0.00067216	0.96313951
MERLOT	ANOVA	Alanine	MILD-CTR	0.00023111	-0.00129162	0.00175384	0.97025809
MERLOT	ANOVA	Alanine	HIGH-CTR	-0.00042222	-0.00194495	0.00110051	0.85057742
MERLOT	ANOVA	Alanine	EXTREME-CTR	-0.00014556	-0.00166829	0.00137718	0.99217098
MERLOT	ANOVA	Alanine	HIGH-MILD	-0.00065333	-0.00251829	0.00121162	0.741784
MERLOT	ANOVA	Alanine	EXTREME-MILD	-0.00037667	-0.00224162	0.00148829	0.93435
MERLOT	ANOVA	Alanine	EXTREME-HIGH	0.00027667	-0.00158829	0.00214162	0.97211922

MERLOT	ANOVA	Arginine	MILD-CTR	0	0	0	NaN
MERLOT	ANOVA	Arginine	HIGH-CTR	0	0	0	NaN
MERLOT	ANOVA	Arginine	EXTREME-CTR	0	0	0	NaN
MERLOT	ANOVA	Arginine	HIGH-MILD	0	0	0	NaN
MERLOT	ANOVA	Arginine	EXTREME-MILD	0	0	0	NaN
MERLOT	ANOVA	Arginine	EXTREME-HIGH	0	0	0	NaN
MERLOT	ANOVA	Isoleucine	MILD-CTR	-2.44E-05	-0.00070968	0.00066079	0.99958066
MERLOT	ANOVA	Isoleucine	HIGH-CTR	0.00100889	0.00032366	0.00169412	0.00375863
MERLOT	ANOVA	Isoleucine	EXTREME-CTR	0.00214889	0.00146366	0.00283412	1.57E-06
MERLOT	ANOVA	Isoleucine	HIGH-MILD	0.00103333	0.0001941	0.00187257	0.01418648
MERLOT	ANOVA	Isoleucine	EXTREME-MILD	0.00217333	0.0013341	0.00301257	1.47E-05
MERLOT	ANOVA	Isoleucine	EXTREME-HIGH	0.00114	0.00030077	0.00197923	0.0070321
MERLOT	ANOVA	Leucine	MILD-CTR	-5.89E-05	-0.00046809	0.00035032	0.97442455
MERLOT	ANOVA	Leucine	HIGH-CTR	0.00068111	0.00027191	0.00109032	0.001329
MERLOT	ANOVA	Leucine	EXTREME-CTR	0.00149444	0.00108524	0.00190365	2.41E-07
MERLOT	ANOVA	Leucine	HIGH-MILD	0.00074	0.00023883	0.00124117	0.00367308
MERLOT	ANOVA	Leucine	EXTREME-MILD	0.00155333	0.00105216	0.00205451	1.81E-06
MERLOT	ANOVA	Leucine	EXTREME-HIGH	0.00081333	0.00031216	0.00131451	0.00166076
MERLOT	ANOVA	Proline	MILD-CTR	0.00053778	-0.00734736	0.00842292	0.99710736
MERLOT	ANOVA	Proline	HIGH-CTR	0.00139444	-0.0064907	0.00927958	0.95436613
MERLOT	ANOVA	Proline	EXTREME-CTR	0.00811778	0.00023264	0.01600292	0.04270188
MERLOT	ANOVA	Proline	HIGH-MILD	0.00085667	-0.00880062	0.01051395	0.99371283
MERLOT	ANOVA	Proline	EXTREME-MILD	0.00758	-0.00207728	0.01723728	0.1497432
MERLOT	ANOVA	Proline	EXTREME-HIGH	0.00672333	-0.00293395	0.01638062	0.22593175
MERLOT	ANOVA	Acetate	MILD-CTR	-0.00015667	-0.00095803	0.0006447	0.93989875
MERLOT	ANOVA	Acetate	HIGH-CTR	0.00012333	-0.00067803	0.0009247	0.96906321
MERLOT	ANOVA	Acetate	EXTREME-CTR	-0.00039	-0.00119136	0.00041136	0.51121851
MERLOT	ANOVA	Acetate	HIGH-MILD	0.00028	-0.00070147	0.00126147	0.83977078
MERLOT	ANOVA	Acetate	EXTREME-MILD	-0.00023333	-0.0012148	0.00074813	0.89882538
MERLOT	ANOVA	Acetate	EXTREME-HIGH	-0.00051333	-0.0014948	0.00046813	0.45221301
MERLOT	ANOVA	Ascorbate	MILD-CTR	0.00863222	-0.00343729	0.02070174	0.20741781
MERLOT	ANOVA	Ascorbate	HIGH-CTR	-0.04096444	-0.05303396	-0.02889493	5.99E-07
MERLOT	ANOVA	Ascorbate	EXTREME-CTR	0.00184222	-0.01022729	0.01391174	0.96977902
MERLOT	ANOVA	Ascorbate	HIGH-MILD	-0.04959667	-0.06437875	-0.03481459	6.90E-07

MERLOT	ANOVA	Ascorbate	EXTREME-MILD	-0.00679	-0.02157208	0.00799208	0.55716532
MERLOT	ANOVA	Ascorbate	EXTREME-HIGH	0.04280667	0.02802459	0.05758875	4.05E-06
MERLOT	ANOVA	Caffeic.acid	MILD-CTR	0.00653333	-0.01043897	0.02350564	0.6843699
MERLOT	ANOVA	Caffeic.acid	HIGH-CTR	0.00651333	-0.01045897	0.02348564	0.6863604
MERLOT	ANOVA	Caffeic.acid	EXTREME-CTR	0.01792333	0.00095103	0.03489564	0.03701666
MERLOT	ANOVA	Caffeic.acid	HIGH-MILD	-2.00E-05	-0.02080674	0.02076674	0.99999999
MERLOT	ANOVA	Caffeic.acid	EXTREME-MILD	0.01139	-0.00939674	0.03217674	0.41364016
MERLOT	ANOVA	Caffeic.acid	EXTREME-HIGH	0.01141	-0.00937674	0.03219674	0.41218568
MERLOT	ANOVA	Lactate	MILD-CTR	0.00042222	-0.00072429	0.00156874	0.71232842
MERLOT	ANOVA	Lactate	HIGH-CTR	0.00112222	-2.43E-05	0.00226874	0.05595389
MERLOT	ANOVA	Lactate	EXTREME-CTR	0.00185222	0.00070571	0.00299874	0.0017276
MERLOT	ANOVA	Lactate	HIGH-MILD	7.00E-04	-0.00070419	0.00210419	0.49169796
MERLOT	ANOVA	Lactate	EXTREME-MILD	0.00143	2.58E-05	0.00283419	0.04532551
MERLOT	ANOVA	Lactate	EXTREME-HIGH	0.00073	-0.00067419	0.00213419	0.45721372
MERLOT	ANOVA	Malate	MILD-CTR	-0.04158556	-0.11648764	0.03331653	0.40274245
MERLOT	ANOVA	Malate	HIGH-CTR	0.03924778	-0.03565431	0.11414986	0.45069546
MERLOT	ANOVA	Malate	EXTREME-CTR	0.03701444	-0.03788764	0.11191653	0.49881866
MERLOT	ANOVA	Malate	HIGH-MILD	0.08083333	-0.01090261	0.17256928	0.09295106
MERLOT	ANOVA	Malate	EXTREME-MILD	0.0786	-0.01313594	0.17033594	0.10513058
MERLOT	ANOVA	Malate	EXTREME-HIGH	-0.00223333	-0.09396928	0.08950261	0.99986637
MERLOT	ANOVA	Succinate	MILD-CTR	-0.00046	-0.00157872	0.00065872	0.63964664
MERLOT	ANOVA	Succinate	HIGH-CTR	-0.00083	-0.00194872	0.00028872	0.18347567
MERLOT	ANOVA	Succinate	EXTREME-CTR	-0.00041333	-0.00153206	0.00070539	0.71032682
MERLOT	ANOVA	Succinate	HIGH-MILD	-0.00037	-0.00174015	0.00100015	0.86005792
MERLOT	ANOVA	Succinate	EXTREME-MILD	4.67E-05	-0.00132348	0.00141682	0.99963488
MERLOT	ANOVA	Succinate	EXTREME-HIGH	0.00041667	-0.00095348	0.00178682	0.81316449
MERLOT	ANOVA	Tartrate	MILD-CTR	0.07984333	-0.03265995	0.19234662	0.21265552
MERLOT	ANOVA	Tartrate	HIGH-CTR	0.08424333	-0.02825995	0.19674662	0.17767932

MERLOT	ANOVA	Tartrate	EXTREME-CTR	0.07547	-0.03703328	0.18797328	0.25259105
MERLOT	ANOVA	Tartrate	HIGH-MILD	0.0044	-0.13338782	0.14218782	0.99969892
MERLOT	ANOVA	Tartrate	EXTREME-MILD	-0.00437333	-0.14216115	0.13341449	0.99970435
MERLOT	ANOVA	Tartrate	EXTREME-HIGH	-0.00877333	-0.14656115	0.12901449	0.99764078
MERLOT	ANOVA	Fructose	MILD-CTR	0.00794111	-0.0110522	0.02693442	0.62777914
MERLOT	ANOVA	Fructose	HIGH-CTR	0.01360778	-0.00538553	0.03260109	0.20625116
MERLOT	ANOVA	Fructose	EXTREME-CTR	0.01068778	-0.00830553	0.02968109	0.39162116
MERLOT	ANOVA	Fructose	HIGH-MILD	0.00566667	-0.01759529	0.02892863	0.89224178
MERLOT	ANOVA	Fructose	EXTREME-MILD	0.00274667	-0.02051529	0.02600863	0.98551
MERLOT	ANOVA	Fructose	EXTREME-HIGH	-0.00292	-0.02618196	0.02034196	0.9827087
MERLOT	ANOVA	Glucose	MILD-CTR	0.04325778	-0.01381277	0.10032833	0.1702058
MERLOT	ANOVA	Glucose	HIGH-CTR	0.08938111	0.03231056	0.14645166	0.00225507
MERLOT	ANOVA	Glucose	EXTREME-CTR	0.05262111	-0.00444944	0.10969166	0.07534141
MERLOT	ANOVA	Glucose	HIGH-MILD	0.04612333	-0.02377353	0.1160202	0.26479957
MERLOT	ANOVA	Glucose	EXTREME-MILD	0.00936333	-0.06053353	0.0792602	0.97915682
MERLOT	ANOVA	Glucose	EXTREME-HIGH	-0.03676	-0.10665687	0.03313687	0.44765326
MERLOT	ANOVA	Sucrose	MILD-CTR	0.03550889	-0.00387025	0.07488802	0.08367142
MERLOT	ANOVA	Sucrose	HIGH-CTR	0.04226222	0.00288309	0.08164136	0.03373079
MERLOT	ANOVA	Sucrose	EXTREME-CTR	0.00345889	-0.03592025	0.04283802	0.99389268
MERLOT	ANOVA	Sucrose	HIGH-MILD	0.00675333	-0.04147606	0.05498273	0.97634307
MERLOT	ANOVA	Sucrose	EXTREME-MILD	-0.03205	-0.08027939	0.01617939	0.25955584
MERLOT	ANOVA	Sucrose	EXTREME-HIGH	-0.03880333	-0.08703273	0.00942606	0.13615999
MERLOT	ANOVA	Choline	MILD-CTR	-0.00023889	-0.00198187	0.00150409	0.97774695
MERLOT	ANOVA	Choline	HIGH-CTR	-0.00135556	-0.00309853	0.00038742	0.15498149
MERLOT	ANOVA	Choline	EXTREME-CTR	-0.00053556	-0.00227853	0.00120742	0.80853751
MERLOT	ANOVA	Choline	HIGH-MILD	-0.00111667	-0.00325137	0.00101804	0.45209559
MERLOT	ANOVA	Choline	EXTREME-MILD	-0.00029667	-0.00243137	0.00183804	0.97684683

MERLOT	ANOVA	Choline	EXTREME-HIGH	0.00082	-0.0013147	0.0029547	0.68574252
MERLOT	ANOVA	Methanol	MILD-CTR	0.00053778	-0.00510758	0.00403202	0.98564978
MERLOT	ANOVA	Methanol	HIGH-CTR	0.00093889	-0.00363091	0.00550869	0.93124914
MERLOT	ANOVA	Methanol	EXTREME-CTR	0.00140222	-0.00316758	0.00597202	0.80915375
MERLOT	ANOVA	Methanol	HIGH-MILD	0.00147667	-0.00412017	0.00707351	0.86796349
MERLOT	ANOVA	Methanol	EXTREME-MILD	0.00194	-0.00365684	0.00753684	0.74775202
MERLOT	ANOVA	Methanol	EXTREME-HIGH	0.00046333	-0.00513351	0.00606017	0.99487001
MERLOT	ANOVA	Trigonelline	MILD-CTR	0	0	0	NaN
MERLOT	ANOVA	Trigonelline	HIGH-CTR	0	0	0	NaN
MERLOT	ANOVA	Trigonelline	EXTREME-CTR	0	0	0	NaN
MERLOT	ANOVA	Trigonelline	HIGH-MILD	0	0	0	NaN
MERLOT	ANOVA	Trigonelline	EXTREME-MILD	0	0	0	NaN
MERLOT	ANOVA	Trigonelline	EXTREME-HIGH	0	0	0	NaN

Table 2: Tukey honesty test results on ANOVA one-way results by Condition (Control or CTR, Mild, High or Extreme) for each variety Varieties (Callet and Merlot)

Variety	type_analysis	metabolite	conditions	diff	lwr	upr	p adj
CTR	ANOVA	Alanine	MERLOT-CALLET	8.33E-05	-0.00077238	0.00093904	0.83904616
CTR	ANOVA	Arginine	MERLOT-CALLET	0	0	0	NaN
CTR	ANOVA	Isoleucine	MERLOT-CALLET	-6.44E-05	-0.00017338	4.45E-05	0.22780303
CTR	ANOVA	Leucine	MERLOT-CALLET	0	-7.02E-05	7.02E-05	1
CTR	ANOVA	Proline	MERLOT-CALLET	-0.00123	-0.0026275	0.0001675	0.08050811
CTR	ANOVA	Acetate	MERLOT-CALLET	0.00060556	0.00032338	0.00088773	0.00032832
CTR	ANOVA	Ascorbate	MERLOT-CALLET	-0.01964333	-0.02820321	-0.01108346	0.00017207
CTR	ANOVA	Caffeic.acid	MERLOT-CALLET	-0.03513	-0.04725428	-0.02300572	1.42E-05
CTR	ANOVA	Lactate	MERLOT-CALLET	-0.00038111	-0.00087379	0.00011156	0.12054334
CTR	ANOVA	Malate	MERLOT-CALLET	0.07603222	0.03785763	0.11420682	0.00064772
CTR	ANOVA	Succinate	MERLOT-CALLET	0.00040556	-0.00031845	0.00112956	0.25237078
CTR	ANOVA	Tartrate	MERLOT-CALLET	-0.02904111	-0.11583217	0.05774995	0.48832203
CTR	ANOVA	Fructose	MERLOT-CALLET	0.00442556	-0.0159915	0.02484261	0.65205254
CTR	ANOVA	Glucose	MERLOT-CALLET	0.00123111	-0.03495577	0.03741799	0.94339936

CTR	ANOVA	Sucrose	MERLOT-CALLET	0.03451	-0.01292282	0.08194282	0.14253397
CTR	ANOVA	Choline	MERLOT-CALLET	-0.00149667	-0.00312845	0.00013512	0.06964813
CTR	ANOVA	Methanol	MERLOT-CALLET	-0.00316556	-0.00621267	-0.00011844	0.04265508
CTR	ANOVA	Trigonelline	MERLOT-CALLET	-0.00032	-0.0005189	-0.0001211	0.00357865
MILD	ANOVA	Alanine	MERLOT-CALLET	4.67E-05	-0.00096173	0.00105506	0.90396327
MILD	ANOVA	Arginine	MERLOT-CALLET	0	0	0	NaN
MILD	ANOVA	Isoleucine	MERLOT-CALLET	-0.00024	-0.00052163	4.16E-05	0.07715139
MILD	ANOVA	Leucine	MERLOT-CALLET	-0.00015667	-0.00042807	0.00011474	0.18426544
MILD	ANOVA	Proline	MERLOT-CALLET	-0.00302	-0.01299813	0.00695813	0.44802027
MILD	ANOVA	Acetate	MERLOT-CALLET	0.00048667	-0.00028599	0.00125932	0.15523671
MILD	ANOVA	Ascorbate	MERLOT-CALLET	-0.00640333	-0.02115958	0.00835291	0.29468114
MILD	ANOVA	Caffeic.acid	MERLOT-CALLET	-0.00053	-0.02131651	0.02025651	0.94696124
MILD	ANOVA	Lactate	MERLOT-CALLET	-0.00034333	-0.0010102	0.00032353	0.22608937
MILD	ANOVA	Malate	MERLOT-CALLET	-4.67E-05	-0.04619641	0.04610308	0.99789434
MILD	ANOVA	Succinate	MERLOT-CALLET	-0.00021	-0.0009433	0.0005233	0.47106071
MILD	ANOVA	Tartrate	MERLOT-CALLET	-0.03784333	-0.1584617	0.08277503	0.4328612
MILD	ANOVA	Fructose	MERLOT-CALLET	-0.02120667	-0.04957334	0.00716	0.10654615
MILD	ANOVA	Glucose	MERLOT-CALLET	-0.03624667	-0.08962478	0.01713145	0.13245781
MILD	ANOVA	Sucrose	MERLOT-CALLET	0.01062333	-0.04369669	0.06494335	0.61597802
MILD	ANOVA	Choline	MERLOT-CALLET	-0.00049	-0.00297037	0.00199037	0.6125296
MILD	ANOVA	Methanol	MERLOT-CALLET	-0.00538667	-0.0075236	-0.00324974	0.00219523
MILD	ANOVA	Trigonelline	MERLOT-CALLET	-0.00057	-0.00089537	-0.00024463	0.00825694
HIGH	ANOVA	Alanine	MERLOT-CALLET	-0.00196667	-0.00457297	0.00063964	0.10423368
HIGH	ANOVA	Arginine	MERLOT-CALLET	0	0	0	NaN
HIGH	ANOVA	Isoleucine	MERLOT-CALLET	-0.00590333	-0.01068482	-0.00112185	0.02658803
HIGH	ANOVA	Leucine	MERLOT-CALLET	-0.00512333	-0.00886851	-0.00137816	0.01913507

HIGH	ANOVA	Proline	MERLOT-CALLET	-0.03998333	-0.07052763	-0.00943903	0.02207339
HIGH	ANOVA	Acetate	MERLOT-CALLET	0.00014333	-0.00078021	0.00106687	0.68874188
HIGH	ANOVA	Ascorbate	MERLOT-CALLET	-0.03256667	-0.04267798	-0.02245535	0.00086708
HIGH	ANOVA	Caffeic.acid	MERLOT-CALLET	0.00557333	-0.0151171	0.02626376	0.49610063
HIGH	ANOVA	Lactate	MERLOT-CALLET	-0.00196667	-0.00386378	-6.96E-05	0.04509288
HIGH	ANOVA	Malate	MERLOT-CALLET	-0.03474667	-0.14583767	0.07634433	0.43416815
HIGH	ANOVA	Succinate	MERLOT-CALLET	-2.33E-05	-0.00102269	0.00097602	0.95142309
HIGH	ANOVA	Tartrate	MERLOT-CALLET	-0.02951	-0.16584345	0.10682345	0.58025083
HIGH	ANOVA	Fructose	MERLOT-CALLET	0.00532	-0.00618165	0.01682165	0.26839717
HIGH	ANOVA	Glucose	MERLOT-CALLET	-0.00616667	-0.13615841	0.12382507	0.90157135
HIGH	ANOVA	Sucrose	MERLOT-CALLET	0.05058	0.00814093	0.09301907	0.02968142
HIGH	ANOVA	Choline	MERLOT-CALLET	-0.00122	-0.00263481	0.00019481	0.07483017
HIGH	ANOVA	Methanol	MERLOT-CALLET	0.00260667	-0.00568299	0.01089632	0.43191281
HIGH	ANOVA	Trigonelline	MERLOT-CALLET	-0.00086	-0.00121156	-0.00050844	0.00245578
EXTREME	ANOVA	Alanine	MERLOT-CALLET	-0.00124667	-0.00297034	0.00047701	0.10482119
EXTREME	ANOVA	Arginine	MERLOT-CALLET	0	0	0	NaN
EXTREME	ANOVA	Isoleucine	MERLOT-CALLET	-0.00485833	-0.0116477	0.00193103	0.10720203
EXTREME	ANOVA	Leucine	MERLOT-CALLET	-0.0032	-0.00693371	0.00053371	0.07209004
EXTREME	ANOVA	Proline	MERLOT-CALLET	-0.02163333	-0.05318397	0.0099173	0.11711714
EXTREME	ANOVA	Acetate	MERLOT-CALLET	-0.00030333	-0.00138081	0.00077415	0.43631182
EXTREME	ANOVA	Ascorbate	MERLOT-CALLET	0.03724167	0.02349902	0.05098432	0.00327854
EXTREME	ANOVA	Caffeic.acid	MERLOT-CALLET	0.02886167	-0.02508111	0.08280444	0.18716829
EXTREME	ANOVA	Lactate	MERLOT-CALLET	-0.00211167	-0.00514304	0.0009197	0.11336929
EXTREME	ANOVA	Malate	MERLOT-CALLET	0.02698167	-0.09561353	0.14957687	0.53410078
EXTREME	ANOVA	Succinate	MERLOT-CALLET	-0.000585	-0.0024754	0.0013054	0.39732148
EXTREME	ANOVA	Tartrate	MERLOT-CALLET	0.03966833	0.02606364	0.05327303	0.00264884

EXTREME	ANOVA	Fructose	MERLOT-CALLET	0.00764333	-0.0491543	0.06444097	0.69734369
EXTREME	ANOVA	Glucose	MERLOT-CALLET	0.01367667	-0.23198784	0.25934117	0.87065884
EXTREME	ANOVA	Sucrose	MERLOT-CALLET	0.03252167	-0.02710177	0.0921451	0.18099002
EXTREME	ANOVA	Choline	MERLOT-CALLET	-0.00397	-0.00648952	-0.00145048	0.01527012
EXTREME	ANOVA	Methanol	MERLOT-CALLET	-0.00869667	-0.01904697	0.00165363	0.07543318
EXTREME	ANOVA	Trigonelline	MERLOT-CALLET	-0.00073	-0.00172626	0.00026626	0.10197111

Figure 1: Results of correlation Log2FoldChange coming from the selected DEG from RNA-seq and Log2FoldChange coming from the RT-qPCR results.

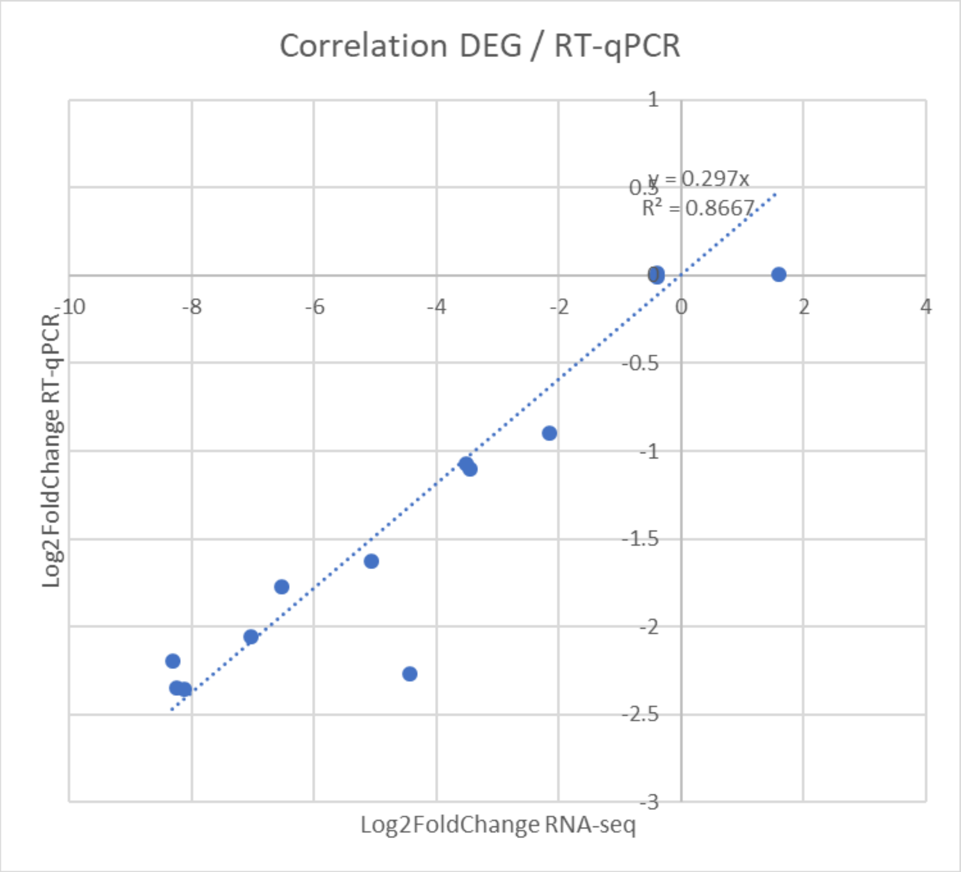


Table 3: Principal Proteins related to genes identified in Principal Component Analysis and their principal functions.

Gene Name	Protein	Homologous Organism	Functions
Vitvi01g00556	NAD(P)-linked oxidoreductase superfamily protein	*Arabidopsis thaliana*	Response to Abscisic Acid, response to ethylene, response to osmotic stress, response to water deprivation
Vitvi01g01351	Leucine-rich repeat protein kinase family protein	*Arabidopsis thaliana*	Anther development, cellular response to peptide, homeostasis of number of meristem cells, phosphorylation
Vitvi01g02258	Ribosomal protein L22p/L17e family protein	*Arabidopsis thaliana*	Translation
Vitvi02g00429	STAY-GREEN-like protein	*Arabidopsis thaliana*	Chloroplast Organization
Vitvi03g01490	NmrA-like negative transcriptional regulator family protein	*Arabidopsis thaliana*	Response to Oxidative stress
Vitvi03g01503	-	-	-
Vitvi04g00312	Phosphorylase superfamily protein	*Arabidopsis thaliana*	Response to water deprivation
Vitvi04g00602	early nodulin-like protein	*Arabidopsis thaliana*	Plasma membrane
Vitvi04g00997	GDSL-like Lipase/Acylhydrolase superfamily protein	*Arabidopsis thaliana*	Macromolecule biosynthetic process
Vitvi04g01368	Dehydrin family protein	*Arabidopsis thaliana*	Response to Abscisic Acid, response to water deprivation
Vitvi04g01633	N-acetylglucosamine-1-phosphate uridylyltransferase	*Arabidopsis thaliana*	UDP-N-acetylgalactosamine metabolic process, UDP-N-acetylglucosamine metabolic process
Vitvi05g00170	galactinol synthase	*Arabidopsis thaliana*	Response to Abscisic acid, response to salt stress, response to water deprivation
Vitvi05g01953	-	-	-
Vitvi05g04032	Heat stable protein	*Arabidopsis thaliana*	Defense response to bacterium, defense response to fungus
Vitvi05g04385	-	-	-
Vitvi06g00666	Asparagine synthetase	*Arabidopsis thaliana*	L-asparagine biosynthetic process, asparagine biosynthetic process
Vitvi06g01346	Tonoplast intrinsic protein	*Arabidopsis thaliana*	Water transport
Vitvi06g01696	Metallothionein	*Helianthus annuus*	Response to Abscisic Acid
Vitvi06g04052	Histone superfamily protein	*Arabidopsis thaliana*	DNA replication, macromolecule methylation, macromolecule modification, regulation of DNA metabolic process, regulation of cell cycle, regulation of nucleobase-containing compound metabolic process
Vitvi07g00792	UDP-Glycosyltransferase superfamily protein	*Arabidopsis thaliana*	Cellulose biosynthetic process, cold acclimation

Vitvi07g02097	Histone H2A protein	*Arabidopsis thaliana*	DNA methylation, environmental cues
Vitvi07g02504	Homolog of Synechocystis YCF37	*Arabidopsis thaliana*	Response to light stimulus
Vitvi07g03083	Cinnamyl alcohol dehydrogenase	*Arabidopsis thaliana*	Lignin biosynthetic process
Vitvi08g01520	Cytosolic enolase	*Arabidopsis thaliana*	Glycolytic process
Vitvi08g02226	Glutatione-S-transferase	*Arabidopsis thaliana*	Light signalling, sensitivity to ABA
Vitvi08g02375	Pectin lyase-like superfamily protein	*Arabidopsis thaliana*	Fruit dehiscence
Vitvi09g00264	GDSL-like Lipase/Acylhydrolase superfamily protein	*Arabidopsis thaliana*	Lipid catabolic process
Vitvi09g04225	Inorganic H pyrophosphatase family protein	*Arabidopsis thaliana*	Regulation of apoplastic pH and auxin transport
Vitvi10g00761	Ribosomal protein L18ae family protein	*Arabidopsis thaliana*	Translation
Vitvi10g04021	-	-	-
Vitvi10g04288	Similar to Polyphenol oxidase	*Oryza sativa Japonica Group*	Protecting Photosynthetic electron transport
Vitvi12g01876	CRA-RmlC-like Proteins	*Arabidopsis thaliana*	Cellular response to abscisic Acid Stimulus
Vitvi12g02394	-	-	-
Vitvi13g02522	Thioredoxin superfamily protein	*Arabidopsis thaliana*	Antioxidant activity
Vitvi14g00322	alpha/beta-Hydrolases superfamily protein	*Arabidopsis thaliana*	Response to gibberellin
Vitvi14g01449	Chalcone and Stilbene synthase	*Arabidopsis thaliana*	Flavonoid biosynthetic process
Vitvi15g00960	O-acyltransferase (WSD1-like) family protein	*Arabidopsis thaliana*	Triglyceride biosynthetic process, wax synthesis
Vitvi15g01110	Plasma Membrane Intrinsic Protein	*Arabidopsis thaliana*	Response to water deprivation, water transport
Vitvi15g01538	Purple acid phosphatase	*Arabidopsis thaliana*	Metal ion binding
Vitvi17g00700	Cytochrome P450 superfamily protein	*Arabidopsis thaliana*	Flavonoid biosynthetic process, response to auxin
Vitvi17g04032	-	-	-
Vitvi18g01072	Cytochrome P450 superfamily protein	*Arabidopsis thaliana*	Indole-containing compound metabolic process
Vitvi18g03215	-	-	-
Vitvi19g00041	Trichome Birefringence-like	*Arabidopsis thaliana*	O-acetyltransferase activity
Vitvi19g01878	Cellulase (glycosyl hydroxilase family 5) protein	*Arabidopsis thaliana*	Organic Substance Metabolic Process
Vitvi19g02056	ZRT/IRT-like protein	*Oryza sativa Japonica Group*	Zinc ion transport

Annex 2: General information of grapevines involved in Chapter I

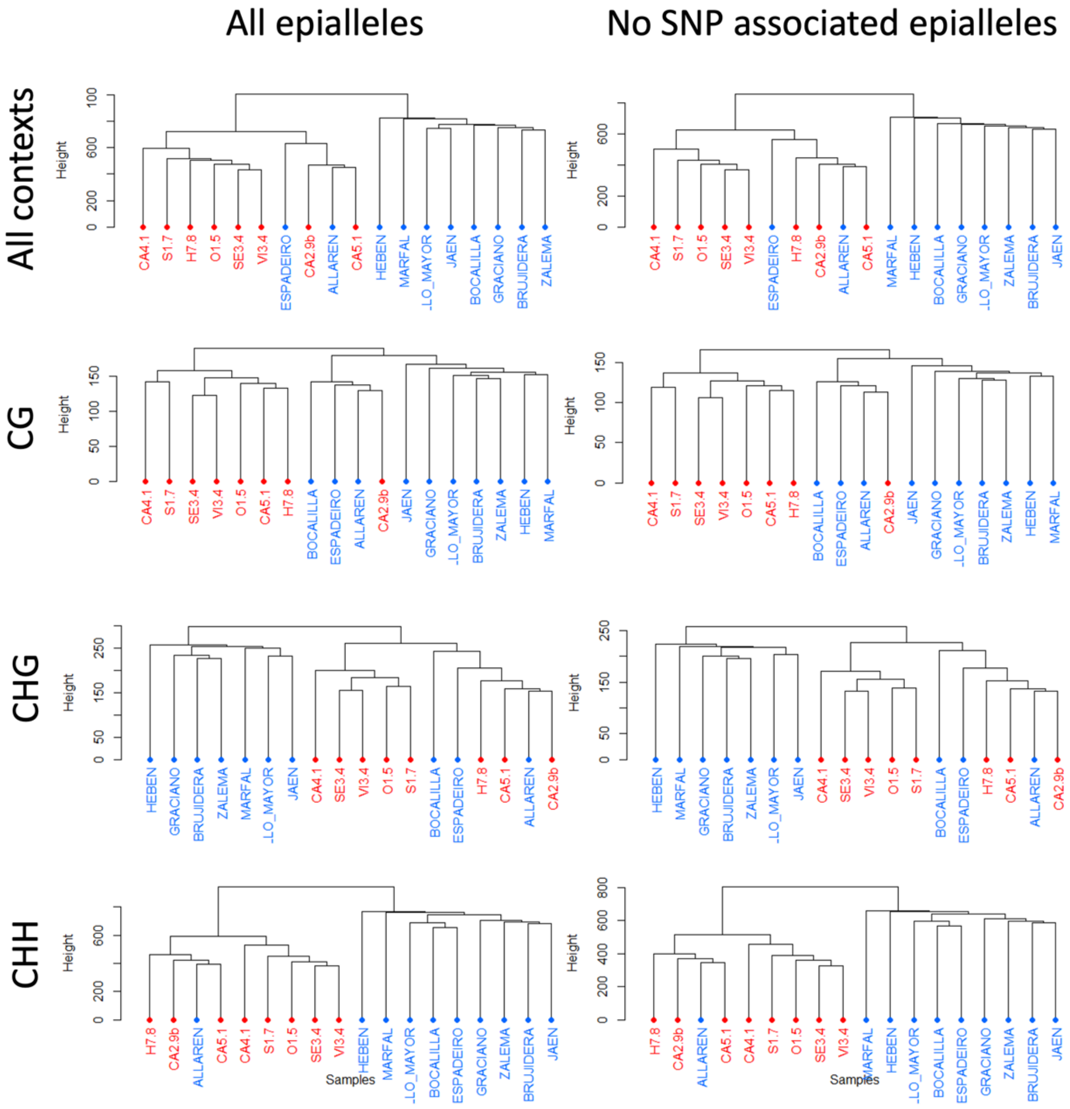
Table 1: Taxonomy and registration of the plants involved in the chapter I.

BGVCAM code	ID	taxonomy ID	genrus	Specie	ssp	accesion	category	registration date
ESP080-BGVCAM3298	CA2.9b	755351	*Vitis*	*vinifera*	*sylvestris*	CA2.9b	wine grape	24/11/2004
ESP080-BGVCAM2930	CA4.1	755351	*Vitis*	*vinifera*	*sylvestris*	CA4.1	wine grape	30/01/2003
ESP080-BGVCAM2948	CA5.1	755351	*Vitis*	*vinifera*	*sylvestris*	CA5.1	wine grape	30/01/2003
ESP080-BGVCAM3014	H7.8	755351	*Vitis*	*vinifera*	*sylvestris*	H7.8	wine grape	30/01/2003
ESP080-BGVCAM3162	O1.5	755351	*Vitis*	*vinifera*	*sylvestris*	O1.5	wine grape	27/02/2003
ESP080-BGVCAM3173	S1.7	755351	*Vitis*	*vinifera*	*sylvestris*	S1.7	wine grape	11/02/2003
ESP080-BGVCAM3051	SE3.4	755351	*Vitis*	*vinifera*	*sylvestris*	SE3.4	wine grape	30/01/2003
ESP080-BGVCAM3207	VI3.4	755351	*Vitis*	*vinifera*	*sylvestris*	VI3.4	wine grape	09/01/2003
ESP080-BGVCAM0929	ALBILLO_MAYOR	29760	*Vitis*	*vinifera*	*vinifera*	Albillo Mayor	wine grape	01/01/1914
ESP080-BGVCAM0934	ALLAREN	29760	*Vitis*	*vinifera*	*vinifera*	Allarén	wine grape	01/01/1914
ESP080-BGVCAM1110	BOCALILLA	29760	*Vitis*	*vinifera*	*vinifera*	Bocalilla	wine grape	01/01/1951
ESP080-BGVCAM0949	BRUJIDERA	29760	*Vitis*	*vinifera*	*vinifera*	Brujidera	wine grape	01/01/1914
ESP080-BGVCAM1867	ESPADEIRO	29760	*Vitis*	*vinifera*	*vinifera*	Espadeiro	wine grape	10/01/1975
ESP080-BGVCAM2134	GRACIANO	29760	*Vitis*	*vinifera*	*vinifera*	Graciano	wine grape	06/03/1981
ESP080-BGVCAM2030	HEBEN	29760	*Vitis*	*vinifera*	*vinifera*	Heben	wine grape	05/01/1980
ESP080-BGVCAM2184	JAEN_TINTO	29760	*Vitis*	*vinifera*	*vinifera*	Jaén Tinto	wine grape	03/02/1982
ESP080-BGVCAM1526	MARFAL	29760	*Vitis*	*vinifera*	*vinifera*	Marfal	wine grape	01/02/1971
ESP080-BGVCAM2119	ZALEMA	29760	*Vitis*	*vinifera*	*vinifera*	Zalema	wine grape	21/01/1981

Table 2: Geographical information for wild and cultivated grapevines involved in Chapter I

ID	country	State	region	Village	Location	UTM X	UTM Y	zone (huso)	orientation
CA2.9b	Spain	Andalucía	Cádiz	El Bosque	Rio Majaceite	277292,94	4072262,21	30	S
CA4.1	Spain	Andalucía	Cádiz	Prado del Rey	Pantano de los Hurones	271556,35	4066522,53	30	S
CA5.1	Spain	Andalucía	Cádiz	Ubrique	Rio Tavizna	277382,04	4067109,15	30	S
H7.8	Spain	Andalucía	Huelva	Almonte	Doñana, Las Algaidas de Meloncillo y Carrizal	718114,61	4113719,79	29	S
O1.5	Spain	Asturias	Asturias	Ponga	Rio Cares	331639,44	4781312,73	30	N
S1.7	Spain	Cantabria	Cantabria	Ramales de la Victoria	Cueva Covalanas	463486,85	4787565,10	30	N
SE3.4	Spain	Andalucía	Sevilla	El Garrobo	Embalse Laminilla	749704,31	4172819,47	29	S
VI3.4	Spain	Euskadi	A⍰lava	Lantaron	Sobron	492316,56	4733273,51	30	N
ALBILLO_MAYOR	Spain	Castilla y León	Palencia	Nd	-	-	-	-	-
ALLAREN	Spain	Castilla y León	León	Nd	-	-	-	-	-
BOCALILLA	Spain	Comunidad de Madrid	Madrid	Madrid	-	-	-	-	-
BRUJIDERA	Spain	Castilla-La Mancha	Toledo	Nd	-	-	-	-	-
ESPADEIRO	Spain	Galicia	Pontevedra	Cambados	-	-	-	-	-
GRACIANO	Spain	Navarra	Navarra	Nd	-	-	-	-	-
HEBEN	Spain	Castilla-La Mancha	Guadalajara	Mondejar	-	-	-	-	-
JAEN_TINTO	Spain	Andalucía	Granada	Nd	-	-	-	-	-
MARFAL	Spain	Extremadura	Cáceres	CaÒamero	-	-	-	-	-
ZALEMA	Spain	Andalucía	Huelva	Moguer	-	691.937,15	4.127.514,17	29	-

Figure 1: Analysis of differences in global levels of DNA methylation in cultivated and wild V. vinifera accessions. Hierarchical cluster analysis of percentage of methylation for all 222,711 epialleles sequenced (left column), or 165,189 epialleles not associated to SNP (right column), in cultivated (blue) and wild (red) *V. vinifera* accessions. Cluster plots show results for methylation analysis results containing all sequence context, and separated by context (e.g., CG, CHG, and CHH).



Annex 3: Heatmaps and analysis corresponding to Chapter II

Figure 1: Heatmaps of known DEGs detected in *Callet/110 Richter* and *Merlot/110 Richter* for High and Extreme drought conditions in leaves and roots: a) Flavonoid pathway genes and b) Stilbene pathway genes. The colors represent in blue scale the downregulated genes and in red scale the upregulated genes (expressed at log2foldChange value).

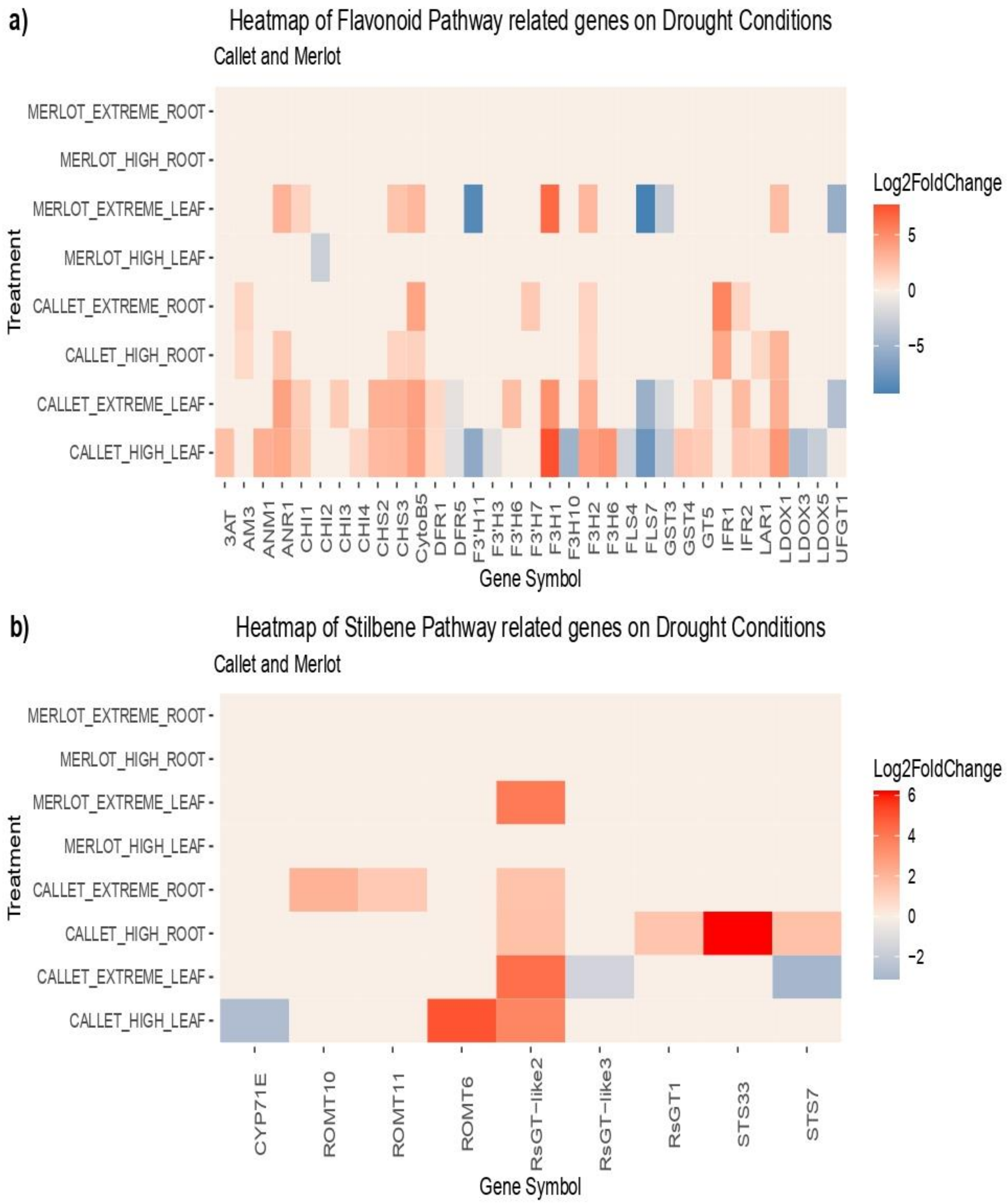
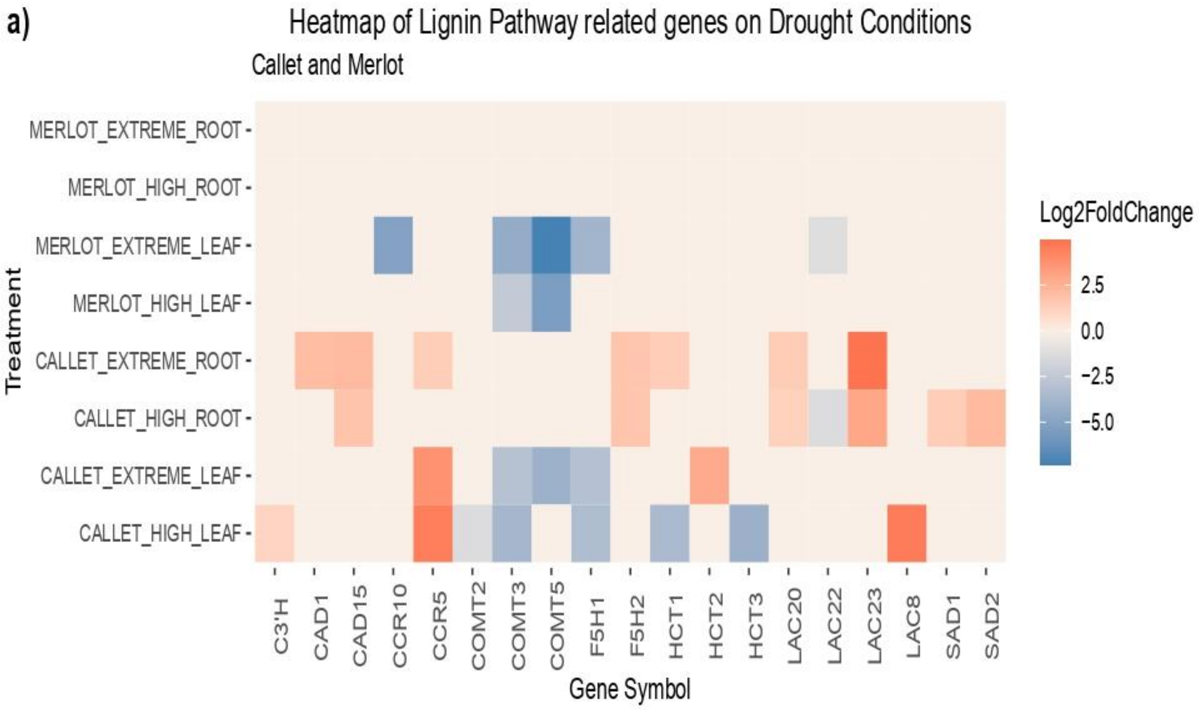


Figure 2: Heatmaps of known DEGs detected in *Callet/110 Richter* and *Merlot/110 Richter* for High and Extreme drought conditions in leaves and roots: a) Lignin pathway genes; and b) Sugar metabolism pathway genes. The colors represent in blue scale the downregulated genes and in red scale the upregulated genes (expressed at log2foldChange value).



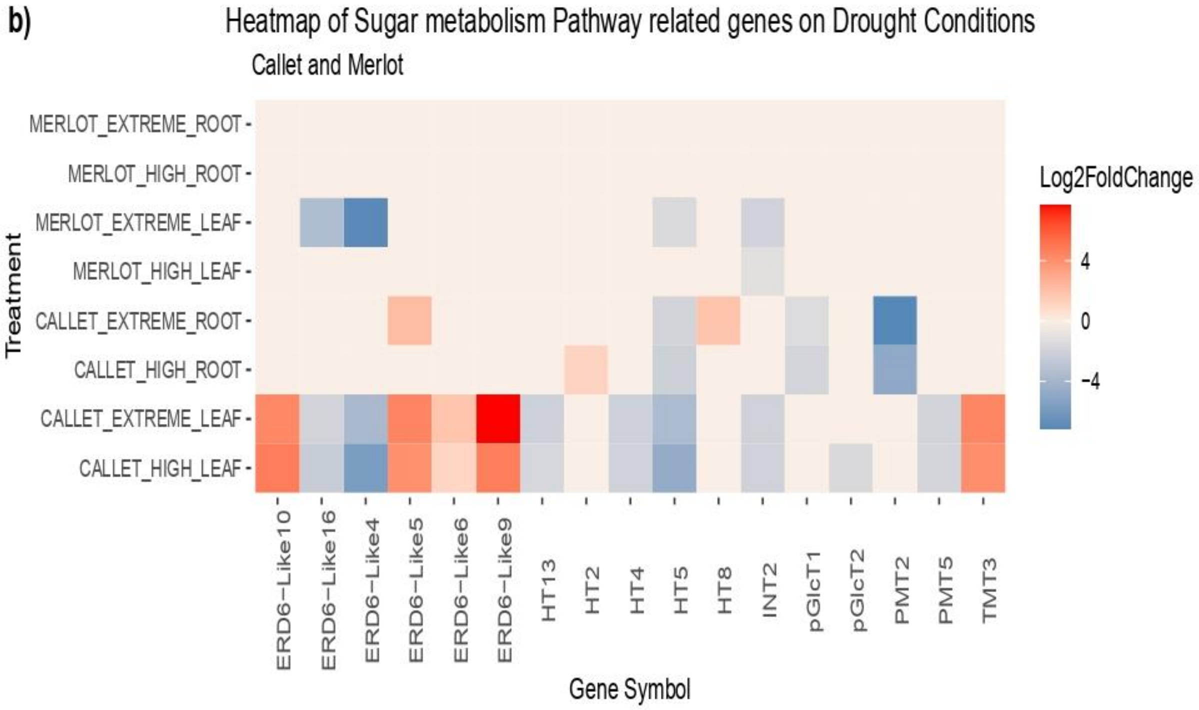


Figure 3: Heatmaps of known DEGs detected in *Callet/110 Richter* and *Merlot/110 Richter* for High and Extreme drought conditions in leaves and roots: a) Phenyl Propanoid pathway genes; and b) WRKYs genes. The colors represent in blue scale the downregulated genes and in red scale the upregulated genes (expressed at log2foldChange value).

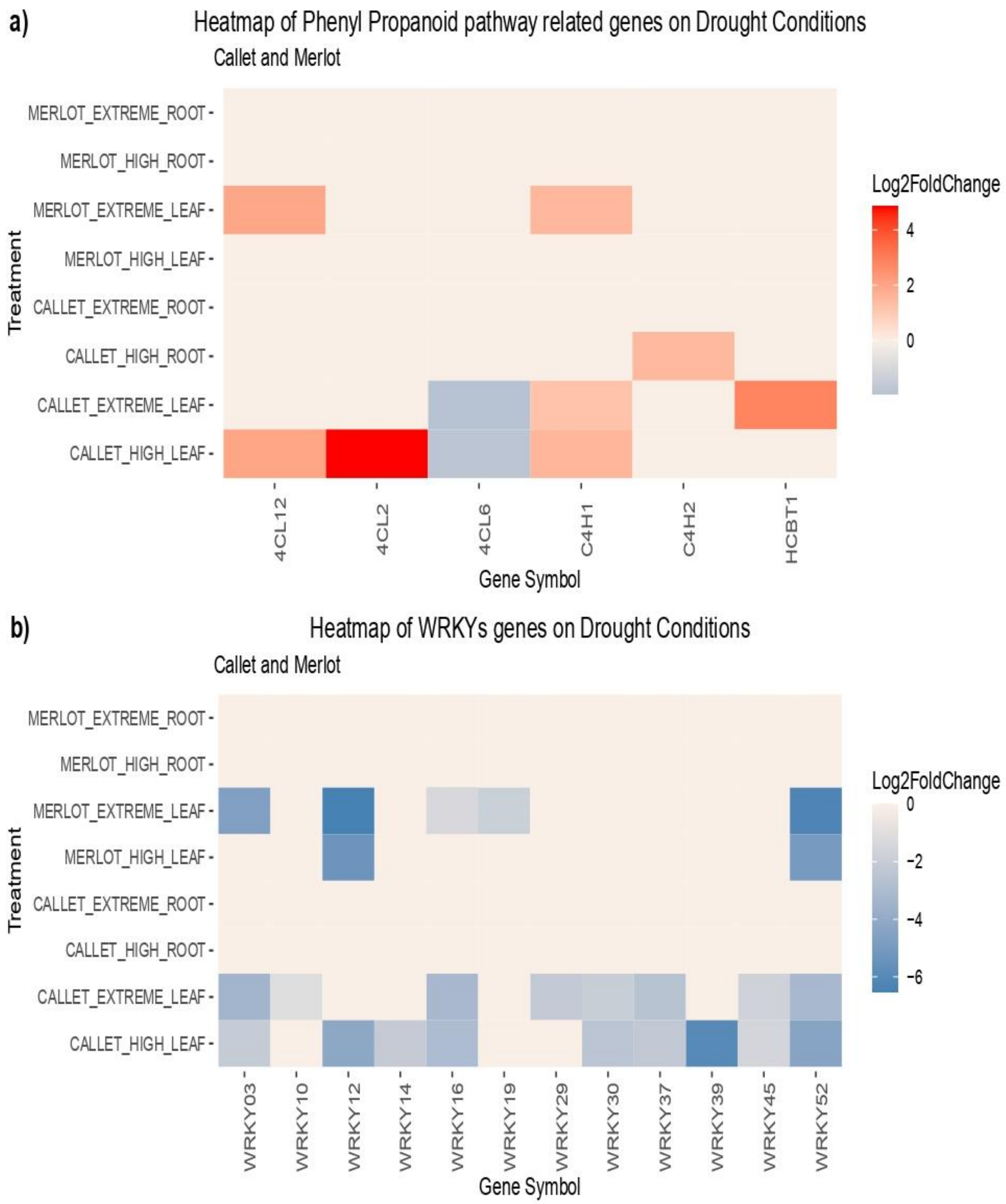


Figure 4: Heatmaps of known DEGs detected in *Callet/110 Richter* and *Merlot/110 Richter* for High and Extreme drought conditions in leaves and roots: a) Caroten pathway genes; and b) bZIPs genes. The colors represent in blue scale the downregulated genes and in red scale the upregulated genes (expressed at log2foldChange value).

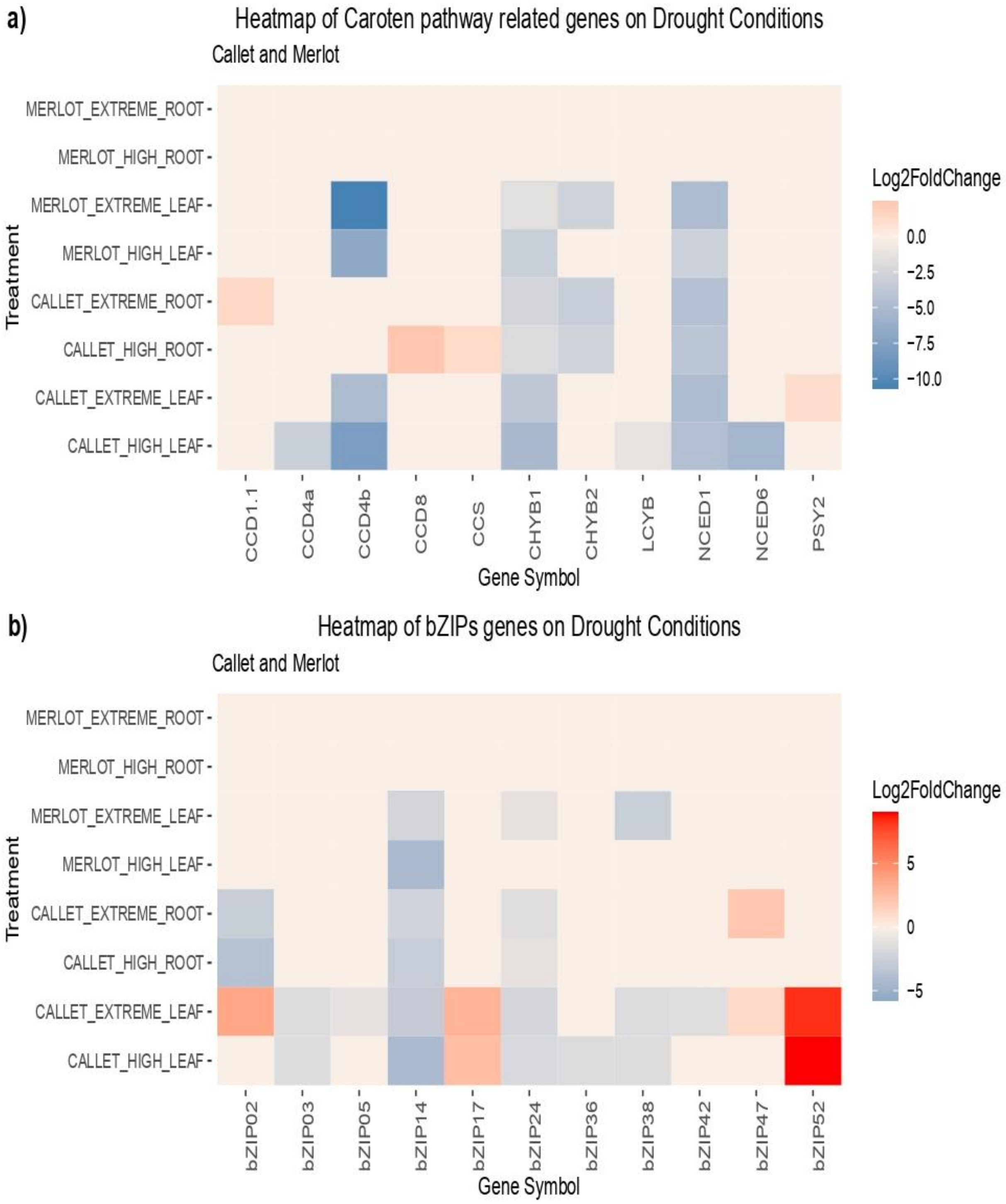


Figure 5: Boxplot of the results of a) Indolacetic Acid (IAA); b) Jasmonic Acid (JA); and c) Salicilic Acid (SA) concentration in leaves (expressed in ng ABA / g dry leaves); on Mild, High and Extreme conditions in 2022 for *Callet/110 Richter* and *Merlot/110 Richter*.

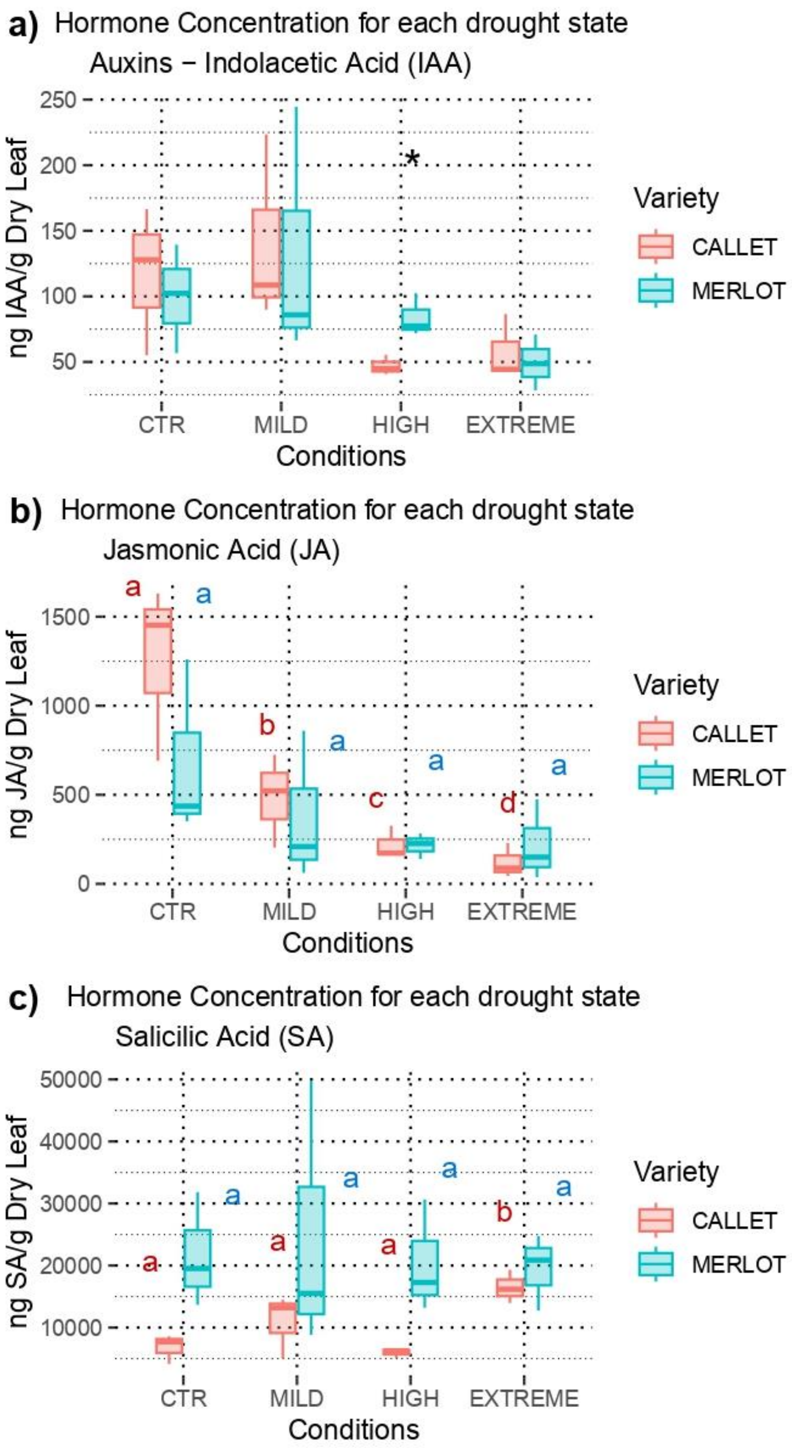
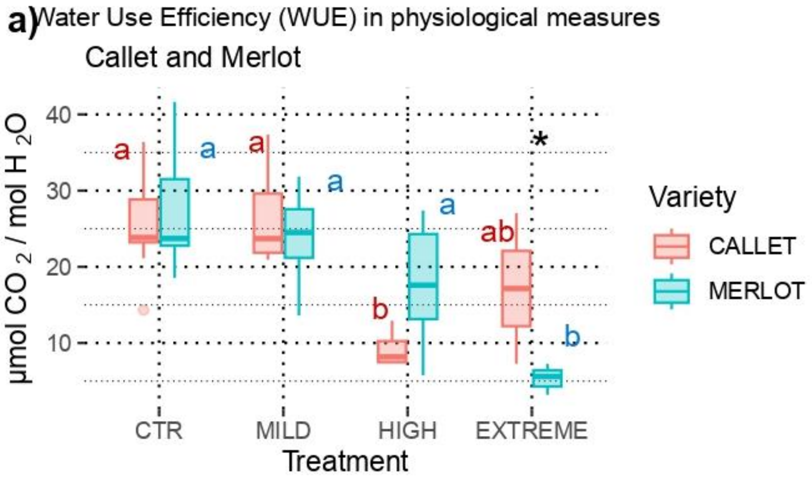
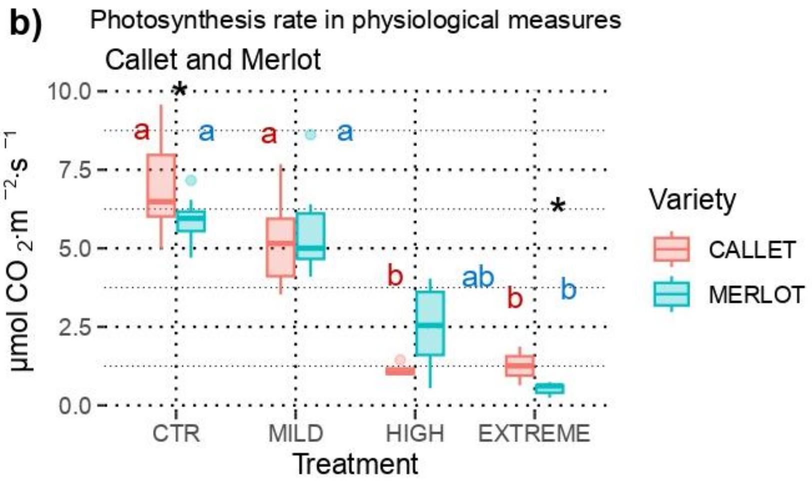


Figure 6: Boxplots of: a) Water Use Efficiency (WUE) expressed in $\mu mol CO_2 / mol H_2O$; b) Photosynthesis Rate expressed in $\mu mol CO_2 \cdot m^2 \cdot s^{-1}$; and c) Stomatal conductance expressed in $mol H_2O \cdot m^{-2} \cdot s^{-1}$; values for *Callet/110 Richter* and *Merlot/110 Richter* on Mild, High and Extreme conditions in 2020 and 2022.





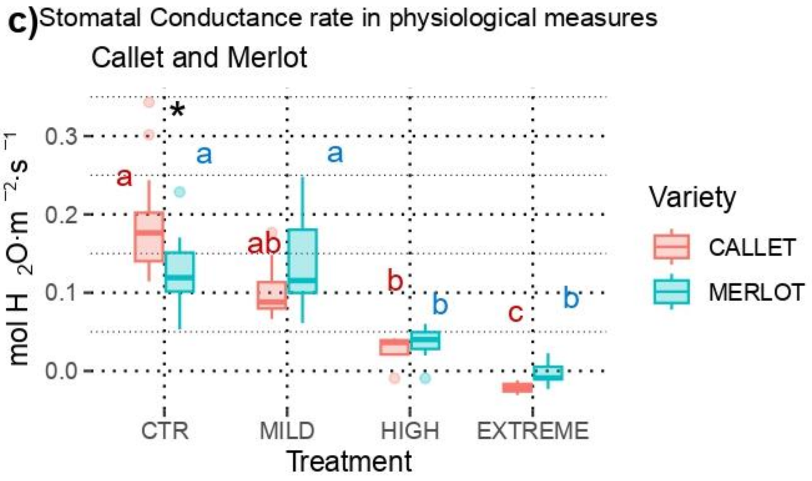
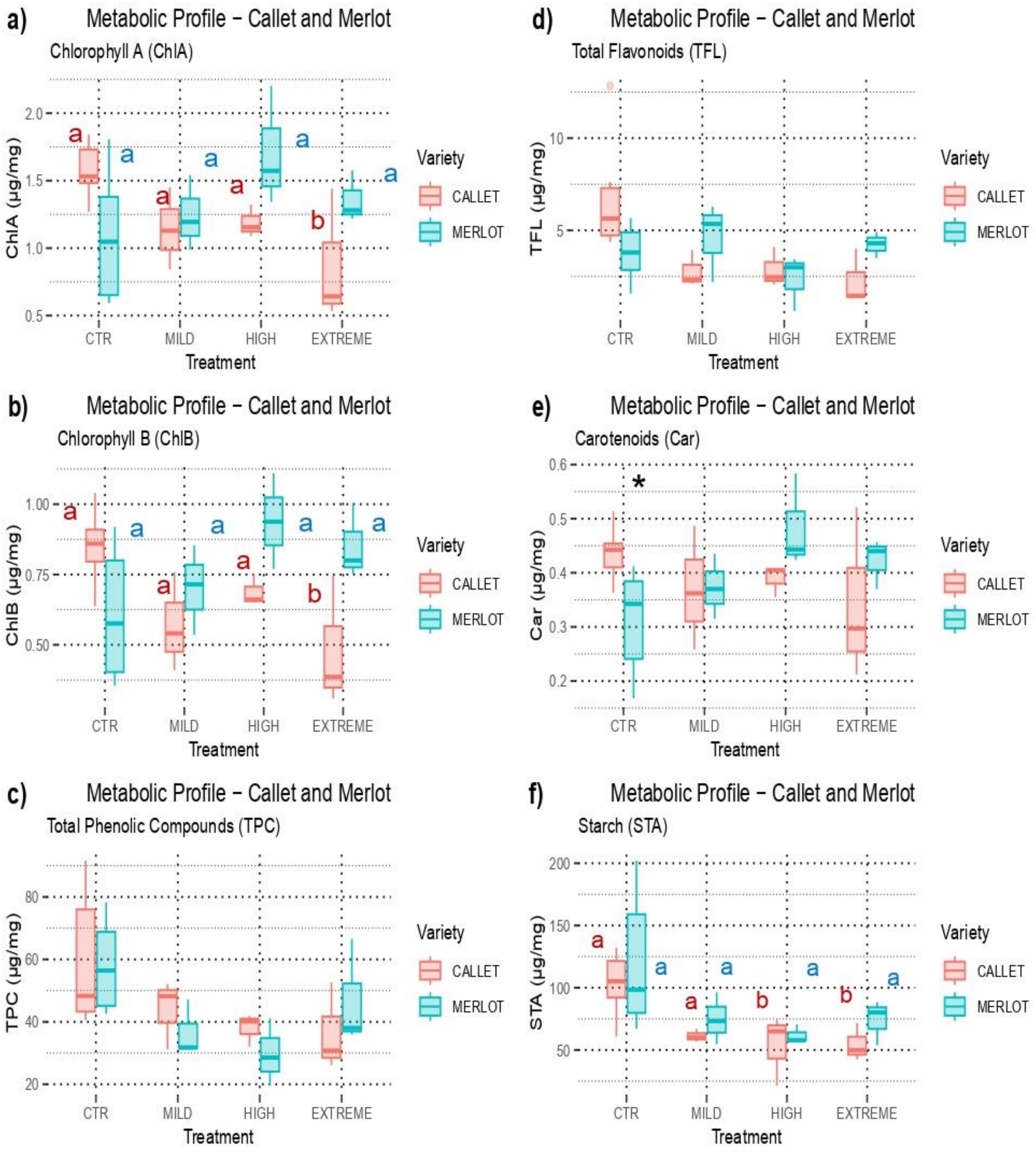


Figure 7: Boxplot of the results for the physiological state of: a) metabolic profile of Chlorophyll A; b) metabolic profile of Chlorophyll B; c) metabolic profile of Total Phenolic Compounds (TPC); d) metabolic profile of Total Flavonols (TFL); e) metabolic profile of Carotenoids (Car); and f) metabolic profile of Starch; all expressed in µg metabolite/mg dry leaves on Mild, High and Extreme conditions in 2020 and 2022 for for *Callet/110 Richter* and *Merlot/110 Richter*.



In figures 5, 6 and 7: * Different letters indicate significant differences (p-value < 0.05) for comparisons between treatments in Callet/110 Richter (in red) and Merlot/110 Richter (in blue). In black, * indicates significant differences between the cultivars.

Annex 4: Gene Ontology profiles and co-expression gene clusters in Chapter II.

Figure 1: Principal functions depending on the condition from the Venn Diagram (figure 5 in the article) for: a) upregulated genes in leaves; b) upregulated in roots; c) downregulated genes in leaves; and d) downregulated genes in roots; for *Callet/110 Richter* and *Merlot/110 Richter*. The x-axis represents the number of detected GOs. The bubble color represents each comparison in the Venn Diagram, and the bubble size indicates the percentage of detected GOs for GO Biological Process in the total universe of GOs.

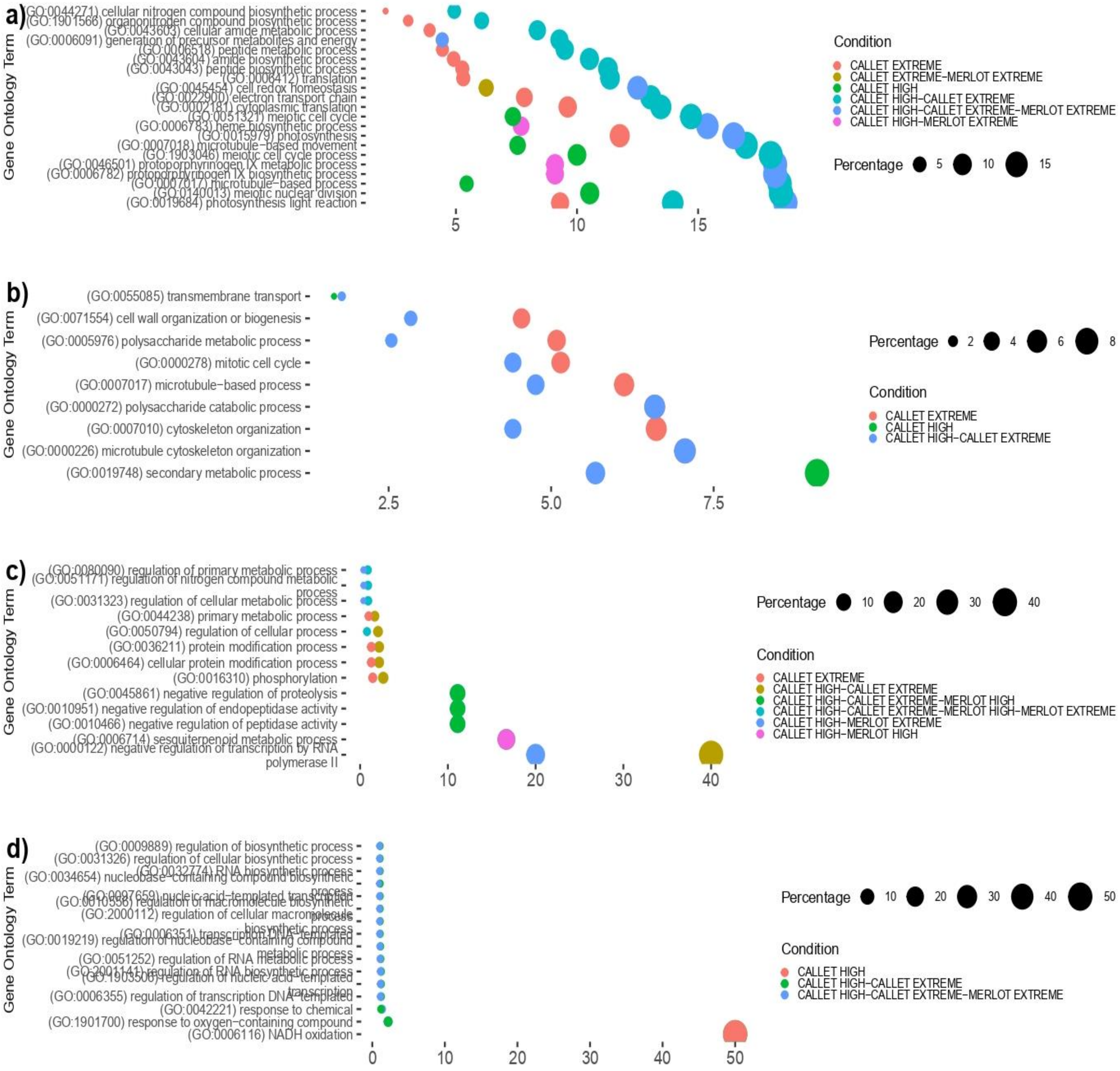


Figure 2: Representation of the principal functions depending on the condition from the Venn Diagram – Downregulated Genes in Leaves: a) Common Biological Processes represented in the Percentage of the represented genes over the total of genes that contain this GO term, and their coincidences between comparisons; b) Representative heatmap of the mentioned conditons depending on the P-value obtained in the Gene Ontology Analysis.

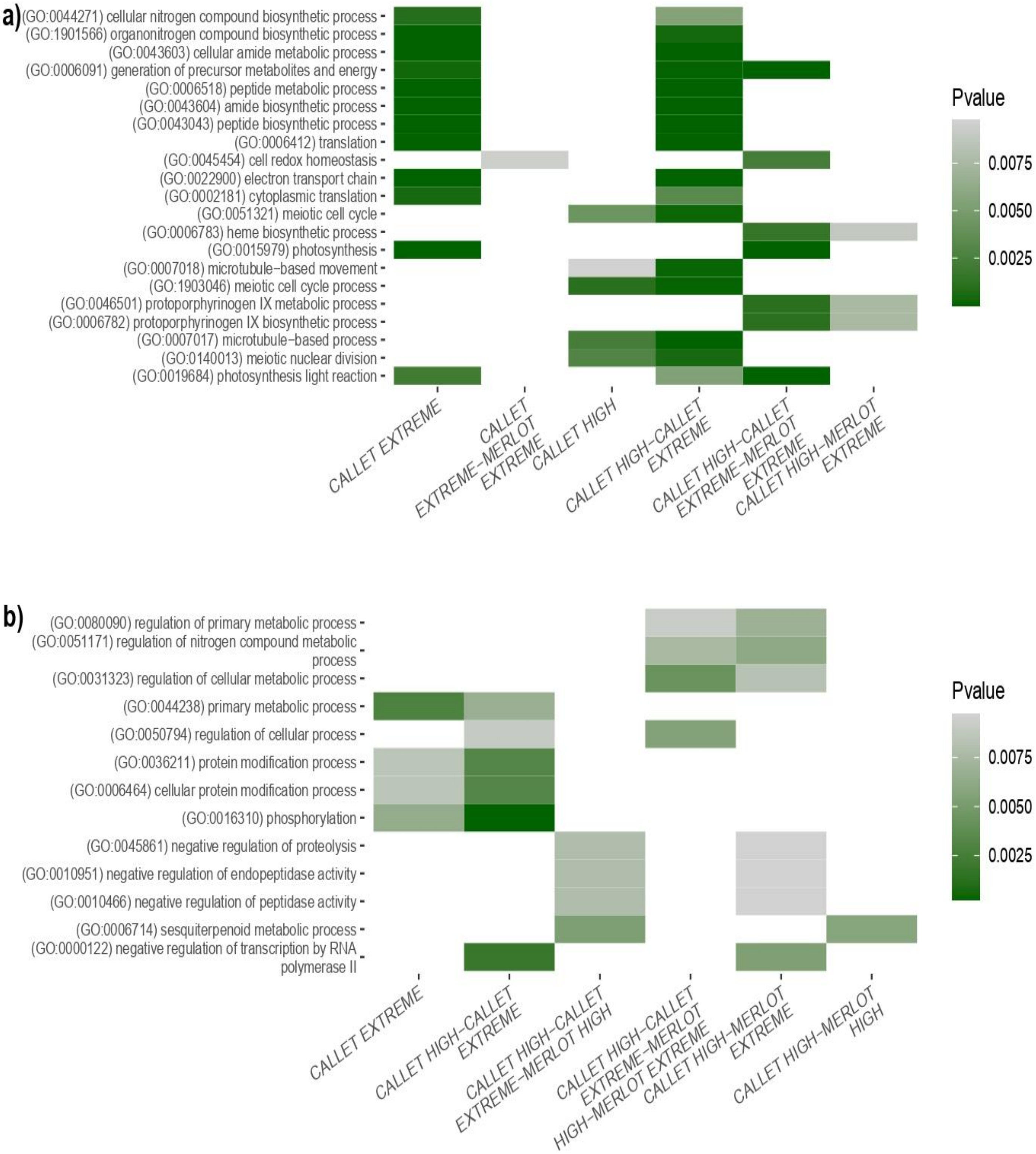


Figure 3: Representation of the principal functions depending on the condition from the Venn Diagram – Downregulated Genes in Leaves: a) Common Biological Processes represented in the Percentage of the represented genes over the total of genes that contain this GO term, and their coincidences between comparisons; b) Representative heatmap of the mentioned conditons depending on the P-value obtained in the Gene Ontology Analysis.

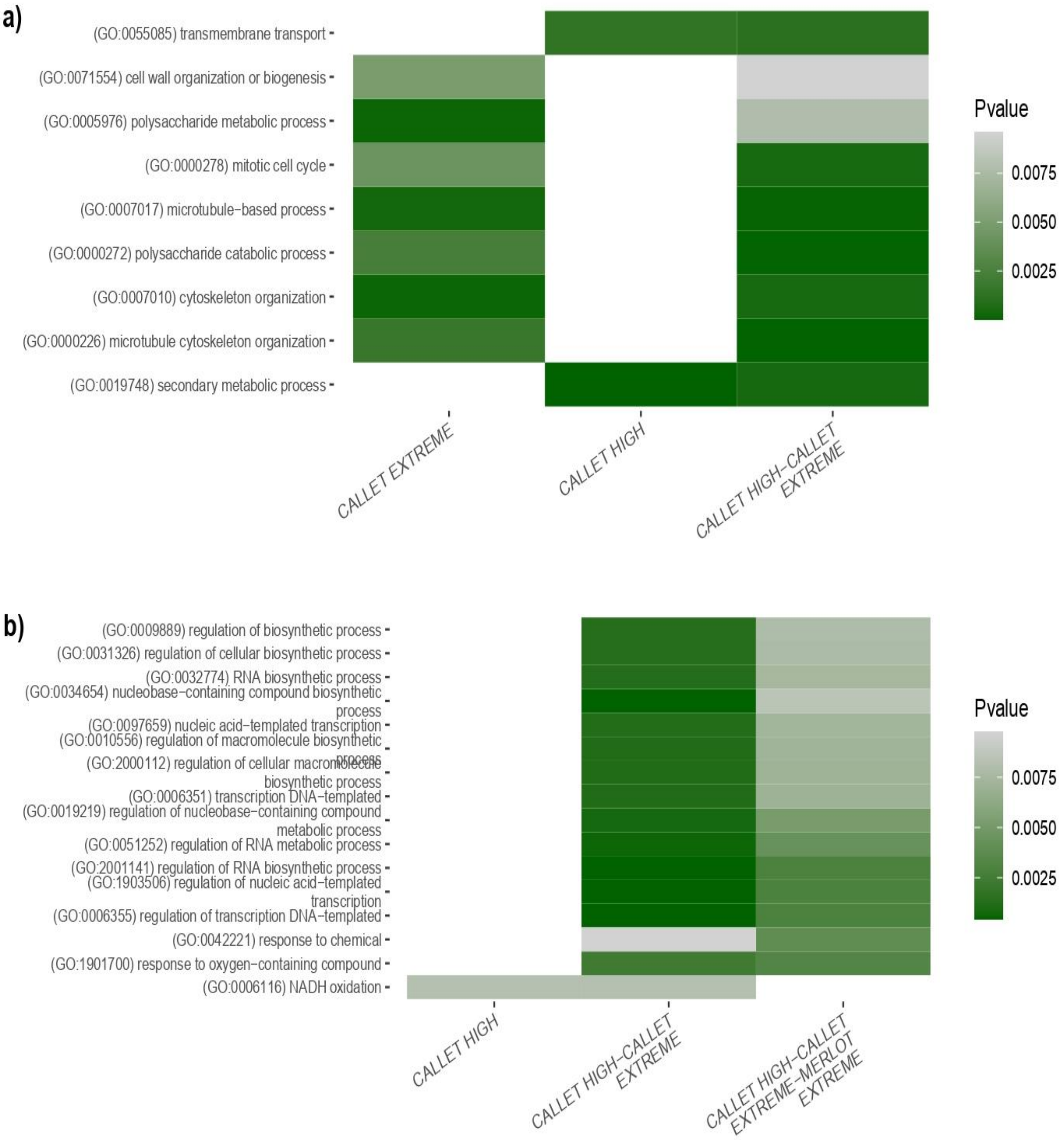


Figure 4: Clusters for co-expression of genes in *Callet/110 Richter* Leaves

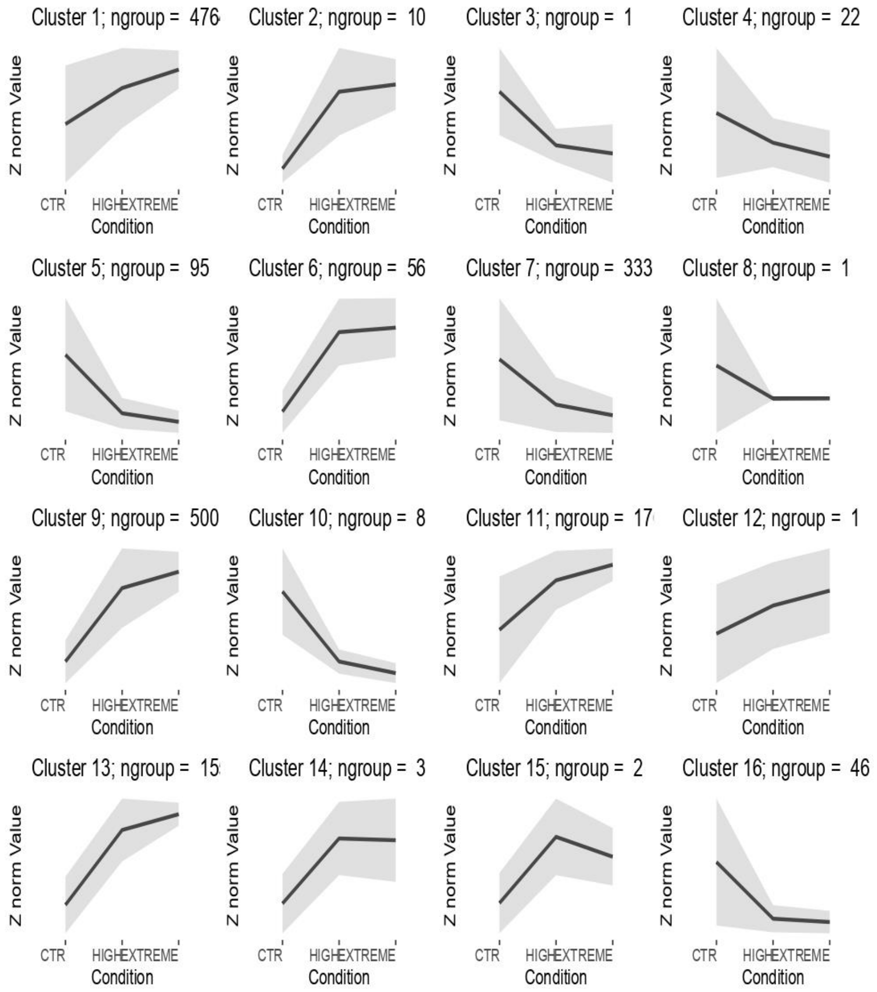


Figure 5: Clusters for co-expression of genes in *Merlot/110 Richter* Leaves

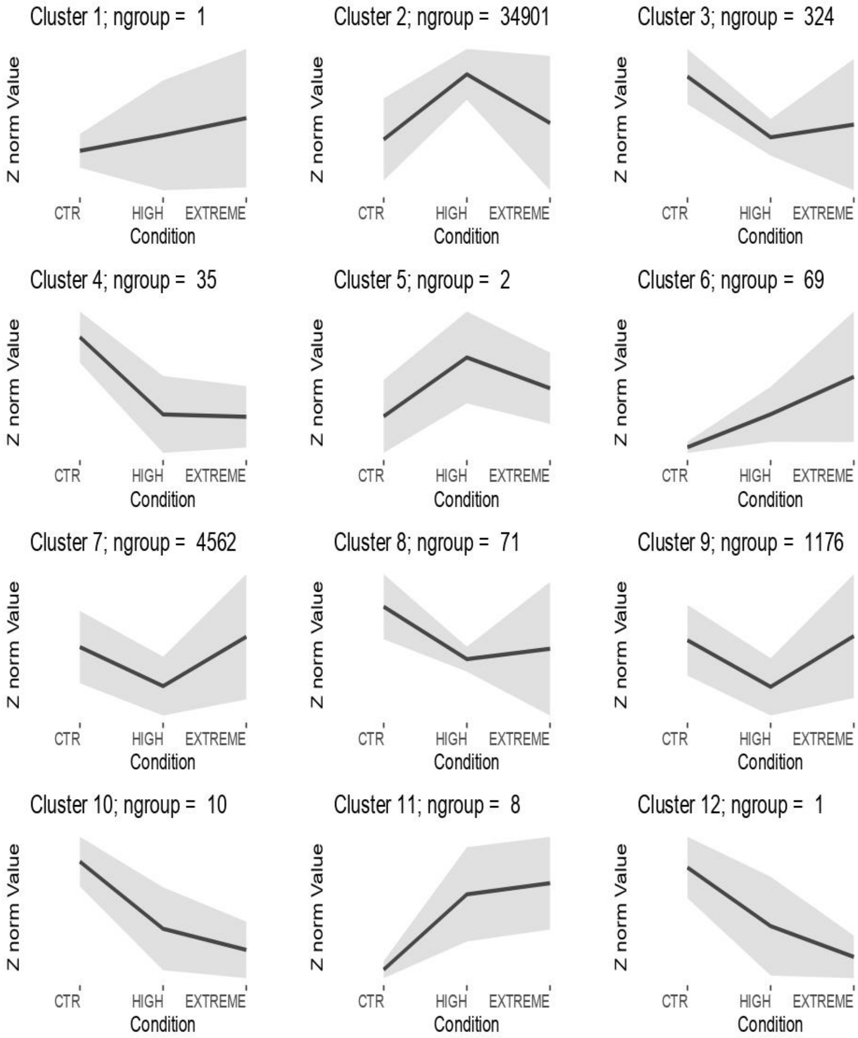


Figure 6: Clusters for co-expression of genes in *Callet/110 Richter* Roots

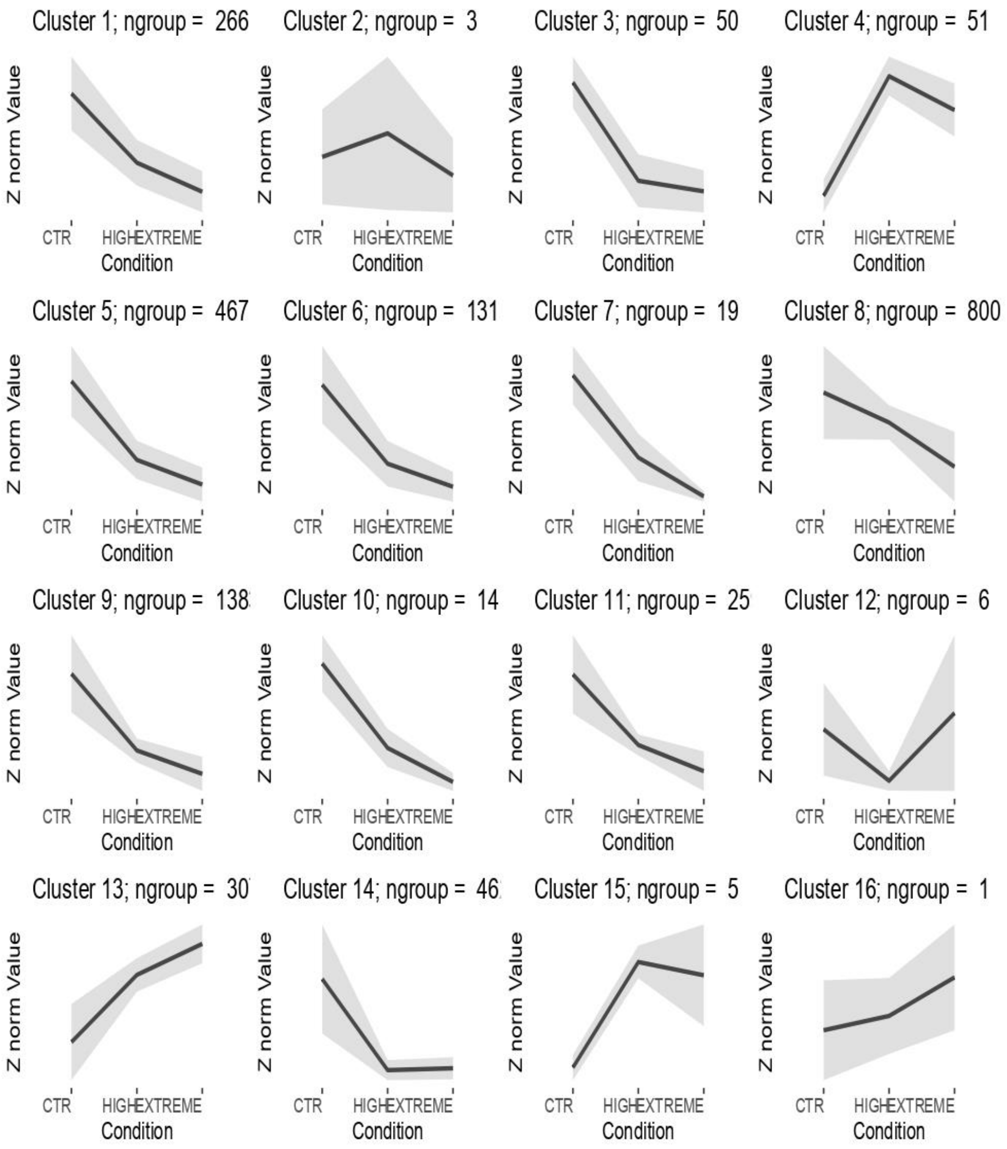
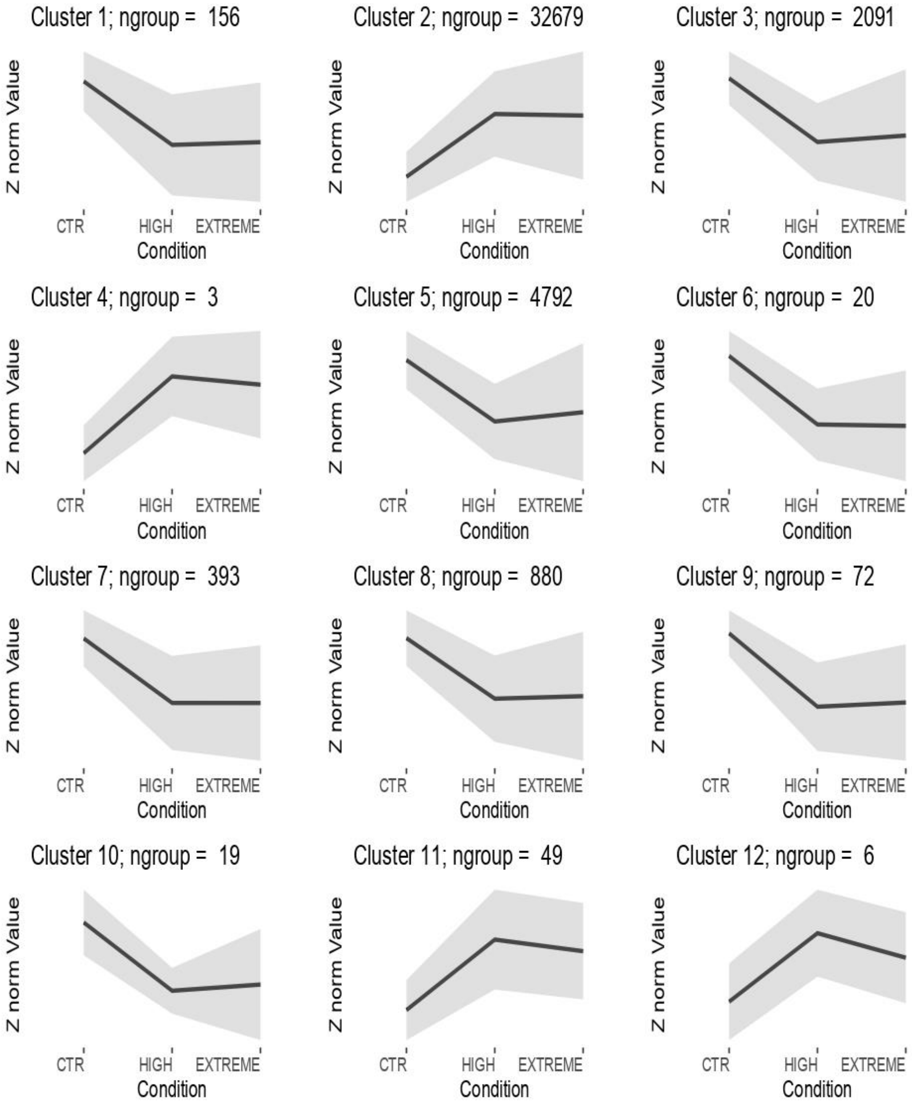


Figure 7: Clusters for co-expression of genes in *Merlot/110 Richter* Roots



Annex 5: Publication 1

plants MDPI

Article

Coastal Wild Grapevine Accession (*Vitis vinifera* L. ssp. *sylvestris*) Shows Distinct Late and Early Transcriptome Changes under Salt Stress in Comparison to Commercial Rootstock Richter 110

David Carrasco [1,†], Andres Zhou-Tsang [1,2,3,†], Alberto Rodriguez-Izquierdo [1], Rafael Ocete [4], María Angeles Revilla [5] and Rosa Arroyo-García [1,*]

[1] CSIC-INIA(CBGP) Centro de Biotecnología y Genómica de Plantas, UPM-INIA, Parque Científico y Tecnológico de la UPM Campus de Montegancedo, CtraM-40, Km 38, Pozuelo de Alarcón, 28223 Madrid, Spain
[2] Waite Research Institute, The School of Agriculture, Food and Wine, Faculty of Sciences, Engineering and Technology, The University of Adelaide, Glen Osmond, SA 5064, Australia
[3] ARC Industrial Transformation Training Centre for Innovative Wine Production, Waite Research Institute, Glen Osmond, SA 5064, Australia
[4] Laboratorio Entomología Aplicada, Universidad de Sevilla, Avenida Reina Mercedes 6, 41012 Sevilla, Spain
[5] Departamento Biología de Organismos y Sistemas, Facultad de Biología, Universidad de Oviedo, 33071 Oviedo, Spain
* Correspondence: rarroyo@inia.csic.es
† David Carrasco and Andres Zhou-Tsang contributed equally to this work.

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Abstract: Increase in soil salinity, driven by climate change, is a widespread constrain for viticulture across several regions, including the Mediterranean basin. The implementation of salt-tolerant varieties is sought after to reduce the negative impact of salinity in grape production. An accession of wild grapevine (*Vitis vinifera* L. ssp. *sylvestris*), named AS1B, found on the coastline of Asturias (Spain), could be of interest toward the achievement of salt-tolerant varieties, as it demonstrated the ability to survive and grow under high levels of salinity. In the present study, AS1B is compared against widely cultivated commercial rootstock Richter 110, regarding their survival capabilities, and transcriptomic profiles analysis allowed us to identify the genes by employing RNA-seq and gene ontology analyses under increasing salinity and validate (via RT-qPCR) seven salinity-stress-induced genes. The results suggest contrasting transcriptomic responses between AS1B and Richter 110. AS1B is more responsive to a milder increase in salinity and builds up specific mechanisms of tolerance over a sustained salt stress, while Richter 110 maintains a constitutive expression until high and prolonged saline inputs, when it mainly shows responses to osmotic stress. The genetic basis of AS1B's strategy to confront salinity could be valuable in cultivar breeding programs, to expand the current range of salt-tolerant rootstocks, aiming to improve the adaptation of viticulture against climate change.

Keywords: wild grapevine accession; rootstock; salinity; salt tolerance; transcriptomic analysis; gene ontology

1. Introduction

Grapevine (*Vitis vinifera L.*) is one of the most extended crops in the world, especially in the Mediterranean area. The value of this crop is represented across agronomical, food, biotechnological, and commercial sectors, among others. The largest grape-growing region dedicated to wine production in the world is Spain, possessing an area of more than 950,000 ha; hence, grapevine has a particularly special importance in Spain, with great economic representation (EUR 1–1.5 billion, 10% of total agricultural production), social value represented by over 400,000 producers (contributing to promote rural population fixation), and great representation in the environment and landscapes [1]. Given the importance of

Annex 6: Publication 2

RESEARCH ARTICLES

PLANT GENETICS

Dual domestications and origin of traits in grapevine evolution

Yang Dong[1,2]†, Shengchang Duan[1,2]†, Qiuju Xia[3]†, Zhenchang Liang[4]†, Xiao Dong[1,2]†§, Kristine Margaryan[5,6]‡, Mirza Musayev[7]‡, Svitlana Goryslavets[8]‡, Goran Zdunić[9]‡, Pierre-François Bert[10]‡, Thierry Lacombe[11]‡, Erika Maul[12]‡, Peter Nick[13]‡, Kakha Bitskinashvili[14]‡, György Dénes Bisztray[15]‡, Elyashiv Drori[16,17]‡, Gabriella De Lorenzis[18]‡, Jorge Cunha[19,20]‡, Carmen Florentina Popescu[21]‡, Rosa Arroyo-Garcia[22]‡, Claire Arnold[23]‡, Ali Ergül[24]‡, Yifan Zhu[1]‡, Chao Ma[25]‡, Shufen Wang[1,2], Siqi Liu[1,2], Liu Tang[1,2], Chunping Wang[1,2], Dawei Li[1,2], Yunbing Pan[1,2], Jingxian Li[1,2], Ling Yang[1,2], Xuzhen Li[1,2], Guisheng Xiang[1,2], Zijiang Yang[1,2], Baozheng Chen[1,2], Zhanwu Dai[4], Yi Wang[4], Arsen Arakelyan[5,26,27], Varis Kuliyev[28], Gennady Spotar[8], Nabil Girollet[10], Serge Delrot[10], Nathalie Ollat[10], Patrice This[11], Cécile Marchal[29], Gautier Sarah[11], Valérie Laucou[11], Roberto Bacilieri[11], Franco Röckel[12], Pingyin Guan[13], Andreas Jung[30], Michael Riemann[13], Levan Ujmajuridze[14], Tekle Zakalashvili[14], David Maghradze[14], Maria Höhn[15], Gizella Jahnke[15], Erzsébet Kiss[15], Tamás Deák[15], Oshrit Rahimi[16], Sariel Hübner[31], Fabrizio Grassi[32,33], Francesco Mercati[34], Francesco Sunseri[35], José Eiras-Dias[19,20], Anamaria Mirabela Dumitru[21], David Carrasco[22], Alberto Rodriguez-Izquierdo[22], Gregorio Muñoz[36], Tamer Uysal[37], Cengiz Özer[37], Kemal Kazan[38], Meilong Xu[39], Yunyue Wang[1], Shusheng Zhu[1], Jiang Lu[40], Maoxiang Zhao[25], Lei Wang[25], Songtao Jiu[25], Ying Zhang[41], Lei Sun[41], Huanming Yang[42], Ehud Weiss[43], Shiping Wang[25], Youyong Zhu[1], Shaohua Li[4]*, Jun Sheng[1,2]*, Wei Chen[1,2]*

We elucidate grapevine evolution and domestication histories with 3525 cultivated and wild accessions worldwide. In the Pleistocene, harsh climate drove the separation of wild grape ecotypes caused by continuous habitat fragmentation. Then, domestication occurred concurrently about 11,000 years ago in Western Asia and the Caucasus to yield table and wine grapevines. The Western Asia domesticates dispersed into Europe with early farmers, introgressed with ancient wild western ecotypes, and subsequently diversified along human migration trails into muscat and unique western wine grape ancestries by the late Neolithic. Analyses of domestication traits also reveal new insights into selection for berry palatability, hermaphroditism, muscat flavor, and berry skin color. These data demonstrate the role of the grapevines in the early inception of agriculture across Eurasia.

The cultivated grapevine (*Vitis vinifera* ssp. *vinifera*, hereafter *V. vinifera*) shares a close relationship with humans (*1*). With unmatched cultivar diversity, this food source (table and raisin grapes) and winemaking ingredient (wine grapes) became an emblem of cultural identity in major Eurasian civilizations (*1*–*3*), leading to intensive research in ampelography, archaeobotany, and historical records to reveal its history (*4*). Early work asserted that *V. vinifera* originated from its wild progenitor *Vitis vinifera* ssp. *sylvestris* (hereafter *V. sylvestris*) ~8000 years ago during the Neolithic agricultural revolution in the Western Asia (*5*, *6*). In recent years, various genetic studies explored this proposition (*6*–*13*), but the critical details of grapevine domestication were often inconsistent. Studies argued for the existence of domestication centers in the western Mediterranean (*13*), Caucasus (*12*, *14*), and Central Asia (*12*), which in turn cast doubt on the popular notion of a single past domestication event (*10*, *11*). Three demographic inferences yielded population split times between *V. vinifera* and *V. sylvestris* to dates between 15,000 and 400,000 years ago, predating the historical consensus on domestication time (*7*–*9*). Because early domesticates spread to other parts of Eurasia through poorly defined migration routes in the ensuing millennia (*5*), the single-origin theory also confounds the origin order between table and wine grapevines. One view proposes a wine grapevine–first model, with the two types diverging ~2500 years ago (*7*, *10*, *11*). Hybridization with local *V. sylvestris* was common in creating extant European wine grapes (*10*, *11*), but when these introgression events occurred is unknown. Several studies suggest that the earliest cultivation of European wine grapes in France and Iberia postdates 3000 years ago (*10*, *15*). These discrepancies primarily result from the inadequate sampling of grapevine accessions and the limited resolution of genetic data in previous analyses. Therefore, we report the genomic variation dataset from a global cohort to systematically delineate the structure of grapevine genetic diversity, explore the origin of *V. vinifera*, deduce a putative dispersal history, and investigate key domestication traits and diversification signatures.

Results

We constructed a chromosome-level reference *V. sylvestris* genome assembly (VS-1 from Tunisia) to attain genomic variations, which shows a higher percentage of anchored chromosomal lengths than PN40024 (fig. S1 and tables S1 to S9) (*16*). From the 3304 assembled accessions from a dozen Eurasian germplasm and private collections, we obtained good-quality Illumina paired-end sequencing data to an average 20× coverage for 3186 grapevine accessions (2237 *V. vinifera* and 949 *V. sylvestris*; tables S10 to S13). The sample selection preferentially included old, autochthonous, and economically important varieties to maximize the spectrum of genetic diversity. We also included genomic data for 339 previously sequenced accessions (266 *V. vinifera* and 73 *V. sylvestris*; table S14) in the analyses (*7*, *8*, *17*), producing the final cohort of 3525 grapevine accessions (2503 *V. vinifera* and 1022 *V. sylvestris*). The alignment of the Illumina reads to the VS-1 reference genome identifies 45,624,306 biallelic single-nucleotide polymorphisms (SNPs) and 7,314,397 biallelic short Indels [≤40 base pairs (bp); 73.2% shorter than 5 bp] (*16*), among which rare alleles (minor allele frequency ≤1%) accounted for the majority (fig. S2 and tables S15 to S22).

Core accessions differentiate by eight distinct genetic ancestries

Clones, mutants, synonyms, and homonyms are common phenomena in grapevine germplasm and collections (*18*). Using the identity-by-state sharing pattern estimators, we found 1534 accessions sharing the genetic profile with at least one other in the cohort, totaling 498 distinct genotypes (fig. S3 and table S23) (*16*). We kept one accession for each distinct genotype, corrected misidentified accessions, and excluded interspecific hybrids for a core cohort of 2448 grapevines (1604 *V. vinifera* and 844 *V. sylvestris*; fig. S3), which remain representative of the major viticultural regions (*19*) in the world (Fig. 1A and fig. S3).

Principal component analysis (PCA) showed that *V. sylvestris* and *V. vinifera* separately spread out along the first two axes (total variance explained: PC1 7.56% and PC2 1.71%), with both displaying a crude Western Asia to Western Europe gradient (Fig. 1B and figs. S4 and S5). The PC3 axis (1.26% variance) separates *V. vinifera* individuals according to their utilization, agreeing with the main table and wine grapevine clades in the maximum likelihood phylogenetic tree and reticulate phylogenetic network (figs. S6 and S7). The *V. vinifera* accessions show a weak isolation-by-distance correlation (Fig. 1C), suggesting a disconnection between the viticultural geographic pattern and the genetic structures in the grapevine

Annex 7: Publication 3

Transcriptome analysis of grapevine under drought conditions and description of adaptation strategies

A. Rodriguez-Izquierdo[1], D. Carrasco[1], J. Bota[2] and R. Arroyo-Garcia[1]

[1]Centro de Biotecnología y Genómica de Plantas (UPM-CSIC, CBGP), Campus de Montegancedo, Autovía M-40 km, 38, 28223 Pozuelo de Alarcón, Madrid, Spain; [2]Research Group on Plant Biology under Mediterranean Conditions, Departament de Biologia, Universitat de les Illes Balears (UIB) - Agro-Environmental and Water Economics Institute (INAGEA), Carretera de Valldemossa Km 7.5, 07122 Palma, Illes Balears, Spain.

Abstract

Grapevine is one of the most important fruit crops in the world. However, the effect of climate change on *Vitis vinifera* L. could dramatically affect the productivity of vineyards, causing serious economic and ecological damage. Some of the most important factors are drought and water availability. Varietal sensitivity to these abiotic stresses is more pronounced in commercial cultivars like 'Merlot' than local cultivars like 'Callet'. In order to better understand the adaptive differences between these two cultivars, we used transcriptomic, metabolic and hormonal analyses, at three different levels of drought stress (mild, high and extreme), using Richter 110 as rootstock. Differential gene expression (DGE) analysis was performed using RNA-seq data for each condition in both roots and leaves. DGE results showed that the response started in roots, with more differential expression at mild drought stress in these two cultivars. However, more differentially-expressed genes (DEGs) were found out in 'Merlot' than 'Callet', with the most significant response to mild drought in 'Merlot'. However, in high and extreme drought conditions, DGE analysis in leaves showed an increase of DEGs in the two cultivars, being more pronounced in 'Callet' than 'Merlot', suggesting a different the strategy of response between them. Hormonal and metabolic results displayed two different pathways in drought responses. While 'Merlot' allocated more resources to maintain cellular turgor, increasing sugar and alditol concentration in cells, 'Callet' produced more secondary metabolites like mono- or sesqui-terpenes, assisting with sugar transport to the fruit and thus increasing berry quality. Furthermore, a higher photosynthetic activity was found in 'Callet' than 'Merlot', showing higher water use efficiency.

Keywords: drought, grapevine, transcriptome, 'Merlot', 'Callet'

INTRODUCTION

Vitis vinifera L. is one of the most important fruit crops in the world, especially in the Mediterranean area. For example, in Spain, with a total surface of 950,000 ha, vineyards generate almost 1,000-1,500 million Euro annually (near 10% of the agricultural production in value). Unfortunately, climatic change and the problem of water availability in Mediterranean area are becoming a serious issue in terms of vineyard management and grape production. That problem increases each year and has to be solved (Hannah et al., 2013). For all, viticulture needs new approaches to ensure the development of vineyards ahead of future challenges. One of the best approaches to develop that adaptation in vitiviniculture depends on the use of cultivars more efficient in water use or more tolerant to drought. The high genetic diversity of grapevine (International Organisation of Vine and Wine Intergovernmental Organisation, 2018), may provide a way to adapt to abiotic stresses (Chaves et al., 2010). For this reason, morphological, molecular, and genetic analyses on *V. vinifera* L. in drought conditions could help to better understand the mechanisms of adaptation to these adverse conditions.

Generally, *V. vinifera* L. is considered to be fairly adapted to drought environments, especially in the Mediterranean area (Lovisolo et al., 2010). However, in some regions, the fact that the drought season is concomitant to high air temperatures high evaporation rates,

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Annex 8: Publication 4

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RESEARCH **Open Access**

Epigenetic differences between wild and cultivated grapevines highlight the contribution of DNA methylation during crop domestication

Alberto Rodriguez-Izquierdo[1], David Carrasco[1], Lakshay Anand[2], Roberta Magnani[2], Pablo Catarecha[1], Rosa Arroyo-Garcia[1*†^] and Carlos M. Rodriguez Lopez[2*†]

Abstract
The domestication process in grapevines has facilitated the fixation of desired traits. Nowadays, vegetative propagation through cuttings enables easier preservation of these genotypes compared to sexual reproduction. Nonetheless, even with vegetative propagation, various phenotypes are often present within the same vineyard due to the accumulation of somatic mutations. These mutations are not the sole factors influencing phenotype. Alongside somatic variations, epigenetic variation has been proposed as a pivotal player in regulating phenotypic variability acquired during domestication. The emergence of these epialleles might have significantly influenced grapevine domestication over time. This study aims to investigate the impact of domestication on methylation patterns in cultivated grapevines. Reduced-representation bisulfite sequencing was conducted on 18 cultivated and wild accessions. Results revealed that cultivated grapevines exhibited higher methylation levels than their wild counterparts. Differential Methylation Analysis between wild and cultivated grapevines identified a total of 9955 differentially methylated cytosines, of which 78% were hypermethylated in cultivated grapevines. Functional analysis shows that core methylated genes (consistently methylated in both wild and cultivated accessions) are associated with stress response and terpenoid/isoprenoid metabolic processes. Meanwhile, genes with differential methylation are linked to protein targeting to the peroxisome, ethylene regulation, histone modifications, and defense response. Collectively, our results highlight the significant roles that epialleles may have played throughout the domestication history of grapevines.

Keywords Domestication, Epigenomics, Grapevine, EpiGBS, Wild, Cultivated, Methylation, Epigenetic memory

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[†] Rosa Arroyo-Garcia and Carlos M. Rodriguez Lopez equal senior authors.

[^] In memoriam of Rosa Arroyo-Garcia.

*Correspondence:
Rosa Arroyo-Garcia
rarroyo@inia.csic.es
Carlos M. Rodriguez Lopez
carlos.rodriguezlopez@uky.edu
Full list of author information is available at the end of the article