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The Transcriptional Landscape of The Berry Skin In Two Novel Resistant Grapevine Varieties

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To my family
Woman, Life, Freedom

Abstract

Successful vineyard management, which guarantees optimum yields and fruits with desirable traits, is intimately related to the creation of high-quality wines. However, grape producers face difficulties, most notably fungal and oomycete diseases including Gray Mold, powdery mildew, and downy mildew that can ruin harvests and have an influence on the wine industry. Chemical pesticide-based traditional defensive strategies are incompatible with integrated crop protection strategies and environmental sustainability goals. New opportunities for grape production with less environmental impact are presented by advances in genetic understanding and biotechnology. Working with resistant varieties involves investigating whether there are inherent or constitutive mechanisms within these varieties that might contribute to their resistance. This exploration aims to uncover basal or pre-existing mechanisms that play a role in conferring resistance traits, shedding light on the underlying factors that make these varieties resistant to conditions or stressors. Conventional breeding has been instrumental in developing desirable traits, yet it suffers from drawbacks. These include time-consuming processes, reliance on limited genetic diversity leading to unexpected genetic modifications, resource-intensive demands, and challenges in addressing complex features. The need for alternate strategies, such as biotechnology, to get over these obstacles and hasten grapevine genetic progress is emphasized in this study. The investigation and analysis of the transcriptional profile of grapevine types showing resistance to oomycete and fungal diseases is the main goal of this research. To gain a better understanding of the genetic basis of resistance in grapevines, this study compares the transcriptional profiles of these resistant varieties with those of other grapevine varieties and elite types. Therefore, it would be possible to better comprehend the genetic basis of resistance in grapevines if we were to become more familiar with the underlying genetic profile of disease resistance.

Keywords: Conventional breeding, Downy Mildew, RNA-Seq, Berry skin, Gray Mold, Powdery Mildew.

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1. INTRODUCTION

1.1. The History of Grapevine

The most common species of grapevine utilized in the world's wine industry is *Vitis vinifera*. It is a member of the Vitis genus, which is part of the Vitaceae family and has 60 interfertile species, the majority of which are found in the northern hemisphere. Its history goes back roughly 65 million years [71]. The cultivation of *V. vinifera* covered approximately 7.4 million hectares in 2020 with a production higher than 69 million tons of grapes [36]. Two sub-species are recognized: the wild form, V. vinifera subsp. sylvestris, and the domesticated one, *V. vinifera* subsp. vinifera (or sativa). *V. vinifera* is notably abundant in secondary metabolites, showcasing an array of flavonoids (such as flavan-3-ols and flavanols), phenolic acids, antho- cyanins, fatty acids, amino acids, and vitamins. Notably, it features the distinctive presence of stilbene derivatives. The diverse qualitative composition of these phytochemicals varies based on the specific morphological parts of the plant [31]. *V. vinifera* has garnered scientific interest due to the diverse array of compounds it harbours. This grapevine species demonstrates antioxidant, antibacterial, anti-inflammatory, and anticancer activities. Moreover, it highlights properties containing cardioprotective, hepatoprotective, and neuroprotective effects [23, 39]. antibacterial properties *V. vinifera* suggest its potential as an alternative to chemical preservatives [50]. fruits of *V. vinifera*, known as grapes, are extensively utilized in the production of wines, juices, and raisins as well as fresh consumption [62].

V. vinifera is believed to originate from V. vinifera spp. sylvestris, a wild grapevine. These wild grapevines still exist in small populations in forested regions near rivers, climbing trees natural environments suitable for their growth. They are dispersed across regions extending from the Atlantic coast of Europe to Tajikistan and the Western Himalayas [60].

Subspecies sylvestris is considered the progenitor of the subspecies *V. vinifera* and, phenotypically, the two subspecies differ in some traits relating to the morphology of flower, seed, and leaf, to berry and bunch size [33]. The grapevine (*Vitis vinifera L*) is attractive for genomic research because it is diploid and has a small genome size of 475.500 Mb, relatively smaller compared to other plants (it is approximately four times the size of Arabidopsis and one sixth the size of the corn genome) [71, 41].

1.2. The Most Important Oomycete and Fungal Diseases Affecting Grapevines

As one of the most significant fruit crops in the world, grapevine (Vitis vinifera L.) is confronted with numerous pathogenic effects that cause infections pre-, mid-, or post-harvest, which have a significant negative impact on commercial cultivars, compromising fruit quality, processing, and export. Bacteria, fungus, oomycete, or viruses with various life cycles, infection methods, and evasion techniques are some examples of potential dangers [3]. Gray Mold, powdery mildew, and downy mildew (DM), among others, are some of the most significant ailments affecting grapevine. These diseases are brought on by *Botrytis cinerea*, *Erysiphe necator*, and *Plasmopara viticola*, respectively [3]. The term "grapevine trunk diseases" (GTDs) refers to a group of disorders affecting grapevines that are brought on by a variety of 26 fungal pathogens inhabiting and colonizing the wood of perennial organs and most importantly produce vascular infections, white decays, wood necrosis [47, 5]. On the other hand, the oomycete *P. viticola*, which caused a fungal- like plant disease native to North America, entered Europe by mistake at the end of the 19th century and significantly harmed the grape crop [29].

Although interactions between plants and pathogens are cycles of resistance and susceptibility, resistance features from natural resources are chosen and explained in breeding and sustainable agriculture for reducing field treatments against pathogens, thus reducing operative costs and enhancing the product healthiness [3].

1.2.1. Downy Mildew

The Downy Mildew, a plant disease brought on by the oomycete *P. viticola*. The northeastern part of the United States was the location where this ailment first attracted notice and was later described. As shown in Figure 1.1, the oomycete that caused the establishment of Downy Mildew was first collected by Schweinitz in 1834 from the northeastern region of the United States and classified as *Botrytis cana* [38]. Among the native wild Vitis species of North America, the causative agent *P. viticola* continues to be endemic. It made its first appearance within the borders of Europe in the year 1878 [29]. Afterward, there was a challenging period of fifty years, during which European

grape crops encountered three significant issues: powdery mildew first, then phylloxera, and finally, Downy Mildew [29].

Starting from the early 20th century, the disease posed a significant challenge for European viticulture [26] and Germany, France, Switzerland along with Italy were experienced serious grape crop lost to *P. viticola* [29]. Infecting grapevines with Downy Mildew need several days of good temperature, about 160°C/days above 8°C, starting from January 1st, measured 2 meters above the ground [27].

The pathogen's life cycle begins with dormant oospores in the soil, becoming active upon encountering a host. It causes primary and secondary infections, overwinters in leaf litter or soil, and emerges in spring. The initial phase remains symptomless until yellow spots appear, later turning brown. Sporangia release spores, prompting further infections, leading to premature leaf shedding and structural distortions in shoots and berries. The entire cycle lasts 5 to 18 days, influenced by environmental factors [38].

Traditionally, its control heavily relies on fungicide use throughout the growing season, which leads to environmental pollution and can result in the emergence of resistant pathogen strains over time. To combat this, there is a growing need to shift towards agroecological methods that emphasize prevention and enhanced agroecosystem resistance [53]. Primary inoculum, such as oospores, significantly influences the prevalence of grapevine downy mildew [28]. Tests focusing on germination have revealed that initial infections can take place continuously during the whole growing season [42].

1.2.2. Powdery Mildew

A crucial period in the history of plant pathology and viticulture was highlighted by the introduction of the grapevine Powdery Mildew pathogen (*E. necator*) from North America to Europe (1845), as well as the quick spread of the infection from England to France in 1847. Due to the destructive effects on grapevines of this pathogen, disease control practices underwent a radical change [13].

The disease symptoms vary across leaves, shoots, and berries. Leaves display white or gray patches on both surfaces, often mistaken for downy mildew, which can enlarge and cause leaf curling. Shoot symptoms are rare and appear as dark patches. Berries may experience flower loss, white fungal

growth resembling powdered sugar or flour, rust-coloured spots, and splitting. In some cases, purple or red berries fail to colour properly and appear blotchy at harvest [49].

Furthermore, Powdery Mildew triggers metabolic reprogramming in its host [51]. At the basic metabolic level, it lowers the amounts of glycolytic, photorespiratory, and photosynthetic proteins [43]. and results in a shift in carbon reserves through increased invertase and alpha-amylase activity [24]. These enzymes degrade starch reserves into glucose and maltose [56]. This metabolic shift coincides with an increase in the transcription of hydroxymethyl-glutaryl- CoA (*HMG-CoA*) and HMG-CoA reductase genes [24]. The HMG-CoA synthase enzyme facilitates the conversion of *Acetoacetyl-CoA* into *3-hydroxy-3-methylglutaryl-CoA*, which, in turn, is transformed into mevalonate by the *HMG-CoA* reductase enzyme. These two molecules play a crucial role in the biosynthesis pathway of terpenes, carotenoids, and sterol compounds [66].

The management of this pathogen predominantly hinges on the application of fungicides, a practice associated with environmental repercussions and escalated production expenses. A sustainable, economically prudent, and environmentally benign alternative for disease control is rooted in the deployment of resistant grapevine varieties. While a substantial proportion of *V. vinifera* cultivars remain susceptible to powdery mildew, noteworthy resilience has been observed in various species within the Vitaceae family [46]. Understanding the genetic foundation of this resistance is paramount for its effective integration into breeding initiatives [46]. Consumers are increasingly advocating for sustainability in food production. As a result, there is a growing need for the development of new grapevine cultivars that possess genetic resistance to powdery mildew. These cultivars can significantly contribute to sustainable viticulture practices, all while preserving yield, fruit quality, and the quality of the resulting wine [64].

1.2.3. Gray Mold

Botrytis cinerea is known to incite one of the most severe diseases affecting above-ground grapevine *V. vinifera* structures in numerous vineyards worldwide [1]. The disease consists of a necrotrophic fungus characterized by a brief biotrophic phase and it is known to infect over 1400 plant species.[80]. Gray Mold (GM) by *B. cinerea* significantly harms important crops like grapes, strawberries, and tomatoes [14].

GM results in considerable economic losses in global viticulture, contributing to 20% to 50% reductions in grape yields. This is primarily due to the spoilage of ripe grape bunches during post-harvest periods [22]. The optimal conditions of high humidity and moderate temperatures throughout the growth cycle of grapevine promote the thriving of this fungal pathogen [79]. This pathogen is a natural part of the microenvironment of vineyard, often found in the soil among dead plant material [19].

During the ripening stage, the disease affects the fruits, leading to necrotic regions with abundant fungal growth, resulting in the typical grey rot appearance. Consequently, the affected grapes become unsuitable for wine production. Berries typically get infected by airborne conidia from overwintered sources [45, 48]. Upon contact with the plant, *B. cinerea* induces cell death by releasing phytotoxins and cell wall degrading enzymes. It manipulates the host's metabolism to aid its colonization [79, 7].

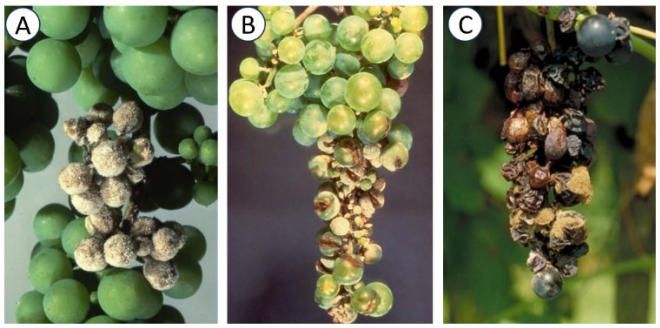

Figure 1.1. Berry cluster infected by: (A): Downy Mildew. (B): Powdery Mildew. (C): Gray Mold.

1.3. Conventional Breeding

The bulk of food crops available in grocery stores owe their existence to conventional plant breeding methods. While seedless watermelon, plots, and tangelos might be misconstrued as products of modern genetic engineering, they are, in fact, outcomes of traditional breeding practices [12, 2]. Commercial conventional breeding introduces hundreds of new crop varieties annually. These endeavours aim to enhance crop productivity, fortify food security, enrich nutritional value, and widen consumer options [21].

Conventional plant breeding involves the identification of parent plants exhibiting desirable characteristics, creating favourable combinations in subsequent generations. This selection process

for superior plants in food, feed, and fiber production has an extensive history of over 10,000 years and has undergone significant refinement in the last century [16, 63].

Early agriculturalists leveraged natural genetic diversity within wild plant populations, choosing specific plants exhibiting desirable attributes. Today's plant breeders expand upon this diversity by selecting genetically varied plants as parents, accounting for factors like geographical isolation and differing maturation rates. To discern the most promising progeny, breeders select plants for desired traits and employ established scientific methodologies to assess crucial parameters specific to each crop [69].

Conventional breeding, an evolving process, has led to a robust framework enhancing crop performance while ensuring the production of safe and nutritious food. It involves a series of strategic decisions, such as the selection of parent plants, their cross-pollination, and the progression of preferred progeny. Unlike animal breeding, plant breeding takes advantage of creating extensive populations, often in the tens of thousands, but only a small fraction exhibiting the desired traits, generally less than 1%, moves forward in the breeding process. This meticulous selection from larger populations is a pivotal aspect of the entire breeding process, involved in trait mapping, introgression, and field-testing stages [69].

The initial grapevine breeding activities commenced around the early 19th century and were predominantly inaugurated in North America. In Europe, more targeted breeding efforts began later, catalysed by the emergence of mildews and phylloxera between 1845 and 1878. Principally, private French breeders initiated extensive breeding programs, aiming to incorporate resistance traits sourced from American wild species into the gene pool of the European cultivars belonging to Vitis vinifera [18].

Crossbreeding exploits genetic variation through controlled sexual repro- duction to obtain diverse progeny. In grapevine, it was initially used in the 18th century to develop "American hybrids" adapted to local conditions in America, surpassing local wild species for winemaking [72]. In Europe, crossbreeding began in the 20th century to combat emerging pests like downy mildew, powdery mildew, and GM. Early attempts led to "first-generation hybrids" but faced quality issues for winemaking. While effective for rootstocks, these hybrids lacked viticultural qualities, prompting regulatory constraints in the EU. Despite these challenges, subsequent breeding in other regions produced successful grape varieties for European cultivation and winemaking [52].

To harness resistance from wild species, gene introgression is used. This involves hybridizing a wild plant carrying the resistance trait with a commercial variety. The resulting plant is then repeatedly backcrossed with the commercial variety while selecting for the desired resistance trait [52].

The Vitaceae family, containing 16 genera and 950 species, is divided into five tribes [81]. The Vitis genus, comprising 75 species, belongs to the *Viteae* tribe. Vitis includes *muscadinia* and Vitis subgenera, including wild *V. vinifera* wild spp. *sylvestris* and cultivated spp. sativa. The similarity between these grape types, shown in between types that are a mix, backs up the idea that our cultivated grapes came from tamed wild ones [32].

The genetic makeup of *V. vinifera* grapes might be less diverse compared to mixed or wild varieties, yet these unique characteristic sparks variations between different types of grapes [8]. These variations become an asset in facing fungal diseases that commonly affect grapevines [74].

The updated regulations permitting hybrids under Protected Geographical Indication (PGI) by EU council in 2009, have revitalized the creation of resistant species through interspecific crossbreeding. These new cultivars, recognized as *V. vinifera* varieties, are labelled, in German, as Pilzwiderstandsfähig (PIWI), meaning "fungal disease resistant" [52].

Originating from Bordeaux, France, elite Cabernet Sauvignon (CS) is a renowned grape variety spread across 160,000 hectares globally. It yields wines with high alcohol content and is vulnerable to certain diseases. Cabernet Cortis (CC), is a PIWI variety, a hybrid of Cabernet Sauvignon and a white variety called Solaris, and it offers winemaking potential and, blending qualities from both parents. The other PIWI variety Cabernet Volos (CV), sharing traits with Cabernet Sauvignon, boasts good resistance to mildews, promising versatility, and winter hardiness for viticulture [73, 76].

Sauvignon Blanc (SB) is globally renowned for producing aromatic, crisp white wines. Widely cultivated, particularly in Bordeaux and Marlborough, this variety emphasizes flavour and aromatic qualities rather than disease resistance, lacking notable resilience compared to some specially bred variants. On the other hand, Sauvignon Nepis (SN) features hairless shoots, compact clusters, and a thick-skinned, flavour-neutral berry. Resilient against powdery mildew and downy mildew, it exhibits remarkable Vigour and winter hardiness, thriving even in temperatures as low as -20°C. Notably, its aromatic profile boasts fruity- floral notes with a hint of spice, making it versatile for various wine types. Finally, Sauvignon Rytos (SR), resembling its Sauvignon parent, displays strong disease

resistance against mildews but it is sensitive to *Botrytis* due to its compact clusters. Despite this, it endures harsh winters and offers balanced sugar and acidity values. With tropical and mineral aromas, SR yields complex Flavours, promising potential for both aged and immediate-consumption wines [54, 77].

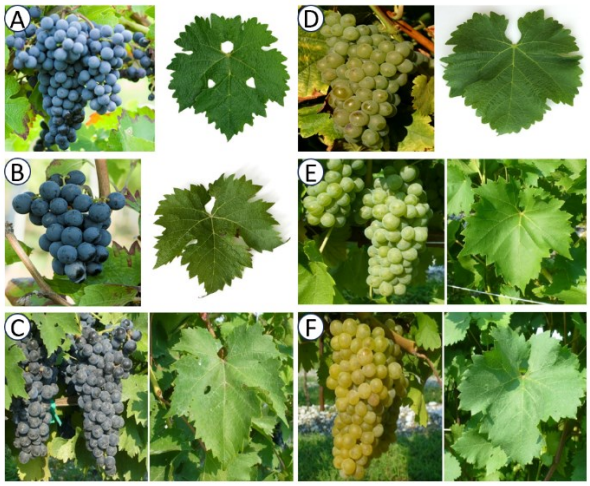

Figure 1.2. Two Elite Grapevine Varieties with their PIWI types: Reds: (A) Cabernet Sauvignon. (B) Cabernet Cortis. (C) Cabernet Volos. Whites: (D) Sauvignon Blanc. (E) Sauvignon Rytos. (F) Sauvignon Nepis.

1.4. The Extraction of Biomolecules

The process of extracting biomolecules like DNA, RNA, and protein is fundamental in molecular biology [67]. It's the starting point for creating diagnostic kits and other products. We can isolate Deoxy ribonucleic Acid (DNA), ribonucleic Acid (RNA), and protein from different biological materials, including tissues, insects, bacteria, and virus particles, for various purposes [67, 83].

DNA and RNA can be extracted from various biological sources, including living, or preserved tissues, cells, viruses, or other specimens, for analytical or preparative purposes [83].

The extraction methods vary based on the intended use and source of genetic material [83]. However, the fundamental solubility properties of RNA and DNA enable their extraction these are molecules highly soluble in water, they precipitate as macromolecules in alcohol-water mixtures, and they are poorly soluble in organic solvents like chloroform or phenol, allowing for relative ease in extracting proteins and hydrophobic components from nucleic acid solutions [83].

In the same vein as DNA extraction, the process for isolating RNA involves the removal of other molecular components, such as proteins and lipids [83]. Just like in DNA extraction, organic solvents are utilized to separate RNA from these impurities [83]. However, RNA necessitates distinct treatment compared to DNA in several aspects not to co-extract it with the other nucleic acids of the sample [83].

Many cell types harbour a significant quantity of RNases in their cytoplasm, coexisting with the mRNA, often the prime target of analytical extraction [83, 17]. RNA experiences rapid degradation following to its extraction, attributed to its inherently short half-life, particularly after cell death [17].

The prime factor contributing to this phenomenon is contamination by RNases, a pervasive class of heat-stable enzymes known for their ability to swiftly degrade RNA [25] To counteract the pervasive influence of these RNases, the isolation procedure must be conducted swiftly, transitioning the cell lysate into an environment that inhibits RNases [83]. Potent denaturing agents like guanidinium isothiocyanate (GTC) solutions are employed for this purpose [83].

Freshly obtained or harvested tissues can be homogenized and largely dissolved in 4 M GTC [83]. The RNA can then be segregated through *CsCl* density gradient centrifugation, effectively separating it from DNA, which does not precipitate under these specific conditions [83]. Following this, a variety of methods can be employed to further purify the RNA pellet.

Alternatively, RNA can also be extracted from homogenized tissues using different denaturing salt solutions, such as 4 M lithium chloride [83]. Subsequently, a phenol extraction is performed to eliminate proteins from the homogenate, after which the RNA is precipitated using alcohol [83].

1.5. Sequencing

In 1977, a significant breakthrough in DNA sequencing emerged, presenting a method reminiscent of Sanger and Coulson's technique. This novel approach utilized 2',3'-dideoxy and arabino nucleoside analogues, demonstrating faster and more accurate DNA sequencing, notably showcased on bacteriophage ϕX174 DNA [57, 58]. Concurrently, A.M Maxam and W. Gilbert proposed an alternative sequencing method, revolutionizing the field by selectively cleaving DNA strands at specific nucleotide sites, fundamentally altering DNA sequencing approaches [44]. The 1990s marked a turning point in grapevine cultivar identification with the introduction of microsatellite markers through Polymerase Chain Reaction (PCR) [78]. Additionally, microarray

technology, pioneered in 1995 by Schena and colleagues, transformed gene expression analysis. Although the initial microarray enabled comprehensive genome-wide gene examination, its limitations in targeting known genes and lacking exon-level detail led to the evolution of exon microarrays for more detailed insights [59, 30, 11].

RNA sequencing (RNA-Seq) was pioneered over a decade ago [20, 40]. Following the discovery of reverse transcriptase [68], it became feasible to convert mRNA and ncRNA into stable DNA, known as complementary DNA (cDNA). Through the utilization of the DNA sequencing system developed by British chemist Sanger in 1975 and the cDNA PCR method, which exponentially amplifies cDNA creatively introduced by Iscove [6], the sequencing of RNA through its synthesized cDNA became achievable.

Genomic sequencing technology has evolved through three distinct generations. The first-generation sequencing, pioneered by Frederick Sanger, utilized chain-termination methods, allowing for the sequencing of DNA. However, it was slow and costly for comprehensive genomic studies. The second-generation sequencing, also known as Next Generation Sequencing (NGS), including Illumina and Ion Torrent, significantly enhanced sequencing capabilities by parallelizing reactions, revolutionizing speed, and reducing costs. In contrast, the third-generation sequencing, exemplified by PacBio and Oxford Nanopore, advanced the field with single-molecule sequencing, offering long-read sequencing and real-time data generation, effectively reducing the necessity for DNA amplification, and enabling more contiguous genome assemblies. Each generation brought pivotal improvements, empowering the genomic sciences with increased speed, accuracy, and cost-efficiency.

The typical laboratory workflow starts with RNA extraction and continues with cDNA synthesis, Messenger RNA (mRNA) enrichment or Ribosomal RNA (rRNA) depletion, and adaptor-ligated sequencing library creation. This library is frequently sequenced on a high-throughput platform like Illumina, with a read depth of 1030 million reads per sample. The final stages are computational and include filtering, normalizing between samples, quantifying reads overlapping transcripts, statistically analysing significant changes in the expression levels of individual genes and/or transcripts among sample groups, and aligning and/or assembling sequencing reads to a transcriptome [65].

With the help of this technological development, the wine grape business was able to enter a new era of accuracy and precision in identifying and tracking the origins of grapevine varietals [78]. Researchers and viticulturists have obtained the ability to reveal complex genetic links across grape types, unravelling the intricate tapestry of their lineage with unmatched detail, by utilizing the power of PCR-based microsatellite markers [78]. This development's subsequent effects on the viticulture, genetics, and enology areas were considerable and allowed for a better knowledge of grapevine diversity, heritage, and evolution [78].

1.6. Gene Expression Analysis

Studying constitutive (baseline) gene expression in uninfected grapevine berries can provide valuable insights into the defence resistance mechanisms of grapevine varieties, particularly when comparing resistant and susceptible varieties. Constitutive gene expression refers to the level of gene expression in the absence of an external stimulus, such as pathogen infection.
Here are a few reasons why analysing constitutive gene expression in uninfected berries makes sense:

Baseline Comparison: Examining the gene expression in uninfected berries allows researchers to establish a baseline for gene expression in the absence of disease. This baseline can then be compared to the gene expression patterns observed during infection, helping identify genes that are specifically induced or suppressed in response to the pathogen.

Identification of Basal Defence Mechanisms: Constitutive gene expression can reveal the presence of genes that are already actively involved in the defense mechanisms of the plant even before an infection occurs. These genes may play a role in providing basal resistance, making the plant less susceptible to pathogens.

Varietal Differences: Comparing resistant and susceptible varieties in terms of constitutive gene expression can highlight genetic differences that contribute to the varying levels of resistance. Understanding these differences at the molecular level can aid in the development of more resistant grapevine varieties through breeding or biotechnological approaches.

Early Detection of Resistance Markers: By identifying genes with constitutive expression patterns associated with resistance, researchers may find potential molecular markers that can be used for early detection of resistance traits in grapevine varieties. It is important to note that gene expression is just one aspect of the complex defence mechanisms in plants.

The interplay of various factors, including genetic, environmental, and physiological, contributes to the overall resistance or susceptibility of a plant to pathogens. Studying constitutive gene expression is a valuable component of a broader investigation into the molecular mechanisms underlying grapevine defence responses.

1.7. Aim of the study

Our objectives in this work were to identification of basal defence mechanisms possess in grapevines to protect themselves against pathogens, pests, or environmental stressors, to investigate the differences amongst varietals in case of gene expression profiles, and to identify early resistance detection markers which can be genetic sequences that are linked to resistance in grapevine varieties.

The grapevine RNA sequencing study encompassed a comprehensive workflow designed to unveil gene expression patterns across distinct grape varieties. RNA extraction following library preparation. Rigorous data processing in RStudio enabled differential gene expression analysis and candidate gene identification. Enrichment analyses unveiled significant biological pathways and functions among differentially expressed genes, while tissue-specificity analysis offered insights into gene expression patterns within the *Vitis* reference genome. Overall, the workflow provided a detailed examination of gene expression variations among grapevine varieties, shedding light on their genetic intricacies to reach our goal.

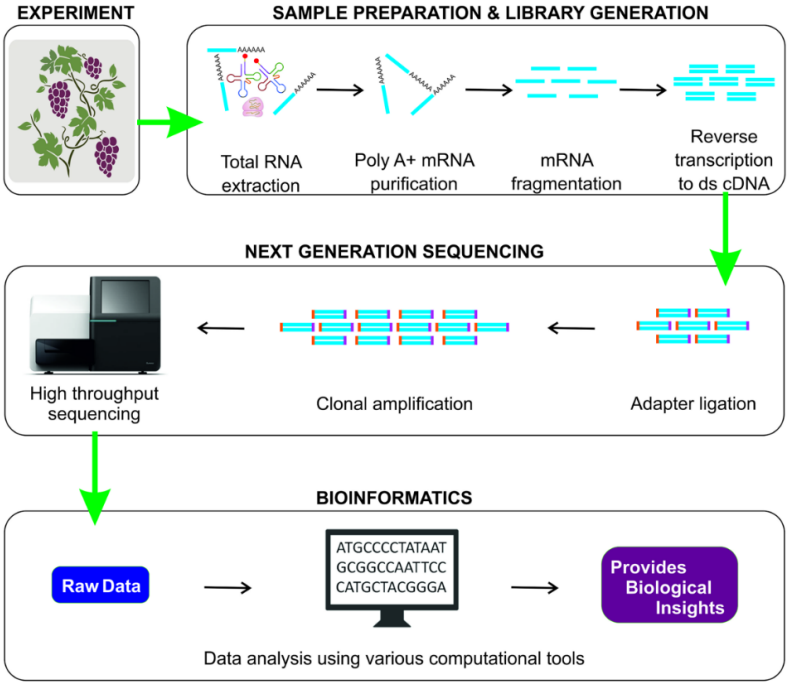

Figure 1.3. Schematic representation of the common transcriptomic analysis walkthrough for RNAseq approach.

2. MATERIALS AND METHODS

2.1. Sample Preparation

2.1.1. Sample Collection

For each grapevine variety considered, (CS, CV, CC, SB, SN, and SR), we collected a total number of 40 berries from 4 plants grown in the same vineyard (Fossalon di Grado) and we stored them at -80 °C before their processing.

Storing RNA at -80°C is crucial due to its delicate nature and vulnerability to degradation. Keeping RNA samples at such low temperatures serves multiple vital purposes. Firstly, it preserves the integrity of RNA molecules, preventing rapid degradation and ensuring an accurate representation of the biological state at the time of collection. Secondly, it slows down enzymatic activity, particularly that of ribonucleases, which are less active at -80°C, reducing the degradation risk. Additionally, maintaining RNA at -80°C offers a stable and consistent environment for long-term storage, critical for extended research or diagnostic needs, thus ensuring high-quality RNA which is essential for various analytical applications such as RNA sequencing.

2.1.2. RNA Extraction

In this process, we employed the Sigma-Aldrich Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St, Louis, MO, USA), containing reagents reporting in figure 2.1 [61]. Sigma's Spectrum Plant Total RNA Kit simplifies RNA extraction from plants by effectively eliminating interfering substances without hazardous solvents. This streamlined process ensures clean RNA from challenging plant species like spruce, cotton, and grape, suitable for various downstream applications like Northern blot and Real-Time PCR (RT-PCR). Additionally, the On-Column DNase I Digest Set efficiently removes residual genomic DNA, enabling recovery of even small RNA molecules like microRNA.

Reagents Provided	Catalog No.	10 Preps	50 Preps	250 Preps
Lysis Solution	L8167	10 ml	50 ml	250 ml
2-Mercaptoethanol	M3148	0.15 ml	0.9 ml	2 × 2 ml
Binding Solution	L8042	10 ml	50 ml	250 ml
Wash Solution 1	W1141	10 ml	50 ml	250 ml
Wash Solution 2 Concentrate	W3261	2.5 ml	15 ml	75 ml
Elution Solution	E8024	1.5 ml	10 ml	50 ml
Filtration Columns	C6866	10 each	50 each	5 × 50 each
Binding Columns	C6991	10 each	50 each	5 × 50 each
Collection Tubes, 2 ml	T5449	4 × 10 each	4 × 50 each	4 × 250 each

Reagents and Equipment Required But Not Provided

- Mortar and pestle
- Liquid nitrogen
- Dry ice
- 2 ml microcentrifuge tubes for weighing tissue
- Microcentrifuge (12,000 × *g* or higher)
- Heat block
- 100% Ethanol, Catalog No. **459836**
- On-Column DNase I Digest Set, Catalog No. **DNASE10** and **DNASE70** (optional)
- Amplication Grade DNase I, Catalog No. **AMPD1** (optional)

Figure 2.1. Sigma-Aldrich Spectrum™ Plant Total RNA Kit reagents used in the manufacturer protocol [61].

Dissection of grapevine berries

The dissection of grape berries for high-quality samples requires meticulous attention to detail. Samples stored at -80°C are delicately retrieved and swiftly placed in liquid nitrogen for preservation. With sterile tools like mortar, pestle, forceps, and a blade, each berry is carefully handled to separate its components efficiently. The process involves carefully removing the skin to obtain pure samples and splitting the pulp to extract seeds, swiftly placing each element into designated tubes. Safety precautions and rigorous cleaning protocols are crucial to maintain a sterile environment and safeguard the integrity of the samples during this procedure, specially focusing on avoiding cross-contamination between samples material.

Grinding of samples

The grinding, or homogenization, of grape berry samples is pivotal in preparing them for RNA extraction. Retrieving the samples from -80°C storage and maintaining their low temperature during the process is pivotal. Pre-cooling the equipment and utilizing aids like liquid nitrogen facilitate the grinding process and preserve RNA integrity. Samples are ground in manageable batches according

to equipment instructions, aiming for a consistent powder. Monitoring and adjusting parameters ensure efficient homogenization. Thorough cleaning of equipment between samples processing prevents contamination. Finally, the homogenized samples are collected and stored at the appropriate temperature for RNA extraction, where sample integrity is paramount for accurate outcomes. Grinding, thus, plays a crucial role in ensuring uniform samples, critical for subsequent analyses.

RNA extraction, quantification, and quality check

In this experiment, 4g of skin grounded material for each variety, in three replicates each, were used for the RNA extraction using the protocol provided by the commercial kit manufacturer. After the RNA extraction step, samples were eluted in 100 μl of elution buffer before quantification and quality assessment.

RNA quality was assessed qualitatively, with a focus on the A260/280 and A260/230 absorbance ratios, using the Nanodrop 2000C spectrophotometer by ThermoFisher Scientific in figure 2.2[70]. The A260/280 ratio highlights protein contamination, with the suitability range being between 1.8 and 2.0. Deviations may suggest protein contaminant. Meanwhile, the A260/230 ratio, suitable when around 2.0 or higher, indicates absence of common impurities such as phenol or salts [70].

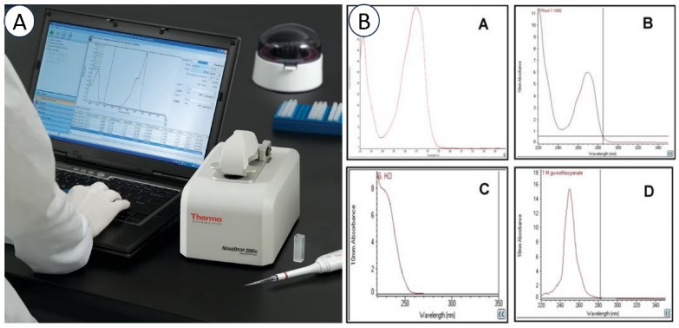

Figure 2.2. (A): Nanodrop 2000C. (B): Common reagents used in the isolation of nucleic acids include: A) *TriZol*. B) *Phenol*. C) *Guanidine Hydrochloride* (HCl). D) *Guanidine Isocyanate*.

2.1.3. Purification

After quality checks, samples showing high contaminants concentrations were further processed through purification step involving precipitation in lithium chloride (LiCl 10 M). This step is divided into two rounds. In the initial round, a 2.5 M LiCl solution was added to the previously extracted RNA solution, with 16.6μl of the 10 M LiCl solution incorporated into each 50μl sample. These

samples were then stored at 4°C in the fridge overnight. In the subsequent round, subjected to centrifugation for 15 minutes at a maximum speed of 14,000 RPM at 4°C. Following it, the supernatant was carefully removed, and samples underwent a washing step with 500μl of 70% ethanol in DEPC (*Diethyl Pyrocarbonate*) water to inactivate possible RNases or enzymes that can degrade RNA. After the purification process, a further assessment by Nanodrop was performed to ensure that samples achieved the desired quality. Upon obtaining satisfactory results, samples were securely stored at -80°C.

2.2. Library Preparation and Sequencing platform

Selecting the appropriate library preparation protocol is crucial when planning an RNA sequencing study, requiring alignment with the study's goals, the specific biological material being studied, and the sequencing requirements [37]. Recent advancements in NGS platforms have expanded read lengths beyond 100 nucleotides, offering enhanced coverage for more comprehensive transcriptome analysis through RNA-seq. To leverage these extended reads effectively, the chosen library preparation protocol must ensure optimal library and insert lengths, aligning with the length of the sequenced RNA fragment [37].

Reverse transcription

Reverse transcription is essential for converting RNA into a cDNA molecule. the enzyme reverse transcriptase plays the main role to synthesize a single-stranded cDNA molecule from an RNA template. It enables the preservation and amplification of RNA molecules. Through this method, the originally single-stranded RNA is converted into a double-stranded cDNA molecule, making it more amenable for downstream sequencing. Reverse transcription is crucial in creating a stable and amplifiable cDNA library.

Fragmentation

cDNA molecules obtained after RT-PCR are fragmented into shorter segments. To accommodate the size restrictions of prevalent sequencing platforms (e.g., <600 bp on Illumina sequencers), Transcripts fragmentation is imperative. This can be accomplished using methods including exposure to alkaline solutions, solutions containing divalent cations like Mg++ or Zn++, and enzymatic

treatments such as RNase III [35]. By breaking down the cDNA into smaller, uniform sections, the sequencer can efficiently handle and read these sequences, enabling a comprehensive analysis of the transcriptome.

Adaptor ligation

During this step, short nucleotide sequences called adaptors are attached to the ends of fragmented RNA or cDNA molecules. These adaptors contain specific sequences vital for binding to the sequencing platform and identifying sequence origin and orientation during sequencing. It facilitates fragment amplification and sequencing by providing sequences necessary for the initiation of the sequencing reaction and aids in differentiating the start and end points of the fragments, ensuring precise analysis and enabling the machine to recognize diverse samples and sequences. This allows efficient and cost-effective simultaneous analysis of multiple samples during sequencing.

PCR amplification

To enhance detection on most sequencers, cDNA libraries must undergo PCR amplification before sequencing [35] for increasing the quantity of sequences attached to adaptors in preparation for sequencing. It works by exponentially replicating the nucleic fragments attached to the adaptors, thus producing sufficient material to be sequenced. The amplified (or enriched) sequences represent the genetic material of interest. Ensuring the adequate amount of DNA for accurate and comprehensive analysis is mandatory.

RNA Sequencing of 6 *Vitis* varieties

The RNA from the 6 selected varieties underwent library preparation and sequencing at an external facility (BMK, Berlin, DE) employing Illumina platforms. For robust statistical analyses, each variety was meticulously examined across three independent biological replicates.

The RNA-Seq process commenced with an evaluation of the integrity, concentration, and quantity of the extracted RNA before proceeding to library preparation. Utilizing TapeStation (Agilent) and Qubit (Thermo Scientific) instruments for rigorous quality assessments, samples were meticulously prepared and pooled to facilitate subsequent Illumina sequencing.

Following sequencing, the raw reads underwent initial filtering to eliminate low-quality data and were subsequently trimmed to remove adapters and barcodes. These preparatory steps preceded the reads mapping and counting, integral to the subsequent bioinformatic analyses. The sequencing company handled the initial stages of sequencing, including the preliminary filtering and demultiplexing of raw reads, before providing the FastQ files for each replicate.

Sequencing Data analysis

Obtained fastq files, derived from the RNAseq of the 18 libraries, followed the quality checks using fastp package [10]. The objective was to eliminate low-quality bases and remove adapter sequences before the reads mapping step. The processed trimmed reads were utilized to map and quantify them against the reference transcriptome of the grapevine. Considering that the grapevine transcriptome has several versions and annotations, some of which are still undergoing publication and revision. The most comprehensive and representative one was identified in that of version 3 (12X.2) of the grapevine transcriptome, including its associated annotations (VCost.v3). The reference files, sourced from the INRA portal [75], served as the basis for mapping and counting the reads. The reads alignment and counting per annotation was conducted using STAR software version 2.7.11a [15] on individual data sets for each variety and biological replica. The resulting reads-count files were then combined in a single dataset for further analyses, which were performed using RStudio platform. This procedure facilitated the generation of a read-count file, subsequently utilized in RStudio software for the differentially expressed gene (DEG) analysis and further investigations [55].

2.3. Differential gene expression analysis and candidate genes identification

A powerful tool in RStudio, called DESeq2 package [85], was used to perform the differential expression analysis with two separate variables: grapevine variety typology ("Elite" or "PIWI") and berry colour ("White" and "Red"). The count file was imported into the R environment after STAR alignment, and the “dds” tool from DEseq2 made facilitated the analysis and identification of genes that were differentially expressed. To prepare for further comparison studies, read count normalization was carried out and the significance level was set at pvalue ≤ 0.01, while the expression level threshold, considered as the Log2 Fold Change (log2FC), were set to $-2 \geq \log2FC \geq 2$. Using

RStudio, the DEG results were visually represented using ggplot2 package, an effective tool for creating a wide range of visualizations suitable for publication. With flexibility and convenience in data exploration and presentation, the user-friendly syntax of R makes it possible to create stunning plots ranging from basic charts to intricate ones and multi-layered visualizations [82].

Combined with the DEG analysis, the gene enrichment analysis was performed using g:Profiler web-tool [84] starting from the DEGs results. This analysis was performed to identify the molecular function (MF), cell compound (CC) and biological pathways (BP) related genes showing higher relevance among the differentially expressed genes identified through the DEGs analyses in the different comparisons between PIWI and the respective elite variety, both for white and red grapes.

A further investigation was conducted adopting the tau (τ) algorithm, which, although it is commonly adopted for animals and human transcriptomic analyses, was used for assessing the tissue-specificity level of each predicted gene within the adopted reference genome of *Vitis*.

3. RESULTS AND DESCUSSION

3.1. Sequencing quality reports

Paired-end reads obtained by RNA sequencing of 18 corresponding to the six varieties under study in three biological replicates were analysed and processed using fastp 0.20.1 [9]. The mean read length before filtering was 149 base pairs (bp) for both directions. After read processing, the mean read length remained unchanged at 149 bp for both directions. The duplication rate was estimated around 31% on average and an insert size peak of 269 bp was determined. The minimum, maximum and average value of total pre-filtering reads was 40.7 M, 46.7 M, and 43.7 M, for red varieties and, 40.2 M for minimum, 44.1 M for maximum, and 42.1 M for white ones. Table 3.1 reports the most important fastp statistics for each variety and its replicates.

Table 3.1. Fastp report results for each replicant\variety showing the total number of reads and bases pre- and post-filtering. High quality bases (Q30) are also reported.

RED Varieties	**CC1**	**CC2**	**CC3**	**CV1**	**CV2**	**CV3**	**CS1**	**CS2**	**CS3**
Total reads pre-filtering	45.4 M	43.9 M	46.7 M	40.7 M	41.2 M	40.6 M	42.3 M	41.7 M	41.6 M
Total reads post-filtering	44.8 M	43.4 M	46.1 M	40.1 M	40.5 M	39.9 M	41.8 M	41.1 M	41.1 M
Q30 pre-filtering	6.3 G (92.8%)	6.1 G (93.5%)	6.5 G (93.3%)	5.7 G (93.2%)	5.7 G (92.9%)	5.6 G (92.2%)	5.9 (93.4%)	5.8 G (93.2%)	5.8 G (93.1%)
Q30 post-filtering	6.2 G (93.3%)	6.1 G (94 %)	6.5 G (93.9%)	5.6 G (93.8%)	5.7 G (93.6%)	5.5 G (92.8%)	5.9 G (93.8%)	5.8 G (93.7%)	5.7 G (93.6%)
Read passed filters	44.8 M (98.7%)	43.3 M (98.8%)	46.1 M (98.6%)	40.1 M (98.6%)	40.5 M (98.4%)	39.9 M (98.3%)	41.8 M (98.9%)	41.1 M (98.7%)	41.1 M (98.7%)
White Varieties	**SN1**	**SN2**	**SN3**	**SR1**	**SR2**	**SR3**	**SB1**	**SB2**	**SB3**
Total reads pre-filtering	44.1 M	45.1 M	40.2 M	41.1 M	40.2 M	40.6 M	40.9 M	40.7 M	43.5 M
Total reads post-filtering	43.4 M	44.4 M	39.6 M	40.5 M	39.7 M	40.4 M	40.2 M	40.2 M	42.9 M
Q30 pre-filtering	6.1G (93.1%)	6.3 G (92.9%)	5.5 G (92.5%)	5.7 G (93.2%)	5.6 G (93.4%)	5.7 G (93.6%)	5.7 G (93.3%)	5.7 G (93.3%)	6.1 G (93.2 %)
Q30 post-filtering	6.1 G (93.6%)	6.2 G (93.5%)	5.5 G (93.1%)	5.7 G (93.7%)	5.6 G (93.9%)	5.7 G (94.1%)	5.6 G (93.9%)	5.6 G (93.9%)	6.1 G (93.6%)
Read passed filters	43.4 M (98.6%)	44.4 M (98.6%)	39.6 M (98.7%)	39.7 M (98.5%)	40.0 M (98.6%)	40.4 M (98.9%)	40.2 M (98.7%)	40.2 M (98.7%)	42.9 M (98.8%)

3.2. Identification of Differentially Expressed Genes between tolerant and susceptible grapevine varieties

. Filtered reads were mapped on the 12x.v2 assembly of the *V. vinifera* PN40024 reference genome using the VCost.v3 annotation. The resulting counts were imported in RStudio and analysed by means of DESeq2 package to identify genes differentially expressed between tolerant and susceptible varieties. As a preliminary test, before computing the differential expression of genes aver different pairwise comparisons, we used the DEseq2 software to ascertain the relationship between replicates and genotypes based on normalized read counts. Plots are shown in Figure 3.1 and Figure 3.2 showing low variance between replicas and good distinctiveness among varieties. In detail, the PC1 explained 43% of variance well separating white genotypes from red ones. The PC2, which explained 21% of variance separated elite varieties from PIQWI ones. Results of these preliminary analyses not only demonstrated the goodness of replicates and therefore the robustness of transcriptional data, but also indicated a clear distinction between the transcriptome of Piwi and elite varieties and of grape and white ones.

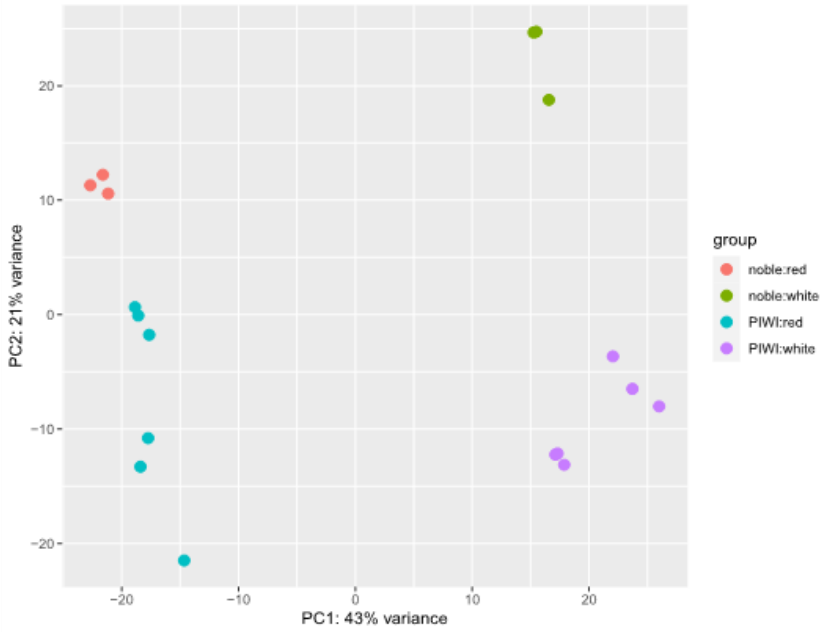

Figure 3.1. PCA representing the overall relationships between replicas and varieties considering the reads count mapped to each annotated gene previous to the DEGs analysis.

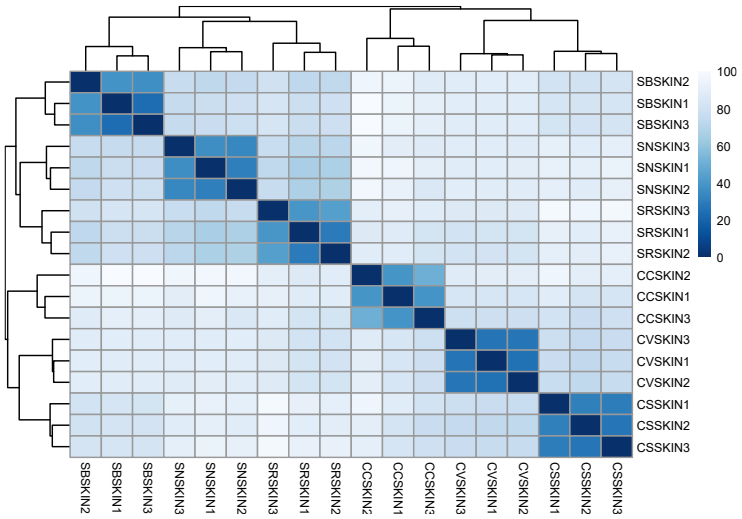

Figure 3.2. Heatmap representing the overall relationships between replicas and varieties considering the reads count mapped to each annotated gene previous to the DEGs analysis.

Based on the refined data derived from DESeq2 results of differential gene expression analysis, a comparative overview reveals the count of up- and down-regulated genes across various varieties as juxtaposed with their respective parental variety. In Figure 3.3, between all PIWIs and Elites regardless of the colour, 77 genes are upregulated, and 191 genes are downregulated. In the comparison between PIWIs and Elites of the considered red varieties, 960 genes were found upregulated, and 878 genes downregulated. Among PIWIs *versus* Elites in the white varieties 994 genes resulted as upregulated, and 995 downregulated.

Considering the single PIWI vs. Elite comparisons (Figure 3.45), the number of DEGs for the red CC is 494 upregulated and 659 downregulated genes, while for CV it is 1495 for upregulated and 1512 for downregulated ones, which coincides with the highest number of DEGs observed in both red and white varieties comparisons. In case of white varieties, in SN, 225 genes resulted upregulated, and 374 genes were downregulated, while, for SR, 1295 and 1345 genes were identified as upregulated and downregulated, respectively., Regarding SR vs. SB, it resulted as the most differential in terms of number of DEGs within white samples. In general, CV and SR showed the highest number of differentially expressed genes compared to the respective elite parent, thus indicating lower similarity with the elite varieties compared to the other two PIWI considered in this study.

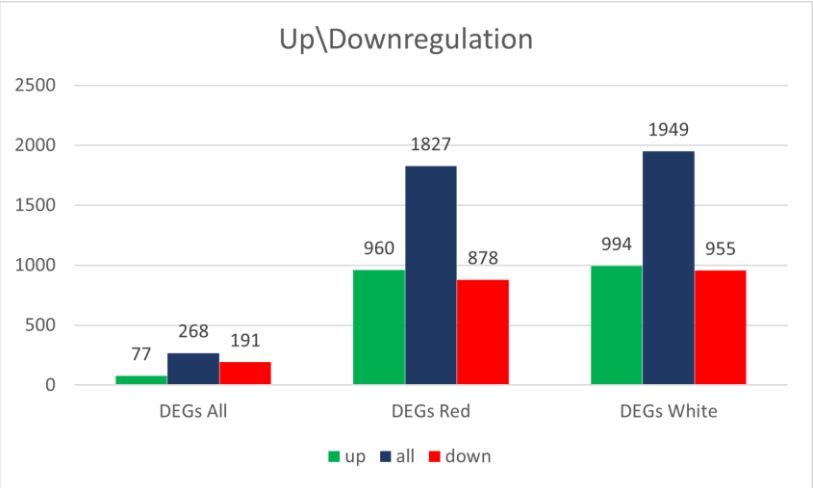

Figure 3.3. histogram reporting the number of upregulated and downregulated genes between samples overall, only red varieties, and only whites ones.

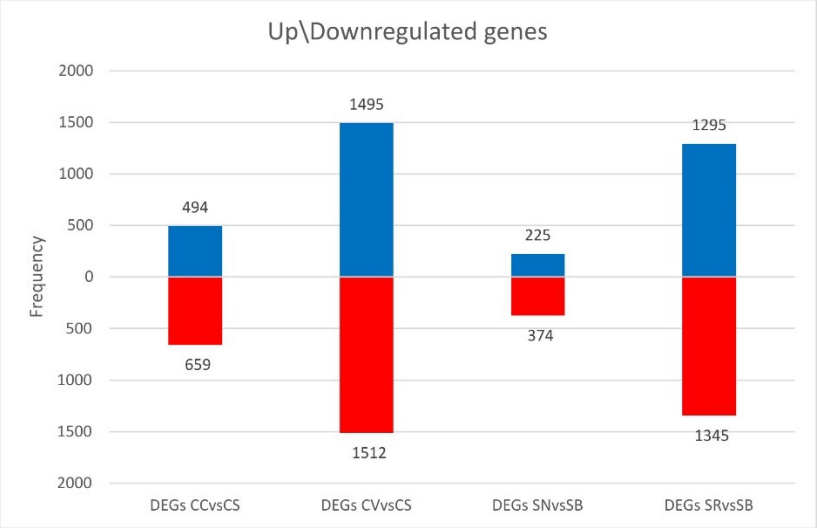

Figure 3.4. histogram reporting the number of upregulated and downregulated genes within red and white varieties considering single variety comparisons to the respective elite parent.

In Figure 3.5, an overview of the samples' similarity is presented that highlights the good correlation occurring between replicas form the same variety, both for Elite and PIWI ones. Higher similarity was indicated by shorter distances, highlighted by the dark blue colour of the heatmap. Specifically, comparisons between the three replicates within the same variety demonstrated notable closer distances, thus indicating high concordance between their respective sequencing results.

Notably, comparisons among white varieties revealed a closer proximity compared to that of the red varieties.

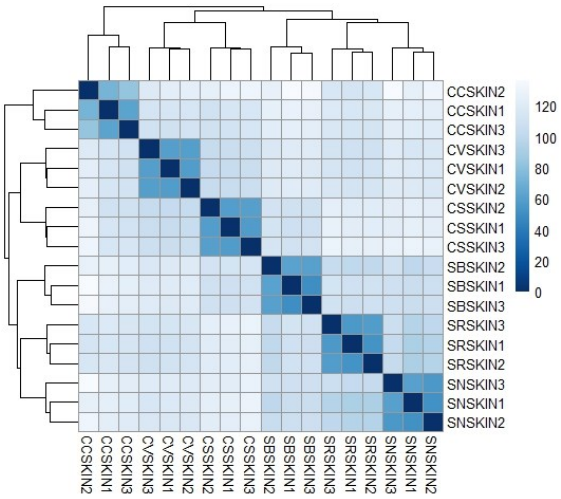

Figure 3.5. Heatmap comparing the differentially expressed genes counts from all the analysed samples and replicas.

In Figure 3.6, a principal component analysis (PCA), the clustering of samples illustrates the grouping of highly correlated replicas sharing similar gene expression profiles. Moreover, the analysis reveals six distinct clusters, each comprising the three replicates of each variety. Notably, the top left quadrant of the chart displays the CS parental replicates, denoting the elite sample among the red varieties and showcasing strong correlation. Similarly, in the top right quadrant of the chart, SB replicates are present exhibiting pronounced correlation.

White samples exhibited a tighter clustering pattern around their respective parental variety, indicating a higher degree of similarity compared to the red ones. This observed pattern was discerned from the heatmap analysis based on the gene expression profiles, where the white samples displayed shorter distances among themselves, thus denoting a stronger resemblance within this group. As the CC3 replica had higher distance compared with other ones, it shows variability or divergence in gene expression compared to the other samples, which are generally grouped depending on the PC2 for what concerns the grape colour and depending on the PC1 for what regards the variety typology ("Elite", or "PIWI").

The axes are arranged in order of significance, with the differences observed along the primary principal component axis (PC1) holding greater importance than those along the secondary principal

component axis (PC2). As a result, variations between clusters along PC1 are more substantial, while similarities in distances are more apparent along PC2. Each principal component captures a certain amount of variability in the data, and the percentage of variance explained by each component indicates its importance in representing the overall variation within the dataset. Therefore, here PC1 accounts for 36% of the total variability observed in the dataset, while PC2 represents the 20%. Overall, the two considered principal components represented 56% of the total observed variance.

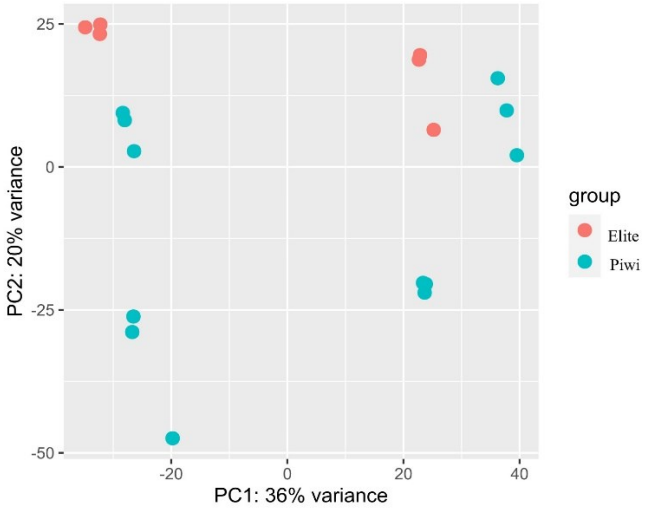

Figure 3.6. PCA plot comparing the expression levels among all the analysed samples. the distance on PC1 axis shows the difference between clusters.

3.3. White varieties

In Figure 3.7, the proximity between the SN and SR PIWI varieties, concerning white grapes, illustrates a closer relationship compared to their distance from the parental variety SB. The distinct similarity of the SR and SN PIWI varieties, as revealed in this figure, emphasizes their respective association and the divergence from the parental variety. Overall variance, represented by the two axes PC1 (59%) and PC2 (35%), is 94%. The same DEGs results used for the construction of the heatmap in Figure 3.7 were used for the creation of the PCA shown in Figure 3.8

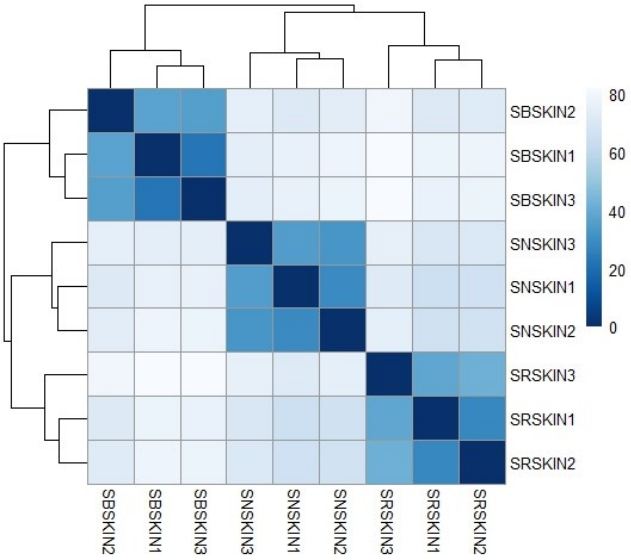

Figure 3.7. Heatmap plot reporting the comparisons between white varieties considering the only DEGs results.

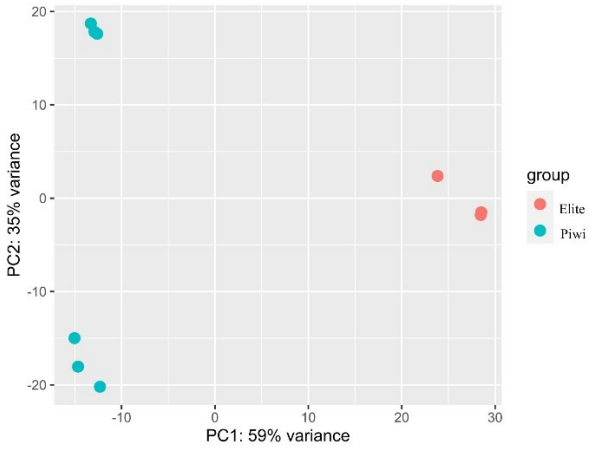

Figure 3.8. PCA plot reporting the comparisons between white varieties considering the only DEGs results.

3.4. Red varieties

In Figure 3.9, the inter-sample distances within the red varieties provide insightful comparisons. Notably, among the red varieties, the distance between CV and CS appears shorter than that between CC and CS. Conversely, the largest distance is observed between CC and the CV, suggesting the lowest similarity between these samples within the red group. From the PCA in Figure 3.10, the PC1

highlighted strong discrimination between CC and CV compared to CS, plus the strong difference occurring between them. Overall variance (93%) is mostly represented by PC1 (63% of variance), which is able to discriminate replicates from the two PIWI varieties. On the other hand, PC2 (30% of variance) is able to show distinctiveness between “Elite” and “PIWI” red varieties.

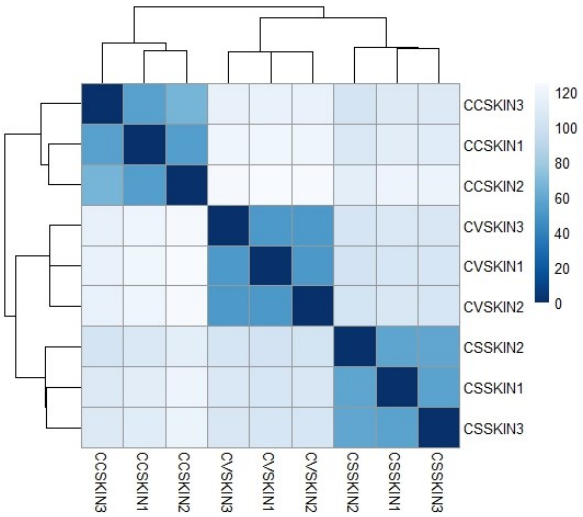

Figure 3.9. Heatmap plot reporting the comparisons between red varieties considering the only DEGs results.

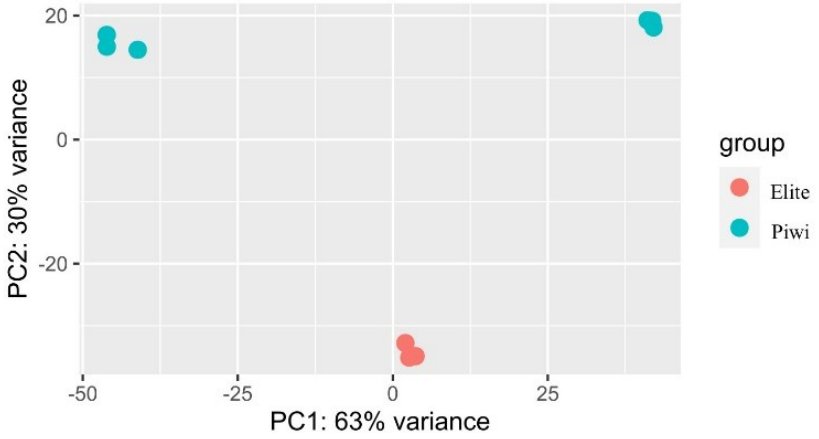

Figure 3.10. PCA plot reporting the comparisons between red varieties considering the only DEGs results.

Based on the DEGs results, a Venn diagram, which represents the relationships and commonalities among different varieties, was created referring of white samples (Figure 3.11). This graph displays the counts of private upregulated and downregulated genes per variety alongside the shared gene counts within each category. Across all white varieties, there were 525 upregulated genes

shared between SN and SR, constituting 40% of the total 1295 upregulated genes. On the downregulated front, the white varieties showed 682 shared genes, contributing to 51% of the total 1345 downregulated genes observed in the white PIWI varieties.

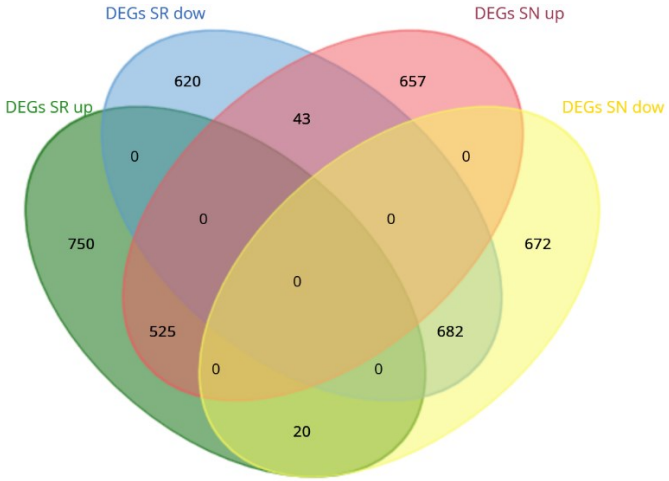

Figure 3.11. Venn diagram of shared upregulated and downregulated gene within white varieties.

On the other hand, within the red varieties, the analysis reveals a mere 46 genes shared between CC and CV among the total 1595 upregulated genes (3%), while, in the realm of downregulated genes, there were 87 shared genes identified within the red varieties out of a total of 1512 (6%) of the total downregulated genes (Figure 3.12).

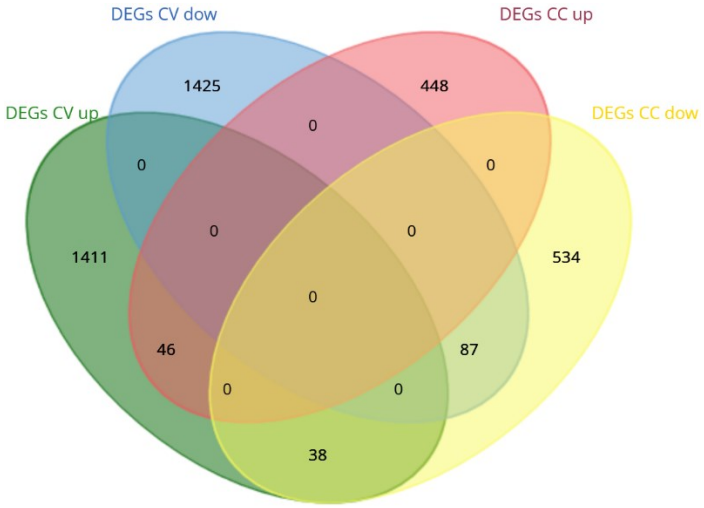

Figure 3.12. Venn diagram of shared upregulated and downregulated gene within red varieties.

3.5. G:Profiler enrichment analysis

Combined with the differential expression analysis, the identification of the molecular functions, biological pathways and cell compounds, which DEGs are related to, was investigated. For this purpose, the DEGs results were analysed using g:Profiler web-tool for each of the considered comparisons.

Figure 3.13 shows the result of the DEGs identified among all the white varieties. In the case of MF the highest enriched terms were related to oxidoreductase activity. For BP the highest P value was observed related to membrane specific genes, and CC group defence response resulted as the highest. KEGG biological pathways related terms resulted on the top. In table 3.3, all most important terms are reported.

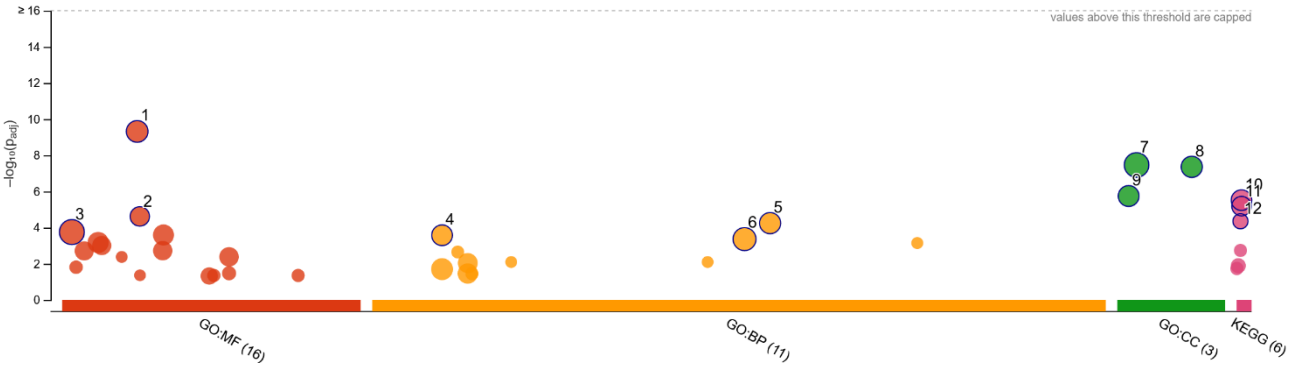

Figure 3.13. G:Profile graph of enrichment analysis of gene sets considering all the white varieties. GO: gene ontology, MF: molecular function, BP: biological process, CC: cellular component. KEGG is biological pathway.

Table 3.2. Top 3 the most important terms identified from the enrichment analysis of genes differentially expressed in white the varieties. Results are divided into each category.

			White
			all
	MF	T	T∩Q (Q)
1	oxidoreductase activity	2191	169 (973)
2	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	653	6a1 (973)
3	Catalytic activity	11701	602 (973)
	CC		
4	Cell periphery	1673	132

			(808)
5	membrane	9855	538 (808)
6	Plasma membrane	1412	111 (808)
	BP		
7	Defence response	1286	97 (840)
8	Transmembrane transport	1627	-
9	Response to another organism	819	-
	KEGG		
10	Metabolic pathways	1235	103 (151)

In Figure 3.14, presenting the g:Profiler results considering all the identified DEGs in the comparison of SN with SB white varieties, in case of MF the highest enriched terms were related to oxidoreductase activity. In BP the highest pvalue was identified for membrane related genes, while for CC terms, defence response was the highest. KEGG related terms highlighted that the metabolic pathways were on the top. On the table 3.3, all most important aspects are mentioned. Figure 3.15 and Figure 3.16, which considered the SN vs. SB DEGs, show the only up- or down-regulated genes enrichments, respectively. Regarding the upregulated genes: MF highest enriched terms were oxidoreductase activity; in BP, the highest pvalue was for transmembrane transport; in CC, cell periphery was the highest; in KEGG, metabolic pathways are on the top. On the table 3.3, all most important aspects are mentioned.

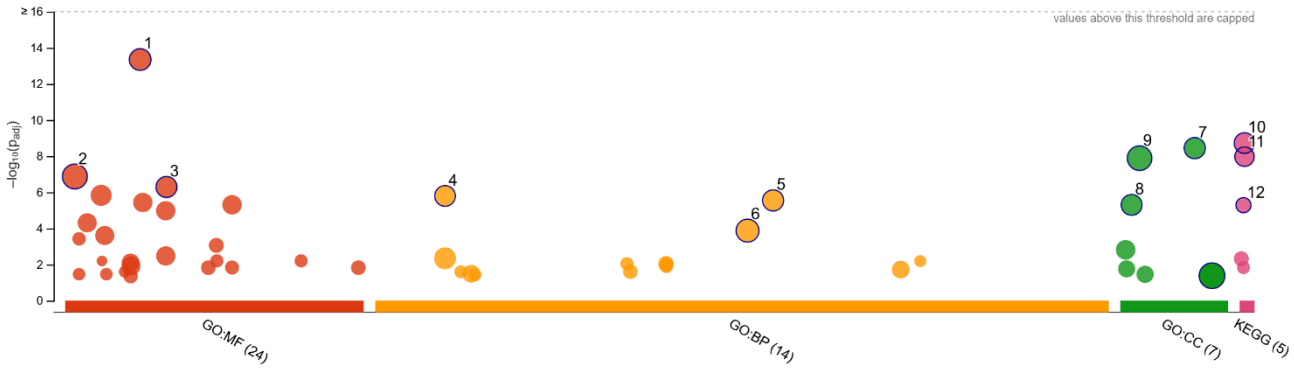

Figure 3.14. g:Profile analysis considering all DEGs in SN.

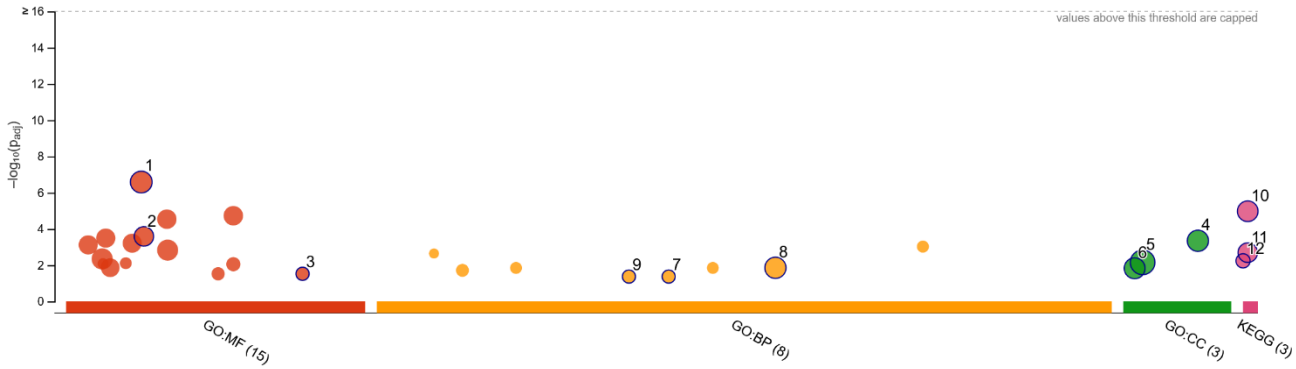

Figure 3.15. g:Profile analysis considering all upregulated DEGs in SN.

Regarding the downregulated genes: MF highest enriched terms were catalitic activity; in BP, the highest pvalue was for defence response; in CC, membrane was the highest; in KEGG, biosynthesis of secondary metabolites are on the top. On the table 3.3, all most important aspects are mentioned.

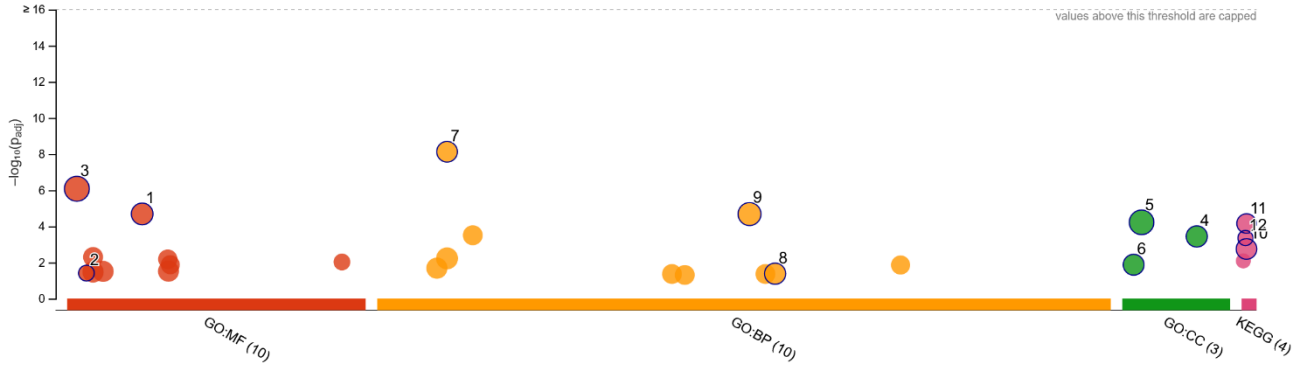

Figure 3.16. g:Profile analysis considering all downregulated DEGs in SN.

Table 3.3. Top three most important enriched terms for each section in SN variety.

		SN		
		all	+	-
MF	T	T∩Q (Q)	T∩Q (Q)	T∩Q (Q)
oxidoreductase activity	2191	235 (1370)	114 (633)	477 (737)
Catalytic activity	11701	852 (1370)	-	-
Transmembrane transporter activity	1530	159 (1370)	-	80 (737)
CC				
Cell periphery	1673	175 (1125)	85 (543)	90 (582)
membrane	9855	733 (1125)	349 (543)	384 (582)
Plasma membrane	1412	142 (1125)	69 (543)	73 (582)
BP				
Defence response	1286	135 (1176)	-	92 (637)
Transmembrane transport	1627	161 (1176)	-	-
Response to stimulus	4152	-	-	-
KEGG				
Metabolic pathways	1235	141 (204)	72 (100)	69 (104)
Biosynthesis of secondary metabolites	681	93 (204)	44 (100)	49 (104)
Phenylpropanoid biosynthesis	70	20 (204)	-	12 (104)

Figure 3.17, computed considering all DEGs from the comparison between SR and SB white varieties, reports for MF the highest enriched term to beoxidoreductase activity, n BP the highest pvalue was identified for defence response,. CC terms matched cell periphery as the highest, and in KEGG, metabolic pathways term is on the top. On the table 3.3, all most important aspects are mentioned.

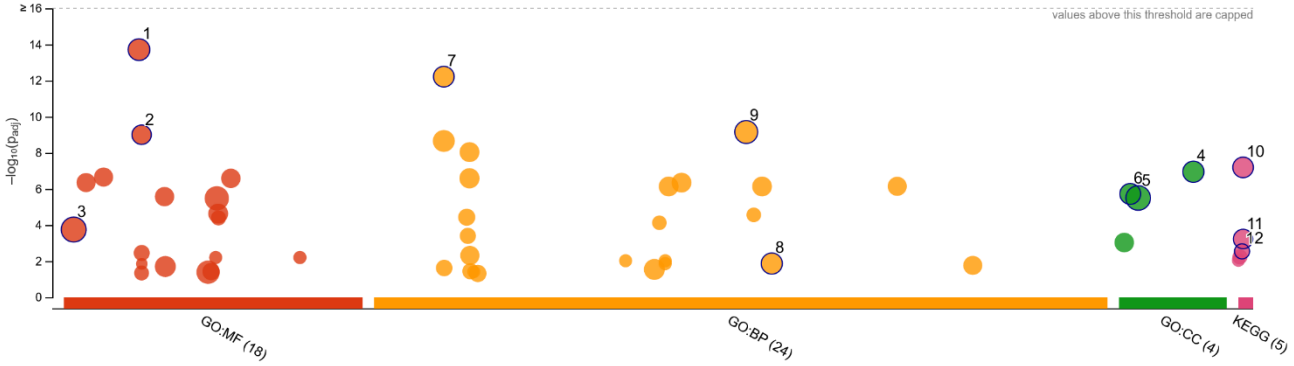

Figure 3.17. g:Profile analysis considering all DEGs in SR.

Table 3.4. top three most important enriched terms for each category referred to the SR variety g:Profiler analysis.

		SR	
			all
	MF	T	T∩Q (Q)
1	oxidoreductase activity	2191	237 (1376)
2	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	653	90 (1376)
3	Catalytic activity	11701	833 (1376)
	CC		
4	Cell periphery	1673	172 (1149)
5	membrane	9855	733 (1149)
6	Plasma membrane	1412	146 (1149)
	BP		
7	Defence response	1286	157 (1189)
8	Transmembrane transport	1627	-
9	Response to stimulus	4152	367 (1189)
	KEGG		
10	Metabolic pathways	1235	152 (230)
11	Biosynthesis of secondary metabolites	681	88 (230)
12	Phenylpropanoid biosynthesis	70	17 (230)

3.6. Gene enrichment and Tau test

In this study, we employed an algorithm commonly utilized in transcriptomic investigations involving animals or humans. However, it had previously been applied in the analysis of tissue-specific genes within the Pinot noir flower, as described by Vannozzi et al. in 2019. This algorithm, denoted as the tau (τ) algorithm [86], was employed to assess the tissue-specificity level of each predicted gene within a given genome. Following the quantile normalization of 23,847 genes, which were selected based on their expression levels, and the subsequent creation of BIN profiles, the τ algorithm was utilized to assign a value ranging from 0 (indicating constitutive expression across all or most tissues) to 1 (indicating absolute specificity for a particular tissue) to each gene. The distribution of τ values across the entire gene set is depicted in Figure 3.18A. In summary, 906 genes exhibited a high degree of specificity (referred to as Highly Specific Genes, HSG, with $\tau > 0.85$), and among them, 570 were identified as absolutely specific genes (Absolute Specific Genes, ASG, with $\tau = 1$). It is important to note that the τ value only characterizes the "specificity" of a gene. To ascertain the specific tissue to which a gene is exclusive, we computed the τ expression fractions (τef).

Among the various grapevine varieties studied, Sauvignon Rytos displayed the highest count of HSG (246) and ASG (157). Conversely, Sauvignon blanc exhibited the lowest number of HSG and ASG values (69 and 22, respectively), as illustrated in Figure 3.18B. As a general observation, the elite grapevine varieties Cabernet Sauvignon and Sauvignon blanc displayed relatively lower counts of ASG and HSG genes. Detailed lists of HSG and ASG for each grapevine variety can be found in Supplementary Table 3.5, whereas Table 3.5 report the top 10 optimal genes for each variety, and their specificity for only the variety in which they are expressed.

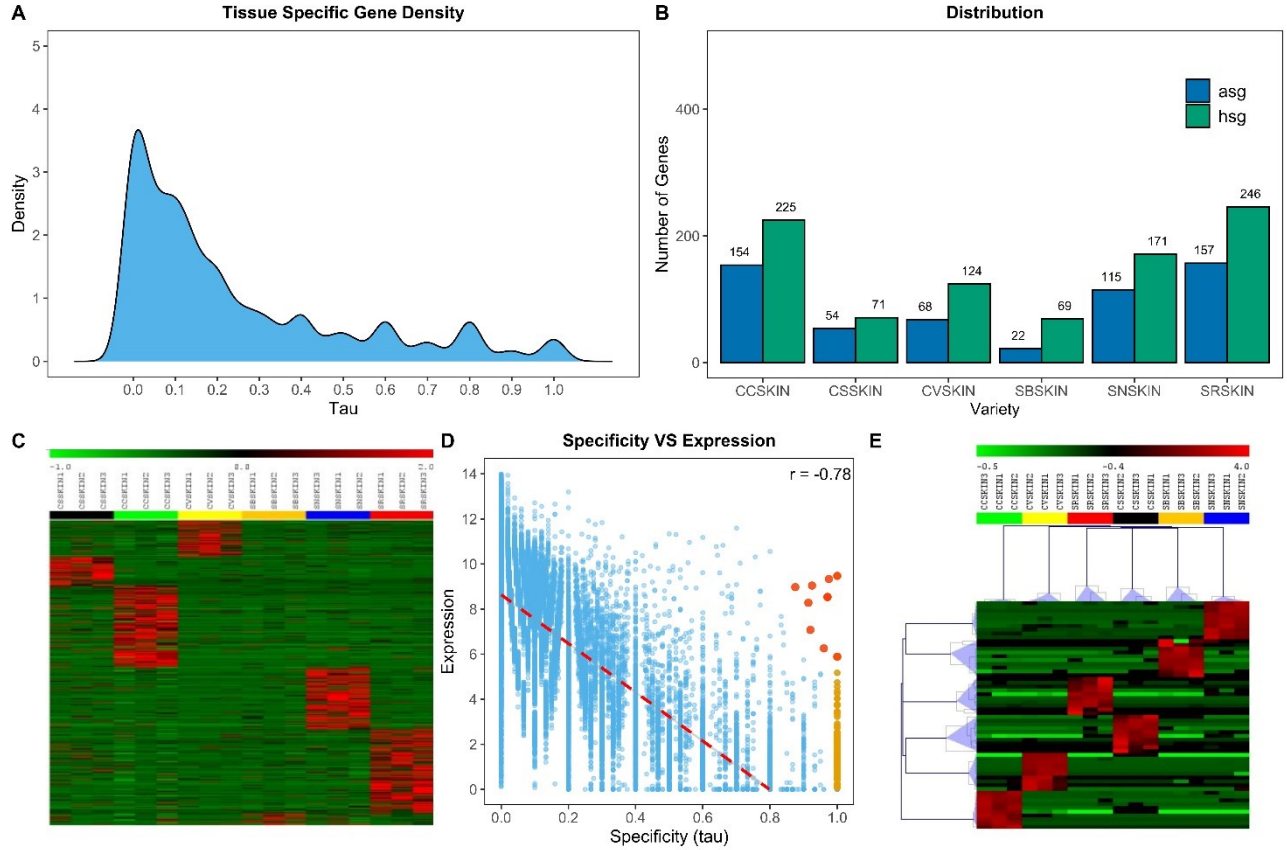

Figure 3.18.

Moreover, highly specific genes (HSG) resulting from the tau analysis were subjected to a GSEA to verify the presence of enriched ontological categories. The obtained results conferred great robustness to the analysis and laid the foundations for a subsequent step aimed at further narrowing down the list of key genes of interest.

Table 3.5. List of ten best ranking genes based on a score value (0-2) corresponding to the sum of the quantile normalized expression of a given gene and its tau expression factor.

Gene ID V3	Gene ID V2	tau	Score	Mean exp	Functional annotation
Cabernet sauvignon					
Vitvi01g02070	VIT_01s0127g00910	0.95	1.265	56,55	AERO1
Vitvi10g00903	-	1.00	1.249	23,71	Leucin-rich repeat protein kinase
Vitvi01g02281	VIT_01s0010g04010	1.00	1.182	9,77	Unknown protein
Vitvi10g02153	-	1.00	1.133	5,16	-
Vitvi07g00496	VIT_07s0005g02310	0.93	1.133	12,47	EXPA17
Vitvi05g02072	-	0.93	1.130	11,93	-
Vitvi10g00183	-	1.00	1.125	4,63	-
Vitvi10g02415	VIT_00s2472g00010	0.85	1.112	27,96	Enhancer of mR-decapping protein 4
Vitvi01g02068	-	1.00	1.111	3,84	-
Vitvi10g02416	-	1.00	1.098	3,25	-
Cabernet cortis					
Vitvi07g02026	VIT_07s0130g00200	1.00	1.423	238,36	***VvGELP21*** - Lipase GDSL
Vitvi19g00082	VIT_19s0014g01060	1.00	1.417	220,86	Sesquiterpene synthase
Vitvi09g01530	VIT_09s0002g01980	0.96	1.325	113,34	Myosin-like protein XIK
Vitvi11g01266	VIT_11s0052g01230	0.93	1.322	153,64	Xyloglucan endotransglucosylase/hydrolase 23
Vitvi09g01648	-	0.96	1.309	91,87	-
Vitvi08g02288	VIT_08s0007g04580	0.86	1.303	319,66	UGT73C2 (UDP-glucosyl transferase 73C2)
Vitvi19g00324	VIT_19s0014g04000	1.00	1.285	40,05	Curculin (mannose-binding) lectin
Vitvi12g02451	VIT_12s0134g00650	1.00	1.274	34,63	Anthocyanin 5-aromatic acyltransferase
Vitvi19g01982	VIT_19s0014g05140	0.95	1.271	64,65	-
Vitvi15g00285	VIT_15s0045g00270	1.00	1.265	30,67	Serine/threonine-protein phosphatase BSL3
Cabernet volos					
Vitvi14g00668	VIT_14s0036g00990	0.86	1.432	1814,13	Polyubiquitin (UBQ4)
Vitvi11g01637	VIT_11s0052g00270	0.85	1.346	652,97	R protein MLA10
Vitvi11g00879	VIT_11s0065g00040	0.89	1.331	321,15	CYP706A12
Vitvi04g00345	VIT_04s0008g04000	0.87	1.295	256,39	Unknown
Vitvi03g01478	VIT_03s0038g04230	0.88	1.240	103,63	Dihydroflavonol 4-reductase
Vitvi08g02374	VIT_08s0007g07760	0.93	1.175	21,69	Polygalacturonase PG1
Vitvi16g01677	-	1.00	1.169	8,42	-
Vitvi08g00789	VIT_08s0058g00650	1.00	1.161	7,72	Aldose reductase
Vitvi11g01568	VIT_11s0065g00740	1.00	1.142	6,04	A -phase-promoting complex subunit 8
Vitvi01g01642	VIT_01s0010g03550	1.00	1.140	5,97	Nuclear transcription factor Y subunit B related
Sauvignon blanc					
Vitvi04g00029	VIT_04s0008g00370	0.85	1.156	57,39	Clavata1 receptor kinase (CLV1)
Vitvi06g01648	VIT_06s0004g02550	0.85	1.150	52,71	Kiwellin Ripening-related protein grip22
Vitvi04g00021	VIT_04s0008g00300	0.85	1.147	50,92	Clavata1 receptor kinase (CLV1)
Vitvi09g01948	-	0.85	1.140	45,92	HcrVf2 protein
Vitvi03g00460	VIT_03s0063g01000	0.85	1.127	39,01	Blue (type 1) copper domain
Vitvi07g01769	VIT_07s0031g00850	0.87	1.094	20,80	Patatin
	VIT_01s0011g01000;				
Vitvi01g01852	VIT_01s0011g00990	0.87	1.080	17,09	RPM1 (resistance to p. syringae pv maculicola 1)
Vitvi10g00005	VIT_10s0116g00150	0.87	1.078	16,85	Receptor kinase RK20-1
Vitvi00g02077	VIT_00s0895g00010	0.87	1.061	13,52	Glucan 1,3-beta-glucosidase
Vitvi16g02124	VIT_00s0294g00100	1.00	1.060	2,29	BR insensitive 1-associated receptor kinase 1
Sauvignon nepis					
Vitvi09g01181	VIT_09s0018g00780	1.00	1.506	710,79	HcrVf1 protein
Vitvi18g02399	VIT_18s0089g01040	0.97	1.474	647,26	Avr9 elicitor response

Gene ID V3	Gene ID V2	tau	Score	Mean exp	Functional annotation
Vitvi10g01863	VIT_10s0003g03530	0.97	1.427	382,88	Lupeol synthase
Vitvi10g01875	VIT_10s0003g03650	0.97	1.427	381,69	Beta-amyrin synthase
Vitvi13g02352	VIT_13s0139g00190	0.92	1.408	529,73	Disease resistance protein RGA2
Vitvi12g02393	VIT_12s0059g01790	0.91	1.356	321,51	Caffeic acid O-methyltransferase
Vitvi02g00721	VIT_02s0012g01610	0.87	1.355	506,49	Beta-1,3-gluca -se precursor
Vitvi03g01757	-	1.00	1.314	63,03	-
Vitvi03g00910	VIT_03s0167g00050	0.92	1.298	140,99	Conca-valin A lectin
Vitvi16g00665	VIT_16s0022g00420	0.96	1.294	81,21	SRG1 (senescence-related gene 1) oxidoreductase
Sauvignon rytos					
Vitvi01g01410	-	1.00	1.326	67,07	-
Vitvi08g00957	VIT_08s0040g00920	0.87	1.280	207,61	Glutathione S-transferase 25 GSTU7
Vitvi14g00080	VIT_14s0060g00990	1.00	1.269	31,94	Unknown
Vitvi13g02566	VIT_13s0156g00390	1.00	1.267	31,10	Myb family
Vitvi18g03265	VIT_18s0089g01000	0.95	1.256	51,80	F-box family protein
Vitvi15g01230	-	1.00	1.229	19,32	-
Vitvi15g01425	VIT_15s0021g01450	1.00	1.224	18,06	No hit
Vitvi13g01636	VIT_13s0158g00050	1.00	1.223	17,85	Serine carboxypeptidase
Vitvi10g01830	VIT_10s0003g02420	1.00	1.217	16,27	SRG1 (senescence-related gene 1) oxidoreductase
Vitvi17g00462	-	1.00	1.216	16,08	-

4. CONCLUSION

This thesis aimed at investigating the transcriptional landscape of grapevine berry skin from resistant varieties, including both PIWI (Cabernet Cortis, Cabernet Volos, Sauvignon Nepis, Sauvignon Rytos) and elite parental varieties (Cabernet Sauvignon, Sauvignon Blanc). This study aimed at shedding light on the basal defence mechanisms specific for each of the analysed varieties. Through RNA sequencing and rigorous bioinformatic analyses, key insights emerged regarding genetic expression patterns and relationships across varieties.

Overall, closer transcriptomic relationships were observed among the white grapevine varieties compared to the red ones. Heatmaps and PCAs revealed tighter clustering of white variety replicates compared to the red ones, suggesting higher similarities in their transcriptomic profiles. Differential gene expression analysis quantified distinct numbers of up- and down-regulated genes when contrasting PIWIs against the respective elite parent, with white PIWIs exhibiting an higher number of DEGs, in general. This result indicates high substantial transcriptional differences in the white PIWIs, thus underlying disease resilience traits.

Interestingly, despite their ancestral connections, low overlap occurred in the actual DEGs induced in red *versus* white PIWI grapes. Cabernet Cortis and Cabernet Volos shared only 3% of total upregulated and 6% of downregulated genes. Meanwhile, 40% of upregulated and 51% of downregulated genes overlapped between Sauvignon Nepis and Sauvignon Rytos, indicating high transcriptional relationships among them. This showcases the intricacy of genetic relationships even among PIWI grapes developed from elite crosses.

Gene enrichment analysis determined overrepresented pathways and functions among DEGs. Oxidoreductase activity, defense response, metabolic processes, and secondary metabolite biosynthesis were consistently featured, aligning with stress resilience roles. The tissue-specificity assessment also revealed divergent trends in highly and absolutely tissue-specific gene counts across varieties. Notably, elite cultivars Cabernet Sauvignon and Sauvignon Blanc harboured fewer of such genes than the respective PIWIs, potentially contributing to disease susceptibility.

Overall, this RNA-Seq study offered meaningful perspectives into the nuanced transcriptomic landscapes distinguishing grapevine varieties, particularly disease-resistant PIWIs and elite cultivars. This study highlighted intriguing differences in genetic relationships and expression patterns even among ancestrally connected grapes. The findings provide valuable leads towards unravelling the

precise transcriptional intricacies governing pathogen resilience in grapevine berries. Further probing of specific genes and pathways hold promise for harnessing disease resistance mechanisms to promote sustainable viticulture.

In conclusion, this thesis fulfilled its central aims through a methodical workflow spanning RNA extraction, sequencing, differential expression quantification and gene enrichment analyses. The outcomes advanced the understanding of basal defence traits in grapevine varieties from transcriptional perspectives. This work sets the stage for more targeted inquiries into precise transcriptomic factors conferring resilience against destructive pathogens for environmentally benign disease control approaches in viticulture. The study affirmed the power of RNA sequencing combined with rigorous bioinformatics for elucidating genetic complexities underlying biological phenomena.

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