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Innovative strategies to enhance grapevine sustainability and resilience against stresses

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TABLE OF CONTENTS

GENERAL INTRODUCTION TO THIS STUDY 4

CHAPTER 1 | A JOURNEY INTO PLANT-MICROBE INTERACTIONS: FROM LAB TO FIELD FOR A SUSTAINABLE AGRICULTURE 8

ABSTRACT 9
UNDERSTANDING THE HOLOBIONT CONCEPT: AN OVERVIEW 10
PLANTS - FUNGI RELATIONSHIPS: A BRIEF OVERVIEW 12
PLANT-BACTERIA INTERACTIONS IN AGRICULTURE: FROM QUORUM SENSING TO SYNCOMS DEVELOPMENT 14
SIZE DOESN'T MATTER! CAN VIRAL ENTITIES CONTROL THE HOLOBIONT? 16
INTEGRATING OMICS APPROACHES TO UNDERSTAND PLANT-PATHOGEN INTERACTIONS AND MICROBIAL DIVERSITY 18
CONCLUSIONS AND FUTURE PROSPECTS 20

CHAPTER 2 | CRISPR/CAS9-DRIVEN DOUBLE MODIFICATION OF GRAPEVINE MLO6-7 IMPARTS POWDERY MILDEW RESISTANCE, WHILE EDITING OF NPR3 AUGMENTS POWDERY AND DOWNY MILDEW TOLERANCE 31

SUMMARY 32
INTRODUCTION 33
RESULTS 36
DISCUSSION 48
CONCLUSIONS 54
EXPERIMENTAL PROCEDURES 54

CHAPTER 3 | EDITING OF *NPR3* REWIRES GRAPEVINE ROOT IMMUNITY TOWARD SYMBIOSIS 73

INTRODUCTION 75
MATERIALS AND METHODS 77
RESULTS 81
DISCUSSION 90
CONCLUSIONS 94

CHAPTER 4 | EPIGENETIC REGULATION AND BEYOND IN GRAPEVINE-PATHOGEN INTERACTIONS: A BIOTECHNOLOGICAL PERSPECTIVE 103

ABSTRACT 104
INTRODUCTION 105
MECHANISMS OF EPIGENETIC REGULATION 106
EPIGENETICS AND PLANT IMMUNITY – ANOTHER LAYER OF REGULATION 109
EPIGENETIC REGULATION IN GRAPEVINE IMMUNITY 111
EPIBREEDING, A NEW FRONTIER IN GRAPEVINE BREEDING 116
CONCLUDING REMARKS AND FUTURE PERSPECTIVES 121

CHAPTER 5 | FUNCTIONAL DISSECTION OF EPIGENETIC MECHANISMS IN GRAPEVINE THROUGH CRISPR/CAS9: INSIGHTS FROM *H2A.Z* AND *DNMT2* 131

INTRODUCTION 131
MATERIALS AND METHODS 134
RESULTS 135
DISCUSSION AND CONCLUSIONS 139

CHAPTER 6 | ABROAD PERIOD AT THE UNIVERSITY OF BRITISH COLUMBIA 145

CHAPTER 7 | CONCLUSIONS 146

PUBBLICATIONS, CONFERENCES, TRAINING AND PROJECTS 148

General introduction to this study

Feeding a growing population under accelerating climate change requires crop production systems that are both higher yielding and more resilient. Biotic constraints alone are estimated to reduce global crop yields by 30% annually, which worth hundreds of billions of dollars (Savary et al., 2019; Ren et al., 2022; Gai and Wang, 2024). Conventional control strategies have relied heavily on agrochemicals, but their efficacy is declining as resistance evolves, while their excessive use raises concerns about environmental sustainability and human health. Stricter regulations and societal demand for eco-friendly practices now call for genetics-first solutions that can ensure durable resistance while reducing chemical inputs. In this context, targeted breeding and biotechnology, and especially CRISPR-based genome and epigenome engineering, offer powerful avenues to generate stress-resilient germplasm and build more sustainable production systems (Zhu et al., 2020; Giudice et al., 2021).

Plants have evolved a sophisticated innate immune system composed of multilayered mechanisms able to perceive and counteract invading organisms. At the cell surface, pattern-recognition receptors detect conserved microbial signatures and trigger pattern-triggered immunity (PTI), while intracellular nucleotide-binding leucine-rich repeat receptors recognize pathogen effectors and activate effector-triggered immunity (ETI). These layers orchestrate local and systemic responses and are strongly modulated by hormonal crosstalk, with salicylic acid playing a central role in defense against biotrophic and hemibiotrophic pathogens. However, the plant immune system is not an isolated trait but functions within a complex ecological context. Plants live as holobionts, interacting continuously with fungi, bacteria, and viruses that can act as pathogens, commensals, or beneficial partners. Arbuscular mycorrhizal fungi and rhizobacteria, for example, contribute to nutrient acquisition and stress tolerance, and synthetic microbial communities (SynComs) are increasingly investigated as tools to promote plant resilience. This holobiont perspective shifts the focus from a purely host-centered defense system to a more integrated view where immunity and symbiosis must be balanced to optimize growth, defense, and sustainability (Barakate et al., 2016; Vandenkoornhuyse et al., 2015; Portal-Gonzalez et al., 2025).

Grapevine (*Vitis vinifera L.*) represents a clear example of the challenges posed by this complexity. It is one of the most important perennial crops worldwide, with immense cultural and economic value, but it is highly susceptible to major fungal diseases such as downy mildew and powdery mildew. Moreover, grapevine also represents a model species for research on woody plants. Viticulture has historically depended on intensive fungicide programs, with copper-based products playing a central role in European vineyards for more than a century. The environmental accumulation of copper and the emergence of fungicide resistance, however, have spurred the search for alternative solutions,

making grapevine a relevant case study for innovative genetic and microbiome-aware strategies (Ren et al., 2022).

In recent years, CRISPR–Cas systems have transformed the landscape of plant biotechnology by enabling precise and efficient modifications at specific genomic loci. Following their first application in plants (Mao et al., 2013), these tools have rapidly expanded to include base and prime editors capable of inducing single-nucleotide changes without double-strand breaks, as well as nuclease-dead Cas proteins fused to regulatory domains for transcriptional control (Devillars et al., 2024). Such innovations provide unparalleled opportunities to dissect gene function and to develop novel breeding strategies, ranging from the inactivation of susceptibility genes to the fine-tuning of immune regulators. In grapevine, CRISPR has already been established as a versatile tool for functional genomics and trait improvement, with the development of plants with enhanced tolerance to major fungal diseases (Ren et al., 2022). These approaches illustrate how targeted manipulation of host genetic pathways can generate durable resistance while avoiding the linkage drag often associated with classical breeding. Plant defence cannot be studied solely from the perspective of pathogen resistance, since enhanced immunity may also influence interactions with beneficial symbionts. A crucial question is whether strengthening immune responses inevitably compromises mutualistic associations such as mycorrhizal colonization, or whether precise genetic interventions can uncouple these processes. Understanding these trade-offs is essential for designing resilient crops that not only withstand pathogens but also maintain or even enhance beneficial plant–microbe partnerships, a prerequisite for sustainable agriculture.

Beyond genetic variation, epigenetic regulation also plays a crucial role in modulating plant interactions with both pathogenic and beneficial microorganisms (Zhu et a., 2016). DNA methylation, chromatin remodelling, histone variants and post-translational modifications, and DNA or RNA methylation modulate the accessibility and expression of genes involved in immunity and stress adaptation. Epigenetic marks are reversible and sensitive to environmental stimuli, offering plasticity that sequence variation alone cannot provide (Lloyd and Lister, 2022). CRISPR technology has now been extended to epigenome editing, where nuclease-dead Cas proteins fused to writers, erasers, or readers of chromatin marks allow the targeted modulation of transcriptional states without altering the underlying DNA sequence (Zhu et al., 2020; Dong et al., 2024). This opens the possibility of "epibreeding," where crops are engineered to maintain favourable epigenetic configurations that enhance resilience to pathogens while preserving growth and yield potential (Dong et al., 2024).

The work presented in this thesis integrates these different layers of plant biology into a coherent framework aimed at enhancing grapevine sustainability. The first part investigates classical immune pathways using CRISPR-mediated editing of key regulators of immunity, not only to assess their

contribution to resistance against fungal pathogens but also to evaluate their impact on beneficial interactions within the holobiont. This includes an introductory chapter that surveys the breadth of plant–microbe interactions, spanning pathogens and beneficial organisms and framing plants as holobionts, followed by a study where CRISPR was applied to target a gene involved in immune regulation to enhance disease tolerance, specifically the Nonexpressor of Pathogenesis Related (*VvNPR3*) gene, and finally an analysis of whether such immune rewiring influences symbiotic associations such as with arbuscular mycorrhizal fungi. The second part shifts focus to the epigenetic dimension, beginning with a perspective article on the role of chromatin dynamics and epigenetic marks in grapevine–pathogen interactions, and continuing with preliminary experimental works where CRISPR was employed to explore the function of key regulators such as the histone variant *VvH2A.Z* and an tRNA methyltransferase (*VvDNMT2*). Together, these complementary approaches highlight the potential of CRISPR-based genome editing to move beyond conventional applications providing innovative strategies for biotechnological applications in viticulture. The ultimate goal is to exploit these technologies to reconcile pathogen resistance with beneficial plant–microbe partnerships, thus contributing to the development of resilient crops that are better equipped to withstand the challenges of global change and meet societal demands for reduced chemical inputs in agriculture.

My doctoral project was supported by a strong collaborative component with the private sector. In particular, the PhD was funded by M.I.V.A. (Moltiplicatori Italiani Viticoli Associati), and within this framework I carried out experimental work on reproductive tissues of nine grapevine cultivars, where embryogenic calli were induced to generate starting material for the application of new breeding techniques aimed at improving traits of commercial interest. Lastly, I carried out a six-month research stay at the University of British Columbia, in the laboratory of Professor Todesco, where I worked on the genetic regulation of branching in sunflower. Beyond the specific biological system, this experience allowed me to train in innovative approaches, such as mRNA single cell sequencing, which hold great potential for future application in grapevine to study the cellular and spatial dynamics of plant–microbe interactions. Together, the activities carried out during the PhD combine fundamental and applied research, bridging academic and industrial perspectives, and strengthening the development of innovative strategies for grapevine resilience and sustainability.

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CHAPTER 1 | A journey into plant-microbe interactions: from lab to field for a sustainable agriculture

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Abstract

The relationship between plants and microorganisms has ancient roots. However, only recently we have begun to appreciate the true complexity of the holobiont and its intricate interactions. Fungi, bacteria, and viruses residing within or around plants play a crucial role in enhancing their resilience to biotic and abiotic stressful factors. The impact of these interactions is not limited to stress resilience but are strictly correlated with the plant's life cycle. The recognition of specific effects on plant growth and resilience has driven research towards utilizing microorganisms as sustainable solutions for agricultural challenges. The application of selected microorganisms, combined with the development of SynCom consortia, marks the beginning of a promising strategy for achieving sustainability in agriculture. This chapter explores the hidden world of plant-associated microorganisms, emphasizing their diverse beneficial impacts and providing evidence to support their potential applications in modern agriculture.

Understanding the Holobiont Concept: An Overview

The symbiotic relationship between plants and microorganisms is widely acknowledged to have ancient origins. However, it was not until 2002 that research revealed a significantly greater diversity of microorganisms colonizing plant roots, highlighting a previous gap in our understanding of root-associated microorganisms (Vandenkoornhuyse et al., 2002).

In 1943, the holobiont theory was empirically formulated by the German theoretical biologist Adolf Meyer-Abich, but it was then in 1991, that the famous symbiosis researcher Lynn Margulis defined the holobiont as a gain, with the holobiont representing an association of partners (bionts) throughout a significant portion of the life host history (Margulis and Fester, 1991).

Nowadays, microbiome research confirmed not only the holobiont theory, but also how it substantially contributed to better insights into host–microbiota relationships and metabolite interplay (Berg et al., 2020; Cordovez et al., 2019). Analyzing the plant microbiome involves the integration of microbial ecology with the biology and functioning of the host plant. Microorganisms within the microbiome serve as a reservoir of additional genes and functions, improving the host plant capabilities (Bulgarelli et al., 2013). Although these interactions might initially seem asymptomatic, the benefits provided by the plant-associated microbiome are crucial. They in fact significantly extend the plant ability to adapt to various environmental conditions and stresses (Bulgarelli et al., 2013), which is of primary importance in view of the sessile lifestyle of plants (Fig. 1).

Nevertheless, our understanding of the role of the plant microbiome remains limited, indeed we are only just beginning to comprehend its ecological functions (Kiers et al., 2011).

The upshot of this better understanding will have substantial impacts on a variety of research investigations and applications, such as possible innovations for crop production (Kiers et al., 2011; Werner et al., 2014).

Nowadays, grasping the holobiont concept is crucial not only for unravelling the coevolution of microorganisms and hosts, but also for enhancing breeding technologies that boost plant resistance (Giudice et al., 2021). With global climate change increasing the frequency of biotic and abiotic stresses, developing new and sustainable crop protection strategies is urgently needed to enhance agricultural resilience. In this context, uncovering plant-microbiome interactions is vital to identifying key factors in the recruitment of beneficial microbes by plants (Giudice et al., 2021, 2022). An example of which are beneficial endophytes, such as plant growth-promoting microorganisms (including actinomycetes and arbuscular mycorrhizal fungi - AMFs), that colonize plant tissues and play a crucial role in protecting plants from stressful factors (Carrión et al., 2019).

Insight into the interactions between plants and endophytic microorganisms can enable the cultivation of well-characterized microbial strains and the development of synthetic microbial communities

(SynComs) that mimic natural microbiome function (Carlström et al., 2019; Sandrini et al., 2022). By studying these SynComs through multi- and interdisciplinary approaches, it is possible to better understand plant microbiome assembly and its effects on plant performance(s) under a variety of environmental conditions (Vannier et al., 2019).

In addition, SynComs not only facilitate basic research into plant-microorganism interactions and the holobiont concept, but also provide practical applications for enhancing plant growth and improving other beneficial traits in host plants (Ma et al., 2021).

Drawing from the concepts explored in this section, the holobiont emerges as a pivotal framework reshaping our understanding of biological systems, emphasizing the intricate relationships between hosts and their microbiomes (Vandenkoornhuyse et al., 2015). Recognizing microorganisms as active participants in host health and resilience, this perspective not only expands our comprehension of evolutionary dynamics, but also opens new pathways for enhancing agricultural sustainability, and environmental conservation efforts. In the future, continued exploration and application of the holobiont concept will revolutionize our approach to investigate and harness biological complexity.

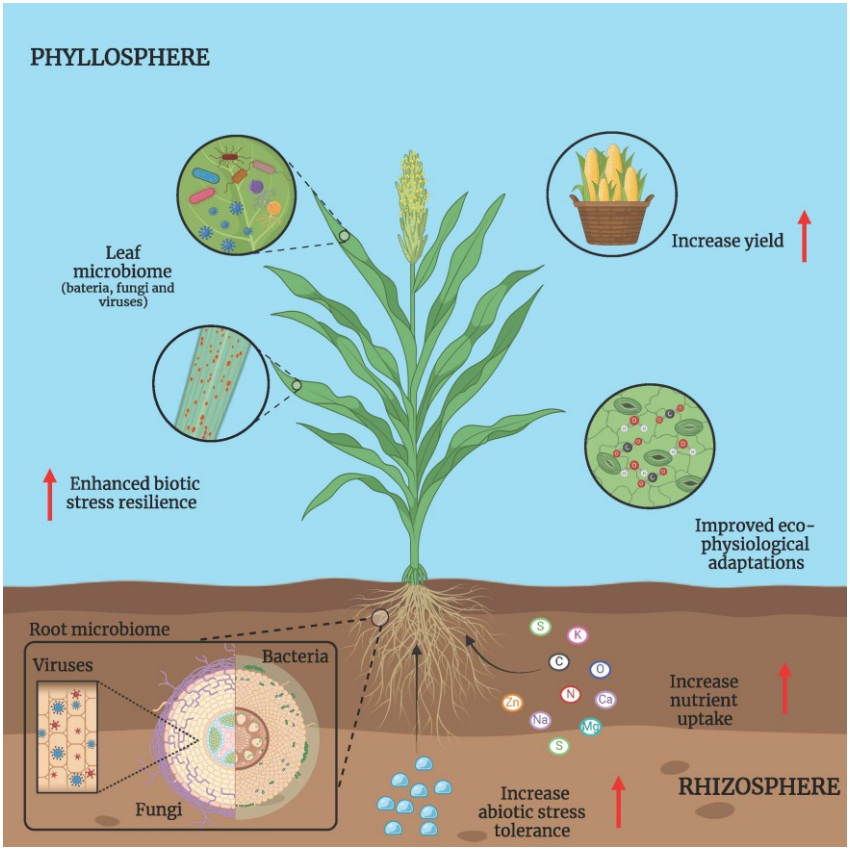

Figure 1. The holobiont concept applied to plants. Schematic overview of the plant holobiont, illustrating the interactions between the host plant and its associated microbiota, including bacteria, fungi, viruses, and other

endophytic microorganisms. These symbiotic partners contribute to nutrient acquisition, stress tolerance, pathogen protection, and overall plant fitness. The holobiont framework emphasizes that the plant and its microbiome function as an integrated biological unit, enhancing adaptation and resilience in changing environments.

Plants - Fungi relationships: a brief overview

Fossil evidence suggests that the symbiotic relationship between plants and fungi, particularly with endophytes and mycorrhizal fungi, have existed for over 400 million years, coinciding with the colonization of land by plants (Anjum et al., 2019; Baron and Rigobelo, 2022; Chadha et al., 2015). These long-standing associations highlights the pivotal role of fungi in plant evolution. Indeed, fungi are ubiquitous and indispensable microorganisms in agriculture, contributing significantly to soil health, plant growth, and ecosystem balance. They are essential for the organic matter decomposition, contributing to nutrient cycling and release of essential elements for plant growth (Frąc et al., 2015). Moreover, fungal hyphae bind soil particles, forming aggregates that improve soil structure, reduce erosion, and enhance water infiltration (Van der Heijden et al., 2015). Additionally, symbiotic associations between fungi and plants, enhance nutrient uptake, drought tolerance, and disease resistance (Newsham, 2011). These combined effects ultimately lead to increased soil fertility and improved plant productivity.

Plants interact with a diverse array of soil fungi, fostering both beneficial and detrimental associations (Thoms et al., 2021). Pathogenic fungi interact negatively with plants, using this connection to their exclusive advantage by infecting both green and woody tissues, causing disease or even death of the host plant. Fungi exhibit diverse lifestyles, which can also be switched during plant infection. Necrotrophs, such as *Alternaria alternata* and *Botrytis cinerea*, are pathogenic agents for several crops and directly kill plant tissues for nutrient acquisition. Biotrophs, such as *Blumeria graminis*, have developed strategies to be capable of weakening the plant's immune system, thus altering the plant cells metabolism, to extract nutrients without causing the plants' death. Finally, Hemibiotrophs, such as *Colletotrichum higginsianum,* well known to colonize leaf tissues of several Brassica and Raphanus species, initially behave like biotrophs, developing hyphae inside leaf epidermal cells, but at a late stage it shifts its behaviour to a secondary necrotrophic phase, penetrating host cells cytoplasm and feeding on both living and dead cells (Yan et al., 2018). Conversely, symbiosis refers to any type of close and long-term interactions between different organisms with a positive, neutral o negative impact, depending on the type of interaction: mutualism, commensalism or parasitism (Martins et al., 2023). Generally, fungi engage in symbiotic relationships with plants through either commensalism, where the fungus benefits from plant without showing apparent beneficial or harmful

effects, or mutualism, where both symbionts gain reciprocal benefits and are usually mutual (Casadevall et al., 2000).

Many endophytic fungi can offer a myriad of benefits to their hosts, including enhanced growth, stress tolerance, and triggering direct (e.g. antibiosis) or indirect defense pathways (e.g. activating host defence responses). They can for example produce bioactive compounds that deter herbivores or inhibit the growth of competing microorganisms (Rodriguez et al., 2009). Moreover, endophytes can facilitate nutrient uptake and improve plant water relations, contributing to overall plant fitness (Guo et al., 2021). Endophytic fungi can inhabit both green and woody tissues, and can exhibit both beneficial and pathogenic lifestyles, influenced by a complex interplay of biotic and environmental conditions (Redman et al., 2001). The endophytic fungus *Trichoderma* (teleomorph *Hypocrea*) has garnered significant attention due to its multifaceted interactions with plants. Found in diverse ecosystems, *Trichoderma* species exhibit a strong antagonistic and mycoparasitic nature, enabling them to suppress plant pathogens in the soil and mitigate disease severity. Furthermore, it has been elucidated the ability of certain *Trichoderma* strains to establish direct interactions with plant roots, leading to enhanced plant growth and disease resistance (Hermosa et al., 2012).

Arbuscular mycorrhizal fungi (AMF), belonging to *phylum Mucoromycota* and *subphylum Glomeromycotina* (Zou et al., 2021), form symbiotic associations with the roots of about 80% of terrestrial plants. They create an extensive hyphal network that efficiently absorbs water and minerals from a large soil volume in exchange of carbohydrates and lipids (Keymer et al., 2017). This enables AMF to provide plants with phosphorus and other mineral nutrients, enhance water uptake, improve leaf photosynthesis, and increase the hydraulic conductivity of roots (Othman et al., 2024; Balestrini et al., 2020; Jacott et al., 2017). These benefits translate to improved plant tolerance towards abiotic stresses, allowing them to thrive under harsh environmental conditions (Porcel et al., 2015; Khaliq et al., 2022). The interaction between soil microorganisms, including AMF and plant growth-promoting rhizobacteria (PGPR), offer a promising avenue for developing a more sustainable agriculture. In fact, several studies report synergistic effects of AMF and PGPR in promoting plant growth and protection against pathogens (Nanjundappa et al., 2019). *Rhizobia*, known for nitrogen fixation in the soil, share signalling pathways with AMF, facilitating their association with plants (Primieri et al., 2021). Recent researches highlight the potential existence of a positive correlation between root AMF colonization and soil microbial diversity (Ferreira et al., 2021). Dissecting and exploiting the interactions between different microorganisms offers potential for developing commercial products, such as biostimulants. Microbial biostimulants can represent sustainable and eco-friendly alternatives to mineral fertilizers. They enhance metabolic and enzymatic processes in plants, improving yield, productivity, crop quality and resistance to abiotic stresses (Sun and Shahrajabian, 2023).

Further research is required to unravel these inter-kingdom communications, moving from lab-controlled to real field conditions. By delving deeper into the ancient alliance between plants and microbes, we can harness its power to cultivate a more resilient and productive agriculture, fostering a healthier planet.

Plant-bacteria interactions in agriculture: From quorum sensing to SynComs development

Plant-associated bacteria can be classified by their interaction with their host, which can be beneficial, deleterious or neutral (Santos et al., 2021). Most of them reside in the rhizosphere, attracted by root exudates that provide energy and nutrients for their reproduction (Kuzyakov and Domanski, 2000). Several of these bacteria are categorized as Plant Growth-Promoting Bacteria (PGPB) and contribute to multiple abiotic stress tolerances in plants through various mechanisms. These mechanisms include the production of phytohormones such as auxins, cytokinins, and gibberellins, which regulate plant growth processes (Maheshwari et al., 2015). PGPB can also solubilize nutrients like phosphorus and iron, thereby enhancing their availability to plants (Etesami et al., 2020). Additionally, under stressful conditions, PGPB synthesize and release stress-induced metabolites, including osmolytes, proline, and antioxidant enzymes, which assist plants in coping with abiotic stress (Subiramani et al., 2020). Beyond abiotic stress tolerance, PGPB also play a crucial role in biotic stress management. Prominent genera of PGPB, like *Pseudomonas*, *Bacillus*, *Azospirillum*, and *Streptomyces,* can behave as biocontrol agents against pathogens by utilizing various mechanisms (Wang et al., 2021). These include direct competition for resources, antibiosis through the production of antibiotics, enzymatic lysis of pathogens, signal interference, and the induction of host plant defences. Furthermore, PGPB can trigger a systemic defence response in plants, known as Induced Systemic Resistance (ISR) (Bukhat et al., 2020). This involves the activation of signalling pathways mediated by jasmonate and ethylene, leading to the production of defence-related enzymes and metabolites, as well as morphological changes that enhance plant resistance to a broad spectrum of pathogens.

All of bacteria traits are ineffective when carried out by single bacteria alone, rather they have the ability to work together in multicellular assemblies. To regulate these group activities, bacteria utilize an interspecific and intraspecific communication process between cells called quorum sensing (QS) (Mukherjee et al. 2019). The most studied is the N-acyl-homoserine lactone (AHL) quorum sensing systems, common in many Gram-negative bacteria (Fuqua et al., 1994). When a critical bacterial concentration, or quorum, is reached, the AHL biosynthesis is induced, leading to the activation of a set of genes essential as nutrient polymer degradation, virulence induction, synthesis of siderophores and biofilm formation (Harmann et. al., 2024). Plants, in a as yet unidentified mechanism can perceive these bacterial signals, and undergo an AHL-mediated effect. Although the mechanisms by which

plants perceive bacterial signalling molecules are not yet fully understood, the impacts of certain AHLs has been documented on several occasions. These impacts include changes in the expression of various genes, alterations in proteomes, and effects on root development (Mathesius et al., 2003; Schuhegger et al., 2006 Schenk et al., 2012; Schikora et al., 2011). The bacteria interaction with the complex microbial biofilms decides about success or failure of beneficial microbes to support plant health, or of pathogens to develop disease. In this context, it is evident that not all bacteria exhibit synergistic effects that are beneficial to the plant. In some cases, pathogens may interact with the plant's native microbiome, thereby increasing their virulence. A notable example is the olive knot disease caused by *Pseudomonas savastanoi* pv. *Savastanoi (Psv).* The disease occurs with tumours (knot) along the branches and twigs within which there is a high concentration of the non-pathogenic bacterium *Erwinia toletana* (Buonaurio et al., 2015). This endophyte produces the same AHLs as *P. savastanoi*, enabling them to exchange signals (Hosni et al., 2011). Co-inoculation experiments between a *P. savastanoi* AHL synthase mutant, which cannot produce AHLs, and the wild-type strain of *E. toletana,* showed that the *P. savastanoi* mutant could induce knot formation in olive plants similar to the wild-type strain. This suggests that the two strains form a consortium where *Et* rescues the virulence of *P. savastanoi* by providing AHLs (Hosni et al., 2011; Buonaurio et al., 2015). Another example, reported by Kim et al.2020, demonstrated that the plant pathogen *Burkholderia glumae* employs a specific type-6 secretion system (T6SS) to interact with rice endophytic microbes. This interaction results in a decreased population of beneficial bacterial genera, such as *Luteibacter* and *Dyella,* which are known to promote plant growth and protect against pathogenic bacteria (Mannaa et al., 2021).

Despite this, the shared lifestyle of bacteria has prompted the development of Synthetic Microbial Communities (SynComs), composed of mixed PGPB species, with the aim of enhancing disease suppression, plant growth promotion, and stress resilience through synergistic interactions (Nerva et al., 2022). In this context, the compatibility between PGPB strains, including factors such as synergistic metabolic activities and complementary modes of action, emerges as a crucial determinant of overall SynCom performance and stability. To fully harness the potential of PGPB-based biocontrol strategies, a comprehensive understanding of the complex interactions within SynComs and their impact on the plant microbiome is imperative. Future research should prioritize unravelling these intricate relationships to optimize SynCom formulations and enhance their effectiveness in promoting sustainable agricultural practices.

Size doesn't matter! Can viral entities control the holobiont?

The viral communities (viromes) associated with organisms exhibit remarkable diversity and complexity. Viruses can infect all cells across all kingdoms of life, with these infections resulting in a spectrum of effects on the host, ranging from beneficial or neutral to detrimental (Roossinck, 2005, 2011). However, the scientific interest around viruses was historically due to their pathogenic effects that results in disease, epidemics and pandemics in different kingdoms from plants to animals. Virology research has recently been strengthened thanks to the new sequencing techniques which allow one to identify the majority of the viruses presents in a cell (Fadiji et al., 2022). The technological advancement opened the door for identifying and appreciating the true complexity of viromes in various organisms, including plants like grapevine (Nuzzo et al., 2022; Shvets et al., 2022), and wheat (Redila et al., 2021). Recently, Rivarez et al. (2023) analysed the virome of healthy and diseased tomato plants, discovering a total of 79 novel viruses. Only 50% of those viruses were associated with the diseases, while the other 50% were either exclusively detected in asymptomatic plants or present in both groups confirming that viruses do not always have pathogenic effects.

Due to the new vision of plants as a holobiont entity (Vandenkoornhuyse et al., 2015), limiting the studies to the plant virome leads inevitably to missing lots of information. Indeed, it was proven that the intimate relationship between plant and endophytes, especially fungi, led to an exchange of viral entities between the two kingdoms that has been defined cross-kingdom viral transmission (Dolja et al., 2020; Sun et al., 2020). This cross-kingdom effect seems to be ancient since plant and fungal viruses shared evolutionary origins (Roossinck, 2019). For instance, it was suggested that the *Ourmiaviruses* infecting plant derived from the *Narnaviruses* infecting fungal mitochondria after the acquisition of the capsid and the movement proteins (Lefeuvre et al., 2019). The close relationship between plant and fungal viruses was initially supported by the discovery that some mycoviruses can replicate within plant cells (Nerva et al., 2017). This finding was further strengthened by evidence suggesting that specific plant movement proteins may facilitate the spread of these mycoviruses throughout plant tissues (Andika et al., 2023; Deom et al., 1990).

Following the holobiont point of view it was recently published the first study that comprehensively analysed the whole endophytic virome of a maize plant (Fadiji et al., 2022). They monitored the variability of the root endophytes virome in maize plants cultivated with different agricultural practises, observing that organic farming increase also the virome biodiversity. The main limitations of these whole virome studies are the lacking information about the functional contributions that these viruses have to the holobiont. Currently, the two main literature-supported beneficial activities of the viruses to the holobiont are drought stress tolerance and hypovirulence (Roossinck, 2015),

Additionally, viruses can participate in tripartite interactions, influencing both the host and the superhost as described by Márquez et al. (2007).

Pagliarani et al. (2022) showed that the pathogenic *Tomato yellow leaf curl Sardinia virus* (*TYLCSV*) encodes a specific C4 protein able to induce stress tolerance in tomato plants. The drought tolerance promotion passes through an ABA independent mechanism; indeed, the accumulation of ABA is four time less in C4 expressing plants than in controls. Another interesting discover was the protein 6K2 from the Turnip mosaic virus (*TuMV*) (Prakash et al., 2023). 6K2 induce drought resistance in *Nicotiana benthamiana* and *Arabidopsis thaliana* plants by promoting salicylic acid accumulation (Miura et al., 2013). An even different pathway was discovered from Wu et al. (2024) on the common bean (*Phaseolus vulgaris*). In this specific case a 21 nucleotides vsiRNA produced from the *Cowpea mild mottle virus* (*CPMMV*) lead to the degradation of the *PvERD15* genes that positive regulates stomatal aperture, ending with an improved drought tolerance.

Hypovirulence is the ability of an infecting virus to reduce host virulence, representing the most studied aspect of mycoviruses (Wagemans et al., 2022). The first interesting application of hypovirulence was made against *Cryphonectria parasitica,* the causative agent of the chestnuts blight. The infection by *Cryphonectria parasitica hypovirus 1* (*CHV1*) strongly reduce the symptoms of the disease and has been effectively used to treat chestnuts blight (Prospero and Rigling, 2016; Rigling and Prospero, 2018; Turina and Rostagno, 2007). Lemus-Minor et al. (2015) discovered a *Fusarium oxysporum f. sp. Dianthi* infected with the Fusarium oxysporum f. sp. Dianthi mycovirus 1 (FodV1) that shown reduced growth and reduce the virulence to carnation compared to the uninfected (Lemus-Minor et al., 2018). This activity was then confirmed in Torres-Trenas et al. (2019) where they microscopically assess the reduction of the growth and virulence in plant.

Moreover, an interesting hypovirus was found infecting *Sclerotinia sclerotium,* one of the most widespread pathogens with a great number of hosts (Bolton et al., 2006). The identified *Slcerotinia sclerotium hypovirulence associated DNA virus 1* (*SsHADV-1*) was able to induce strong hypovirulence effect on the host and moreover was able to modify the host vegetative incompatibility to favour his spread though normally incompatible hosts (Yu et al., 2010). Recently Zhang et al. (2020) analysing the effect of *SsHADV-1* strain infected in *Brassica napus* discovered that the virus has much more than just an hypovirulent effect to the host. Their molecular analysis showed that the mycovirus infection reduce all the molecular pathogenic pathways in *S. sclerotium* such as the cell-wall degrading enzymes and all the effectors able to inhibit plant PTI. TEM (Transmission Electronic Microscopy) analysis proved that the infected *S. sclerotium* strain has an endophytic growth without causing any damage. These effects result in an enhancement of plant growth and increased resistance

to other fungal pathogens due to the priming effect, concluding that SsHADV-1 is able to transform a parasitic host into a beneficial associated organism.

Viruses present exciting opportunities for researchers to discover and harness new beneficial functions within holobionts revealing key roles in host physiology and functionality. However, our understanding of complexity and their precise roles remains in their early stages, highlighting the need for further research.

Integrating OMICs approaches to understand plant-pathogen interactions and microbial diversity

The advent of OMICs technologies has revolutionized our understanding of plant-pathogen interactions and microbial diversity, offering novel strategies for sustainable agriculture. Genomics, transcriptomics, proteomics, and metabolomics provide an in-depth perspective on host-microbe interactions, aiding in the identification of key microbial players involved in plant resilience (Nerva et al., 2022).

OMICs in plant-pathogen interactions

Plant-pathogen interactions involve complex signalling networks that determine disease resistance or susceptibility. Genomics and transcriptomics have been instrumental in identifying resistance genes and regulatory pathways activated in response to pathogen attacks (Sandrini et al., 2022). For instance, transcriptomic studies have revealed differential gene expression patterns in plants inoculated with beneficial microbes, highlighting the activation of defense-related pathways. Similarly, metagenomics approaches have provided insights into microbial community shifts upon pathogen infection, enabling the identification of microbial consortia with biocontrol potential (Nerva et al., 2022). In the context of plant-pathogen interactions, OMICs data have been instrumental in unravelling the intricate immune responses of plants. For instance, Jiang et al. demonstrated that Arabidopsis exhibits distinct immune responses to different pathogens, such as *Golovinomyces orontii* and *Botrytis cinerea*, highlighting the role of specific proteins in mediating these responses (Jiang et al., 2016). Similarly, Abuqamar et al. (2016), emphasized the importance of high-throughput sequencing technologies in understanding the genomic variability and pathogenic diversity of *B. cinerea*, which can inform strategies for enhancing plant resistance. These studies underscore the potential of OMICs to provide a systems biology perspective on plant immunity, allowing for the identification of key genes and pathways that can be targeted for crop improvement.

Proteomic and metabolomic analyses complement these findings by characterizing protein expression changes and metabolite shifts in infected plants. Recent studies have demonstrated that beneficial

microbes can induce systemic resistance through the production of volatile organic compounds and secondary metabolites that modulate host immunity. Furthermore, multi-OMICs approaches integrating metabolomic and transcriptomic data have been applied to uncover plant biochemical responses to fungal infections, elucidating key pathways involved in disease suppression (Mendes et al., 2013; Sandrini et al., 2022 and references therein), enabling the development of predictive models for managing plant diseases (Velasquez-Camacho et al., 2022).

The role of SynCom in enhancing microbial diversity and plant health

As cited above, the SynCom approach further enhances our understanding of plant-microbe interactions by allowing researchers to manipulate and study specific microbial consortia in controlled environments. Vorholt et al. (2017), highlighted the potential of SynComs to establish causality in plant-microbe interactions, providing insights into how specific microbial members contribute to plant health. This approach not only aids in identifying beneficial microbes but also facilitates the development of microbial inoculants that can be used to promote plant growth and disease resistance in agricultural settings. However, selecting compatible microbial strains that can effectively colonize the plant rhizosphere and confer growth benefits remains a challenge. OMICs tools have been pivotal in optimizing SynCom compositions by characterizing microbial interactions at both the taxonomic and functional levels (Nerva et al., 2022).

Metagenomics and metabarcoding have been extensively used to assess microbial diversity in SynCom applications. These approaches allow for the identification of microbial species present in complex soil and root-associated microbiomes, enabling the design of SynComs that mimic natural microbial communities (Sandrini et al., 2022b). Meta-transcriptomics and meta-proteomics further enhance this understanding by providing functional insights into the metabolic activities of SynCom members, allowing researchers to optimize community composition for improved plant performance (Yin et al., 2021; Nerva et al., 2022b).

Advancing OMICs integration in microbial-based solutions: what's next?

Despite significant advances, several challenges remain in the application of OMICs to plant-microbe interactions. One major limitation is the difficulty in integrating multi-OMICs datasets to generate holistic models of microbial function and plant response (Nerva et al., 2022). The development of standardized bioinformatics pipelines and machine-learning algorithms is essential for extracting meaningful insights from OMICs data (Yin et al., 2021; Sandrini et al., 2022b; Crossa et al., 2025). Additionally, future research should focus on applying SynCom formulations under field conditions to validate their effectiveness beyond controlled environments. Combining high-throughput

sequencing with metabolomics will allow for the identification of key microbial metabolites that drive plant growth and stress resilience, paving the way for next-generation microbial-based biofertilizers. The integration of OMICs technologies with microbial diversity assessment and SynCom applications holds great potential for advancing sustainable agriculture. By unravelling the intricate mechanisms governing plant-microbe interactions, researchers can develop targeted microbial solutions to enhance plant resilience against biotic and abiotic stresses (Sandrini et al., 2022b).

Conclusions and future prospects

The intricate interactions between plants and their microbiomes, alongside the complex communication networks between plants and microorganisms, as well as within the microbiome itself, present significant challenges in studying and understanding these systems. However, recent technological advances have enabled the identification and characterization of specific interactions within the holobiont, unveiling the beneficial effects that can arise from these relationships. Only a few beneficial fungi and a limited number of bacterial consortia are commercially available for agricultural use, as the complexity of the plant-associated microbiome limits their effectivness. Fortunately, researchers worldwide are developing and implementing innovative techniques, including microbial-based solutions, to address agricultural challenges on a larger scale. SynComs, in particular, show great promise for enhancing sustainability. Future research should aim to expand the applicability of these green, bio-based technologies.

In addition to bacterial and fungal microorganisms, the often-overlooked viral entities, particularly mycoviruses (viruses that infect fungi), offer potential for pest control and broader agricultural applications.

Continued research is essential to deepen our understanding of the complex interactions within the holobiont. Fortunately, many promising initiatives are emerging from various research groups, with several poised to translate their findings into practical applications for farmers and the agricultural industry.

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CHAPTER 2 | CRISPR/Cas9-driven double modification of grapevine MLO6-7 imparts powdery mildew resistance, while editing of NPR3 augments powdery and downy mildew tolerance

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Summary

The implementation of genome editing strategies in grapevine is the easiest way to improve sustainability and resilience while preserving the original genotype. Among others, the *Mildew Locus-O* (*MLO*) genes have already been reported as good candidates to develop powdery mildew-immune plants. A never explored grapevine target is *NPR3*, a negative regulator of the systemic acquired resistance. We report the exploitation of a cisgenic approach with the Cre-lox recombinase technology to generate grapevine-edited plants with the potential to be transgene free while preserving their original genetic background. The characterisation of three edited lines for each target demonstrated immunity development against *Erysiphe necator* in *MLO6-7*-edited plants. Concomitantly, a significant improvement of resilience, associated with increased leaf thickness and specific biochemical responses, was observed in defective *NPR3* lines against *E. necator* and *Plasmopara viticola*. Transcriptomic analysis revealed that both *MLO6-7* and *NPR3* defective lines modulated their gene expression profiles, pointing to distinct though partially overlapping responses. Furthermore, targeted metabolite analysis highlighted an overaccumulation of stilbenes coupled with an improved oxidative scavenging potential in both editing targets, likely protecting the *MLO6-7* mutants from detrimental pleiotropic effects. Finally, the Cre-loxP approach allowed the recovery of one MLO6-7 edited plant with the complete removal of transgene. Taken together, our achievements provide a comprehensive understanding of the molecular and biochemical adjustments occurring in double *MLO*-defective grape plants. In parallel, the potential of *NPR3* mutants for multiple purposes has been demonstrated, raising new questions on its wide role in orchestrating biotic stress responses.

Keywords

Vitis vinifera, *Erysiphe necator*, Stilbenes, New Plant Breeding Techniques (NPBTs), *Plasmopara viticola*

Significance Statement

Edited grapevine lines for MLO6-7 and NPR3 were generated; MLO mutants showed a quasi-immunity phenotype only against *Erysiphe necator* while NPR3 mutants displayed a higher tolerance to *E. necator* and *Plasmopara viticola*. Additionally, NPR3 lines showed an improved regeneration efficiency, suggesting the existence of an overlapping between immune response and embryo development.

Introduction

Climate change, due to its detrimental effects on agriculture and consequently on food production and the global economy, stands as one of the greatest challenges of the century (Malhi et al., 2021). It causes significant shifts in temperature, precipitation, and corresponding alterations in soil characteristics, adversely affecting crop performances (Anderson et al., 2020). Furthermore, these changes may exacerbate the proliferation of pests and pathogens, altering their interactions with plants (Singh et al., 2023). To counteract the development of pathogens, numerous agrochemicals are used, even if their excessive application revealed negative impacts on the environment and human health (Kim et al., 2017). To mitigate pathogen infections and preserve environmental biodiversity and integrity, alternative strategies must be identified. This necessity is driving researchers to explore innovative solutions, including the development of resilient plant varieties through several approaches. Among other techniques, plant breeding, a historically successful method for crop development, has advanced significantly with the advent of New Plant Breeding Techniques (NPBTs) (Giudice et al., 2021).

The identification of candidate genes is the first critical step for the application of NPBTs (Nerva et al., 2023). Indeed, the development of resistant varieties through conventional breeding relies on harnessing resistance (*R*) genes to trigger the plant immune system and act as the primary defence barrier against pathogens. Upon detecting effectors, plants induce the effector-triggered immunity (ETI) pathway that typically results in localized cell death or in a hypersensitive response (Yuan et al., 2021; Zaidi et al., 2018). An alternative approach for developing disease-resilient crops involves susceptibility (*S*) genes, which play a pivotal role in pathogen-induced diseases, facilitating infection and supporting compatibility between a pathogen and its plant host (Zaidi et al., 2018). In contrast to *R* genes, mutation or loss of function of *S* genes confers resistance to pathogens (van Schie and Takken, 2014). *Mildew Locus O* (*MLO*) genes are acknowledged *S* genes, prominently associated with powdery mildew (PM) susceptibility. They were initially characterized in barley (Büschges et al., 1997; Piffanelli et al., 2002), then their role in the regulation of PM susceptibility has been extensively corroborated across a broad spectrum of plant species, including *Arabidopsis* (Consonni et al., 2006; Consonni et al., 2010), tomato (Zheng et al., 2013), wheat (Li et al., 2022), apple (Pessina, Angeli, et al., 2016), pea (Humphry et al., 2011) and grapevine (Pessina et al., 2016; Wan et al., 2020). *MLO* genes encode proteins characterized by a seven-transmembrane domain (including a CaM-binding domain) structure localized on the plasma membrane and acting as Ca_2^+ channels (Kim *et al.*, 2002; Reddy *et al.*, 2003; Gao *et al.*, 2022). The MLO family is divided into seven phylogenetic clades, among these only two encompass *S* genes: clade IV, housing all monocot *S*-genes, and clade V, containing all dicot *S*-genes (Acevedo-Garcia *et al.*, 2014). While not all members of clades IV

and V exhibit *S*-gene features, potential candidates can be discerned during early stages of PM infection due to heightened expression levels. For instance, in grapevine, three out of four clade V-*MLO* genes *(VvMLO7, VvMLO11*, and *VvMLO13*) show an early increased expression after PM infection (Goyal et al., 2021). Consequently, exploiting RNA interference, the simultaneous knockdown of the genes *VvMLO7* with *VvMLO6* and *VvMLO11* led to a substantial decrease in PM severity (Pessina et al., 2016). These findings suggest that the knockdown of *VvMLO7*, in combination with *VvMLO6* and *VvMLO11*, plays a crucial role in enhancing PM resilience, whereas the contribution of *VvMLO13* appears less significant. Recent studies have demonstrated the successful application of CRISPR/Cas9 technology to achieve targeted mutagenesis of *VvMLO3* and *VvMLO4* genes in grapevine (Wan et al., 2020). The edited lines exhibited varying phenotypes: while some displayed normal growth patterns, others showed senescence-like chlorosis and necrosis damages. These observations highlight that pleiotropic effects can arise in MLO-edited lines. Notably, four of the *VvMLO3*-edited lines showed increased resistance to PM, a resistance mechanism based on host cell death, cell wall apposition, and the accumulation of H_2O_2.

Systemic Acquired Resistance (SAR) is a key component of plant immunity (Vlot et al., 2021) and salicylic acid (SA) is the key phytohormone accumulated in tissues upon pathogen infection and orchestrating SAR responses (Peng et al., 2021). The *Nonexpressor of Pathogenesis Related* (NPR) gene class is a key regulator of SA-mediated signal transduction triggered after SA binding. In *Arabidopsis*, this protein family comprises numerous genes, including *NPR1*, *NPR2*, *NPR3* and *NPR4*. According to the literature, *NPR1* and *NPR2* positively regulate SA association with WRKY and TGA (TGACG-binding) transcription factors in the nucleus, enhancing the expression of *Pathogenesis Related* (PR) genes by binding to their promoter sequences (Liu *et al.*, 2020). Many studies in various species, such as *Arabidopsis* (Wang et al., 2020; Wu et al., 2012), periwinkle (Sung et al., 2019) and citrus (Dutt et al., 2015; Gómez-Muñoz et al., 2017), confirmed the pivotal role of *NPR1* in inducing *PR* gene expression and pathogen tolerance. Conversely, *NPR3* and *NPR4*, despite being paralogues of *NPR1*, exhibit an antagonistic role, functioning as transcriptional co-repressors of key immune regulators such as SARD1 and WRKY70, as well as adaptors of Cullin3 (CUL3)-based E3 ligase, which mediates the degradation of NPR1 (Chang et al., 2019; Liu et al., 2020). This opposite role was demonstrated in previous experiments performed on *Theobroma cacao* in which the knock-out of *TcNPR3* resulted in enhanced resilience to *Phytophthora capsici* and *Phytophthora tropicalis*, accompanied by elevated expression of downstream defence genes (Fister et al., 2018; Shi et al., 2013). We chose a similar approach in grapevine with the aim of reducing the susceptibility against different fungal pathogens. A similar approach in grapevine would potentially allow reducing

susceptibility against not only PM but also *Plasmopara viticola*, the causative agent of downy mildew (DM).

CRISPR/Cas9 stands out among the NPBTs as a powerful and precise tool, which can be used to enhance tolerance to biotic and abiotic stresses in plants and also to functionally characterize putative genes involved in plant immunity and environmental adaptation (Giudice et al., 2021; Zhu et al., 2020). Despite the global economic significance of grapevines, there are currently few studies on CRISPR/Cas9-mediated genome editing (Li et al., 2020; Scintilla et al., 2022; Wan et al., 2020). This is due to a long and challenging process that encompasses many difficult steps, like the necessity to develop embryogenic calli, the recalcitrance of such tissue to regenerate embryos and the following regeneration of plants form the developed embryos (Nuzzo et al., 2022). Furthermore, the difficulties in developing marker-free edited grape plants limit the application of genome editing. Indeed, conventional transformation approaches based on *Agrobacterium* spp. rely on the presence of foreign genes (*i.e.*, the Cas9 protein, the gRNAs and the selection marker), which then turn the plants into genetically modified organisms (GMOs) (Nerva *et al.*, 2023). As such, GMOs undergo specific regulations (very restrictive in Europe) and meet resistance from both the society and politicians (Ceasar and Ignacimuthu, 2023). For this reason, the exploitation of transgene-free approaches is the only way to avoid the GMOs regulation and to develop marketable products. In such context the exploitation of DNA-free genome editing (Gambino *et al.*, 2024) or the cisgenic-like approach (Nerva *et al.*, 2023) are the only two available strategies for preserving the original genotype avoiding the generation of GMOs. In specific, the cisgenic-like approach relies on the exploitation of recombinase systems able to remove the transgenes once the editing event is achieved (Dalla Costa *et al.*, 2016; Ye *et al.*, 2023). Among the different recombinase systems, the Cre/loxP recombination system has been extensively documented for its use in eliminating selectable marker genes across various species (Gilbertson, 2003; Ye *et al.*, 2023). The expression of Cre recombinase can be controlled by inducible promoters that respond to heat, cold, drought, or chemical stimula (Zuo *et al.*, 2001; Éva *et al.*, 2018; Khattri *et al.*, 2011; Wang *et al.*, 2005).

This study reports an efficient approach to overcome these difficulties and provides novel insights into the biological function of genes involved in pathogen-resilience through application of the CRISPR/Cas9 technique. Notably, knock-out grapevine plants with double mutation of *VvMLO6* and *VvMLO7* and *VvNPR3* mutants exhibited significantly enhanced tolerance against PM, with the double mutants resulting almost immune. Interestingly, only NPR3 mutants exhibited also an improved resilience against downy mildew (DM), disease caused by the fungal pathogen *Plasmopara viticola*. Finally, the Cre-loxP system for transgene excision displayed a good efficiency in reducing the copy number and allowed to recover a MLO6-7 edited plant without the presence of transgenes.

Results

Cre-lox recombinase system allows the transgene copy number to be lowered up to ten times

Phylogenetic placement of VvMLO6 and VvMLO7 protein sequences confirmed their belonging to MLO Clade V (Supplementary Figure 1). Similarly, the VvNPR3 protein grouped within the clade of NPR3/4 from *Arabidopsis* and very close to NPR3 from *T. cacao* (Supplementary Figure 2). Employing a conventional *Agrobacterium*-mediated transformation protocol, we induced the differentiation of somatic embryos of *Vitis vinifera* cv Chardonnay and 18 putatively transgenic embryos were obtained. We successfully regenerated 17 plants harbouring the *MLO6/MLO7* silencing construct, all of which exhibited amplification with Cas9 primers. Evaluation of editing efficiency was assessed on PCR amplicons using TIDE software, revealing successful events in 13 (out of 15) of the selected plants. Concurrently, for the *NPR3* construct, 129 somatic embryos of 'Chardonnay' were obtained after *Agrobacterium*-mediated transformation. Forty-eight plants were regenerated from embryos, 53 of which exhibited successful transformation (positive to Cas9) and 33 displayed targeted editing. Subsequent screening of the target regions of gRNAs VvNPR3g2, VvMLO6g7 and VvMLO7g25 via high-throughput sequencing unveiled some editing frequencies exceeding 90% (Figure 1). Three lines for each construct were selected (editing characteristics of each line are reported in Figure 1) and subsequently exploited for further analyses. Specifically, for NPR3g2, the lines NPR3#4, NPR3#9, NPR3#15 exhibited editing frequencies of 88.71%, 89,82% and 90.35% respectively (Figure 1e). Regarding mutations in the *MLO* genes, the percentage of modified reads around VvMLO6g7 was consistently higher than 85% across all lines (Figure 1a). A larger variation in editing frequencies was observed for MLO7g25, as illustrated in Figure 1c. The mutations associated to the lines selected for further analyses and experiments are illustrated in Figure 1b, d and f. Off-target analysis using Sanger sequencing revealed that exclusively the target sequence has been mutated for each gRNA. After confirming successful genome editing, selected edited lines (MLO#1, #3, #9 and NPR#4, #9, #15) and wild type (WT) plants underwent micropropagation and acclimatization (25 plants for each) for further analysis. No phenotypic differences of *MLO6-7*- and *NPR3*- edited lines in comparison to WT have been observed (representative plants are shown in Supplementary Figure 3).

Edited plants were subjected to high temperature in order to activate the excision Cre-loxP system. To evaluate the copy number variation (CNV) in acclimatized plants of *NPR3*- and *MLO6/7*-edited lines compared to the original regenerated ones (still *in-vitro*), qPCR was performed on at least 10 plants for each line. Interestingly, following the heat treatments, some plants displayed a copy number up to 10 times lower than the original plant from which they derived (Table 1). Later in the season, once the plants were acclimatized, the CNV was checked again, and one MLO6-7#3 plant resulted

with a copy number of 0. To further confirm the absence of transgene PCR reactions at 35, 40 and 45 cycles have been run (Supplementary Figure 4). The plant was checked again with the Sanger sequencing method and the editing status was confirmed.

Table 1. Copy number variation (CNV). Plants subjected to heat treatments were analyzed to determine the final copy number and relative percentage reduction. CN: copy number. The original copy number is reported beside the line name.

Target - *VvMLO6* and *VvMLO7*				Target - *NPR3-like*			
Line	**Plant**	**CN**	**Reduction (%)**	**Line**	**Plant**	**CN**	**Reduction (%)**
MLO6-7#1		1.0		**NPR3#4**		1.7	
	1	0.4	60%		1	0.4	71%
	2	0.4	60%		2	0.6	57%
	3	0.3	70%		3	0.5	64%
	4	0.3	70%		4	0.3	79%
	5	0.3	70%		5	0.4	71%
	6	0.5	46%		6	0.4	71%
	7	0.3	66%		7	0.5	64%
	8	0.6	40%		8	0.4	71%
	9	0.6	44%		9	0.4	71%
	10	0.6	41%		10	0.4	71%
	11	0.4	60%		11	0.6	57%
	12	0.3	70%		12	0.3	79%
	13	0.3	70%		13	0.3	79%
MLO6-7#3		1.0		**NPR3#9**		2.0	
	1	0.2	80%		1	0.4	77%
	2	0.1	90%		2	0.4	77%
	3	0.3	70%		3	0.5	71%
	4	0.1	90%		4	0.3	82%
	5	0.1	90%		5	0.5	71%
	6	0.2	80%		6	1.7	4%
	7	0.2	80%		7	1.6	8%
	8	0.1	90%		8	1.6	8%
	9	0.1	90%		9	1.1	35%
	10	0.1	90%		10	1.0	41%
	11	0.2	80%		11	1.1	35%
	12	0.1	90%		12	1.1	35%
	13	0.0	100%		13	1.3	23%
MLO6-7#9		1.0		**NPR3#15**		1.4	
	1	0.4	60%		1	0.2	90%
	2	0.4	60%		2	0.5	75%
	3	0.5	50%		3	0.6	72%
	4	0.3	70%		4	0.5	73%
	5	0.4	60%		5	0.6	72%
	6	0.5	50%		6	0.4	80%
	7	0.5	50%		7	0.6	70%
	8	0.6	40%		8	0.5	75%
	9	0.4	60%		9	2.0	0%
	10	0.4	60%		10	0.3	85%
	11	0.5	50%		11	0.3	85%
	12	0.4	60%		12	0.4	80%
	13	0.5	50%		13	0.3	85%

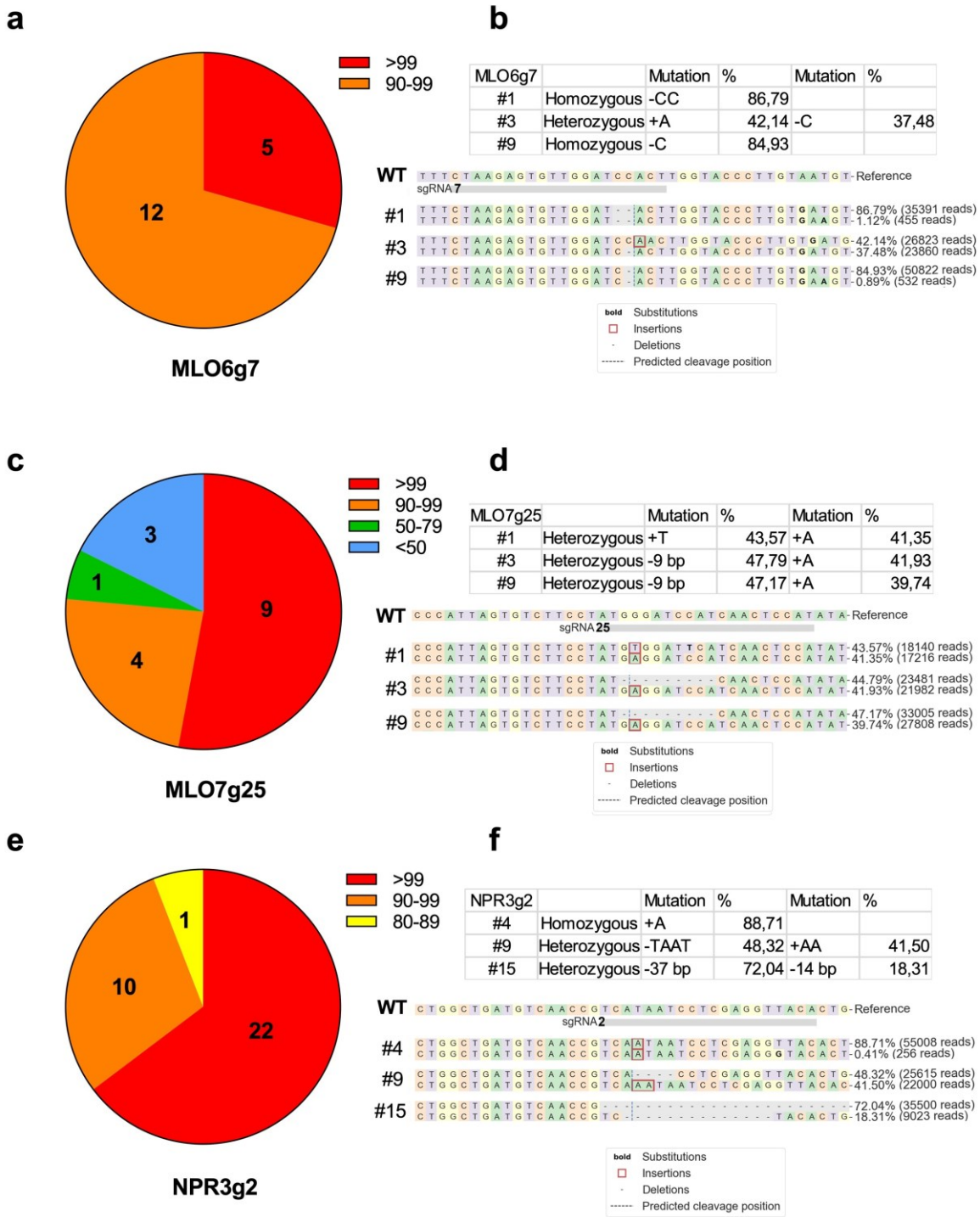

Figure 1 - Alignment and editing frequencies. a-c-e) Frequencies of editing percentages for each guide RNA (gRNA) and target gene. Numbers within the pie charts represent the number of plants belonging to the specific class. For each target gene, three lines have been selected for subsequent experiments. The editing event for each line is reported for MLO6g7 (b), MLO7g25 (d) and NPR3g2 (f).

Edited plants displayed from increased tolerance to immunity to Erysiphe necator*, depending on the edited target*

Initial signs of spontaneous PM infection were observed in WT plants at the end of August 2023 (Figure 2). When all WT plants exhibited symptoms on at least three leaves, disease indices were assessed. WT plants displayed the highest susceptibility to PM, with an incidence of 84% and a severity of 13% (Figure 2a,b). In contrast, the edited lines, particularly the *MLO*-edited ones, showed significantly reduced infection rates. Specifically, the MLO6-7#1, MLO6-7#3 and MLO6-7#9 lines exhibited markedly lower incidences of 0.5%, 0% and 4.5%, respectively, all statistically different from the WT (Figure 2a). Correspondingly, these MLO-edited lines demonstrated significantly lower disease severities, ranging from 0% to 0.2% (Figure 2b). Similarly, the *NPR3*-edited lines also exhibited reduced infection, though to a lesser extent than the MLO-edited lines. Notably, NPR3#4 and NPR3#9 lines showed a significantly lower incidence than the WT, while NPR3#15 showed an incidence of 75.3%, not significantly different from the WT plants (Figure 2a). However, all *NPR3*-edited lines displayed significantly lower severities of 3.1%, 1.7% and 6.7%, respectively, compared to the WT plants (Figure 2b). Representative examples of leaf infections are reported in Figure 2c-e. Evaluation of leaf ecophysiological parameters, including measurement of the chlorophyll content index (CCI), maximum quantum efficiency of photosystem II by determination of the Fv/Fm ratio and leaf thickness, was conducted for both WT and edited plants (Figure 2f-h). CCI analysis (Figure 2f) indicated a slightly lower, though not statistically significant, value in *NPR3* edited lines in comparison to both WT and *MLO6-7* mutated plants. Fv/Fm values did not significantly vary across edited and WT plants (Figure 2g), likely suggesting that PSII efficiency was not altered. Conversely, leaf thickness showed a significant increase only in *NPR3* edited plants, while no significant differences were recorded between MLO6-7 edited lines and WT plants (Figure 2h).

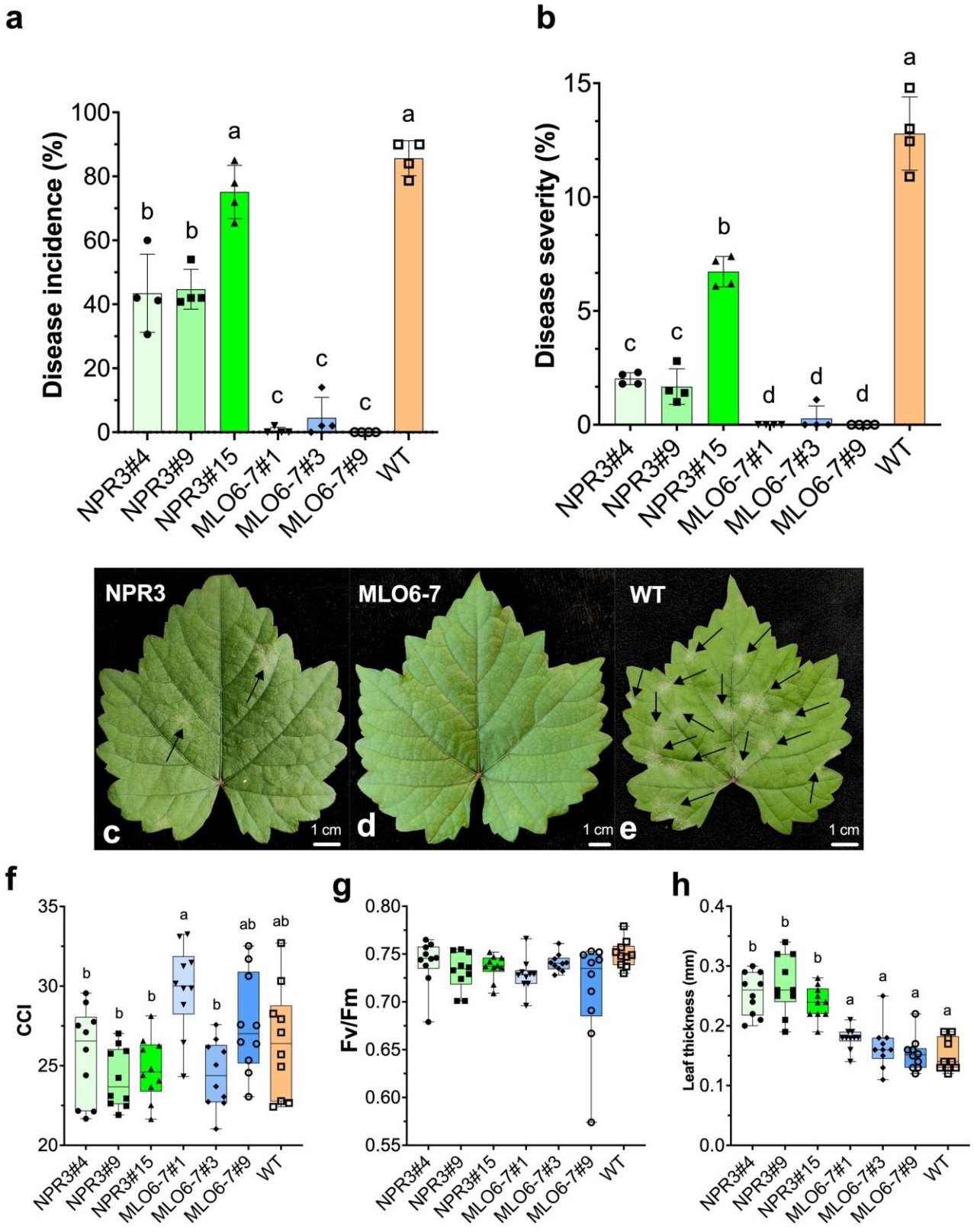

Figure 2 – Evaluation of powdery mildew infection and leaf ecophysiological traits. Disease indices of powdery mildew-infected plants were collected through the grape assess mobile application. a) incidence and b) severity percentages of leaf infections (n=4, each biological replicate is composed by at least 50 observations). c-d-e) representative leaf of *NPR3* -edited, *MLO6-7*-edited and WT-plants, respectively. Red

arrows indicate sites of infection. Leaf ecophysiological traits, including: f) Chlorophyll Content Index (CCI) (n=10), g) maximum quantum efficiency of PSII (Fv/Fm) (n=10) and h) leaf thickness (n=10) were collected using a PhotosynQ apparatus. Lowercase letters above bars indicate significant differences among the samples, as determined by ANOVA followed by Tukey post-hoc test. All data in bar charts represent mean values ± standard deviation bars. In box-plot charts, box limits indicate the range of the central 50% of the data, with a central line marking the median value and lines extend from each box to capture the range of the remaining data.

Only NPR3-*edited plants displayed increased tolerance to* Plasmopara viticola

Considering the improved resilience observed while the spontaneous infection of PM was occurring, a controlled experiment using DM was initiated during the summer 2024. Once confirmed that the non-inoculated leaves did not show any sign of infection after 7 days of incubation, we proceeded to evaluate the inoculated leaves following the OIV descriptor 452-1. The experiment was repeated twice in order to confirm the results with two independent replications. Results showed that WT and MLO-edited lines displayed a similar distribution across the disease resistance classes in both experiments (Figure 3a-b). In fact, widespread visible signs of infection were observed both in WT and MLO-edited lines by the emergence of *P. viticola* from the abaxial surface of the leaves (Figure 3c). On the contrary, both the independent replication confirmed a higher disease resistance score for the *NPR3*-edited lines (Figure 3a-b), with a highly significant difference from the WT (p-val < 0.01). In fact, only mild signs of infection were observed with small and localized emergence of DM (Figure 3c).

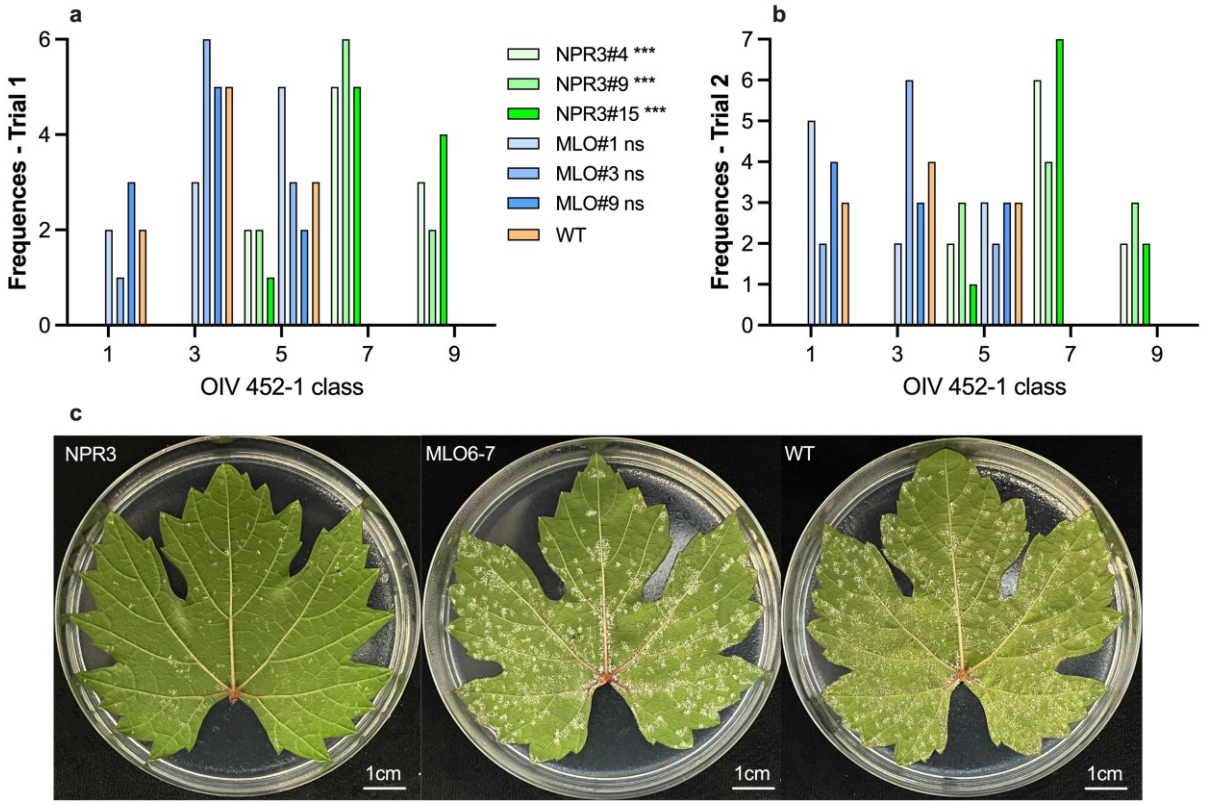

Figure 3 – Evaluation of downy mildew infection. Assessment of leaf resistance to *P. viticola* using the OIV 452-1 descriptors (the higher the value, the higher the resistance). Distribution of class frequences recorded during the first trial 1 (a) and trial 2 (b) (n=10 for each trial). The two trials were carried out 15 days one from the other. Asterisks (***) aside the legend denote highly significant differences as attested by the chi-squared test on the class frequencies (p-value ≤ 0.001). c) representative leaf of *NPR3*-edited, *MLO6-7*-edited and WT-plants, respectively.

Leaf-associated fungal community composition is influenced by the edited target

To further confirm the results obtained with the visual scoring of PM incidence and severity we also evaluated the presence of *Erysiphe* in terms of relative abundance through the ITS metabarcoding (Figure 4). Regardless of whether the single edited lines (supplementary Table S1) or the editing targets (averaging the three independent lines) (supplementary Table S2) were evaluated, the relative abundance of *Erysiphe* was consistently lower in the mutagenized plants. Furthermore, the sequencing results confirmed a stronger inhibition of *Erysiphe* in *VvMLO6* and *VvMLO7* edited lines, which displayed an average log2 fold-change (FC) of -3.65 (p-value 0) when compared to the WT (Fig. 4 supplementary Table S2). Similarly, a significant reduction in *Erysiphe* relative abundance

was also observed in *VvNPR3* edited lines but to a lesser extent (average FC of -0.72) (Fig. 4; supplementary Table S2).

Looking at the whole fungal community responses, both types of editing (i.e., *VvMLO6-7* or *NPR3*) modify fungal taxa abundances. For each editing event, a total of 126 taxa displayed significant differences in at least one line (supplementary Table S1). More in detail, when considering the target gene as the variable, only 49 taxa still display a significant difference when comparing *VvMLO6-7* or *NPR3* mutated lines against the WT (supplementary Table S2). When we consider the latter, we can see that some modifications are shared, like for example the significantly lower abundance of *Hannaella* (FC of -2.65 and -2.85 in *VvMLO6-7*-edited and *NPR3*-edited plants, respectively) and *Rhodotorula* (FC of -3.35 and -4.21 in *VvMLO6-7*-edited and *NPR3*-edited plants, respectively) genera, between both editing targets (supplementary Table S2). Other shifts in the fungal community are instead exclusively related to a single type of modification, like for example a significantly lower abundance of amplicon sequence variants (ASV) assigned to *Dactylonectria*, *Ilyonectria* and *Pleosporales incertae sedis* genera (FC -0.21, -0.36 and -2.55, respectively) which was observed only in the *NPR3* edited lines (supplementary Table S2). Conversely, a higher abundance of *Chaetomium* and *Colletotrichum* (FC 2.32 and 1.68, respectively) was observed on the leaves of *VvMLO6-7*-edited plants (supplementary Table S2) (Fan et al., 2023; Hassan et al., 2022). Finally, it is worth noting that the relative abundance of the *Ampelomyces* genus is stable in the *NPR3* edited lines while it is significantly lower in *VvMLO6-7* edited ones (supplementary Table S2).

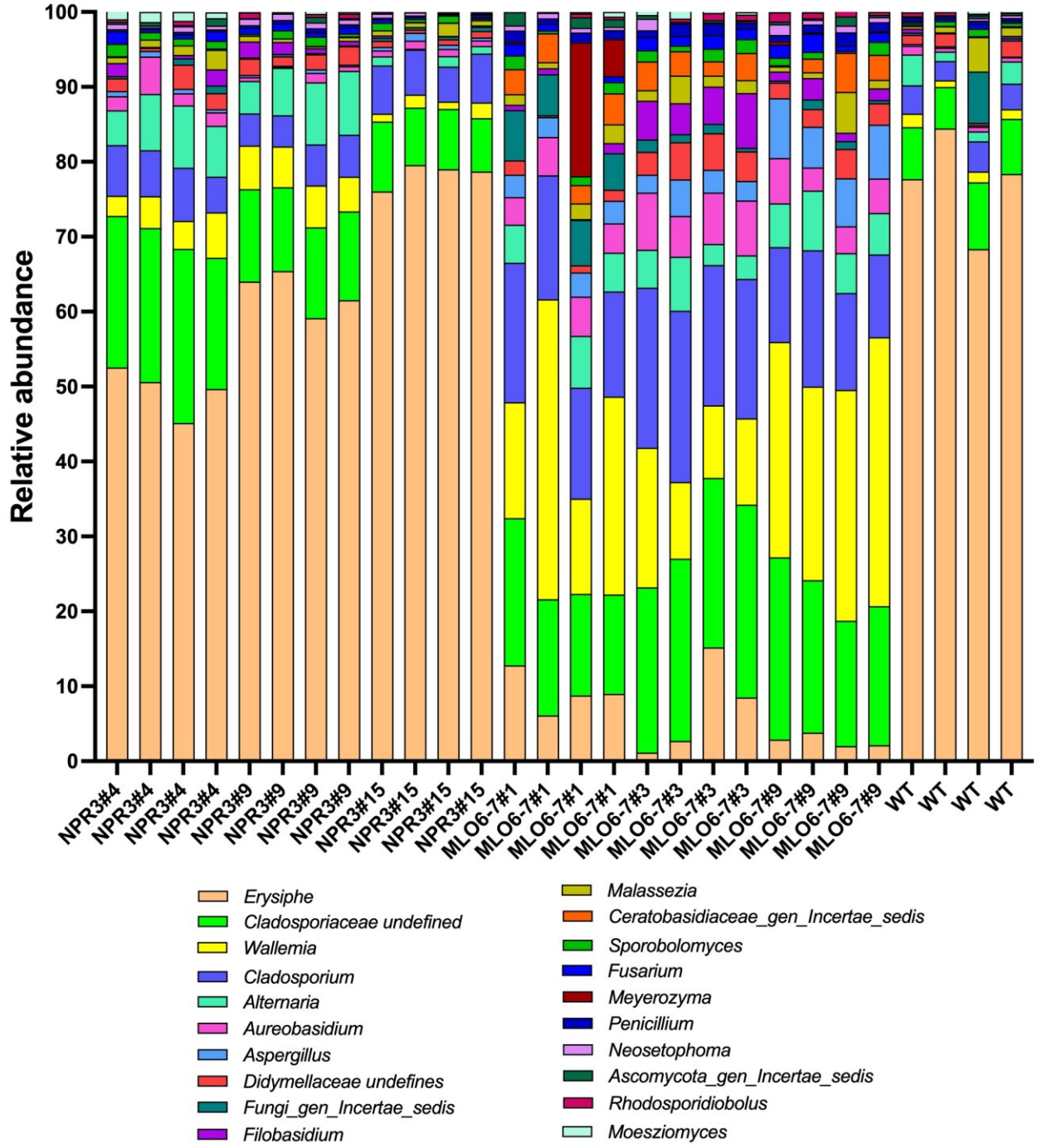

Figure 4 – Analysis of the leaf-associated fungal population in edited and WT plants. Fungal genera were identified through the qiime2-ITSexpress pipeline and compared among samples (n=4). Only the top 20 representative genera among the total classified amplicons were retained in the graphic representation.

Leaf molecular responses of VvMLO6-7 and NPR3 edited plants are partially overlapping

To understand the systemic responses resulting from editing events and pathogen attack, we collected leaves from plants infected by *E. necator* 7 days after the appearance of the first symptoms. The leaves were then used to profile transcriptome reprogramming events through RNAseq.

Looking at the *MLO6-7* edited lines, 376 genes were significantly differentially regulated (FC > 1) in comparison to the WT plants (supplementary Table S3). The majority of the genes (211 over 376) show downregulation, with an over-representation of genes belonging to the energy metabolism cluster, photosynthesis and transport (supplementary Figure 5a-c). Instead, all the *MLO6-7* edited lines displayed an upregulation of genes belonging to the carbohydrate metabolism (supplementary Figure 5 a-c).

When considering the *NPR3-like* edited lines, the number of significantly differentially regulated genes (FC > 1) in comparison to WT plants was 1,216 (supplementary Table S4). Contrary to what was observed in *MLO6-7* edited plants, a slight majority of genes were upregulated (646 over 1,216). Among them we observed an overrepresentation of genes belonging to the metabolic process cluster (including three polyphenol oxidases -VIT_00s0480g00060, VIT_00s0480g00070, VIT_10s0116g00560), particularly those involved in maltose, starch, polysaccharide and thiamine-containing compound metabolism (supplementary Figure 6d-f). Similarly, *NPR3-like* edited lines displayed a downregulation of genes belonging to energy metabolism, photosynthesis and transport (supplementary Figure 5d-f). Genes belonging to the alpha-expansin group (VIT_17s0053g00990, VIT_06s0004g07970, VIT_14s0108g01020, VIT_13s0067g02930) as well as auxin responsive genes (VIT_03s0038g01120, VIT_03s0038g00950, VIT_05s0062g00850, VIT_11s0016g00640) were strongly downregulated. Interestingly, two isoforms of *MLO* genes (namely *MLO1* and *MLO7*) were downregulated in *NPR3* edited plants (supplementary Table S4).

Finally, a comparison between *MLO6-7* and *NPR3-like* edited lines revealed 153 genes with a significant differential expression pattern between the two conditions (supplementary Table S5). Among them, 62 displayed exclusively downregulation, while 91 displayed a stronger upregulation in *MLO6-7*-edited compared to *NPR3*-edited plants. Looking at the processes downregulated in *MLO6-7*-edited lines, an enrichment of genes involved in transport (e.g., anion transport and inorganic anion transport) and response to abiotic stimuli (e.g., spermine and polyol metabolism) was observed (supplementary Figure 5g-i). In parallel, an upregulation of genes related to peculiar processes in *MLO6-7*-edited lines was observed, specifically those involved in maltose and starch metabolism and cell homeostasis (iron homeostasis and RNA/mRNA modification) (supplementary Figure 5g-i).

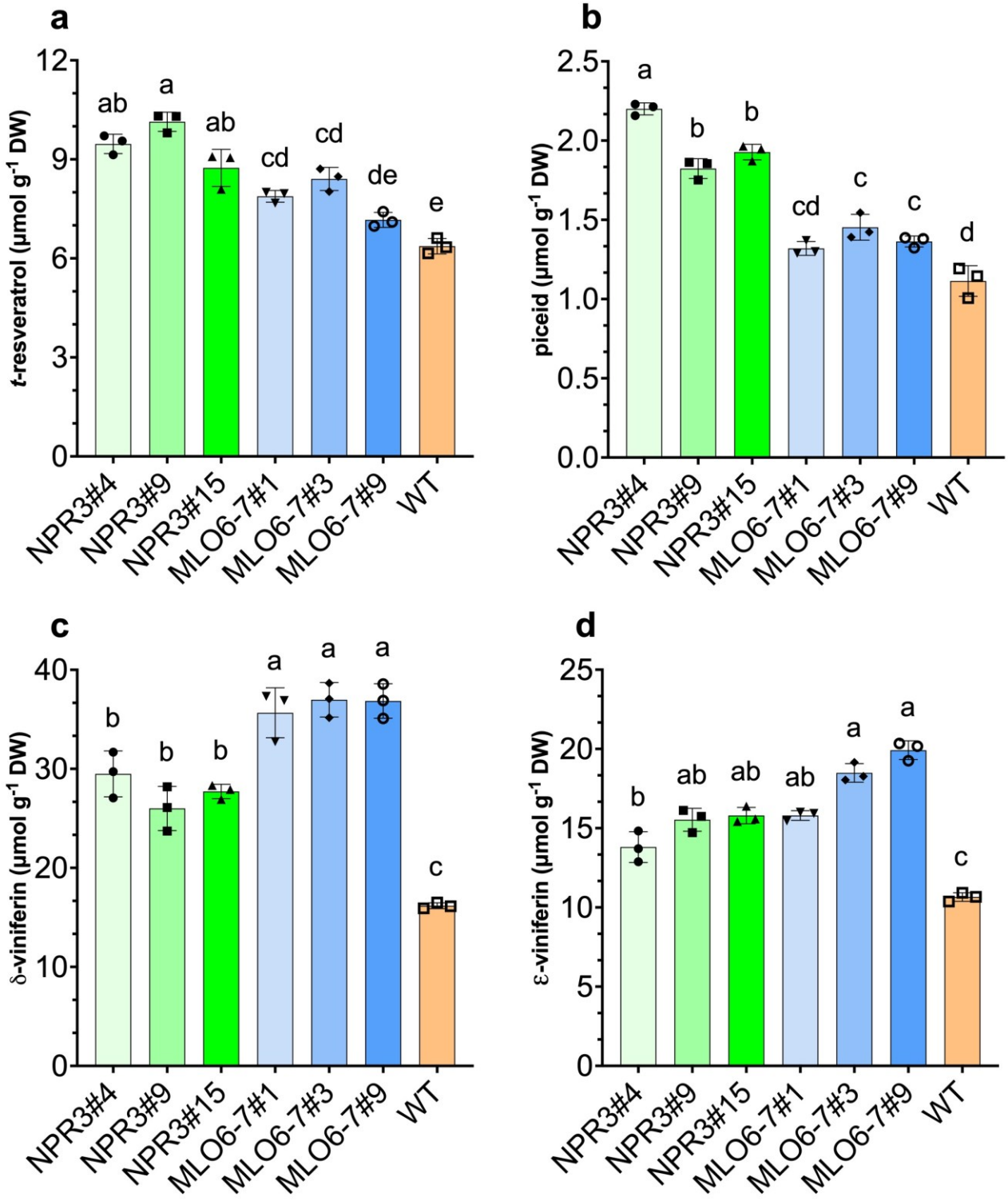

Figure 5 – Quantification of leaf stilbenes in edited and WT plants. Contents of trans-resveratrol (a), piceid (b), δ-viniferin (c), and ε-viniferin (d) quantified in leaf samples of *Vitis vinifera* wild type (WT), MLO6-7-edited or NPR3-like plants (n=3 for each line and compound). Data are expressed as µmol g^{-1} of dry weight (DW). Lowercase letters above bars indicate significant differences among the samples, as determined by

ANOVA followed by Tukey post-hoc test. All data in bar charts represent mean values ± standard deviation bars.

Stilbenes are differentially accumulated in MLO6/7 *and* NPR3 *edited plants*

In order to assess alterations in key target defence-related metabolites (i.e., stilbenes), HPLC-DAD-MS/MS analysis was conducted on edited MLO and NPR lines compared with WT plants. HPLC-MS/MS analyses revealed the presence of *t*-resveratrol (Figure 5a) and its glycosylated form (piceid) (Figure 5b), along with two different oxodimers of resveratrol (δ-viniferin and ε-viniferin) (Figure 5c-d). Looking at the whole dataset, our values were similar to those measured in the leaves of different grape cultivars (Mohammadparast et al., 2024). As a general trend, the lowest levels were found in WT conditions, while the *MLO6-7* and *NPR3* mutant lines exhibited slightly higher contents. On average, the leaves of plants mutated for these genes showed an increase in total stilbene content compared to the WT (Figure 5a), of +1.87 and +1.60, respectively. Specifically, while the rise in *t*-resveratrol content was similar between *MLO6-7* and *NPR3* plants (about +1.4), edited lines resulted in different profiles for the more complex forms of stilbenes. Specifically, the *MLO6-7* mutant plants showed a substantial increase in the polymeric forms of *t*-resveratrol, with increments of +2.3 and +1.73 for δ-viniferin and ε-viniferin (Figure 5c-d), respectively. Instead, the *NPR3* mutant plants, despite an increase in δ-viniferin (+1.91), exhibited a significant increase in piceid, with an increment of +1.65 (+1.71) (Figure 5b).

To comprehensively analyse the oxidative profile of WT, *MLO6-7* and *NPR3* leaves, the total peroxide content (supplementary Figure 7a), total polyphenol content (supplementary Figure 7b), and the enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) (supplementary Figure 7c-d) were determined. Under our experimental conditions, a significant reduction in total peroxide content was observed in the mutated lines. Specifically, values decreased from 1.55 ± 0.22 µmol of H_2O_2 to approximately 1.04 ± 0.09 and 0.89 ± 0.05, for *MLO6-7* and *NPR3* plants, respectively (supplementary Figure 3a). In contrast, a complementary trend was observed for the total polyphenol content (TPC) measured through the molybdenum and tungsten salt reduction assay (supplementary Figure 7b). TPC ranged from 165.80 ± 10.5 (WT) to 446.46 ± 19.22 (NPR3#9). Although variations were evident among the mutated plant lines, WT plants exhibited a significantly lower polyphenol content, approximately -2.05 (in comparison to *MLO6-7*) and -2.55 (in comparison to *NPR3*) times lower. From the enzymatic point of view, both SOD and CAT activities were significantly reduced in WT plants compared to the edited lines (supplementary Figure 7c-d). However, while SOD appeared to be more active in the *MLO6-7* edited lines (supplementary Figure 7c), the *NPR3* lines exhibited higher CAT levels (approximately +2.05) compared to WT (supplementary Figure 7d).

Discussion

The development of gene-edited plant varieties able to cope with environmental stresses represent a promising tool to improve agricultural sustainability (Giudice et al., 2021). Considering the specific case of grapevine, implementing genome editing strategies emerges as the most straightforward method for enhancing sustainability and resilience while maintaining the original genotype (Giudice et al., 2021). Gene editing allows to disrupt as little as possible the genome architecture and the gene pool of "elite" varieties while improving their stress resilience features (Sheridan, 2024).

Despite the great potential of genome editing, its application in grapevine is still limited, mainly due to three bottlenecks. The first issue is related to the knowledge of gene functions. Even though the grapevine genome has been available for about 20 years (Jaillon et al., 2007; Velasco et al., 2007; Wang et al., 2024) most genes are still annotated only *in-silico*, limiting the available pool of targets (Nerva et al., 2023). A second limitation concerns the development of embryogenic calli, the explant required as a starting point to perform either *Agrobacterium*-mediated transformation or DNA-free transfection of protoplasts (Gambino et al., 2024; Najafi et al., 2023; Scintilla et al., 2022; Tricoli and Debernardi, 2024). The last limitation, but equally important, is the ability of the calli to regenerate (Nuzzo et al., 2022). The latter two limitations are primarily associated to genetic features, meaning that each specific variety possesses its own ability to produce embryogenic calli and its own regeneration efficiency (Gribaudo et al., 2017; Nuzzo et al., 2022). Collectively, these limitations led to the application of editing approaches predominantly in easy-to-work genotypes, like for example table grape varieties (e.g., Crimson seedless or Sugraone (Clemens et al., 2022; Scintilla et al., 2022)) or raisin grapes (e.g., Thompson seedless (Najafi *et al.*, 2023; Olivares et al., 2021)), which represent only a small area of cultivated lands. A few examples of gene editing in elite wine grape cultivars are beginning to emerge in the literature (Gambino et al., 2024; Tricoli and Debernardi, 2024), and here we report its application in the worldwide cultivated Chardonnay cultivar.

To preserve the original genetic background and avoid the issues related to DNA-free or stable transformation approaches (Nerva et al., 2023), we decided to exploit a recombinase mechanism based on the Cre-loxP system (Chong-Pérez et al., 2012; Gilbertson, 2003). The recovered data showed that we reduced the transgene copy number by 90%, applying a single cycle of heat-shock, and that lately in the season a complete transgene removal was achieved at least in one MLO6-7#3 plant. This result is in contrast from what previously observed in grapevine using flp-frt recombination, which did not result in a significant reduction of copy number in transgenic grape plants (Dalla Costa et al., 2020), suggesting a more efficient activity of the Cre-loxP system. This result is very encouraging since, as previously mentioned, the editing of grapevine protoplasts, and

the subsequent regeneration of plants, is still very challenging. Thus, by avoiding such a step we will be able to improve the number of exploitable genotypes while reducing the time for generation of edited grapevine plants.

Considering the limited gene function information, we opted to exploit already available information on *VvMLO6* and *VvMLO7* to investigate the effects of a double mutation. Indeed, previous studies took into consideration the role of the different *MLO* genes in grapevine (Feechan et al., 2008) confirming that *MLO6* and *MLO7* were the two most promising targets (Pessina et al., 2016). Our results highlight that the double mutation led to the almost complete loss of susceptibility to PM. Indeed, both disease indices (incidence and severity) remained close to zero, with just one *MLO6-7*-edited line showing very mild infection symptoms. It is worth noting that, even if *MLO* mutants were already generated in transgenic grapevines using RNA interference (Pessina, Lenzi, et al., 2016), no studies have looked at the metabolic shift induced by the disruption of these genes. Indeed, the understanding that mutations in *MLOs* can elicit broader effects beyond conferring resistance to PM has long been reported (Wolter et al., 1993). In particular, *MLO* mutants of herbaceous species present a premature decay of photosynthesis associated to early decline in photosynthetic performance, an altered transcriptional profile, and abnormal levels of secondary metabolites derived by tryptophan (Consonni et al., 2010). All these modifications led to spontaneous mesophyll cell death, probably reflecting an uncontrolled senescence programme (Piffanelli et al., 2002). Additionally, a recent work conducted on *Arabidopsis* linked the emergence of pleiotropic effects to the nitrogen deficiency, pointing out that also external factors can alter the establishment of deleterious effects (Freh *et al.*, 2024). Our findings show that grapevine mutants for *MLO6* and *MLO7* display a decline of molecular processes related to photosynthesis process, partially overlapping to what was observed in *Arabidopsis* (Consonni et al., 2010). Nonetheless, *MLO* grape mutants displayed a transcriptional reprogramming of genes involved in carbohydrate metabolism, a response usually linked to plant immunity (Trouvelot et al., 2014), suggesting a possible trade-off towards growth-defence. Additionally, the biochemical profiling revealed an overaccumulation of stilbenes, which have been consistently identified as primary defence responses to pathogen attacks (including PM) (Schnee et al., 2008), and largely recognized as markers for disease resistance in grape plants (Viret et al., 2018). Collectively, these findings suggest that also in grapevine *MLO* mutants, as in other species where *MLOs* were studied, an enhancement of some defence responses was established, at least considering changes in the stilbene profile. Further investigation on this subject is definitely needed to better elucidate the whole metabolomics changes controlling such defensive mechanisms.

In parallel to mutations of *MLOs*, we decided to explore the effects of the loss of function mutants of *NPR3-like*. This gene belongs to the plant-conserved Nonexpresser of Pathogenesis-Related (NPR) protein family and is classified within clade II, which, together with members of clade I, plays prominent roles in orchestrating plant defence responses (Zhou et al., 2023). Their activity is mediated by the ability to bind salicylic acid, but with an opposite outcome. While members of clade I are essential for activating the SAR responses (Glazebrook et al., 1996), members of clade II impair the activation of defence responses (Ding et al., 2018). In fact, loss of function mutants for *NRP3* and *NPR4* in *Arabidopsis* showed an increased activation of the immune system and a concomitant upregulation of PR proteins, leading to an increased tolerance to pathogen attacks (Fu et al., 2012). Similarly, the transient knock-out of *NPR3* in *Theobroma cacao* leaves limited the spread of *Phytophthora tropicalis* (Fister *et al.*, 2018). Considering this information, and that in grapevine there is only one member of clade I (*VvNPR1*) (Le Henanff et al., 2009) and one member of clade II (*NPR3*) (Zhou et al., 2023), we opted to evaluate an editing approach targeting the *NPR3* gene. From the susceptibility point of view, the *NPR3*- edited lines displayed a significant improvement in terms of resilience against PM but to a lesser extent if compared to *MLO* mutants. In fact, based on disease incidence, only two out of three *NPR3-like* mutant lines (*i.e.*, NPR3#4 and NPR3#9) displayed a significant difference when compared to WT plants. Conversely, the disease severity index was always significantly lower than in WT. These findings are in line with our expectations since in *MLO*-edited plants we disrupted the production of proteins involved in recognition between the fungus and its host, thereby inhibiting fungal growth and tissues colonization. On the other hand, in edited *NPR3-like* plants we blocked the production of a protein that negatively regulates the plant's defence responses, but which is not involved in the early interaction stages between the pathogen and its host. Nonetheless, a significant downregulation of *MLO1* and *MLO7* was observed, suggesting that NPR3 downregulation can indirectly impair the transcription of *MLO* genes. The exploitation of a pathogen independent defence mechanism allowed the *NPR3*-edited lines to show, besides improved tolerance to PM, an improved resilience against *P. viticola*, strongly suggesting a much wider resilience to biotic stresses. This feature makes this approach more suitable for generating a broad-spectrum disease tolerance, as also suggested by the metabarcoding analyses which highlighted a significant reduction in the relative abundance of potential pathogens. In addition to the reported molecular and biochemical adjustments, we observed an improvement in leaf thickness probably related to the molecular adjustment (*i.e.*, downregulation of genes encoding alpha-expansins and auxin-responsive factors) involving more complex metabolic processes. Such a trait was already associated to disease tolerance in other species, including woody plants such as *Populus* clones, where those with greater leaf thickness were more tolerant to rust infections (Fernàndez-Martínez et al., 2013). For the first

time in genome editing studies, we demonstrated here that the outcomes of a lower infection rate can be confirmed by ITS metabarcoding. Indeed, this approach highlighted a significantly lower presence of *E. necator* on the leaves of both *NPR3-like* and *MLO*-edited plants. In parallel, the relative abundance of *Ampelomyces* was reduced in the *MLO6/7* mutants but not in *NPR3*-edited lines. It is worth noting that *Ampelomyces* represent a natural biocontrol agent of PM, able to parasitize the mycelium and chasmothecia in turn limiting PM spread. Its reduced abundance in *MLO6/7* lines can be due to the lower colonization of PM (its natural hosts). On the other hand, the unaffected relative abundance in *NPR3* lines suggests that, even though *NPR3*-edited lines showed improved defence responses, such adjustments are not influencing the *Ampelomyces* activity. Finally, a much wider effect on the immune system of *NPR3*-edited lines can be envisaged by the improved resilience against DM and by an exclusive reduction of *Dactylonectria*, *Ilyonectria* and *Pleosporales incertae sedis* genera, all hosting well-known grape pathogens (Probst et al., 2019), only in *NPR3*-edited plants.

Interestingly, *NPR3*-edited plants exhibited an enhanced regeneration efficiency, suggesting an indirect effect on the embryogenesis process. Indeed, if compared to *MLO*-edited plants we were able to recover a higher number of embryos even if the starting material was the same (same embryogenic calli stock and same starting quantity). Such a result was quite unexpected since, as previously reported (Dal Santo et al., 2022), the elicitation of stress responses and secondary metabolism was associated to a reduced embryogenic potential in grapevine. This unforeseen finding is quite interesting, as recalcitrance to regenerate is one of the main bottlenecks impairing the wide application of gene editing in many plant species (Atkins and Voytas, 2020). A more in-depth evaluation of the effects of *NPR3-like* editing during the regeneration phase is needed in order to uncover the molecular and biochemical signals controlling the observed enhancement of embryo regeneration.

Finally, to improve the understanding of the complex responses occurring in the edited lines, leaf samples were subjected to molecular and biochemical analyses. In particular, both *NPR3* and *MLO*-edited lines accumulated higher levels of stilbenes and polyphenols. Specifically, MLO6/7 mutants displayed a significant overaccumulation of polymerized stilbenes (viniferins). This observation can be explained by the fact that the overproduction of secondary metabolites can lead to polymerization within specific cellular environments. Although the exact mechanism remains unclear, the polymerization is a well-known process occurring for catechins (yielding proanthocyanidins and tannins) (Mannino et al., 2021) and resveratrol (yielding viniferins) (Fuloria et al., 2022). These obtained molecules possess improved stability and bioavailability compared to their monomeric equivalents, potentially enhancing their efficacy in preventing and ameliorating

various pathological conditions (Langcake, 1981). In our experimental conditions, we observed a substantial reduction in total peroxide content for both mutant types, coupled with a concurrent increase in total polyphenol content. The elevation in total polyphenol contents closely correlates with the increased levels of phytoalexins. In *NPR3*-edited lines the increase in polyphenols also correlates with the strong overexpression (FC > 2) of three polyphenol oxidase-encoding genes (VIT_00s0480g00060, VIT_00s0480g00070, VIT_10s0116g00560). Additionally, phenolic compounds are renowned for their antioxidant properties, and along with other secondary metabolites could have contributed to maintain the cellular redox balance in edited grapevine plants by efficiently mitigating free radicals (Amarowicz et al., 2008). The derivatives of *t*-resveratrol, such as piceid and viniferins, also hold robust antioxidant capabilities. In fact, if the greater stability and aqueous solubility of the piceid compared to *t*-resveratrol allows a greater bioavailability, viniferins show an even greater reductive capacity due to their polymeric architecture enhancing their ability to sequester oxidizing agents (Delmas et al., 2011). Moreover, both resveratrol and its derivatives augment the activity of endogenous antioxidant enzymes within cells, including superoxide dismutase (SOD) and catalase (CAT), thereby further fortifying cells' resilience against oxidative stressors. SOD and CAT work synergistically neutralizing free radicals and peroxides, thereby mitigating oxidative damage to cells and safeguarding their structural integrity (Gülçin, 2010). In edited lines, in particular in *NPR3* ones, we observed an overexpression of catalase genes and several *Glutathione S-Transferase* (*GST*) genes (Supplementary Tables 4 and 5) encoding a large protein family that catalyzes the conjugation of reduced glutathione (GSH) to various substrates preventing the oxidative stress (Neuefeind et al., 1997). For example, *GST* expression was higher in asymptomatic leaves of grapevine canes infected by esca disease (Valtaud et al., 2009). Resveratrol and its derivatives possess the ability to modulate the activity of SOD and CAT through various mechanisms. Firstly, as previously discussed, these compounds can act as direct antioxidants, effectively neutralizing free radicals and reducing peroxide generation. Furthermore, resveratrol and its derivatives can directly shield SOD and CAT from inactivation induced by oxidative agents (Gülçin, 2010). The observed reduction in total peroxide content in our experimental conditions could likely be ascribed experimentally to the combined action of both enzymatic activity and polyphenol content (Tang et al., 2010). Finally, the accumulation of stilbenes and higher activity of the antioxidant enzymes, both having a cytoprotective outcome, could also explain the reason for more stable Fv/Fm values, potentially mitigating the pleiotropic effects of *MLOs* knock-out mutants (as depicted by SPAD and Fv/Fm values) as reported in other species (Consonni et al., 2010).

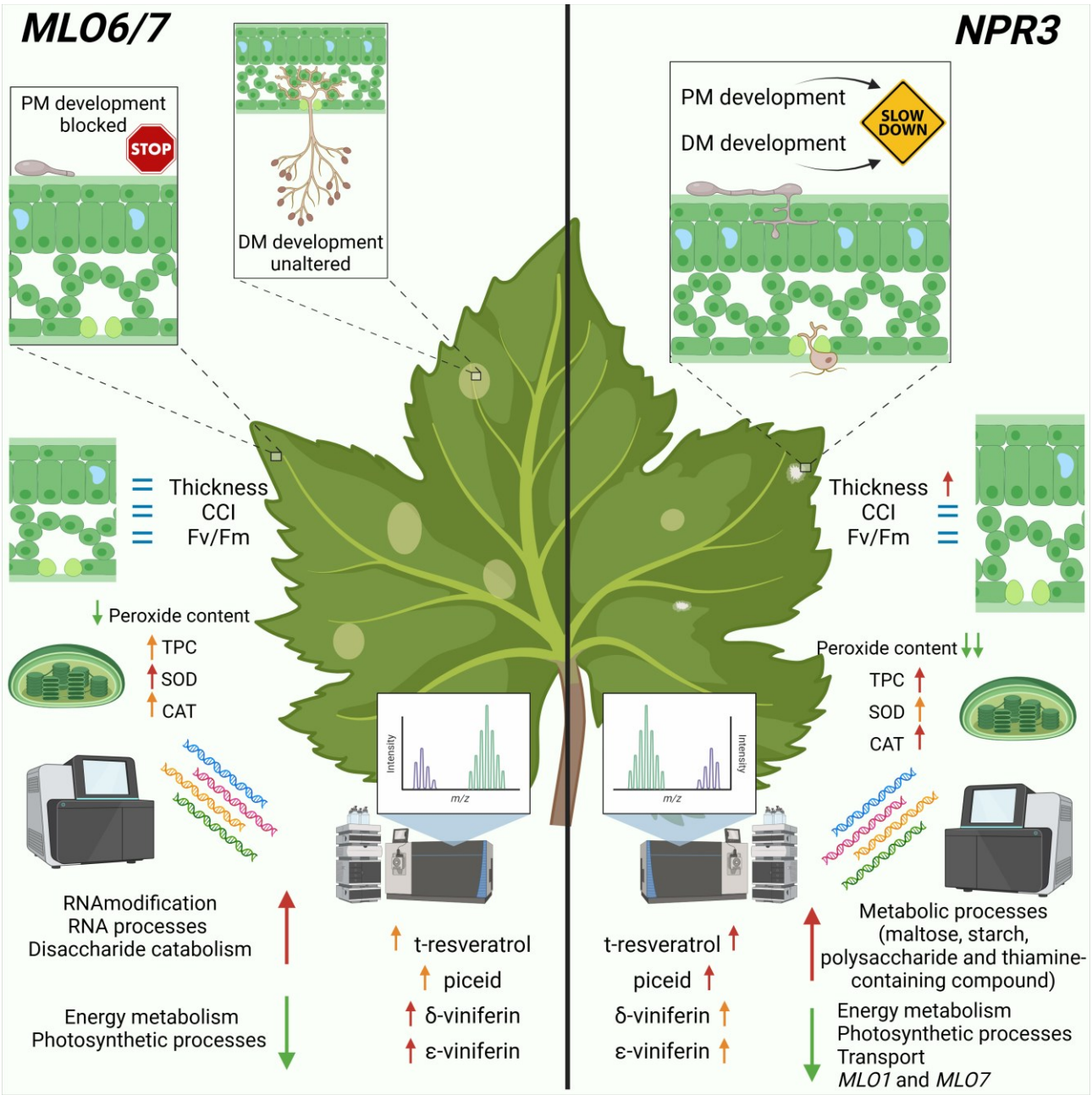

Figure 6 – Overview of the main findings. Summary of the susceptibility degree to powdery mildew (PM) and of the molecular, physiological and biochemical adjustments observed in *MLO6/7* edited (left side) and *NPR3* edited (right side) plants. Metabarcoding analysis revealed that, while in *MLO6/7* plants PM development was almost impaired, in *NPR3* plants PM development significantly lower than in wild-type plants (WT) but not that much as in *MLO6/7*. Additionally, while in *MLO6/7* plants no influence was observed on the relative abundance of other pathogens, in *NPR3* ones a significant reduction was observed. From the physiological point of view, leaf thickness, chlorophyll content index and photosystem II efficiency (Fv/Fm) were evaluated, highlighting a thicker leaf in *NPR3* plants. From the molecular point of view, similarity between *MLO6/7* and *NPR3* edited plants were observed. From the biochemical point of view we observed an enhancement of stilbenes in both type of plants but involving different type of molecules. In *MLO6/7* plants increase in δ- and ε-viniferin was observed while piceid and resveratrol increased more in *NPR3* plants. Finally,

the oxidative status was monitored revealing an increase of scavenging potential in both type of plants but with a larger extent in *NPR3* plants.

Conclusions

The recent legislative proposal adopted by the European Parliament (https://www.europarl.europa.eu/doceo/document/TA-9-2024-0067_EN.html) reflects a consensus among scientific institutions, industry and farmers on the need to exploit biotechnological approaches to start a new green revolution in agriculture (Sheridan, 2024). Indeed, gene editing represents a novel chance to exploit the potential of plant biotechnology in Europe, allowing the amelioration of cultivated plants in terms of nutritional profile, resistance to stress, and yield (Zhan et al., 2021). The present work, exploiting a Cre-lox recombinase approach, demonstrated the huge potential of biotechnology to improve viticulture sustainability producing a transgene-free edited grape plant. Indeed, by targeting two different sets of genes, we obtained immune plants as well as plants showing an improved tolerance to both *E. necator* and *P. viticola* (Figure 6). Metabolic analyses revealed a possible correlation with the accumulation of phenolic compounds, including diverse stilbenes, in both *MLO6/7* and *NPR3-like* mutants. Such an improvement not only positively influenced resilience against PM and DM, but potentially mitigated the pleiotropic effects of *MLO6-7* defective plants already reported in other species. These results suggest that the application of NPBTs for the obtainment of improved genotypes with high level of these strong fungitoxic compounds could help to improve biotic stress resilience. Finally, we uncovered an intriguing side effect of *NPR3-like* defective calli, which displayed an improved ability to regenerate embryos when compared to *MLO*-edited calli. Additional studies are warranted to elucidate the underlying mechanisms contributing to such an effect.

Experimental procedures

Embryogenic calli

From late April to the beginning of May depending on the phenological stage, flower clusters of *V. vinifera* cv. Chardonnay were harvested from a vineyard located in Rauscedo (PN) and managed by Vivai Cooperativi Rauscedo (VCR). Only the basal half of the inflorescence was retained during collection. The right developmental stage of pollen was assessed following established methodologies (Gambino *et al.*, 2007; Gribaudo *et al.*, 2004). Subsequently, the collected flowers underwent a sterilization process involving a 15 minutes treatment with a sodium hypochlorite solution (1.5% available chlorine) containing 100 µL L^{-1} of Tween 20, followed by multiple rinses with sterile distilled water. Ovaries and anthers, including their filaments, were excised and cultured on PIV starter calli induction medium [Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6%

sucrose, 0.3% Gelrite, 4.5 µM 2,4-Dichlorophenoxyacetic acid (2,4 D), and 8.9 µM 6-Benzylaminopurine (BAP), pH of 5.8] (Dhekney *et al.*, 2019; Franks *et al.*, 1998; Gambino *et al.*, 2007; Gambino *et al.*, 2021). Three months after callus induction, embryogenic calli were visually identified and transferred onto C1 proliferation medium (Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6% sucrose, 0.5% Gelrite, 5 µM 2,4 D, and 1 µM BAP, pH of 5.8) and transferred monthly onto fresh medium (Gribaudo *et al.*, 2004).

Vector construction

Guide RNAs (gRNAs) targeting the *VvMLO7*, *VvMLO6* and *VvNPR3* genes (Vitvi13g00578, Vitvi13g04070 and Vitvi10g01335) (Shi et al., 2023) were evaluated using the online tool CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/) (Lei et al., 2014). The gRNAs (Supplementary Table S6) were selected based on their on-target efficiency with a single gRNA designed for each gene. Additionally, an off-target analysis was performed using CRISPOR (Concordet and Haeussler, 2018) and Cas-OFFinder to ensure specificity and minimize potential off-target effects (Bae *et al.*, 2014). Used guides are listed in Supplementary Table S6.

Phylogenetic placement of VvMLO6, VvMLO7 and NPR3 was calculated as previously described (Brambilla *et al.*, 2023; Nerva *et al.*, 2022). Briefly, sequences of *MLO* and *NPR* genes were retrieved from repositories (TAIR for *Arabidopsis*, SolGenomics for tomato, NCBI for others) and aligned using Muscle (Edgar, 2004). For MLO, 143 sequences were used and for NPR 20 sequences were used (including the *VvACT* as outgroup for both analyses). Alignments in fasta format are made available as Supplementary Data 1 (MLO) and Supplementary Data 2 (NPR). Phylogenetic relationship were then calculated using the IQ-TREE webserver (Trifinopoulos *et al.*, 2016) and then visualized in MEGA (Kumar *et al.*, 2016). The pDIRECT_22c vector (Čermák et al., 2017) was customized, and a recombinase system based on the Cre-lox system combined to the heat-shock inducible promoter (Chong-Pérez et al., 2012; Gilbertson, 2003) was integrated (Supplementary Figure 8 and sequence reported in Supplementary Data 3). The vector assembly involved the insertion of a gRNA targeting each gene. In detail, for MLO knock-out, one gRNA targeting *VvMLO6* and one targeting *VvMLO7* were co-integrated, otherwise a gRNA was inserted to achieve *VvNPR3* knock-out. PCR products of gRNAs obtained following the procedure described by Čermák et al., 2017 were cloned into the previously constructed plasmid following the protocols by Lampropoulos et al., 2013. Competent cells of *Agrobacterium tumefaciens* strain GV3101 were used to transform embryogenic calli as previously described by Dhekney et al., 2008.

A. tumefaciens was initially cultured on LB agar and subsequently sub-cultured in MG/L medium to reach a final optical density (OD_{600}) of 0.8, according to Amarowicz et al., 2008 and Li et al., 2008,

with only minor modifications: after MG/L culture, the cell suspension was centrifuged, resuspended in LCM medium supplemented with 100 µM acetosyringone and incubated at 28 °C and 210 rpm for 3 hours.

Embryogenic calli, previously conditioned according to Gribaudo et al., 2004, were co-cultured with *A. tumefaciens* (Torregrosa et al., 2015) using 600 mg L^{-1} of cefotaxime as selective agent in LCM medium to rinse embryogenic calli. Subsequently, the embryogenic calli were dried on sterile filter paper, transferred onto GS1CA medium [Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6% sucrose, 1% Agar, 4.5 µM 2,4 D, 1 µM BAP, 10 µM 2-Napthoxyacetic acid (NOA); 20 µM Indole-3-acetic acid (IAA) pH of 5.8] (Franks et al., 1998; Gambino et al., 2007) supplemented with 450 mg L^{-1} cefotaxime, and maintained at 26 °C for 9 days. Calli were sub-cultured monthly on GS1CA media supplemented with 450 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin for 4 months. Thereafter, calli were transferred monthly onto GS1CA medium supplied with 100 mg L^{-1} kanamycin. Embryos at the torpedo stage were transferred onto MS-SH medium (Murashige and Skooge basal salt, Murashige and Skooge vitamins, 1.5% sucrose, 0.4% Gelrite, and 10 µM BAP, pH of 5.6). After two months, embryos were transferred onto half-strength MS basal salts at 26 °C, 200 µmol m^{-2} s^{-1} (PPFD), and a 16/8-hour day/night photoperiod. When 5 leaves were fully expanded, each node was transferred onto half-strength MS basal salts supplemented with 4 µM BAP for one month. Subsequently, the nodal explants were moved to MS supplemented with 2.5 µM NAA and 2.5 µM IBA for 20 days to induce root formation. The resulting explants were transferred to half-strength MS basal salts for one month and then to sterilized peat pellet. Among all regenerated plants, three lines for the double mutants *MLO6/MLO7* and three for the *NPR3-like* were retained for further experiments. In total 25 plants for each selected line, including wild type embryogenic-derived plants (WT), were acclimatized in a greenhouse at the beginning of June 2023.

Editing efficiency analysis

Genomic DNA was extracted from a single leaf of each plant using the Genomic DNA Isolation Kit NORGEN (Norgen Biotech Corp). The DNA concentration was determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific) and subsequently diluted to a final concentration of 10 ng $µl^{-1}$. The diluted DNA samples were used for PCR reactions using DreamTaq Green DNA polymerase (Thermo Fisher Scientific) using specific primers for Cas9 (supplementary Table S7). The initial editing efficiency was assessed through Sanger sequencing (BioFab Research srl, Italy) of the genomic regions targeted by gRNAs (supplementary Table S7). The sequencing data were analysed using TIDE (https://tide.nki.nl/) software (Brinkman et al., 2014).

The *VvMLO7*, *VvMLO6* and *VvNPR3* target regions of the greenhouse-acclimatized plants were screened by high-throughput sequencing. The target regions were amplified using primers and overhang Illumina adapters to generate Illumina library amplicons (supplementary Table S7). Subsequently, the library was sequenced by the Illumina NovaSeq 6000 platform at the IGA (Udine, Italy). Approximately 100,000 reads per sample were produced. Raw paired-end reads were analysed using the CRISPResso pipeline (https://crispresso2.pinellolab.org/) and removing reads with low quality (Clement et al., 2019).

Susceptibility assay and leaf ecophysiology

After 3 months in the greenhouse, plants were attacked by a naturally occurring PM infection. When all control plants had at least three leaves with clear powdery mildew symptoms, the disease indices (incidence and severity) were collected using the Grape Assess Mobile App (University of Adelaide). For each line, four independent biological groups of observation were considered. Each biological group was made up by at least 50 observations. Five non-infected leaves for each plant were collected, lyophilized and stored at -80 °C for further analyses.

Besides PM evaluation, a controlled infection using downy mildew was performed. Ten leaves from each regenerated line were randomly collected during July. Leaves were surface washed using water and paper towel and then laid on Petri dishes containing sterile water-agar (1%). The inoculum of *P. viticola* was prepared collecting sporangia from infected leaves and then diluting to $5*10^5$ sporangia/mL. After inoculation (1mL per leaf) leaf were incubated at 24°C with a 16-8 light-dark cycle. Development of leaf symptoms (*i.e.*, emergence of sporangia from the leaves) were checked 7 days after inoculation. Descriptor 452-1 of the International Organisation of Vine and Wine (OIV) were used to determine leaf resistance to downy mildew. Briefly, each leaf was visually checked and assigned to a class among the following: class 1 – 61 to 100% of surface covered with sporulation, class 2 – 41 to 60% of surface covered with sporulation, class 3 – 21 to 40% of surface covered with sporulation, class 4 – 1 to 20% of surface covered with sporulation, class 5 – 0% of surface covered with sporulation. Control leaves (n=3) were kept without inoculation to exclude previous infections naturally occurring in the greenhouse. The experiment was repeated twice with a 15 day-delay between the two trials, in order to confirm the results.

Measurements to determine chlorophyll fluorescence parameters (Fv/Fm), relative chlorophyll content (CCI) and leaf thickness were carried out using a MultispeQ v2.0 device controlled by PhotosynQ software (Kuhlgert et al., 2016). Records were taken on ten fully formed non-senescing leaves for each line, selecting one leaf per plant growing between the 4^{th} and 6^{th} shoot from the top.

Nucleic acid extraction, sequencing and bioinformatics

For molecular assays, three independent biological replicates were obtained for each condition by pooling five asymptomatic leaves randomly selected every five vines of the 25 acclimatized plants (1 leaves × 5 plants × 3 biological replicates: each set of five plants therefore represented an independent biological replicate). Attention was paid to randomly collect asymptomatic leaves also from untreated plants. This approach was adopted with the aim of investigating the specific defence responses established in the edited vines regardless of the occurrence of visible symptoms.

Genomic DNA and total RNA were isolated from lyophilized leaves using Genomic DNA Isolation Kit NORGEN (Norgen Biotech Corp) and Spectrum plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA), respectively, according to the manufacturer's instructions.

DNA and RNA concentrations were evaluated using a NanoDrop one Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and integrity was confirmed on agarose gel. To proceed with RNA-seq analysis, at least 2 µg for each sample was sent to Macrogen Inc. (Europe) for cDNA library construction and sequencing using NovaSeq. Furthermore, Illumina tag screening of the ITS2 region of the rDNA was performed by Macrogen Inc. (South Korea) using primers ITS3-ITS4 on a MiSeq Illumina apparatus. Trimmed sequences were analyzed with DADA2 and sequence variants were taxonomically classified using the UNITE database, as previously described (Nerva et al., 2021). Analysis of transcripts was performed using the Artificial Intelligence RNA-seq Software AIR (accessible at https://transcriptomics.cloud). Transcripts were annotated using the V1 annotation of the grapevine genome (Grimplet et al., 2012); functional classification, GO enrichment analysis and identification of differentially expressed genes (DEGs) were performed as previously described (Nerva et al., 2022).

Heat Shock treatments and evaluation of copy number variation through qPCR

Three-weeks old grapevine plants acclimatized in the greenhouse and displaying knock-out of target genes were incubated in a temperature-controlled chamber at 45 °C for 24 hours for three cycles with a 48-hour interval between treatments. To assess the CNV resulting from heat treatments, a single leaf (developed after the heat treatment) from each plant was collected, lyophilized, and the genomic DNA was extracted and used for qPCR analysis using a custom-made calibration curve (Dalla Costa et al., 2009; Lee et al., 2006). Quantification of the *NPTII* copy number was employed to quantify T-DNA insertions (Table 1).

Calibration curves were constructed by amplifying the *VvCHI* fragment from Chardonnay leaf using primers listed in supplementary Table S7. Briefly, PCR product was cloned into pGEM-T Easy vector, which already contained a fragment of the *NPTII* gene cloned from pDIRECT_22c. The final

plasmid pGEM:CHI:NPTII was purified and quantified using NanoDrop One Spectrophotometer and its molarity was calculated using the NEBioCalculator online tool (version 1.15.5). A 10-fold serial dilution series of the pGEM:CHI:NPTII plasmid was used to construct the calibration curve according to Dalla Costa et al., 2009.

Each amplification was followed by melting curve analysis (60–94 °C) with a heating rate of 0.5 °C every 15s. All reactions were performed in duplicate with nuclease-free water as negative control. Estimation of the *NPTII* insertion copies in transgenic grapevines was calculated using the following formula: (*NPTII* gene copy number/*VvCHI* copy number) × 2.

Biochemical analyses

About 100 mg of lyophilized leaves (the same used for RNAseq analysis) were used for the biochemical analyses. Briefly, extraction was performed using 1 mL of a 1:1 (*v/v*) mixture of Methyl-t-Butyl Ether (MTBE) and Methanol (MetOH), as previously described (Liu et al., 2013). Following vortex mixing, the samples were sonicated for 15 minutes at 4 °C. Subsequently, 0.5 mL of 0.1% (*v/v*) HCl was added. After a 5-minute incubation at 4 °C, the samples were centrifuged at 10,000 g for 10 minutes at 4 °C. The upper MTBE phase was transferred, dried under nitrogen flow at constant flow, and resuspended in 100 µL of 50% (*v/v*) propanol. Accordingly, samples were injected into a HPLC-DAD-ESI-MS/MS apparatus and the analytical separation of phytoalexins was conducted employing a binary solvent system consisting of water acidified with 0.1% (*v/v*) formic acid (solvent A) and methanol acidified to 0.1% (*v/v*) with formic acid (solvent B). A Kinetecs C18 column (130 Å, 1.7 µm, 2.1 × 100 mm) was utilized for the separation. The gradient profile was as follows: 0–8 min with 95% (*v/v*) A, 8–12 min decreasing A to 75% (*v/v*), 12–16 min decreasing A to 55% (*v/v*), 16–32 min decreasing A to 25% (*v/v*), and subsequently reduced to 5% (*v/v*) at 45 min. Solvent A concentration was maintained for 10 min. Throughout the chromatographic run, the flow rate was set at 0.2 mL min^{-1}, with a 10 µL injection volume. Chromatographic conditions were restored to initial conditions and maintained for 8 min before the next injection (Sun et al., 2006). Phytoalexins were quantified using different concentrations of *t*-resveratrol, δ-viniferin and ε-viniferin by plotting peak areas against concentrations, and linear regression was applied for curve fitting. Original standards of *t*-resveratrol (purity ≥ 99%), piceid (purity ≥ 99%) and viniferins (purity ≥95%) purchased from Merck KGaA were used to prepare the calibration curves. Limits of detection (LOD) and limits of quantification (LOQ) were calculated for each standard following the method described by (Cao et al., 2020).

Total phenolic content (TPC) was quantified preparing the ethanolic extracts of the lyophilized leaves and assessed using the Folin-Ciocalteu method, which entails the reduction of phosphotungstic-

phosphomolybdic acid to blue pigments in an alkaline medium. This method follows the procedure outlined by Folin and Denis, as previously described by Singleton, 1966. For quantification, a calibration curve with gallic acid (GA) was used, and the results were expressed as milligrams of GA equivalents (GAE) per 100 grams of fresh weight (FW). Each sample was measured in triplicate to ensure accuracy and reliability of the data. This approach allows for a precise evaluation of the phenolic content, which is crucial for understanding the antioxidant properties of the extracts.

The total peroxide content was measured using a Peroxide Assay Kit from Abnova (St. Louis, MO, USA). To begin, 50 mg of lyophilized leaves were ground in liquid nitrogen and extracted with milli-Q water at a ratio of 1:10 (w/v). Following extraction, the samples were centrifuged at 15,000 × g for 10 minutes, and the resulting supernatant was used for the assay. For the assay, 200 µL of detection reagent was added to each 40 µL sample in a flat-bottom 96-well plate. After a 30-minute incubation period, the absorbance was measured at 600 nm using a microplate reader. The H_2O_2 content was determined by comparing the absorbance values to a standard curve generated from serial dilutions of a 3% H_2O_2 standard.

Lyophilized leaves were pulverized and homogenized with a mortar and pestle to determine enzyme activities. The ground material was then extracted in a solution containing 62.5 mM Tris-HCl (pH 7), 10% (v/v) glycerol, 2% (w/v) Sodium Dodecyl Sulfate (SDS), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was centrifuged at 5000 g for 10 minutes at 4 °C, and the supernatant was collected for subsequent enzymatic assays. The total protein concentration was measured using the Lowry method to normalize the enzymatic activities (Assink Junior et al., 2023). Enzymatic activities for Superoxide Dismutase (SOD; ab65354) and Catalase (CAT; ab83464) were determined using commercial kits from Abcam, following the manufacturer's instructions and according to (Agliassa et al., 2021).

Statistical analysis

For the PM disease indices, biochemical data, and MultispeQ data, analysis of variance (ANOVA) was conducted using the SPSS software package (v. 23, SPSS Inc). For DM disease resistance analysis chi-squared test was calculated. The Tukey's HSD post hoc test was used when ANOVA showed significant differences at a probability level of $P \leq 0.05$. The standard deviation (SD) of all means was calculated.

Accession numbers

The GenBank accession numbers of the sequences reported in this paper are:

RNA-seq study: BioProject PRJNA1120233, BioSample SAMN41684953 to SAMN41684955, SRA SRR29288783 to SRR29288785.

ITS metabarcoding: BioProject PRJNA1119720, BioSample SAMN41663148 to SAMN41663150, SRA SRR29272512 to SRR29272514.

NGS amplicon on target genomic sequences: BioProject PRJNA1119700, BioSample SAMN41662708 to SAMN41662739, SRA SRR29272386 to SRR29272402.

All the other study data are included in the article and supporting information.

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Figure 6 has been created in BioRender (https://biorender.com/z80s157).

Conflict of Interests

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal participants.

Author contributions

L.N. and W.C. conceived and supported this study; L.N., W.C. and L.M. designed the experiments; L.M. performed research with assistance from G.M., I.B., G.G., I.P., C.P., C.B., A.S., E.Z., R.V., W.C. and L.N.; L.M., G.M., G.G., W.C. and L.N. analysed the data; L.M., G.M., I.B., W.C. and L.N. wrote the manuscript; and all authors reviewed and edited the manuscript.

Short legends for Supporting Information

Supplementary Figure 1 – Phylogenetic placement of VvMLO proteins.

Supplementary Figure 2 – Phylogenetic placement of VvNPR proteins.

Supplementary Figure 3 – Picture of representative plants.

Supplementary Figure 4 – Gel electrophoresis of the Cre and Cas PCR products.

Supplementary Figure 5 – Downregulated metabolic pathways as identified through RNAseq analysis.

Supplementary Figure 6 – Upregulated metabolic pathways as identified through RNAseq analysis.

Supplementary Figure 7 – Redox-active balance measured through UV/Vis determination.

Supplementary Figure 8 – Map of the modified pDIRECT_22C vector.

Supplementary Table S1. Differential abundances of taxa across edited lines.

Supplementary Table S2. Differential abundances of taxa across edited targets.

Supplementary Table S3. List of differentially expressed genes in MLO6-7 edited lines.

Supplementary Table S4. List of differentially expressed genes in NPR3-like edited lines.

Supplementary Table S5. List of differentially expressed genes in MLO6-7 vs NPR3-like edited lines.

Supplementary Table S6. Selected gRNAs.

Supplementary Table S7. Oligonucleotides.

Supplementary Data 1 – Alignment of MLO proteins.

Supplementary Data 2 – Alignment of NPR proteins.

Supplementary Data 3 – Vector sequence.

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CHAPTER 3 | Editing of *NPR3* rewires grapevine root immunity toward symbiosis

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Abstract

The capacity of plants to balance immune activation with the accommodation of beneficial microbes is critical for their survival and performance. In grapevine, the salicylic acid receptor NPR3 acts as a negative regulator of systemic defense, but whether its modulation also influences symbiotic interactions has remained unclear. To address this, we generated *NPR3*-edited lines and evaluated their ability to recruit the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis*. Our analysis revealed that edited roots showed a marked enhancement of arbuscule formation, a hallmark of functional symbiosis. This enhanced colonization was accompanied by transcriptional reprogramming of defense and cell wall remodeling pathways, as well as metabolic adjustments in stilbenes and flavonoids, creating a root environment favoring to fungal accommodation. Notably, the strong induction of the phosphate transporter *VvPT4*, together with increased fungal gene activity linked to nutrient exchange, confirmed the establishment of a more efficient symbiotic interface. These findings expand the functional scope of *NPR3* beyond immunity, uncovering its role as a regulator of the trade-off between defense and AM symbiosis.

Introduction

Plants, being sessile organisms, are constantly exposed to diverse environmental challenges, encompassing both abiotic and biotic stresses. Their adaptive responses are highly sophisticated and stimulus-specific, relying on complex signaling networks orchestrated by various phytohormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Li et al., 2019). These pathways are not independent but rather operate in a highly interconnected manner, enabling the fine-tuning of defense strategies to optimize survival and maintain fitness in fluctuating environments (Huot et al., 2014). A crucial aspect of these defenses involves interactions with microorganisms, which can be either beneficial or pathogenic. Upon pathogen recognition, plants activate defense responses through key phytohormones. Biotrophic and hemi-biotrophic pathogens, which require living host tissue, typically trigger SA-dependent defenses (Peng et al., 2021). SA is also heavily involved in the systemic acquired resistance (SAR), a plant immunity system which starts upon a localized pathogen infection and induces a broad-spectrum and long-lasting defense response throughout the plant (Vlot et al., 2021) (Klessig et al., 2018). Central to this process is the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES (NPR) protein family, which consists of numerous genes, classified into two classes with opposing functions (Backer et al., 2019). Among them, NPR1-like proteins functions as a master regulator of SAR (Zavaliev and Dong, 2024). Indeed, in response to increased SA levels, NPR1 undergoes conformational modifications, moves to the nucleus, and interacts with TGACG-binding factors (TGAs), thereby promoting the transcription of defense-related genes, including those encoding PATHOGENESIS-RELATED (PR) proteins like PR-1 (Kumar et al., 2022). Additionally, NPR1 contributes to cellular homeostasis and limits excessive cell death by forming SA-induced condensates (SINCs), which are involved in proteasomal regulation during effector-triggered immunity (ETI) (Zavaliev et al., 2020). Conversely, NPR3-like proteins act as negative regulators under non-stress conditions, repressing SA-responsive genes. They also regulate NPR1-like proteins stability, promoting their degradation through the proteasomes (Liu et al., 2020). Upon SA perception, the inhibitory effects of NPR3-like are relieved, facilitating the activation of SAR. Emerging models suggest that NPR3-like may independently function as co-repressors of defense responses. The critical roles of these NPR proteins have been highlighted through genetic studies. For instance, previous studies on *Arabidopsis, Theobroma cacao* and grapevine showed that knockout of *NPR3-like* triggers SAR enhancing defense systems against a broad spectrum of pathogens (Shi et al., 2013 ; Fister et al., 2018 ; Moffa et al., 2025), while *NPR1* silencing, as shown in *Catharanthus roseus,* disrupts defense gene activation, underscoring its essential role (Sung et al., 2019; Wang et al., 2020).

The interaction between plants and pathogenic microorganisms is only one side of the coin. Indeed, plants actively attract beneficial microorganisms to exploit the advantages of symbiosis, which are widely recognized within the concept of ecological services. However, the domestication process has often reduced plants' capacity to associate with beneficial microbes, a phenomenon referred to as "domestication syndrome" (Nerva et al., 2022). Among other beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) are widely recognized for their ability to enhance plant resilience aginst biotic and abiotic stresses (Balestrini et al., 2024). AMF account for approximately 50,000 species across multiple phyla, forming symbiotic relationships with plants (Genre et al., 2020). AMF, belonging to the phylum *Glomeromycota* are highly widespread, forming symbiotic associations with over 80% of plant species. They develop arbuscules within the root cortex, a specialized structures essential for nutrient exchange, through which they supply plants with phosphorus and nitrogen in return for photosynthetically derived carbon (Bonfante and Genre, 2010; Berruti et al., 2016; Balestrini and Lumini, 2018; Balestrini et al., 2020). Beyond nutrient acquisition, AMFs also enhance plant tolerance to environmental stresses like drought, salinity, and metal toxicity (Chitarra et al., 2016; Dastogeer et al., 2020; Riaz et al., 2021; Romero-Munar et al., 2024). The plant-AMF symbiosis is tiny regulated by both partners, with fungi modulating plant immunity to enable colonization and triggering mycorrhiza-induced resistance (MIR) (Cameron et al., 2013). On the other side plants use innate immunity to detect microbe-associated molecular patterns (MAMPs) and activate defense mechanisms. Plants detect diverse MAMPs, including peptides and carbohydrates such as chitin, whose activity depends on chain length and acetylation (Sánchez-Vallet et al., 2015). In arbuscular mycorrhizal fungi (AMF), Myc factors like short-chain chito-oligosaccharides and lipo-chito-oligosaccharides trigger symbiosis by inducing Ca^{2+} influx(Berger and Gutjahr, 2021; Fiorilli et al., 2024) (Barker et al., 2017). AMF also secrete effectors that suppress host immunity and facilitate colonization (Lo Presti et al., 2015). During early colonization, defense responses remain mild, reflecting AMF's limited repertoire of cell wall-degrading enzymes and broad effector arsenal (Fiorilli et al., 2024). Nevertheless, during the early stages of AM colonization, plants show a transient upregulation of defense-related genes although this response is weaker than that induced by pathogens. Hormones modulate this balance: SA shows a biphasic response (Benjamin et al., 2022; Herrera Medina, 2003). while JA levels increase, enhancing protection against herbivores and necrotrofic pathogens (Kadam et al., 2020).

To assess how immune modulation influences symbiosis in *Vitis vinifera*, we generated *VvNPR3* knockout lines via CRISPR/Cas9. Since *NPR3* regulates defense responses and stilbene biosynthesis in grapevine (Moffa et al., 2025) , we investigated whether its loss affected interactions with the AMF *Rhizophagus irregularis*.

Materials and Methods

Embryogenic calli induction and maintenance

Flower clusters of *Vitis vinifera* cv. Chardonnay were collected from a vineyard operated by Vivai Cooperativi Rauscedo – VCR, in Rauscedo (Pordenone, Italy), between late April and early May 2020. Only the basal half of the inflorescence was harvested. After sterilization with a sodium hypochlorite solution 33% containing 100 µL L^{-1} Tween 20, ovaries and anthers were excised and cultured on PIV starter callus induction medium (2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, 0.3% Gelrite, 4.5 µM 2,4-Dichlorophenoxyacetic acid (2,4 D), and 8.9 µM 6-Benzylaminopurine (BAP), pH of 5.8) (Gambino et al., 2007). After three months, the embryogenic calli were transferred to C1 proliferation medium (2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, 0.5% Gelrite, 5 µM 2,4 D, and 1 µM BAP, pH of 5.8) and subcultured at monthly intervals as previously reported (Moffa et al., 2025).

Vector Construction and Callus Transformation

To generate the *VvNPR3* gene knockout, a modified pDIRECT vector was used as previously described in Moffa et al., (2025). Briefly, the gRNA targeting *VvNPR3* (Vitvi05_01chr10g23050) was designed using CRISPR-P) (Lei et al., 2014), tested for off-target with CRISPOR (Concordet and Haeussler, 2018), and cloned into the pDIRECT vector following the protocols previously described (Čermák et al., 2017). Transformation of the embryogenic calli was carried out using *Agrobacterium tumefaciens* strain GV3101 (Kumar et al., 2019). *A. tumefaciens* was cultured on Luria broth (LB) agar, transferred to MG/L medium (5.0 g l^{-1} mannitol, 1.0 g l^{-1} glutamate, 5.0 g l^{-1} tryptone, 2.5 g l^{-1} yeast extract, 5.0 g l^{-1} NaCl, 0.15 g l^{-1} KH_2PO_4, 0.10 g l^{-1} $MgSO_4 \cdot 7H_2O$, 20 ug l^{-1} biotin, pH 7) and cultured until reaching an OD_{600} of 0.8 (Amarowicz et al., 2008.; Li et al., 2008) and then resuspended in LCM medium (2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, pH 5.8) with 100 µM acetosyringone for a 3-hour incubation at 28 °C and 210 rpm. The calli were co-cultivated with *A. tumefaciens* in LCM medium containing 600 mg L^{-1} cefotaxime (Duchefa Biochemie) to eliminate excess bacteria. Following co-cultivation, calli were dried, transferred to GS1CA medium (N 2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, 10 µmol napthoxyacetic acid, 1.0 µmol 6-Benzylaminopurine (BAP), 20 µmol Indole-acetic acid (IAA), pH 6.2 with 0.25% activated charcoal, pH 5.8), and maintained at 26 °C for 9 days (Franks et al., 1998; Gambino et al., 2007). Then calli were subcultured monthly in the presence of 450 mg L^{-1} of cefotaxime and 100 mg L^{-1} of kanamycin (Duchefa Biochemie) for 4 months and subsequently transferred to medium containing only 100 mg L^{-1} of kanamycin. Embryos at the torpedo stage were transferred to Murashige and Skoog (MS) medium supplemented with 10

µM BAP. After one months, embryos were transferred onto half-strength MS basal salts at 26 °C, 200 µmol m^{-2} s^{-1} (Photosynthetic Photon Flux Density, PPFD), and a 16/8-hour day/night photoperiod. This procedure resulted in the generation of several edited lines. In parallel, still starting from callus, we also regenerated wild-type lines.(http://crispr.hzau.edu.cn/CRISPR2/) (Lei et al., 2014), tested for off-target with CRISPOR (Concordet and Haeussler, 2018), and cloned into the pDIRECT vector following the protocols previously described (Čermák et al., 2017). Transformation of the embryogenic calli was carried out using *Agrobacterium tumefaciens* strain GV3101 (Kumar et al., 2019). *A. tumefaciens* was cultured on Luria broth (LB) agar, transferred to MG/L medium (5.0 g l^{-1} mannitol, 1.0 g l^{-1} glutamate, 5.0 g l^{-1} tryptone, 2.5 g l^{-1} yeast extract, 5.0 g l^{-1} NaCl, 0.15 g l^{-1} KH_2PO_4, 0.10 g l^{-1} $MgSO_4 \cdot 7H_2O$, 20 ug l^{-1} biotin, pH 7) and cultured until reaching an OD_{600} of 0.8 (Amarowicz et al., 2008.; Li et al., 2008) and then resuspended in LCM medium (2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, pH 5.8) with 100 µM acetosyringone for a 3-hour incubation at 28 °C and 210 rpm. The calli were co-cultivated with *A. tumefaciens* in LCM medium containing 600 mg L^{-1} cefotaxime (Duchefa Biochemie) to eliminate excess bacteria. Following co-cultivation, calli were dried, transferred to GS1CA medium (N 2,07 g l^{-1} Nitsch and Nitsch basal salt, 0,103 g l^{-1} Murashige and Skooge vitamins, 6% sucrose, 10 µmol napthoxyacetic acid, 1.0 µmol 6-Benzylaminopurine (BAP), 20 µmol Indole-acetic acid (IAA), pH 6.2 with 0.25% activated charcoal, pH 5.8), and maintained at 26 °C for 9 days (Franks et al., 1998; Gambino et al., 2007). Then calli were subcultured monthly in the presence of 450 mg L^{-1} of cefotaxime and 100 mg L^{-1} of kanamycin (Duchefa Biochemie) for 4 months and subsequently transferred to medium containing only 100 mg L^{-1} of kanamycin. Embryos at the torpedo stage were transferred to Murashige and Skoog (MS) medium supplemented with 10 µM BAP. After one months, embryos were transferred onto half-strength MS basal salts at 26 °C, 200 µmol m^{-2} s^{-1} (Photosynthetic Photon Flux Density, PPFD), and a 16/8-hour day/night photoperiod. This procedure resulted in the generation of several edited lines. In parallel, still starting from callus, we also regenerated wild-type lines.

Editing efficiency analysis

Genomic DNA was extracted from individual leaves using the Genomic DNA Isolation Kit (NORGEN, Norgen Biotech Corp) and following manufacture's protocol. The isolated DNA was then quantified using a NanoDrop One Spectrophotometer (ThermoFisher) and diluted to 10 ng μL^{-1} for PCR amplification with Cas9-specific primers (Table S1). Editing efficiency was initially assessed by Sanger sequencing (BioFab Research srl, Milan, Italy) and analyzed using TIDE software (https://tide.nki.nl/) (Brinkman et al., 2014). For high-throughput sequencing, the target regions of *VvNPR3* were amplified from greenhouse-acclimatized plants, and sequencing libraries were

prepared with Illumina adapters. Sequencing was performed on the Illumina NovaSeq 6000 platform, generating approximately 100,000 reads per sample. Data were analyzed using the CRISPResso pipeline (Clement et al., 2019).

In vitro *explants multiplication and inoculation of AMF spores*

Two edited lines, selected for their high editing efficiency (see results section), and a WT line were propagated by nodal cuttings. Each node was cultured on half-strength MS basal salts supplemented with 4 µM BAP for one month to induce shoot proliferation. Then, in order to promote root formation, nodal explants were transferred to MS medium supplemented with 2.5 µM NAA and 2.5 µM IBA for 4 days. The esulting explants were transferred to sterilized peat pellets, and upon root emergence, plants were aseptically inoculated with 80 spores per plant of the AMF *R. irregularis* following the manufacturer's instruction (MycAgro Lab). Two weeks after inoculation, both inoculated and uninoculated plants (Myc and NoMyc, respectively) were acclimatized and moved in a partially climate-controlled greenhouse, with natural light and photoperiod conditions. In summary, plants were arranged into six groups of 12 individuals each: WT NoMyc, NPR3#15 NoMyc, NPR3#28 NoMyc, WT Myc, NPR3#15 Myc, and NPR3#28 Myc. At the time of sampling, each group was further divided into three pools, and each pool was subjected to different analyses.

Assessment of root AMF colonization and sampling of root tissues

At the end of the experiment, after 45 days from inoculation, root samples were simultaneously collected. For Myc samples, a portion of the roots from at least three randomly selected WT and edited lines plants were stained with cotton blue to assess mycorrhizal colonization according to Trouvelot et al. (1986). Three parameters were considerd: (i) frequency of mycorrhiza in the root system (F%); (ii) intensity of mycorrhizal colonization (M%); and (iii) arbuscule abundance (A%). The remaining part of the same samples were immediately freeze dried and stored at −80 °C until molecular and biochemical analyses.

RNA-Seq and Data Analysis

Total RNA was isolated from lyophilized roots using the Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. RNA concentrations were evaluated using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was confirmed on an 1% agarose gel. For RNA-seq analysis, at least 2 µg of RNA from each sample was used for cDNA library construction and sequencing using the NovaSeq platform (Macrogen, Inc. Europe). Transcript analysis was performed using the Artificial Intelligence RNA-seq Software AIR (accessible at https://transcriptomics.cloud). Transcripts annotation,

functional classification, GO enrichment analysis, and identification of differentially expressed genes (DEGs) were performed as previously described (Moffa et al., 2025).

Quantitative real time PCR analysis on target R. irregularis genes

The same RNA from Myc root samples used for RNA sequencing was also used as template to synthesize cDNA employing the SensiFAST cDNA Synthesis Kit (Meridian Life Science, Memphis, TN, USA) starting from 300 ng of total RNA (Nerva et al., 2022). Quantitative PCR (qPCR) were performed using SYBR® Green (Bio-Rad Laboratories Inc., Hercules, CA, USA) as previously described by Chitarra et al (2018). qPCR reactions were carried out in a final volume of 10 µL using 1:5 diluted cDNA as template in the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each reaction was conducted in at least three technical replicates. Relative gene expression was quantified using the ΔΔCt method normalizing against the Elongation factor (*RiEFα*) for fungal genes, as previously described by Nerva et al. (2022). Expression levels were calculated as as expression ratio (Relative Quantity) to WT_Myc samples. Primer sequences used in this study are provided in Table S1.

HPLC-DAD-MS/MS analysis

Bioactive metabolites were extracted from lyophilized roots using 1:10 (w/v) ratio and 90% (v/v) as extraction solvent, as previously described (Moffa et al, 2025). The chromatographic analysis was carried out using an Agilent 1200 HPLC system equipped with a diode array detector (DAD) and coupled to an Agilent 6330 Series Ion Trap LC-MS/MS. Separation was achieved on a reverse-phase Luna C18 column (Phenomenex; 3.00 µm particle size, 150 mm × 3.0 mm I.D.), maintained at 25 °C within an Agilent 1100 HPLC G1316A column compartment, operating at a constant flow rate of 0.2 mL min^{-1}. Instrumental conditions, including chromatographic and MS settings, were optimized to ensure precise identification and quantification of the target compounds, following the methodology previously reported by Moffa et al. (2025).

Statistical Analysis

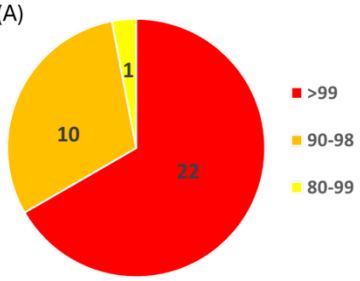
For the MultispeQ, qPCR and root colonization data, analysis of variance (ANOVA) was performed using SPSS software (v. 23, SPSS Inc). The Tukey's HSD post-hoc test was applied when ANOVA revealed significant differences at a probability level of $p \leq 0.05$. The standard deviation (SD) of all means was calculated.

Results

Generation and selection of NPR3-*edited lines*

As previously reported (Moffa et al., 2025), *Agrobacterium*-mediated transformation of *Vitis vinifera* cv Chardonnay resulted in the generation of 129 somatic embryos. From these, 48 plants were successfully regenerated from embryos, which 33 tested for Cas9, confirming successfully transformation. Among them, 15 plants exhibited targeted editing, as determined by Sanger sequencing and analysis of editing efficiency. Subsequent high-throughput sequencing of gRNA *VvNPR3g2* target region showed editing frequencies exceeding 90%. Consequently, for the present study, two lines, specifically NPR3#15 and NPR3#28, were selected based on their high editing efficiency (>99%) (Figure 1).

(A)



(B)

NPR2g2		Mutation	%	Mutation	%
#15	Heterozygous	-37 bp	72,04	-14 bp	19,31
#28	Heterozygous	-4 bp	44,98	-1 bp	44,8

Figure 1. **Characterization of Gene Editing Outcomes at the VvNPR3g2 Target Site**. (A) Distribution of editing outcomes among transgenic plants edited using the guide RNA NPR2g2. The numbers within the pie chart indicate the number of plants corresponding to each editing category; (B) Detailed editing profile of the two selected lines (NPR3#15 and NPR3#28).

Root Colonization by R. irregularis *in WT and* NPR3-*edited lines*

According to the Trouvelot method (Fig. 3), Myc edited lines showed higher F% values (NPR3#15 = 14.4%; NPR3#28 = 19.4%) compared to WT (10.3%), although with no significant differences (Fig. 2A). A similar trend was observed for M%, with increased values in NPR3#15 (4.8%) and NPR3#28 (6.8%) respect to WT (2.3%), again without statistical differences (Fig. 2B). Conversely, A% was significantly ($p < 0.01$) higher in both the edited lines compared to WT (Fig. 2C).

No traces of AMF were detected in any of the NoMyc plants, either in WT or edited lines, confirming the absence of background AMF contamination in the growth substrate (Fig. 2F).

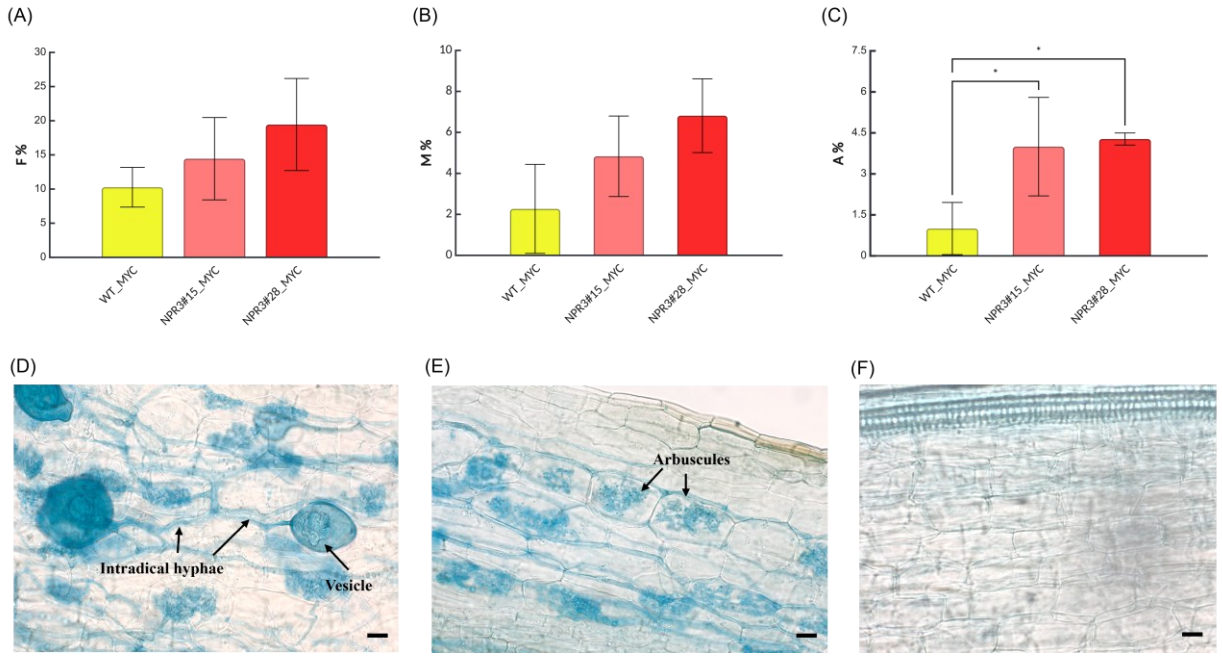


Figure 2. Evaluation of Arbuscular Mycorrhizal Colonization in Root. (A-C) Quantification of root colonization parameters using the Trouvelot method: (A) frequency of mycorrhiza in the root system (F%); (B) intensity of mycorrhizal colonization (M%); and (C) arbuscule abundance (A%). Statistically significant differences among groups, determined by one-way ANOVA, are indicated by asterisks. (D, E) Representative magnified view of R. irregularis-colonized roots of an edited line, showing characteristic AM fungal structures. (F) Root without R. irregularis *inoculation*. The bars stand for 20 µm.

Root transcriptomic reprogramming in NoMyc edited lines

Root transcriptomes were analyzed to investigate the impact of *NPR3* gene editing and AM colonization on global gene expression patterns. In NoMyc plants, *NPR3*-edited lines showed 693 differentially expressed genes (FC > 1) compared to WT_NoMyc plants (Table S2). Of these, 249 genes were upregulated, many of which were associated with cell wall remodelling, primary metabolism, and symbiotic signalling (Fig. 3A). In contrast, 444 genes were downregulated, with a significant enrichment of genes associated with inorganic anion transport and metabolite transmembrane transport, and inositol phosphate metabolism (Fig. 3B). Among the upregulated genes, several were involved in cell wall restructuring, including cellulose synthases (*VvCSLE1*, *VvCSLG3*), *pectinesterases* (*VvPME1*), and xyloglucan endotransglucosylase/hydrolases (*VvXTH23*, *VvXTH16*). Additionally, several genes related to carbohydrate catabolism were also upregulated, including alpha-amylase, pullulanase, beta-1,3-glucanase, alpha-glucan phosphorylases, and members of the galactosidase and mannosidase families. Additionally, components associated with

calcium signalling and symbiotic competence, such as calmodulin and the symbiosis-related kinase *SYMRK*, were upregulated. Among the downregulated genes, several were involved in diverse metabolic processes. In particular, genes associated with the inositol phosphate metabolic process, such as Inositol polyphosphate-related phosphatase (VIT_01s0011g06310), Inositol 1,3,4-trisphosphate 5/6-kinase (VIT_18s0001g08310), and 1-phosphatidylinositol-3-phosphate 5-kinase (VIT_11s0016g00360), were consistently repressed.

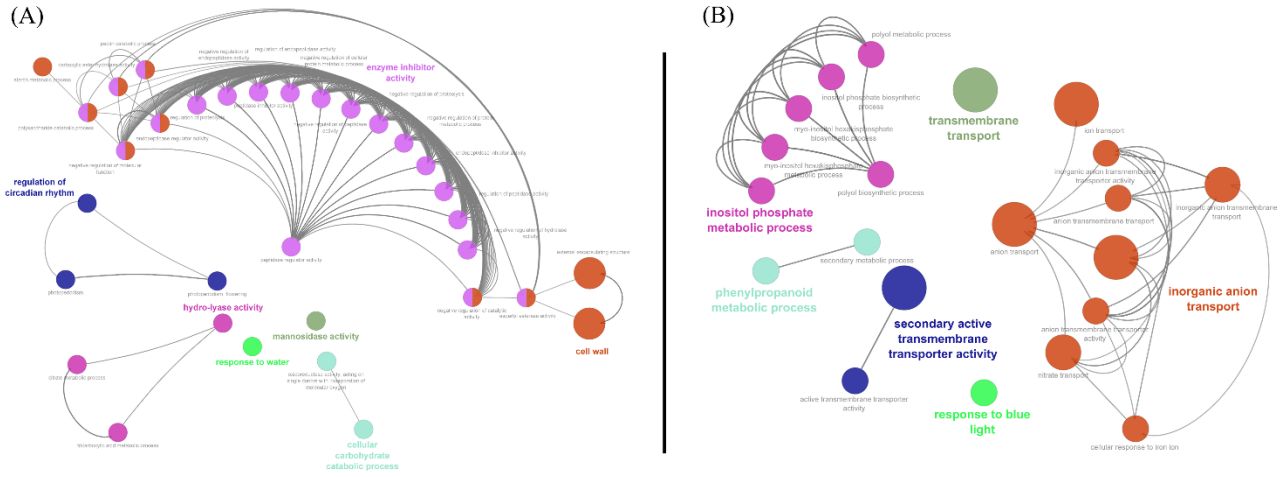


Figure 3. Functional enrichment of differentially expressed genes in non-mycorrhizal plants. Network representation of enriched GO terms among genes (A) upregulated and (B) downregulated in NPR3-edited lines NoMyc compared with WT NoMyc. Colors indicate clusters of functionally related terms.

Still, among the top 10 upregulated genes (Table 1) in the NoMyc edited lines compared to the WT_NoMyc, several were associated with cell wall remodelling, including cellulose synthase, cinnamyl alcohol dehydrogenase and endoglucanase. Others were related to the development regulation (e.g., *VvPIN8*, *VvCOL14* and *VvELF4*). Additionally, genes involved in secondary metabolism, including *ARR1* typeB, Isochorismatase hydrolase, 2-hydroxyacyl-CoA lyase 1 and Galactinol-raffinose galactosyltransferase were also upregulated. Conversely, among the top 10 downregulated genes in the edited lines, were found genes involved in transport and genes involved with microorganism interaction (e.g., the organic cation transporter 1 (*VvOCT1*), Nodulin *MtN21*, Nodulin *MtN3* and Subtilisin serine endopeptidase (*VvXSP1*); genes related to secondary metabolism, UDP-glucose glucosyltransferase and Reticuline oxidase; signal transduction components, including receptor serine/threonine kinase and Chloroplast nucleoid DNA-binding protein; genes involved in protein metabolism, such as Subtilisin serine protease; and stress response, such as *VvACI112*.

Table 1. Top differentially expressed genes (DEGs) in non-mycorrhizal NPR3-edited plants. The ten most strongly upregulated and downregulated genes were selected for each edited line based on log_2 fold change relative to the WT. For each gene, functional annotation and functional class are reported.

Gene ID	logFC_N#15	logFC_N#28	Functional annotation	Functional class
Top upregulated genes				
VIT_00s0469g00020	4,25	3,87	Cellulose synthase CSLE1	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_06s0004g05120	2,89	4,22	ARR1 typeB	HORMONE METABOLISM & SIGNALLING
VIT_19s0015g01540	3,36	3,15	2-hydroxyacyl-CoA lyase 1	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_03s0038g00360	1,90	2,78	Isochorismatase hydrolase	SECONDARY METABOLISM
VIT_13s0067g00620	2,22	2,47	Cinnamyl alcohol dehydrogenase	SECONDARY METABOLISM
VIT_05s0077g00840	2,55	2,00	Galactinol-raffinose galactosyltransferase	CARBOHYDRATE METABOLISM
VIT_13s0067g00860	1,63	2,66	Early flowering 4	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_14s0068g01380	1,81	2,55	Constans-like 14	DEVELOPMENT
VIT_15s0046g03010	2,31	2,08	Barwin endoglucanase	RESPONSE TO STRESS
VIT_14s0108g00020	2,24	2,08	PIN8 (PIN-formed 8); auxin:hydrogen symporter/ transporter	HORMONE METABOLISM & SIGNALLING
Top downregulated genes				
VIT_00s0425g00030	-7,55	-7,62	Receptor serine/threonine kinase	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_12s0034g00040	-5,81	-5,58	UDP-glucose glucosyltransferase	CARBOHYDRATE METABOLISM
VIT_10s0003g05570	-4,91	-6,46	Reticuline oxidase	SECONDARY METABOLISM
VIT_04s0008g07080	-4,99	-6,02	Chloroplast nucleoid DNA-binding protein	NUCLEIC ACID METABOLISM & REGULATION
VIT_17s0119g00080	-5,49	-4,87	Organic cation transport protein OCT1	TRANSPORT
VIT_15s0048g01180	-4,53	-4,15	Subtilisin serine protease	PROTEIN METABOLISM
VIT_15s0048g01200	-3,79	-5,15	Subtilisin serine endopeptidase (XSP1)	TRANSPORT
VIT_01s0026g00550	-3,64	-5,28	Nodulin MtN21 family	TRANSPORT
VIT_13s0084g00310	-3,36	-6,17	ACI112	RESPONSE TO STRESS
VIT_16s0050g02540	-3,83	-4,19	Nodulin MtN3 family	DEVELOPMENT

Transcriptomic changes in root responses to Myc colonization

Overall, 626 genes were differentially expressed (FC > 1) in *NPR3*_Myc lines compared to WT_Myc plants, with 346 genes downregulated and 280 upregulated (Table S3). Upregulated genes were enriched in categories related to cell wall modification, including cellulose synthase, pectinacetylesterase, xyloglucan endotransglucosylase/hydrolases, β-1,3-glucanases, expansin β 2, and extensin, supporting active cell wall remodelling. Proton pumps and associated transporters, such as H^+-ATPase, ABC transporters, and MATE efflux proteins, were also induced, reflecting enhanced ATP-dependent transmembrane activity likely linked to ion and metabolite transport under symbiotic or stress-related conditions (Fig. 4A). In addition, multiple proteolytic regulators were upregulated, including serine-type peptidases (subtilisins, serine carboxypeptidases, cysteine proteases) and their inhibitors (α-amylase/subtilisin inhibitors and Kunitz-type inhibitors), consistent with a tightly regulated proteolytic environment. Conversely, downregulated genes were mainly associated with nutrient transport and secondary metabolism (Fig. 4B). Notably, nitrate transporters (*VvNTP3*, lysine/histidine transporters, amino acid permeases) and transition metal ion transporters (copper transporter 2, zinc-binding proteins, metallothioneins) showed reduced expression, suggesting global suppression of inorganic ion uptake. In parallel, genes involved in phenolic compound biosynthesis, including caffeoyl-CoA O-methyltransferase, caffeic acid O-methyltransferase, and sinapyl alcohol dehydrogenase, were repressed, indicating a decrease in coumarin metabolism and phenylpropanoid-derived defense metabolites such as stilbenes (resveratrol, viniferin) and lignin monomers (e.g., sinapyl alcohol). These changes point to a reprogramming of both structural and metabolic pathways,

balancing enhanced wall remodelling and transport activity with reduced secondary metabolite biosynthesis.

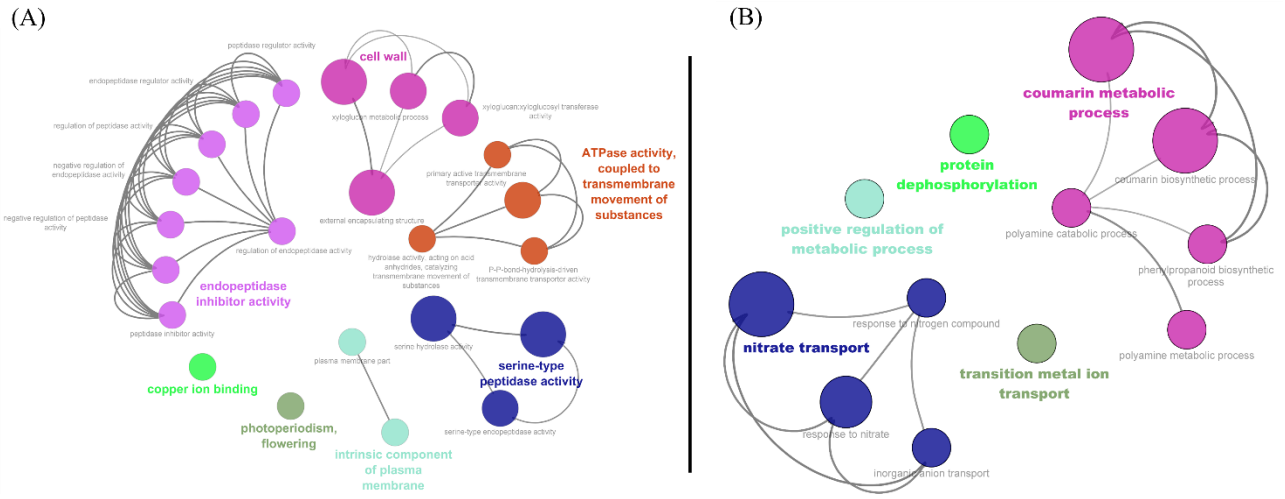


Figure 4 Functional enrichment of differentially expressed genes in mycorrhizal plants. Network representation of enriched GO terms among genes (A) upregulated and (B) downregulated in NPR3-edited lines Myc compared with WT Myc. Colors indicate clusters of functionally related terms.

Among the top regulated genes in Myc plants (Table 2), those upregulated in the edited lines included genes associated with plant development, such as *VvMYB* family transcription factors and *VvEF*-hand proteins ; nutrient transport, including the inorganic phosphate transporter *VvPT4* and a subtilisin protease; cell wall biogenesis and remodeling, such as *VvCSLG3* coding for a cellulose synthase, a lyase, and an acyltransferase; and protein metabolism, including subtilase, cysteine proteinase, and prolyl carboxypeptidase. Conversely, the top downregulated genes comprised regulators of development and light signalling, such as *Constans 2*, *SPA1-related 3* and Zinc finger (B-box type), genes related to microorganism interaction and transport, such as the organic cation transporter 1 (*VvOCT1*), Nodulin *MtN3* family and the translocon at the inner chloroplast envelope 55 (*VvTic55*), in energy metabolism such as *VvLHCA3* and secondary metabolism, including UDP-glucose glucosyltransferase and alanine-related enzyme.

Table 2. Top differentially expressed genes (DEGs) in NPR3-edited lines colonized by R. irregularis. For each edited line, the ten most upregulated and ten most downregulated genes were selected according to log_2 fold change relative to WT. Functional annotation and functional class are reported.

Gene ID	logFC_N#15	logFC_N#28	Functional annotation	Functional class
Top upregulated genes				
VIT_10s0116g01620	3,90	3,43	Lyase	UNKNOWN
VIT_18s0001g10340	2,92	2,67	Subtilisin protease	TRANSPORT
VIT_09s0070g00410	2,85	2,51	Myb family	TRANSCRIPTION FACTORS
VIT_16s0050g02380	3,15	1,95	Inorganic phosphate transporter PT4	TRANSPORT
VIT_12s0059g00340	2,61	2,66	EF hand	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_02s0025g01870	2,69	2,52	Cellulose synthase CSLG3	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_18s0001g10320	3,07	1,84	Subtilase	PROTEIN METABOLISM
VIT_12s0028g02290	2,90	2,18	Cysteine proteinase	PROTEIN METABOLISM
VIT_01s0026g01210	2,96	1,76	Acyltransferase	UNKNOWN
VIT_06s0061g00960	2,73	2,02	Prolylcarboxypeptidase	PROTEIN METABOLISM
Top downregulated genes				
VIT_16s0050g02540	-2,27	-4,15	Nodulin MtN3 family	DEVELOPMENT
VIT_14s0083g00640	-2,10	-3,59	Constans 2 (COL2)	DEVELOPMENT
VIT_12s0028g03570	-2,42	-2,90	SPA3 (SPA1-related 3)	PROTEIN METABOLISM
VIT_15s0024g00040	-2,20	-2,99	LHCA3 (Photosystem I light harvesting complex gene 3)	ENERGY METABOLISM
VIT_12s0034g00040	-1,65	-5,19	UDP-glucose glucosyltransferase	CARBOHYDRATE METABOLISM
VIT_08s0058g00930	-2,08	-3,09	Alanine--glyoxylate aminotransferase 2 3, mitochondrial	AMINO ACID METABOLISM
VIT_05s0020g00420	-1,75	-3,78	Polygalacturonase GH28	CELLULAR PROCESSES, SIGNALLING & HOMEOSTASIS
VIT_04s0008g07020	-1,85	-3,37	Tic55 (translocon at the inner envelope membrane of chloroplasts 55)	TRANSPORT
VIT_17s0119g00080	-1,53	-5,20	Organic cation transport protein OCT1	TRANSPORT
VIT_05s0102g00750	-1,80	-3,23	Zinc finger (B-box type)	TRANSCRIPTION FACTORS

Analysis of R. irregularis *target genes in Myc plants*

qRT–PCR assays targeted key *R. irregularis* genes associated with different stages of colonization, including a high-affinity phosphate transporter (*GintPT*), a crinkler effector (*RiCRN1*), a "preferentially expressed in planta" gene (*RiPEIP1*), a dipeptide transporter (*RiPTR2*), a monosaccharide transporter (*RiMST2*), and a member of the 14-3-3 protein family (*Ri14-3-3*). Overall, all genes displayed a similar expression pattern, with higher transcript levels in both edited lines compared to the WT (Fig. 5). In particular, *GintPT* was significantly upregulated in the edited lines, showing a 3.4-fold and 4.4-fold increase in NPR3#15 and NPR3#28, respectively, relative to the WT_Myc samples. Likewise, *Ri14-3-3* was also significantly upregulated in edited lines compared to WT_Myc plants. Although not statistically significant, *RiCRN1*, *RiPEIP1*, *RiPTR2*, and *RiMST2* consistently showed a trend toward higher expression in the edited lines relative to the WT.

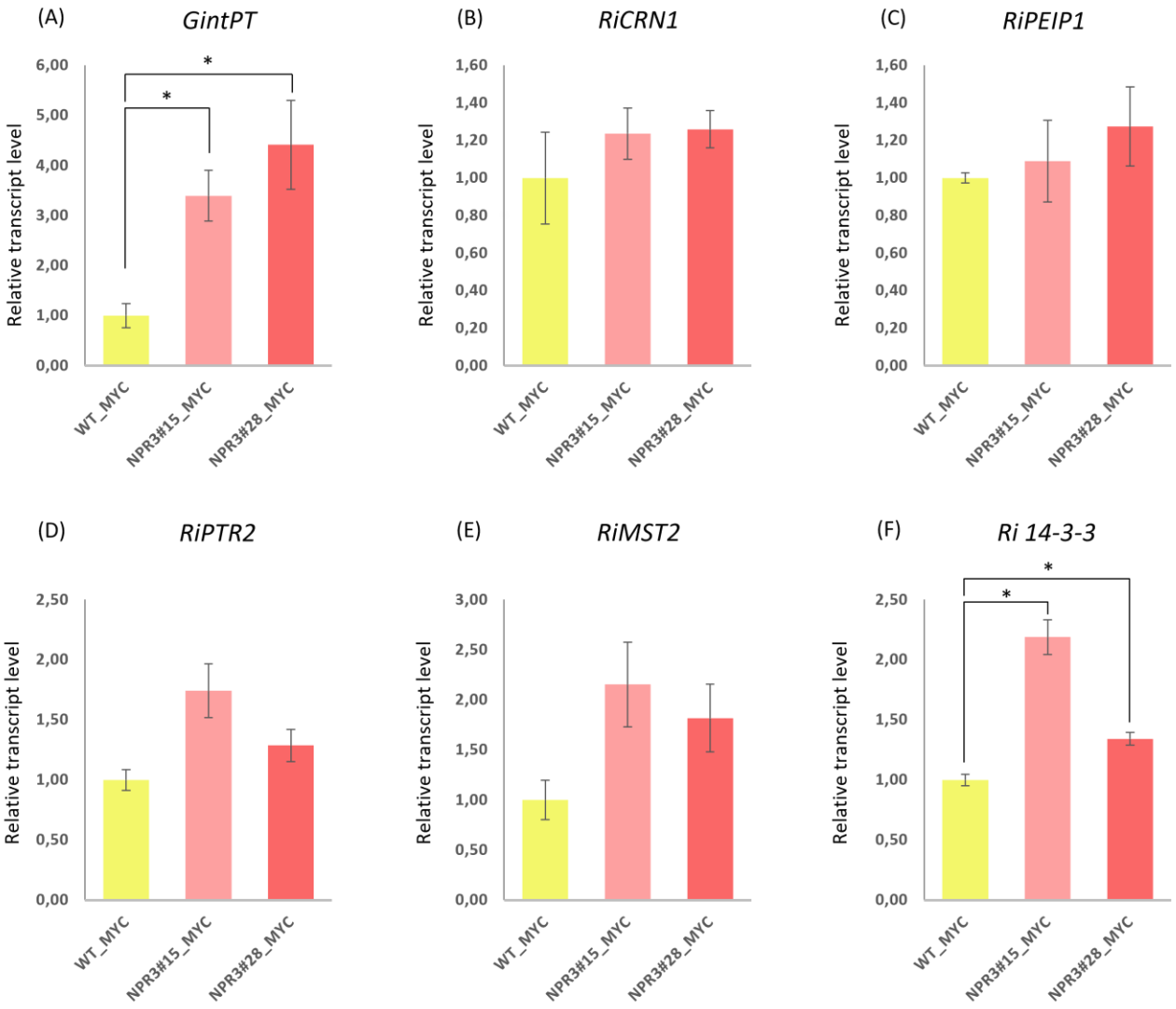


Figure 5. Expression analysis of R. irregularis target genes in roots: RT-qPCR analyses for *GintPT*, *RiCRN1*, *RiPEIP1*, *RiPTR2*, *RiMST2*, and *Ri14-3-3* transcripts (A-F) in WT_Myc and NPR3-edited lines (NPR3#15_Myc and NPR3#28_Myc) after inoculation. *Elongation factor 1α* (*EF-1α*) was used as endogenous control gene for normalization. Asterisks indicate statistically significant differences ($P < 0.05$) according to one-way ANOVA followed by Tukey's test; bars represent standard errors of the mean (n=3).

HPLC analysis of metabolites in MYC and NPR_MYC

HPLC analysis of target metabolites revealed distinct differences between WT and NPR samples, also considering Myc inoculation (Fig. 5A-C). In particular, fold change analysis clearly showed that phenolic compounds and viniferin derivatives undergo the most marked variations between the control samples and the NPR samples (Fig. 5A). In this context, dPACA, VinE, and VinD showed the highest log2(FC) values, indicating a significant accumulation following NPR3 genome modification. These are followed by dPACB, tPACB, catechin and, with more moderate but still

significant variations, resveratrol. Heatmap analysis visualization with cluster analysis showed marked percentage variations between the control and NPR3#15_NoMyc and NPR3#28_NoMyc samples. Catechin increases by 30–40% compared to the control, while proanthocyanidins show even more consistent increases: dPACA grows by over 250% in NPR3#5_NoMyc and by about 300% in NPR3#28_NoMyc, dPACB increased by 10–20%, and tPACB reaches increases close to 40%. In contrast, resveratrol had positive average variations of about 10%. In viniferins, VinD doubles in levels from about 16–18 to over 30 (+90–110%), while VinE grows more modestly, with increases between 20 and 40% (Fig. 5B). On the other hand, the hormonal profile showed different trends. Particularly, SA and ABA were high in the control, partially increased in NPR3#15_NoMyc and NPR3#28_NoMyc. Gibberellins tend to decrease, while JA remains high, peaking in NPR samples. Zeatin remains stable, while IAA shows strong variability without a clear trend. Of particular note is the consistent increase in brassinosteroid in both NPR3 conditions (Fig. 5B). Overall, these specific changes enabled a clear separation of NPR samples from CTRL in the orthogonal PCA model, with NPR3#15_NoMyc and NPR3#28_NoMyc segregating from their respective controls along positive T scores (Fig. 5C).

Regarding Myc samples, HPLC analysis of target metabolites revealed significant differences also between Myc and NPR3_Myc (Fig. 5D-F). Fold change analysis clearly showed that Cat, ABA and zeatin showed consistent changes for positive Log2(FC), while SA, brassinolide, resveratrol, IAA and viniferin D for negative log2(FC) (Fig 5C). Specifically, catechin increases significantly in NPR3_Myc samples compared to simply Myc, rising approximately of 30–50%. Conversely, dPACA had a reduction of 10–25%, while dPACB showed moderate variations, with an average increase of 10–15%. On the other hand, resveratrol, VinD and VinE showed a decrease in NPR3_Myc (Fig 5E). These specific changes in metabolites and hormones allowed NPR3_Myc samples to be clearly distinguished from Myc in orthogonal PCA models, with modified samples segregating along the principal axes (Fig 5F).

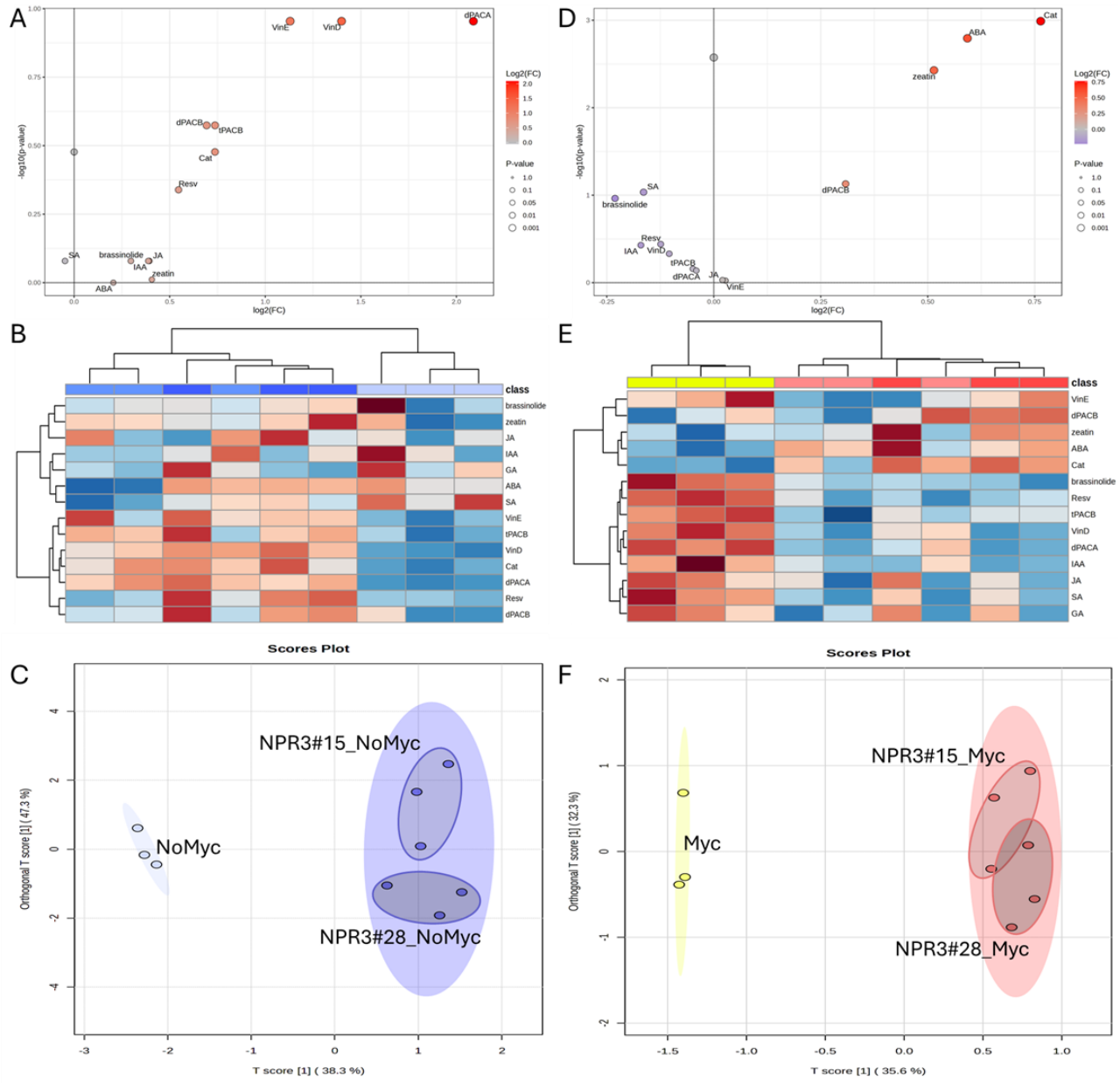


Figure 6. Multivariate analysis of differentially accumulated metabolites in NoMyc and inoculated with Myc. (A, D) Volcano plot of metabolites and target hormones in NPR_NoMyc (A) and NPR_Myc (D) samples compared to their respective controls (NoMyc or MYC). The x-axis represents the log2(fold change) of metabolites normalized to the mean of the controls, while the y-axis indicates the −log10(p-value). Differences between groups were evaluated using one-way ANOVA with FDR correction. (B, E) Heatmap with hierarchical clustering analysis of normalized HPLC data (log2 transformed and scaled according to Pareto rotation), highlighting distinctive patterns of accumulation of the analyzed compounds between NPR_NoMyc and NoMyc samples (B) or NPR_Myc and Myc samples (E). (C, F) Orthogonal PCA of analyzed metabolites in NPR_NoMyc and their respective NoMYC controls (E) or NPR_Myc and their respective Myc controls (**F**).

Discussion

Given the central role of roots as the interface between plants and the soil microbiome, we explored whether *VvNPR3* editing could reshape the symbiotic dialogue with AMF, key mutualists driving nutrient acquisition and plant resilience (Balestrini et al., 2018). Here, we compared WT and *NPR3*-edited lines through an integrative approach combining transcriptome profiling, target metabolomics and fungal colonization features. The results revealed a coordinated reprogramming of gene expression in the edited lines, paralleled by a striking stimulation of *R. irregularis* arbuscule development. This enhanced formation of symbiotic structures suggests that NPR3 may act as a regulatory node at the crossroads of immunity and mutualism, with its removal tilting the balance toward a more functional AM symbiosis.

Editing of NPR3 *remodels root defense and cell wall biogenesis without* PR *gene induction*

RNA-seq analysis revealed that the editing of *NPR3* strongly affected root gene expression in NoMyc plants. *NPR3* is known to act as a negative regulator of *NPR1* via SA-dependent degradation. Therefore, its deletion was expected to stabilize *NPR1*, enhancing SAR induction and expression of PR genes (Backer et al., 2019). Surprisingly, in our experimental conditions no PR proteins were found to be upregulated. On the contrary, several PR-related proteins, including the *VvPRF*, the pathogenesis-related protein 1 precursor (*VvPRP1*), the R protein *VvMLA10* and an NBS-LRR R protein (VIT_13s0156g00460), were downregulated in roots. Additionally, several disease resistance-responsive proteins (e.g. *VvDIR33*, *VvDIR38* and *VvDIR34*) also showed reduced expression. Notably, among the downregulated genes we found the Defective in Induced Resistance 1 (*VvDIR1*) gene, around 7.9-fold decrease in expression compared to the WT, which encodes a putative apoplastic lipid transfer protein expressed in sieve elements and companion cells of the phloem. Previous studies have shown that *VvDIR1* is required for long-distance signaling during SAR (Lascombe et al., 2008). Indeed, *dir1* mutants fail to develop SAR in distal, non-inoculated leaves following local pathogen challenge (Maldonado et al., 2002). Taken together, we hypothesize that *VvNPR3* deletion might impair *VvDIR1* expression in roots by preventing the arrival of signaling molecules required to activate SAR and, consequently, favoring the AMF colonization.

Alongside defense genes, we also identified transcriptional changes affecting cell wall remodeling, a key interface for both immunity and beneficial root interactions. Notably, a Beta-1,3-glucanase the cellulose synthase *VvCSLE1* and a pectinesterase (VIT_04s0044g01020) were upregulated, suggesting both structural reinforcement and chemical modification of the cell wall components. These alterations may enhance defense readiness while reshaping the apoplastic environment to

accommodate microbial partners (Kawa et al., 2022; Dore et al., 2022). Furthermore, we found that five chalcone synthase genes were significantly downregulated. *VvCHS* catalyzes the entry point into flavonoid biosynthesis, which shares precursors with the lignin pathway. Its repression may indicate a metabolic shift away from lignification, potentially resulting in a more flexible, less lignified, cell-wall that favors AMF colonization (Zuk et al., 2016).

Transcriptional reprogramming in NPR3*_Myc edited plants supports functional symbiosis establishment*

The analysis of the transcriptomes of WT_Myc and NPR3_Myc-edited lines revealed a significant influence of the colonization on the whole root transcriptome. Overall, following inoculation, there was no clear trend indicating a consistent activation of SAR. This is reflected in the regulation of R protein genes, key immune receptors, in edited lines. Notably, *VvDIR1* was downregulated by approximately 2.3-fold and 5.3-fold in the two edited lines compared to the WT, as also observed under No_Myc conditions. These results may suggest that, in roots, *NPR3*-editing leads to a shift in the defense response away from the canonical SAR signaling, at least in grapevine, potentially supporting the establishment and development of arbuscular mycorrhizal colonization. Consistently, recent field studies indicate that grapevine immunity often relies less on conventional SAR/ISR frameworks and more on a stilbene-centered response, with SynCom- or AMF-inoculated plants showing enhanced accumulation of resveratrol and related derivatives (Sandrini et al., 2024; Nerva et al., 2023).

Still following the same trend that was found in the NoMyc roots, also in the Myc ones many genes involved in cell wall remodeling were found to be regulated. Specifically, as the upregulation of cellulose synthase (*VvCSLG3*), pectinacetylesterase, xyloglucan endotransglucosylase/hydrolases, beta-1,3-glucanases, expansin beta 2 and extensin support the idea of an active cell-wall restructuring. Furthermore, the activation of cell wall–modifying enzymes may helped to reshape the apoplastic compartment, reducing physical barriers and creating a more permissive interface for *R. irregularis* colonization (Balestrini et al., 2014).

Among the most strongly induced genes, the inorganic phosphate transporter *VvPT4* was found to be upregulated by approximately 8.9-fold and 3.9-fold in NPR3#15_Myc and NPR3#28_Myc, respectively, compared to the WT_Myc line. This transporter is well characterized in *Medicago truncatula*, where *MtPT4* is exclusively expressed in mycorrhizal roots and specifically activated in cells containing arbuscules (Harrison et al., 2002; Serrano et al., 2024). In grapevine as well, *VvPT4* has been reported to be upregulated upon colonization by various AMF species, and it is commonly

used as a molecular marker of functional symbiosis (Valat et al., 2018; Wang et al., 2025). Notably, *VvPT4* expression has been shown to be functionally associated with *RiMST2*, which was also found upregulated in the *NPR3_Myc*-edited lines. In *R. irregularis*, silencing of *RiMST2* has been shown to disrupt arbuscule development and to reduce *MtPT4* expression, highlighting a coordinated regulation between sugar and phosphate transport during mycorrhizal colonization (Garcia et al., 2016). Accordingly, the strong upregulation of *VvPT4,* mirroring the arbuscule frequency (A%), provides further evidence for the enhanced arbuscule abundance observed in the NPR3_Myc-edited lines, reinforcing its function as a molecular marker of symbiosis.

Interestingly, several aquaporins (e.g., *VvPIP1-4*, *VvPIP2-3*, *VvPIP2-5*, *VvPIP2-4*, *VvPIP1-1*) and a gene encoding a proline-rich protein (VIT_00s0259g00100) were found to be downregulated. Although AMF are generally known to improve plant tolerance to abiotic stresses, such as drought, partly by modulating aquaporin expression and enhancing proline biosynthesis (Cheng et al., 2020; Pavithra et al., 2018), the downregulation observed in the edited lines may be attributed to a more efficient water uptake. This enhanced water absorption capacity is likely due to the greater extent and functionality of AMF colonization, which was particularly evident in these genotypes. Consistently, previous studies have shown that aquaporin regulation in mycorrhizal roots is context dependent: some isoforms (e.g., *LeNIP3;1*) are upregulated in arbuscule-containing cells, while others (e.g., *LePIP1;1* and *LeTIP2;3*) can be downregulated under prolonged stress or in AM-colonized plants (Giovannetti et al., 2012; Chitarra et al., 2016). These findings support the idea that reduced aquaporin expression in our edited lines does not necessarily impair water relations but may rather reflect a compensatory mechanism linked to improved symbiotic water transport efficiency. Finally, it is worth noting the differential regulation of genes related to the circadian clock. Among them, circadian 1 (*CIR1/RVE2*), circadian clock associated 1 (*VvCCA1*), and *VvLCL1* (*LHY/CCA1-like 1*) were found to be downregulated both in Myc and NoMyc plants. On the other hand, GIGANTEA (*VvGI*) and Pseudo-response regulator 7 (*VvAPRR7*) were consistently upregulated under both conditions. Interestingly, *VvGI* and *VvPRR7* had already been reported in trifoliate orange (*Poncirus trifoliata* L.) by Ding et al. (2022) to be upregulated at specific times of day following *R. irregularis* inoculation, pointing to a possible interaction between AMF colonization and the host circadian rhythm. The circadian clock is also known to influence plant immunity, and *NPR1* has been directly linked to this system. In particular, the monomeric form of *NPR1* has been shown to accumulate during the night, suggesting that its nuclear translocation, regulated by oscillations in endogenous SA, might contribute to the rhythmic expression of clock-associated genes. Moreover, *NPR1* has been proposed to act as an upstream regulator of the clock component *TOC1*, in response to the rhythmic accumulation of SA (Goodspeed et al., 2012; Zhang et al., 2019). Based on these considerations, it is

reasonable to speculate that *NPR3* editing may have altered the balance of the *NPR1* pathway and impacted circadian regulation, thereby contributing to the creation of a physiological context more permissive to *R. irregularis* colonization. Such a connection between immune signaling and the circadian machinery suggests that *NPR3* disruption could not only alter canonical defense outputs but also reshape temporal regulatory networks, ultimately favoring the synchronization of host physiology with symbiotic cues. This integration of circadian and immune pathways may represent an additional layer through which *NPR3* editing enhances arbuscular mycorrhizal accommodation.

Enhanced arbuscule formation underpin strengthened AM symbiosis in NPR3-edited lines

Comparison of mycorrhizal colonization levels between WT_Myc and NPR3_Myc-edited lines, revealed a greater tendency toward colonization rates in the edited lines, coupled with a significantly higher abundance of arbuscules. This pattern was further corroborated by qPCR-based expression analyses, which revealed consistently higher transcript levels of *R. irregularis* genes in the edited lines relative to the WT. Among these, *GintPT*, a high-affinity phosphate transporter gene expressed over the fungal progression during rhizodermal penetration stage and linked to the formation of finely branched arbuscules (Fiorilli et al., 2013; Duret et al., 2022), was indeed upregulated in the two edited lines. In parallel, *RiMST2*, encoding for a monosaccharide transporter and well-established symbiotic marker gene, was found upregulated in the Myc edited lines. *RiMST2* is primarily expressed in intra-radical structures and is crucial for proper arbuscule development, as its silencing impairs arbuscule morphology. Notably, *RiMST2* is currently the only known transporter directly mediating sugar uptake at the root-mycorrhizal interface (Helber et al., 2011; Garcia et al., 2016). Also, the *14-3-3* gene, which encodes proteins involved in arbuscule formation during AM symbiosis (Sun et al., 2018), was significantly upregulated in *NPR3*-edited lines compared to the WT. In contrast, other fungal genes such as *RiCRN1, RiPEIP1,* and *RiPTR2* showed only a modest, non-significant upregulation in the edited lines. This partial response may reflect the distinct functional roles and temporal regulation of these genes during the symbiotic process: while nutrient transporters like *GintPT* and *RiMST2* closely track arbuscule abundance and functionality, effector genes and peptide transporters often display more transient or condition-dependent expression. Moreover, variability in fungal colonization patterns across replicates and the relatively low transcript abundance of some of these genes may have limited statistical resolution. Nevertheless, the overall upward trend observed across all markers supports the conclusion that *NPR3* silencing enhances fungal activity within roots. This result, along with the upregulation of plant symbiosis-related genes supports the higher abundance of arbuscules. Furthermore, the upregulation of multiple fungal genes associated with arbuscule development and nutrient exchange suggests not only a quantitative increase in colonization but also a more functionally active symbiotic stage in the *NPR3*_Myc-edited lines.

Root metabolic reprogramming in NPR3-edited plants supports AM symbiosis accommodation features

Targeted HPLC analysis of root metabolites revealed marked chemical changes in the modified *NPR3* edited lines, consistent with the transcriptional reprogramming observed in the RNA-seq data. Under NoMyc conditions, the most significant changes involved phenolic compounds and viniferin derivatives. In particular, dPACA, VinE, and VinD showed the highest fold change values, with dPACA increased by more than 250–300% in NPR#15 and NPR#28, and VinD doubled compared to WT. Other compounds such as dPACB, tPACB, and catechin showed more moderate increases, while resveratrol showed a more modest increase (~10%). These results suggest a general activation of flavonoid and stilbene derivatives, consistent with a shift in metabolic flux from lignification to soluble phenolic metabolites, potentially contributing to a more permissive apoplastic environment for colonization by AMF (Lone et al., 2024). However, it is important to note that the accumulation of viniferins, known antifungal stilbenes, could also represent a fine-tuning of chemical defense, allowing fungal reception without fully activating SAR (Kolupaev et al., 2025).

The hormonal profile showed elevated levels of SA and ABA in WT roots, with a slight increase in *NPR3* edited lines, while gibberellins tended to decrease and JA remained elevated, especially in *NPR3* samples. Noteworthy is the consistent increase in brassinosteroids following *NPR3* editing, suggesting a possible role in modulating root growth and symbiotic interactions. IAA levels showed variable trends, while zeatin remained stable. However, after inoculation with Myc, HPLC profiles further highlighted metabolic differences between WT_Myc and NPR3_Myc. Catechin, ABA, and zeatin increased in NPR3_Myc roots, while SA, brassinolide, resveratrol, and VinD decreased (Fig. 5D–F). Interestingly, dPACA levels decreased by 10–25% under Myc conditions, while dPACB increased moderately, suggesting a dynamic remodeling of the flavonoid pool in response to AMF colonization. These metabolic changes, while remaining correlative, reinforce the transcriptional trends observed, including the upregulation of enzymes involved in cell wall modification and phosphate transporters, indicating an integrated network of chemical and structural modifications that could promote arbuscules development (Li et al., 2022).

Conclusions

This study reveals an unexpected dimension of *NPR3* function in grapevine, highlighting its role not only in immune system reprogramming but also in the regulation of AM symbioses. Generating NPR3-edited lines, we demonstrated extensive transcriptional reprogramming in roots, including alterations in cell wall organization and defense-related pathways. These molecular changes were accompanied by a significant increase in arbuscule abundance and the upregulation of fungal genes

associated with nutrient exchange, indicating a shift toward a more advanced and functionally active stage of AM colonization. The strong induction of canonical symbiotic markers such as *VvPT4*, *GintPT*, and *RiMST2* further supports the notion that *NPR3* disruption generates a favorable root 'environment' for a functional AM symbiosis development. Collectively, our findings broaden the functional framework of *NPR3*, establishing it not only as a negative regulator of *NPR1* but also as an unexpected modulator of AMF interactions. This dual role suggests that targeted manipulation of *NPR3* could represent a promising strategy to simultaneously strengthen pathogen resilience and enhance symbiotic functionality in perennial crops.

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CHAPTER 4 | Epigenetic regulation and beyond in grapevine-pathogen interactions: a biotechnological perspective

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Abstract

As one of the most important crop plants worldwide, understanding the mechanisms underlying grapevine response to pathogen attacks is key to achieving a productive and sustainable viticulture. Recently, epigenetic regulation in plant immunity has amounted significant traction in the scientific community, not only for its role in gene expression regulation but also for its heritability, giving it an enormous biotechnological potential. Epigenetic marks have been shown to be dynamically modulated in key genomic regions upon infection, with some being maintained after such, being responsible for priming defense genes. In grapevine, however, knowledge of epigenetic mechanisms is still limited, especially regarding biotic stress responses, representing a glaring gap in knowledge in this important crop plant. Here, we report and integrate current knowledge on grapevine epigenetic regulation as well as non-epigenetic non-coding RNAs in the response to biotic stress. We also explore how epigenetic marks may be useful in grapevine breeding for resistance, considering different approaches, from uncovering and exploiting natural variation to inducing it through different means.

Keywords

Grapevine; epigenetic regulation; non-coding RNAs; plant immunity; epibreeding, plant-pathogen interaction

Introduction

The current agricultural landscape sits at a crossroads. While securing productivity is crucial to meeting the ever-increasing societal demands, agriculture is facing threats from various fronts, which jeopardize it. Among them, diseases are one of the most significant factors that come to compromise both productivity and sustainability as they are fought with the extensive use of environmentally deleterious phytopharmaceuticals. Because of this, the search for new mechanisms and methodologies that aim to increase plant resilience towards disease has become increasingly important, enabling the development of more tolerant crops to mitigate the use of these products.

Epigenetic regulation consists of modifications occurring at the DNA level that alter gene function without altering the underlying genomic sequence, and that are also inherited both mitotically and meiotically (Deans and Maggert 2015). These modifications, such as DNA methylation or histone modifications, play a key role in transposable element (TE) silencing and gene expression regulation, among other processes (Pikaard and Mittelsten Scheid 2014). Considering the two main features of epigenetic regulation – gene expression control and heritability - it is no surprise that it has garnered significant attention in the context of plant-pathogen interactions. On one hand, transcriptional reprogramming is one of the hallmarks of the early stages of an infection, often dictating the outcome of the interaction (Birkenbihl et al. 2017). On the other hand, memory of infection at different timescales is of relevance to the fate of subsequent attacks, whether it be in the same generation (i.e., somatic memory), the next one (i.e., intergenerational memory) or many generations after the initial exposure (i.e., transgenerational memory) (Mladenov et al. 2021; Hannan Parker et al. 2022; Gallusci et al. 2023). When considering biotechnological approaches, inter- and transgenerational memory are essential for either annual plants or clonally propagated crops if the breeding process involves sexual reproduction. Conversely, long-term somatic memory in clonally propagated crops is of greater importance, although it was recently argued that this form of propagation is not as conducive to maintaining a memory state (Ibañez and Quadrana 2023).

Grapevine (*Vitis vinifera* L.) is renowned for its quality and finds extensive application in wine production. One of the greatest threats to yields in viticulture are its common diseases, which are fought with the extensive use of phytopharmaceuticals, representing one of the main contributing factors to the unsustainability of this sector (Fouillet et al. 2022). It is therefore worthwhile to better understand how grapevine responds to common pathogens at the molecular and genetic levels, with epigenetics posing an interesting study target for this very aim. Indeed, grapevine has emerged as a promising woody crop plant model for studying epigenetic processes due to the availability of a reference genome rich in TEs, which is more prone to silencing via DNA methylation (see section Mechanisms of epigenetic regulation). Considering this high percentage of TEs (*circa* 40% of the

genome; The French–Italian Public Consortium for Grapevine Genome Characterization 2007), one would expect that DNA methylation modulation would be of greater significance in grapevine than in plants with lower amounts of TEs like Arabidopsis (circa 14% of the genome; The Arabidopsis Genome Initiative 2000).

Although still in its infancy, epigenetic research in grapevine is gaining traction in the scientific community. In fact, epigenetic regulation and its potential for improving the response of grapevine to stress has recently been discussed both in the scope of climate change (Berger et al. 2023; Tan and Rodríguez López 2023) and in its agronomical potential as a whole (Venios et al. 2024). However, the potential role of epigenetics in enhancing grapevine resilience to diseases remains unclear and has not been addressed within the scope of the current research. In this review, we aim to discuss the prospect of epigenetics in grapevine breeding for increased tolerance to diseases. We summarize current knowledge on epigenetic regulation in grapevine-pathogen interactions, as well as other non-epigenetic regulatory mechanisms, and speculate on ways these mechanisms can be harnessed in breeding, along with their feasibility in grapevine.

Mechanisms of epigenetic regulation

Different epigenetic mechanisms and pathways contribute to epigenetic state stability and modification in plant cells and tissues during development and in response to the environment. Epigenetic states are characterized by different combinations of epigenetic marks, which include DNA methylation, histone post-translational modifications (HPTM) and regulation by regulatory non-coding RNAs (ncRNAs) like small interfering RNA (siRNA) and long non-coding RNA (lncRNA) (Figure 1).

In plants, DNA methylation status is mediated by two enzyme families, DNA methyltransferases and DNA demethylases, which catalyze the addition of a methyl group to cytosines and excision of 5-methylcytosine (5mC) from DNA, respectively. Unlike mammals, DNA methylation in plants occurs in three distinct sequence contexts: CG, CHG and CHH (H = A, T, G). DNA methyltransferases can be divided into 3 distinct classes: methyltransferase 1 (MET1), chromomethylases (CMT), and domains rearranged methyltransferase (DRM) (Zhang et al. 2018), with each class playing a specific role. For instance, CMT and MET1 are linked to maintenance methylation (i.e., assuring full methylation of hemi-methylated sites), MET1 is linked to CG maintenance, while CMT3 and CMT2, albeit to a lesser extent, are linked to CHG and CMT2 and DRM to CHH in a siRNA-dependent mechanism (Zhang et al. 2018). Furthermore, *de novo* DNA methylation, assured by DRM2, is directed by siRNAs loaded into ARGONAUTE4/6 (AGO4/6) in the RNA-directed DNA methylation (Erdmann and Picard 2020). Active DNA demethylation is mediated by DNA demethylases, namely

DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DME-like 2 (DML2) and DME-like 3 (DML3), which, unlike DNA methyltransferases, appear to not have an affinity for particular sequence contexts (Parrilla-Doblas et al. 2019).

Methylation occurs in various genomic regions, achieving different biological functions depending on where it is present. A canonical function for DNA methylation is the repression of TEs by both blocking their movement and inhibiting their insertion in other regions, contributing to genomic stability. As for its role in gene expression, it appears to be more complex. For instance, promoter methylation is widely associated with transcriptional repression due to either inhibition of transcription factor binding or the binding of methylation-associated transcriptional repressors (O'Malley et al. 2016; Charvin et al. 2023). Interestingly. the reverse case has been described for *ROS1* expression in *Arabidopsis* (Lei et al. 2015; Williams et al. 2015) and in tomato ripening-associated genes (Lang et al. 2017), suggesting other mechanisms could interplay with DNA methylation. The role of intragenic methylation is also not fully known, with possible different roles ascribed to where inside the gene it is located. For example, exon methylation (known as gene body methylation, gbM), initially thought to be a positive regulator of gene expression, is hypothesized to play many more roles, from preventing aberrant transcription to regulating splicing. Furthermore, a role in protecting the coding regions of genes from unwanted TE insertion has also been put forth (reviewed in Muyle et al. 2022). To date, in grapevine, only DNA methyltransferases have been characterized at both the bioinformatic and expression level, in the context of infection with downy mildew (Pereira et al. 2022). DNA demethylaseshave been studied in the context of fruit ripening (Shangguan et al. 2020), but have not been characterized in terms of pathogen infection.

Regarding the role of histones in epigenetic regulation, it is important to consider both the role of histone variants and their post-translational modifications. The nucleosome is composed of four histone types (H2A, H2B, H3 and H4) consisting of a core (H3-H4)2 tetramer and two H2A-H2B dimers (Luger et al. 1997). The amino acid tails of these histones can be modified by writers and erasers of histone marks, impacting chromatin accessibility and gene expression states. Perhaps the most studied modifications are histone methylation and acetylation. Histone methylation is added by the writers Histone methyltransferases (HMT) and removed by the erasers Histone demethylases (HDMT) (Hu and Du 2022). Depending on the methylated amino acid residue and genomic location, different modifications can play different roles. For instance, the marks H3K4me2/3, generally deposited in gene promoters, are associated with opposite roles in gene expression regulation, with dimethylation linked to repression and trimethylation associated with activation. In addition, marks such as H3K9me2 and H3K4me1 have been linked to crosstalk with DNA methylation, the former being involved in CMT2/3-mediated non-CG methylation, and the latter in the recruitment of RdDM

machinery (Niu et al. 2021; Hu and Du 2022). Acetylation, on the other hand, is added by Histone acetyltransferases (HAT) and removed by histone deacetylases (HDAC). As opposed to methylation, histone acetylation is typically associated with transcriptionally active chromatin, due to the disruption of the DNA-histone interaction by the acetyl group (Kumar et al. 2021). Additionally, nonallelic sequence variants of canonical histones that have introns exist in plant genomes, particularly of H2A and H3, such as H2A.Z or H3.1, respectively, and that can be incorporated into nucleosomes in a targeted and replication-independent manner. Due to their sequence divergence from canonical histones, histone variants give specific properties to the nucleosomes they occupy and can affect epigenetic states, transcription and genome stability (Foroozani et al. 2022). Perhaps the best-characterized histone variant in the context of biotic stress is H2A.Z, which is predominantly deposited by the SWR1-C complex in the nucleosomes of environmentally responsive genes (ERGs). Indeed, loss of function of H2A.Z, as well as components of the SWR1-C complex, were noted to impair effector-triggered immunity (ETI), as well as salicylic acid (SA)- and jasmonic acid (JA)-associated hormonal signalling (Berriri et al. 2016). Furthermore, H2A.Z may also play a key role in the priming of defense genes, interplaying with RdDM-related mechanisms (Hannan Parker et al. 2022) (see Epigenetics and plant immunity – another layer of regulation)

In grapevine, both HATs and HDACs have been characterized bioinformatically and in the expression of their genes (Aquea et al. 2010). Another study, using a similar approach to the previous, identified not only HATs and HDACs but also HMTs and HDMTs, relating their expression patterns to various biological processes (Wang et al. 2020).

The third pillar of epigenetics is the regulation by ncRNAs, of which the most relevant for epigenetic processes are siRNA and lncRNA. Small interfering RNAs can originate from both endogenous and exogenous sources and can play roles in both PTGS and RdDM, depending on whether they are loaded in AGO1 or AGO4/6, respectively (Sanan-Mishra et al. 2021; Waheed et al. 2021). Endogenous siRNA can also emerge from the canonical RdDM pathway, resulting from DICER-LIKE3-mediated cleavage of dsRNAs transcribed from RNA Pol IV, or from the cleavage of miRNA precursors (Erdmann and Picard 2020). Some long non-coding RNA, on the other hand, can interact with and modulate chromatin (reviewed in Chen et al. 2021). Beyond epigenetic regulation, other ncRNAs also play important roles in gene expression regulation, a quintessential example being micro RNAs in the post-transcriptional gene silencing (PTGS) pathway (Yang et al. 2021). Although not epigenetic in nature, such examples will be discussed alongside other cases.

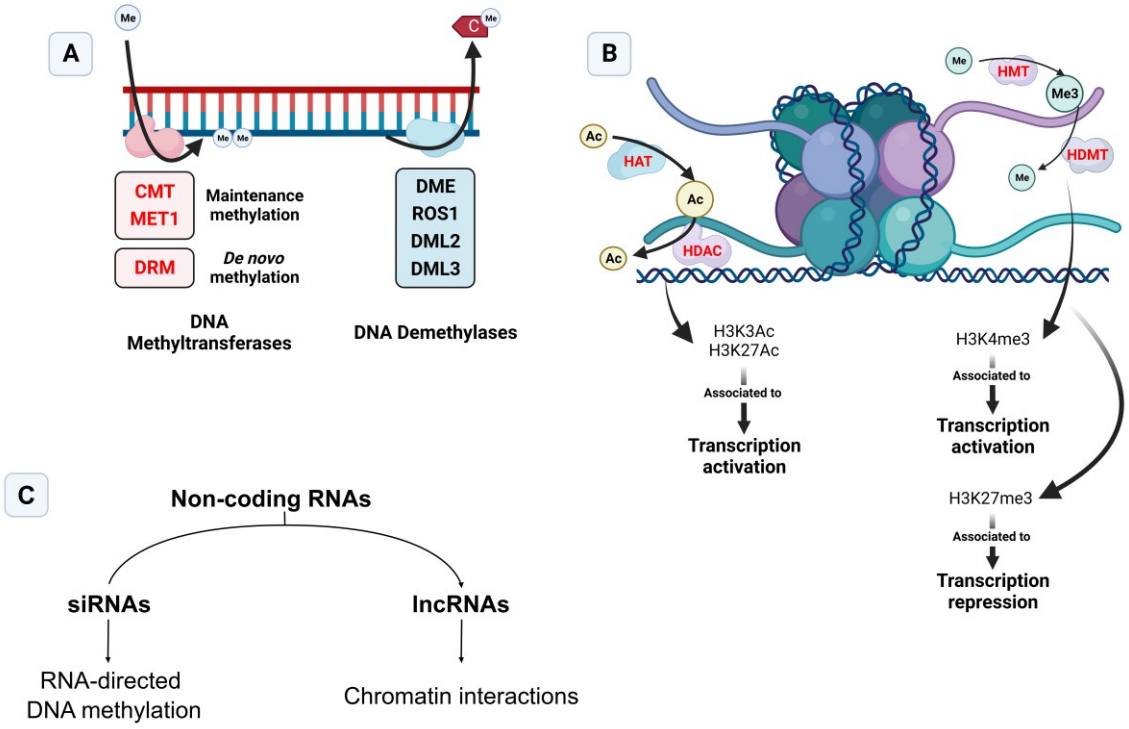

Figure 1. Main mechanisms of epigenetic regulation. A – mechanisms of DNA methylation; B – mechanisms of histone modifications; C – mechanisms of epigenetic regulatory ncRNA and their functions. Text in red represents gene families and functions already identified in grapevine. CMT – Chromomethylase; MET1 – Methyltransferase 1; DRM – Domains rearranged methyltransferase; DME – Demeter; ROS1 – Repressor of silencing 1; DML2 – Demeter-like 2; DML3 – Demeter-like 3; HAT – Histone Acetyltransferase; HDAC – Histone Deacetylase; HMT – histone methyltransferase; HDMT – Histone demethylase; siRNA – small interfering RNA; lncRNA – long non-coding RNA; Ac – acetyl group; Me – methyl group. Created with BioRender

Epigenetics and plant immunity – another layer of regulation

From the model plant Arabidopsis to crop plants, the role of epigenetic regulation plays in plant immunity is being unraveled at an increasingly faster pace. DNA methylation is among the better studied epigenetic mechanisms in this context, with hypomethylation appearing to be important in improved immune responses (Geng et al. 2019; Lee et al. 2023), although the opposite has also been observed for necrotrophic pathogens (López Sánchez et al. 2016). This hypomethylation may also be actively imposed, as evidenced by the fact that mutants lacking DNA demethylases are left more susceptible to pathogens (López Sánchez et al. 2016; Zeng et al. 2021; Huang et al. 2022; Zhang et al. 2022). The general line of reasoning follows that hypomethylation is behind the transcriptional

activation. This remodeling is also strongly linked to TEs, since their DNA methylation patterns are known to be modulated by stress. Indeed, the case of *TNL RESISTANCE METYLATION GENE* 1 (*RMG1*) is representative of this case, where it was shown that ROS1 actively demethylates a TE embedded in its promoter upon infection. This leads to the derepression of *RMG1,* contributing to resistance against *Pseudomonas syringae* pv. *tomato* (Halter et al. 2021). Despite this form of proximal regulation of DNA methylation, termed *cis*-regulation, a considerable amount of evidence also points towards these patterns governing the expression of distal genes (i.e., *trans*-regulation) (Hannan Parker et al. 2022). This form of regulation is also reliant on TEs, but this case is mediated by siRNAs that are generated from TE cleavage during their re-silencing after an initial infection. Besides targeting TEs for RdDM, these siRNAs are also involved in promoting defense gene expression, with this mechanism being hypothesized to be mediated by AGO1 H2A.Z in gene coding regions (Cambiagno et al. 2018; Liu et al. 2018; Hannan Parker et al. 2022).

Histone tail modifications have too been implicated in the plant immune response: from acetylation acting as a key regulator of JA signaling (Zhou et al. 2005; Vincent et al. 2022) to methylation playing a role in the JA-salicylic acid (SA) crosstalk (Alvarez-Venegas et al. 2007). In addition to histone modifications *per se*, other chromatin remodelers play important roles in the plant immune response, being frequently linked to transcriptional regulation of NLR genes. This has already been reported in Arabidopsis, for example, for Chromatin remodeler 5 (CHR5) (Zou et al. 2017), Splayed (SPY) (Johnson et al. 2015), and Switch/sucrose non-fermentable (SWI/SNF)-associated protein (SWP73A) (Huang et al. 2021), the latter two being constituents of the SWI/SNF complex, evidencing the importance of chromatin remodeling in the establishment of ETI.

Despite the importance of chromatin changes that take place throughout the infection course, what arises after this *rendezvous* is also of significance. Indeed, plants are able to hold a molecular memory after infection, which produces stable changes from the epigenetic and transcriptional to metabolic levels, culminating in an induced resistance phenotype (Harris et al. 2023). One quite essential example of priming against pathogens is found in systemic acquired resistance (SAR), where the plant develops a systemic response after a local infection, and is able to hold a memory at the somatic level in the short-term (Fu and Dong 2013). Incidentally, epigenetics has long been hypothesized to play a role in SAR establishment, with some (albeit limited) evidence even pointing towards a possible transgenerational transmission of this state (Luna and Ton 2012; Luna et al. 2012). At the (epi)genetic level, TEs play once again a central role in the establishment of priming states. Indeed, it has been hypothesized that miRNAs and siRNAs produced from TEs, which are stress-activated, can remain bound to AGO1 after stress. The AGO1-sRNA complex then accumulates at the promoters of defense genes and recruits RNA Polymerase II, although it is kept paused by H2A.Z,

which is deposited after the stress dwindles. Because of this, when a subsequent stress is imposed, primed defense genes are more quickly activated, thus establishing a sensitized state at the genetic level (Hannan Parker et al. 2022).

However, priming can also be performed without exposing plants to stress, since treating plants with priming molecules such as PAMPs, specific hormones or other related signaling molecules can produce similarly effective results. In fact, both DNA methylation and histone modifications have been implicated in memory establishment, both somatically and transgenerationally, after pathogen challenge or chemical elicitation (Harris et al. 2023).

As it stands, knowledge regarding epigenetic processes in plant-pathogen interactions is quickly compounding, although more focused on chromatin dynamics that take place during the infection course. However, understanding the memory component of epigenetics is essential, not only at a fundamental level but also within the context of plant immunity (i.e., priming), something that is currently lacking. In Harris et al. (2023), the authors outline key questions such as the lack of knowledge around causality of epigenetic marks and priming, as well as how specific the induced changes are, depending on which stress is being applied. Alongside the stability of these marks after priming, these amount to the largest questions when it comes to epigenetic stress memory. Answering these questions will surely lead to a better understanding of this complex regulatory mechanism and will also enable researchers to harness it for future biotechnological applications.

Epigenetic regulation in grapevine immunity

Although a relatively new study topic in the field of grapevine research, epigenetic regulation of the immune response has recently garnered increased attention from all facets (summarized in Table 1). The role of DNA methylation, for instance, has been highlighted in the context of grapevine downy mildew (DM), caused by the obligate biotroph oomycete *Plasmopara viticola*. It was shown that genotypes tend to present lower levels of DNA methylation compared to their susceptible counterparts (Azevedo et al. 2022; Pereira et al. 2022). Furthermore, differences at the transcriptional level were also uncovered, with genotypes carrying a Rpv3 background presenting a stronger downregulation of DNA methyltransferase gene expression during infection (Pereira et al. 2022). This is in line with the current understanding of the role of DNA methylation in plant-pathogen interactions, where hypomethylation is generally linked to transcriptional activation and, subsequently, to an improved immune response (Zhang et al. 2018; Tirnaz and Batley 2019). Furthermore, this suggests that grapevine tolerance to *P. viticola* may also be explained at the epigenetic level. Nevertheless, more detailed knowledge regarding this is much needed, since global methylation is not informative enough for conclusions to be drawn. Questions such as (1) which genes

or other genomic regions are differentially methylated, (2) if and how different are these regions between different genotypes, or (3) if these patterns are maintained after the infection are central regarding the role of epigenetics in the plant immune response.

When it comes to long-lasting epigenetic changes in response to pathogens, only one study has explored the importance of epigenetics, DNA methylation in particular, in two-year recovered grapevine plants previously infected with *Candidatus Phytoplasma vitis*, the causal agent of *Flavescence dorée* (FD) (Pagliarani et al. 2020). In this study, methylation landscapes were first noted to be distinct between healthy and two-year recovered plants, with most of the difference being present at the gene body level when compared to promoters. Simultaneously, it was found that genes related to sugar and flavonoid metabolism were downregulated in recovered plants compared to healthy ones, along with photosynthesis-related genes. Many of these genes also showed hypermethylation, indicating a possible role for DNA methylation in transcriptome remodeling post-infection, and suggesting a memory can last up to two years after FD onset. However, true priming effects were not evaluated since no re-infection was performed. Understanding if the response of these plants to re-infection was improved would be crucial in better understanding the mechanisms of priming against disease in grapevine and in ascribing a role for DNA methylation in this process.

Grapevine has also received considerable attention regarding priming with elicitors against pathogens, with the epigenetic component having recently been implicated in priming with melatonin. Indeed, Gao and associates (2020) found that melatonin treatment improved the response against *Botrytis cinerea*, while inducing hypomethylation at the promoter level of defense-related genes, correlating with their upregulation. Like the previously mentioned work concerning FD, one cannot conclude true priming effects arise from melatonin since no short- or long-term memory was tested. Nevertheless, the observed transcriptional and methylome reprogramming events are noteworthy, showing the *cis*-regulation of defense genes in response to elicitation, and provide basis for further work in this crop plant. It would be of relevance to assess actual priming responses, which could be approached by temporally spacing infection events or infection from elicitation, evaluating the short-term somatic memory these marks possess.

As for the role of histone modifications in grapevine immunity, none of the more commonly studied marks have been directly studied, with the only clues consisting of gene expression of histone-modifying enzyme-encoding genes. For instance, grapevine leafroll-associated virus 3 (GLRaV-3) infection induced changes in the expression of HAT genes (Aquea et al., 2011a), while infection with *Erysiphe necator*, the causal agent of powdery mildew (PM), also triggered upregulation of several genes of this family 12 hours after infection, including *VvHAC1*, *VvHAG4* and *VvHAG23* (Wang et al. 2020). Histone acetylation is generally linked to euchromatic regions and expressed genes

(Shahbazian and Grunstein 2007; Maeshima et al. 2024), while deacetylation was noted to negatively affect immunity upon pathogen challenge in Arabidopsis (Yang et al. 2020) and rice (Ding et al. 2012). Histone acetylation could therefore be a possible mechanism involved in transcriptional activation upon infection in grapevine, although more pathosystems must be studied and the epigenetic states directly assessed. Indeed, no other works aimed to characterize histone-modifying enzymes in the context of grapevine-pathogen interactions, and no studies exist whatsoever where specific histone modifications are directly analyzed, representing a glaring gap in epigenetic research in this specific context.

As to the role of ncRNAs in grapevine immunity, the distinction between epigenetic and non-epigenetic mechanisms is once again worth noting. For instance, while miRNAs are generally not epigenetic, rather than being an integral part of PTGS, siRNA and lncRNA can be epigenetic (see section Mechanisms of epigenetic regulation). Currently, studies relating the role of siRNA with chromatin dynamics in grapevine are next to non-existent. Indeed, up to our knowledge, there is only one work where siRNAs are linked to DNA methylation modulation via *de novo* methylation, performed in grapevine embryogenic *calli.* In it, it was shown that CHH hypermethylation arises in heterochromatin of grapevine *calli*, which was attributed to *de novo* methylation driven by heterochromatic siRNA that concomitantly accumulated in this tissue (Lizamore et al. 2021). This could also represent an interesting approach for introducing novel epigenetic regulation during tissue culture, something that could be harnessed for future breeding programs.

Beyond this, the only work with ncRNAs in the field of grapevine immunity is restricted to post-transcriptional control of gene expression, representing a glaring gap in the knowledge of the role of these molecules in epigenetic processes. Nevertheless, they represent another important layer of gene expression regulation.

Bioinformatic prediction of trans-acting siRNAs (tasiRNA) encoded in the *VvTAS7* locus points towards the regulation of several genes that may be linked to immune responses, including a leucine-rich receptor-like protein kinase, a histone acetyltransferase, and a fatty acid desaturase (Zhang et al. 2012). All these genes have been linked to plant immunity against many taxa, in grapevine or otherwise, which provides clues for the role of these tasiRNAs in this same context (Couto and Zipfel 2016; Ramirez-Prado et al. 2018; Laureano et al. 2021). Another interesting role of tasiRNAs (and all sRNAs alike) is their role in host-pathogen crosstalk. It has been found that *VvTAS3* encodes siRNAs that specifically target *P. viticola* transcripts, which may indicate that these sRNAs may act not only within the plant, but also as a direct mechanism of immunity. Likewise, *P. viticola*-derived sRNAs targeting grapevine transcripts have also been uncovered, one of which is putatively targeting an LRR-like protein kinase, an important family of proteins in plant immunity, while grapevine-

derived sRNAs originate mostly from disease-related genes (Brilli et al. 2018). A scenario in which cross-kingdom sRNA crosstalk can alter the fate of grapevine-pathogen interactions opens new ways to tackle this problem since novel endogenous plant sRNAs could be engineered to target specific pathogen genes, hindering their pathogenicity.

Besides sRNAs, novel evidence has shown that lncRNAs may likely play a role in grapevine immunity against various pathogens. One study explored lncRNA profiles upon infection by *Lasiodiploidia theobromae*, where it was possible to discriminate the tolerant cultivar 'Merlot' from the susceptible 'Cabernet Franc' based on lncRNA profile alone. Additionally, many identified lncRNAs were positively correlated with the expression of genes related to chitin catabolism and cell wall dynamics, which have been shown to be of importance in plant-fungal interactions (Xing et al. 2019). Although the authors did not explore this regulatory role further, this is of particular interest in the scope of epigenetic regulation, given the role some lncRNAs have in chromatin remodeling. Recently, it was found that the Arabidopsis lncRNA *salicylic acid biogenesis controller 1 (SABC1)* acted as a negative regulator of SA-mediated immune responses by silencing a NAC transcription factor, *NAC3*, that promotes SA synthesis. This repression was due to the recruitment of the PRC2 to the locus of NAC3, which resulted in the deposition of the repressive H3K27me3 mark. Interestingly, SABC1 is repressed during infection, releasing the repression of *NAC3* and allowing SA accumulation (Liu et al. 2022). It is thus possible that lncRNAs correlated with transcriptional activation of other genes may imply that chromatin remodeling can occur, although this would require a finer analysis of the systems at hand. A similar case emerged in the interactions with *P. viticola*, *E. necator* and *B. cinerea*, where many functionally related genes appeared positively regulated to lncRNAs modulated by the infection with these pathogens. For example, *P. viticola* infection was shown to increase lncRNAs correlated to JA signaling-associated genes, while *E. necator* triggered the ones correlated to SA signaling (Bhatia et al. 2021), two phytohormones that play key roles in the interactions with these pathogens (Weng et al. 2014; Guerreiro et al. 2016). Conversely, in the interaction with *B. cinerea*, a putative association to processes such as chitin degradation or stilbenoid biosynthesis was attributed to some lncRNA molecules (Bhatia et al. 2020). Interestingly, both works uncovered a potential role for pathogen-responsive lncRNAs as decoys for miRNAs implicated in PTGS of defense-related genes. Indeed, DM- and PM-responsive lncRNAs may bind miRNAs targeting MYB transcription factors, which are important molecular players in immune responses (Yu et al. 2023). Interestingly, PM, DM, and *B. cinerea* infections triggered the accumulation of a lncRNA found to target vvi-miR482, a miRNA associated with NLR gene silencing. These findings suggest an important role of lncRNAs in regulating PTGS in response to pathogens, and suggests a conserved function in grapevine immune responses (Bhatia et al. 2020, 2021).

Table 1. Summary of current research in grapevine-pathogen interactions, from the scope of epigenetic regulation and non-epigenetic, non-coding RNAs

Pathogen	Studied regulatory mechanism	Epigenetic marks directly assessed?	Biological effect	Reference
Epigenetic mechanisms				
P. viticola	DNA methylation	Yes, but global methylation only	Lower global methylation levels in tolerant genotypes in response to infection. Reprogramming of DNA methylation- and chromatin remodeling-associated genes.	(Azevedo et al. 2022; Pereira et al. 2022)
Candidatus *P. vitis*	DNA methylation	Yes	Differential methylation patterns in two-year recovered vs healthy plants.	(Pagliarani et al. 2020)
B. cinerea	DNA methylation	Yes	Melatonin elicitation reprograms methylation in promoters of immunity-related genes and increases tolerance to *B. cinerea*	(Gao et al. 2020)
GLRaV-3	Histone acetylation	No	Early upregulation of HAT genes in response to viral infection	(Aquea et al. 2010)
Non-epigenetic mechanisms				
P. viticola	phasiRNA, siRNA	N/A	Various grapevine-derived sRNAs target *P. viticola* transcripts, and vice-versa, including siRNA, phasiRNA, and miRNA	(Brilli et al. 2018)

L. theobromae	lncRNA	N/A	Differential accumulation of lncRNAs between tolerant and susceptible, targeting immunity-related genes	(Xing et al. 2019)
P. viticola	lncRNA	N/A	Grapevine lncRNA coexpresses with genes linked to JA and chalcone biosynthesis, genes encoding PR proteins and WRKY TFs.	(Bhatia et al. 2021)
E. necator	lncRNA	N/A	Grapevine lncRNA coexpresses with genes linked to SA and cell wal degradation, genes encoding PR proteins and WRKY TFs.	
B. cinerea	lncRNA	N/A	Co-expression of pathogen-responsive lncRNA targeting genes associated with stilbenoid biosynthesis, chitin degradation. Potential decoys for miRNAs targeting PR protein-encoding genes	(Bhatia et al. 2020)

Epibreeding, a new frontier in grapevine breeding

Although in-depth knowledge of epigenetic regulation of grapevine immunity remains elusive, there are now robust clues that implicate these mechanisms in this ever-important aspect of grapevine biology. Accordingly, it has become particularly enticing to speculate on how these mechanisms could be employed in grapevine breeding (termed epibreeding), with this exercise already having been done very recently for environmental changes in general (Berger et al. 2023; Tan and Rodríguez López 2023) and to other aspects of grapevine biology (Venios et al. 2024). Although modern breeding techniques, which rely on the deployment of resistance genes, can be extremely effective, having another layer of regulation represents another tool in the arsenal of breeders to improve resilience. Epibreeding can also be valuable in preserving genetic landscapes and their variability, as it may not require genome editing. However, it is important to understand not only the possible

benefits of epibreeding in the scope of improving grapevine resilience to disease but also its limitations in the agronomical context it exists in. Therefore, in this section, we aim to pose new perspectives on epibreeding applied to grapevine immune responses while critically analyzing the feasibility of different strategies (summarized in Figure 2).

As it stands, there are two possible ways to approach epibreeding: 1) to explore naturally occurring epigenetic variability; and 2) to induce new epigenetic marks. Exploring natural variability is generally associated with epigenome-wide association studies (EWAS), the discovery of epigenetic quantitative trait loci (epiQTL) or epialleles – in essence, the correlative study of preexisting epigenetic marks that are widespread in populations and a given phenotype.

Very recently, a naturally occurring, heritable epiallele regulating the expression of two adjacent NLR genes was uncovered and was linked to heightened clubroot disease tolerance in Arabidopsis (Gravot et al. 2024). The existence of naturally occurring, epigenetically regulated expression polymorphisms of defense-related genes sets a precedent for investigating epigenetic variability in other populations, including crop plants.In grapevine, natural epigenetic variability has been a subject of study, and is a possible factor behind the observed phenotypic plasticity (Xie et al. 2017; Varela et al. 2021). Furthermore it has been demonstrated that environmentally-induced DNA methylation marks are not only maintained up to three years after transplantation (Varela et al. 2024), but also in plants that recovered from FD (Pagliarani et al. 2020). With these aspects in mind, it becomes enticing to enquire if there are naturally occurring epigenetic marks associated with increased tolerance to diseases in grapevine, how they exert their biological role and how stable they are on a multi-year scale. To approach this in a robust manner, it is essential to remove as much of the genotypic effect as possible, which is particularly relevant in grapevine due to its high intervarietal genetic diversity. One way to possibly overcome this is to study grapevine at the intravarietal level (i.e., different clones of the same genotype), which also displays significant phenotypic diversity that arose due to longstanding practices of vegetative propagation (Gonçalves and Martins 2022). This diversity has been harnessed in the past in breeding programs for viticultural and oenological traits (Gonçalves and Martins 2022), as well as for drought stress tolerance (Carvalho et al. 2020), but never in the scope of increased tolerance to disease. Although it is known that clonal diversity can be linked to epigenetics (Guarino et al. 2015), including grapevine (Varela et al. 2024), its effect on phenotypic diversity was never assessed in the scope of immunity or any other type of stress. For this, directing efforts towards extensive epi-genotyping and phenotyping in consecutive seasons is key, with particular care towards the environmental context in which these are done, as well as the genetic background of the studied populations. For instance, susceptibility assays in grapevine are often performed in leaf disk, and the inoculation is performed under tightly controlled conditions. If the point of these efforts is to uncover

epigenetic variation among individuals, it is possible that this procedure may introduce biases in these same mechanisms, possibly leading to confounding effects.

Exploring the naturally occurring heritable epigenetic changes is, however, not the last frontier of epibreeding since these changes can be artificially induced. Some strategies, ranging from the development of epigenetic recombinant inbred lines (epiRIL) or the induction of novel marks using epigenetic inhibitors (e.g., DNA methylation or HDAC inhibitors) to more cutting-edge methods of epigenome editing, could be employed for epibreeding in grapevine.

A promising new technique for inducing changes to the epigenome in a site-specific can be found in epigenome editing. These strategies generally rely on harnessing dead Cas (dCas), a mutated Cas protein without nuclease activity, in fusion with an epigenetic writer or eraser to direct the modulation of epigenetic marks to specific loci (Gardiner et al. 2022). This is especially powerful if the knockout or overexpression of genes is not adequate for a crop system since these can have pleiotropic effects and hinder other important agronomical traits (Derbyshire et al. 2024). Rather, epigenome editing can act as a dial for gene expression, rather than the binary effect (i.e., overexpression and knockout) that is found in traditional genome engineering approaches. For instance, the silencing of a sucrose transporter in cassava, whose promoter was known to be unmethylated, via targeted DNA methylation was sufficient to improve resilience to bacterial blight (Veley et al. 2023).

The use of epigenome editing is, of course, dependent on the knowledge of immune-related genes that are under epigenetic control which, in grapevine, is still limited. As an initial approach, it is essential to uncover immune-related genes that are under control of chromatin mechanisms and if these are maintained under epigenetic memory. Although both cases have separately been achieved, providing some evidence to this end, a unified vision of epigenetically regulated immunity genes is still required. Epigenome editing also suffers from the same hindrance of transformation, although no crossing is required, making it a more feasible alternative to EpiRILs. It is also important to note that, although difficult, a lot of progress has been made regarding grapevine transformation and regeneration, including boosting resilience to disease (Moffa et al. 2024). Additionally, the advent of the New Genomic Techniques (NGT) has paved the way for increased acceptance of genetic engineering in crop species, although the legal framework is yet to be set. Once more knowledge on which marks are epigenetically regulated in the context of immune responses, novel targets can be elected for these approaches.

Conversely, the induction of new epigenetic marks without resorting to genome editing techniques, although perhaps less targeted, may prove more fruitful for future breeding efforts. For instance, the use of epigenetic inhibitors could prove to be more feasible, since it only requires *in vitro* culture of grapevine and their treatment with said inhibitors. Indeed, this technique has already been reported

in grapevine somaclones treated with DNA methylation inhibitors, although the clones are still awaiting phenotyping (Berger et al. 2023). This strategy is particularly enticing because it generates a considerable amount of epigenetic variability, albeit randomly, that could be harnessed for various aspects of grapevine biology: from development to stress tolerance. Furthermore, inducing DNA hypomethylation is made even more interesting in the context of grapevine-pathogen interactions, having been previously reported to be associated with tolerance (Azevedo et al. 2022; Pereira et al. 2022), in line with the scientific consensus. Nevertheless, the barrier of grapevine *in vitro* culture is worth bearing in mind, especially when considering its general recalcitrance due to high genotype and explant specificity (Nuzzo et al. 2022).

Another promising facet of induction of epigenetic reprogramming lies in immune priming. Although priming is not a novelty for the potentiation of plant immunity, the notion of epigenetics playing a major role in transcriptional reprogramming and memory maintenance has been gaining traction. Indeed, both DNA methylation and chromatin-associated mechanisms, such as nucleosome occupancy or histone modifications, have been linked to priming memory, with specific marks being associated with different types of memory (Harris et al. 2023). In grapevine, various immune priming methods have been studied, from chemical priming agents such as laminarin (Gauthier et al. 2014), fatty acids (Laureano et al. 2024) or lipopolysaccharides (Castro et al. 2023), to biopriming (i.e., priming with beneficial microbes) (Jeandet et al. 2023; Langa-Lomba et al. 2023). Often these works examine not only the immediate effects of priming, but also the short-term memory modulation that takes place, generally at the transcriptional level. However, there are currently no studies that examine if and how epigenetic marks are modulated, their role in transcriptional reprogramming and their stability in the short-, medium- and long-term.

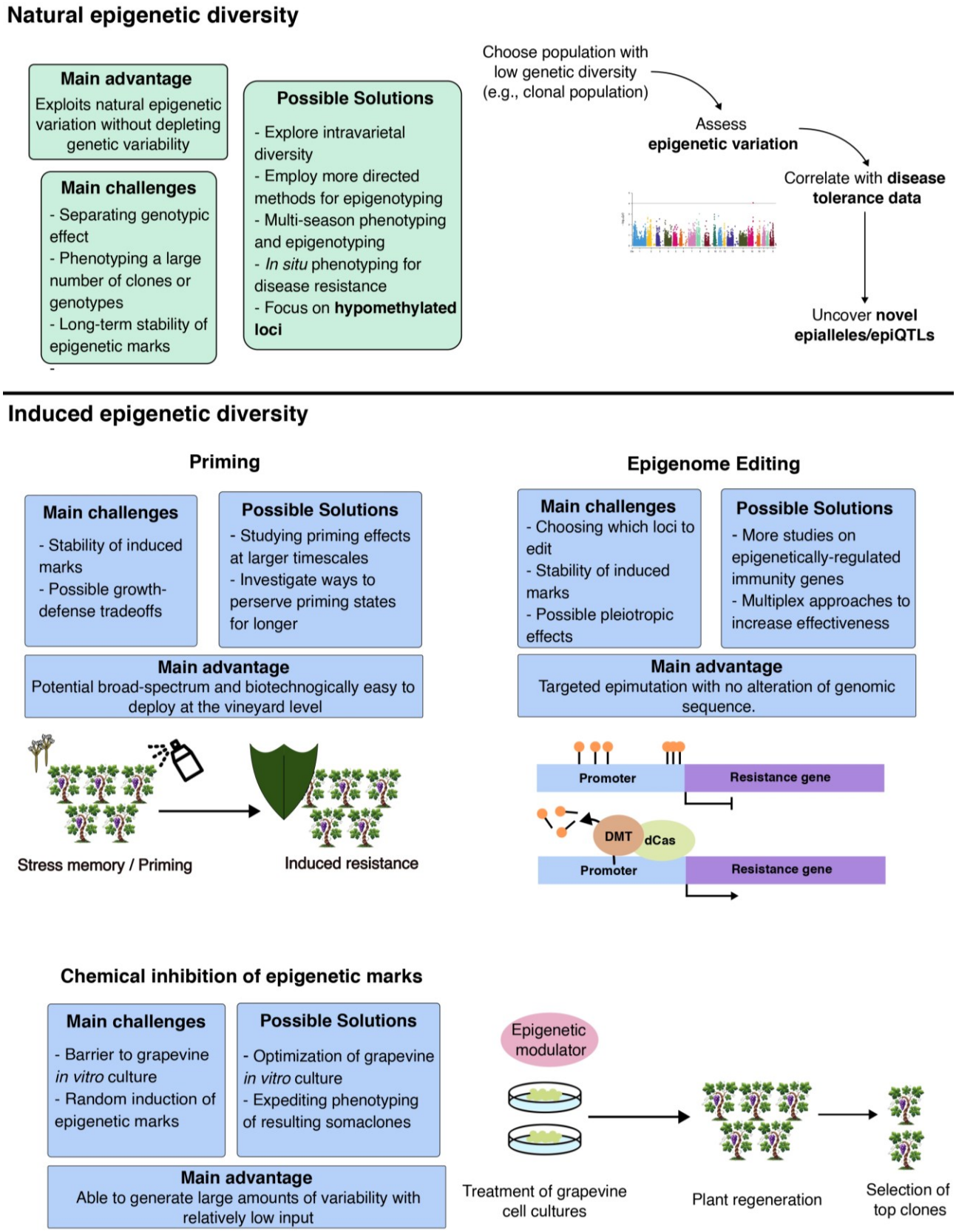

Figure 2. Possible paths of introducing epigenetic marks in grapevine breeding, from harnessing natural epigenetic diversity (top pannel) to inducing it (bottom pannel). epiQTL – epigenetic quantitative trait locus; DMT – DNA demethylase; dCas – dead Cas.

Concluding remarks and future perspectives

Although much is yet to be understood, it has become clear that epigenetic marks and all their facets play a key role in plant immune responses, from gene expression regulation to memory. Moreover, some non-epigenetic players, as some lncRNAs, have also been shown to be important to the outcome of plant-pathogen interactions. Most of this knowledge is, however, still circumscribed to the model plant Arabidopsis, while mechanisms in crop plants are significantly less elucidated, grapevine being no exception.

Recently, some progress has been made regarding these very mechanisms in grapevine. Differential responses at the DNA methylation level in a genotype-dependent manner were described, along with gene expression regulation of other writers, erasers and readers of chromatin, in response to downy mildew. Elicitation was also found to reprogram DNA methylation at the promoters of immunity-regulated genes, and strong clues for epigenetic memory were uncovered in plants recovered from FD. Albeit limited, this provides a robust foundation for pursuing further research in this crop plant, which we propose should be directed towards two key points:

1. Understand the responsiveness of epigenetic marks to infection and their stability in its aftermath

Are there disease-responsive genes that are controlled by epigenetic mechanisms, and is this control genotype-dependent? The coupling between genetic and epigenetic components has been previously described in other models (Taudt et al. 2016) and reconciling this with the differential response between genotypes suggests the same could occur in the grapevine immune response. It is also enticing to investigate the stability of these marks, distinguishing them from chromatin regulation of gene expression.

2. Explore epigenetic diversity in inter- and intra-varietal diversity

Are there stable epigenetic marks that confer increased tolerance to disease in grapevine? Could those explain part of the observed phenotypic diversity among clones or even varieties? Searching for natural epigenetic variation in grapevine populations could hold tremendous potential in the scope of plant immunity, but it is not without its difficulties. First, it is important to establish if grapevine intravarietal diversity observed in other important traits also extends to susceptibility to disease, requiring robust phenotyping of selected varieties. By looking at this level, genetic variation is minimized and solely the epigenetic effect can be assessed. Nevertheless, it could also be of interest to assess if epigenetics also plays a role in intervarietal diversity, possibly interplaying with the already well-known genetic diversity. Novel methods, such as the one developed by Lesur and associates (2024), enable the study of epigenetics at the population level, considering naturally occurring genetic variations and reconciling strong epigenomic characterization with a reduced

operational cost. This approach could also be applied to grapevine populations, enabling higher resolution analyses, which have relied on low-throughput techniques like MSAP in the past (Varela et al. 2024).

Author contributions

JPP, IB and SV wrote the manuscript. JPP prepared the figures. All authors have read and approved the manuscript.

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Data availability statement

Non-applicable.

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CHAPTER 5 | Functional dissection of epigenetic mechanisms in grapevine through CRISPR/Cas9: insights from *H2A.Z* and *DNMT2*

Introduction

Plants are sessile organisms continuously challenged by a broad range of environmental and biotic stresses. Their survival relies on multi-layered defence systems that integrate canonical signalling pathways with additional regulatory layers, including epigenetic regulation, chromatin dynamics and RNA modifications. In recent years, epigenetics has emerged as a central regulator of plant development, stress adaptation, and immunity, offering mechanisms that can shape transcriptional reprogramming and contribute to plant plasticity and stress memory (Lloyd and Lister, 2022; Ramirez-Prado et al., 2018). Epigenetic marks, including DNA methylation, histone modifications, histone variants, and RNA methylation, are dynamic and reversible, enabling plants to fine-tune their responses to environmental challenges. Grapevine represents a particularly interesting model for studying epigenetic regulation in crops. As a perennial woody species propagated clonally, grapevine preserves its genetic background across generations while still exhibiting phenotypic diversity that is likely influenced by epigenetic variation (Pelsy, 2010; Fortes and Gallusci, 2017). Moreover, as a long-lived species constantly exposed to pathogens and abiotic stresses, grapevine provides an opportunity to study how epigenetic states are maintained and reshaped during development and environmental interactions (Quadrana and Colot, 2016). Epigenetic mechanisms not only regulate gene expression but also contribute to the regulation of stress-responsive pathways, thereby linking developmental plasticity with adaptation to biotic and abiotic pressures (Hemenway and Gehring, 2023). Although our knowledge of the grapevine epigenomes is still limited compared with those of model species, studies on DNA methylation pathways, chromatin remodelling complexes, and histone modifiers have already highlighted their involvement in berry development, response to viral infection, and varietal diversity (Aquea et al., 2010; Magris et al., 2019; Shangguan et al., 2020). This makes grapevine a promising system to investigate epigenetic determinants of plant–microbe interactions and their potential exploitation for sustainable viticulture. An important component of chromatin regulation is the existence of histone variants, which replace canonical histones in the nucleosome to confer specific structural and functional properties. Variants of H2A, H2B, H3, and H4 have been described, and they play roles in DNA repair, chromosome segregation, transcriptional regulation, and stress responses (Probst et al., 2020; Wu et al., 2023). These proteins are not mere substitutes but can profoundly alter nucleosome stability, histone modification landscapes, and higher-order chromatin architecture, thereby influencing how genomes respond to developmental and environmental cues. Among them, H2A.Z is one of the most conserved and functionally versatile histone variants. Its incorporation into chromatin is mediated by the SWR1 chromatin remodelling

complex, which catalyses the replacement of canonical H2A with H2A.Z at transcription start sites (March-Díaz et al., 2007). This process modulates nucleosome stability and positioning, thereby influencing gene accessibility. Functional studies in Arabidopsis have shown that double mutants in *HTA9* and *HTA11* display pleiotropic developmental defects, early flowering, and widespread transcriptional misregulation (March-Díaz et al., 2007). H2A.Z also contributes to abiotic stress adaptation: its eviction under drought and heat conditions facilitates activation of stress-responsive genes (Sura et al., 2017; Kumar and Wigge, 2010). In crops such as wheat, variants of H2A.Z have been linked to improved drought tolerance and ABA-dependent stomatal regulation (Xu et al., 2016). In addition to these roles, H2A.Z is strongly implicated in plant–microbe interactions. Mutants defective in SWR1 components such as PIE1, ARP6, or SWC6 exhibit constitutive activation of systemic acquired resistance (SAR) markers, spontaneous cell death, and enhanced resistance to *Pseudomonas syringae* (Berriri et al., 2016). Conversely, H2A.Z depletion increases susceptibility to necrotrophic pathogens like *Sclerotinia sclerotiorum*, due to impaired activation of WRKY33 and the ERECTA signalling pathway (Cai et al., 2021). These findings suggest that H2A.Z functions as a critical regulator balancing growth and defence, fine-tuning transcriptional responses during plant–pathogen encounters. Beyond chromatin-based regulation, plants rely on RNA methylation as an additional epigenetic layer that controls RNA metabolism. The most abundant internal modification in mRNA is N^6-methyladenosine (m^6A), which regulates splicing, stability, translation, and transport (Hu et al., 2019; Shinde et al., 2023). Its deposition is mediated by RNA methyltransferases, often referred to as “writers,” while demethylases remove the mark and “readers” interpret it to determine RNA fate (Amara et al., 2023). These enzymes are essential for development, as demonstrated by embryonic lethality in Arabidopsis mutants lacking the core writer MTA (Zhong et al., 2008). Within this large family, TRDMT1/DNMT2 is a particularly interesting case because it acts as a tRNA methyltransferase. This protein catalyzes the methylation of cytosine 38 (m^5C38) in the anticodon loop of tRNA^Asp and other selected tRNAs, a modification conserved across eukaryotes (Goll et al., 2006). The presence of m^5C38 stabilizes the tRNA, ensuring proper anticodon–codon recognition and efficient translation under stress conditions. Loss of DNMT2 activity in Arabidopsis mutants results in hypomethylated tRNA^Asp, leading to a strong reduction in its accumulation (Wadhwa et al., 2023). Proteomic studies have revealed that DNMT2 deficiency reshapes the plant proteome, with more than 300 proteins differentially regulated compared to wild type. Many of these proteins are directly or indirectly involved in stress responses, including iron homeostasis, innate immune signalling, ROS metabolism, stomatal regulation, and cell wall modifications (Wadhwa et al., 2023). For instance, Allene Oxide Cyclase 2 (AOC2), a key enzyme in jasmonic acid biosynthesis, is strongly upregulated in trdmt1 mutants, while transcriptional co-

activators such as MBF1B, which contribute to stress tolerance, are downregulated. Importantly, many differentially expressed proteins also fall into categories directly linked to pathogen defence, such as antiviral responses, programmed cell death, and regulation of stress hormones including abscisic acid and jasmonic acid. The connection with innate immunity is further reinforced by the observation that DNMT2 localizes not only in the nucleus but also in RNA processing bodies and stress granules, where it may influence RNA stability and degradation under infection conditions (Wadhwa et al., 2023).

Recent work in *Physcomitrium patens* has further highlighted the broad role of TRDMT1/DNMT2 in coordinating developmental and stress-related pathways. In this early land plant, loss of DNMT2 function impairs not only methylation at C38 but also at additional sites (C48 and C49) of tRNA^Asp, leading to global defects in protein translation, deregulation of housekeeping and stress-related pathways, and enhanced sensitivity to abiotic stresses (Sharma et al., 2025). Intriguingly, among the differentially expressed proteins and transcripts, several are linked to secondary metabolism and jasmonate biosynthesis, including allene oxide cyclases, suggesting that TRDMT1-mediated RNA methylation can directly modulate pathways central to plant–pathogen interactions.

Similarly, studies in rose (*Rosa chinensis*) revealed that DNMT2 belongs to a conserved family of C5-methyltransferases responsive to environmental stresses, with stress-related cis-elements enriched in their promoters (Gangwar et al., 2025). This points to a conserved role for DNMT2 in integrating epigenetic RNA modifications with stress- and hormone-responsive transcriptional networks.

Together, these findings indicate that TRDMT1/DNMT2-dependent tRNA methylation is not only crucial for translational homeostasis but also shapes the proteomic and transcriptional landscape in ways that impact immune signalling, hormone pathways, and secondary metabolism. By tuning these responses, DNMT2 may contribute to the fine balance between growth and defence, ultimately influencing how plants respond to and resist pathogenic microorganisms. Based on these considerations, this part of the thesis explored the role of two epigenetic regulators in grapevine: the histone variant *VvH2A.Z* and the tRNA methyltransferase *VvDNMT2*. In particular, these represent two distinct projects with complementary approaches. Indeed, in the first we investigated how perturbation of *VvH2A.Z* affects grapevine responses to microbial pathogens, focusing on the role of this histone variant in balancing development and immunity. In the second, conducted in collaboration with the Grapevine Pathogens Systems Lab of Prof. Andreia Figueiredo (University of Lisbon), we induced the knockout of a grapevine ortholog *VvDNMT2* to test whether disruption of tRNA methylation alters the plant's capacity to respond to pathogens. In both cases, we employed CRISPR/Cas9-based targeted knockouts, a precise and innovative genome-editing strategy, to dissect the contribution of these regulators to grapevine immunity.

Materials and methods

Embryogenic calli induction and maintenance

Flower clusters of *Vitis vinifera* cv. Chardonnay were collected in 2022 from vineyards managed by Vivai Cooperativi Rauscedo – VCR, in Rauscedo (Pordenone, Italy). Only the basal portion of the inflorescences was harvested and subsequently sterilized with a sodium hypochlorite solution containing Tween 20. After sterilization, ovaries and anthers were excised and placed on PIV starter callus induction medium (Gambino et al., 2007). After approximately three months of culture, embryogenic calli were obtained and transferred to C1 proliferation medium, where they were maintained and subcultured at monthly intervals, as previously reported (Moffa et al., 2025).

Vector Construction and Callus Transformation

Guide RNAs (gRNAs) targeting the *VvDNMT2* gene (Vitvi05_01chr04g06210) and two homologs of *VvH2A.Z* (VIT_200s0179g00340 and VIT_204s0008g04700) were designed and evaluated using the CRISPR-P web tool (http://crispr.hzau.edu.cn/CRISPR2/) (Lei et al., 2014). For *VvDNMT2*, two gRNAs were selected, whereas for *VvH2A.Z* one gRNA was used per gene. The complete list of guides is provided in Supplementary Table S1. Homologous sequences of *H2A.Z* were identified by generating a phylogenetic tree including all *H2A* histone variants of grapevine and *Arabidopsis thaliana*. Sequences were aligned with MUSCLE (Edgar, 2004), and phylogenetic relationships were inferred with MEGA (Kumar et al., 2016). For *VvDNMT2* editing, the pDIRECT_22c vector (Čermák et al., 2017) was used to carry two gRNAs. A similar strategy was initially employed for *VvH2A.Z*, but due to the absence of regenerated plants after a first transformation event, a second transformation was later performed using the same construct added with the GRF4–GIF1 complex, which has been reported to enhance regeneration efficiency (Debernardi et al., 2020). Agrobacterium tumefaciens strain GV3101 was used to deliver the constructs into embryogenic calli according to standard procedures (Dhekney et al., 2008; Torregrosa et al., 2015). Cultures were maintained and transferred through the established regeneration steps (Gribaudo et al., 2004; Gambino et al., 2007), until plantlets were obtained and acclimatized in the greenhouse. In total, three independent edited lines for *VvDNMT2* together with one wild-type control were successfully transferred to peat pellets and established in the greenhouse. In the case of *VvH2A.Z*, plants have reached the stage suitable for transfer to peat pellets, but the acclimatization step has not yet been performed.

Editing efficiency analysis

Genomic DNA was isolated from single leaves using the Genomic DNA Isolation Kit (NORGEN, Norgen Biotech Corp) according to the manufacturer's instructions. DNA concentration and purity

were measured with a NanoDrop One Spectrophotometer (ThermoFisher), and samples were adjusted to 10 ng μL^{-1} for subsequent PCR amplification with Cas9-specific primers (Table S1). The presence and efficiency of editing were first evaluated through Sanger sequencing (BioFab Research srl, Milan, Italy), and the resulting chromatograms were analyzed using the Inference of CRISPR Edits (ICE) analysis (https://ice.synthego.com/). Subsequently, a newly generated open reading frame (ORF) was identified using the ORF Finder tool available at NCBI, and the corresponding protein structures were modeled with SWISS-MODEL (https://swissmodel.expasy.org/) to predict the structural consequences of the edits.

Susceptibility assay

A controlled infection assay with *P. viticola* was carried out on three grapevine lines edited for *VvDNMT2* and on a wild-type control line. In July, twelve leaves from each selected line were randomly sampled, rinsed with water, gently dried with paper towels, and placed on Petri dishes containing sterile water-agar (1%). The inoculum of *P. viticola* was prepared by collecting sporangia from naturally infected leaves and adjusting the suspension to a final concentration of 5×10^5 sporangia mL^{-1}. Each leaf was inoculated with 1 mL of suspension and incubated at 24 °C under a 16/8 h light/dark cycle. Symptom development, evaluated as sporulation on the leaf surface, was assessed six days after inoculation. Resistance levels were determined according to descriptor 452-1 of the International Organisation of Vine and Wine (OIV). Leaves were visually scored and classified into the following categories: class 0 – no sporulation; class 1 – 1 to 10% of the surface affected; class 2 – 11 to 30%; class 3 – 31 to 50%; class 4 – 51 to 75%; class 5 – 76 to 100%. For each line, three non-inoculated leaves were used as negative controls to verify the absence of pre-existing infections.

Results

Generation of VvH2A.Z *and* VvDNMT2 *Edited Lines*

A phylogenetic analysis was conducted to classify the histone H2A gene family in grapevine and to determine their evolutionary relationships with Arabidopsis thaliana orthologs. Protein sequences from both species were aligned using MUSCLE (Edgar, 2004), and the resulting phylogenetic tree was generated and visualized with MEGA (Kumar et al., 2016). This analysis allowed the subdivision of the *V. vinifera* H2A variants into distinct groups and enabled the identification of three genes encoding the H2A.Z histone variant (Figure 1). To minimize the risk of pleiotropic effects potentially associated with a complete knockout of this variant, two homologs were selected as targets for genome editing: VIT_200s0179g00340 and VIT_204s0008g04700.

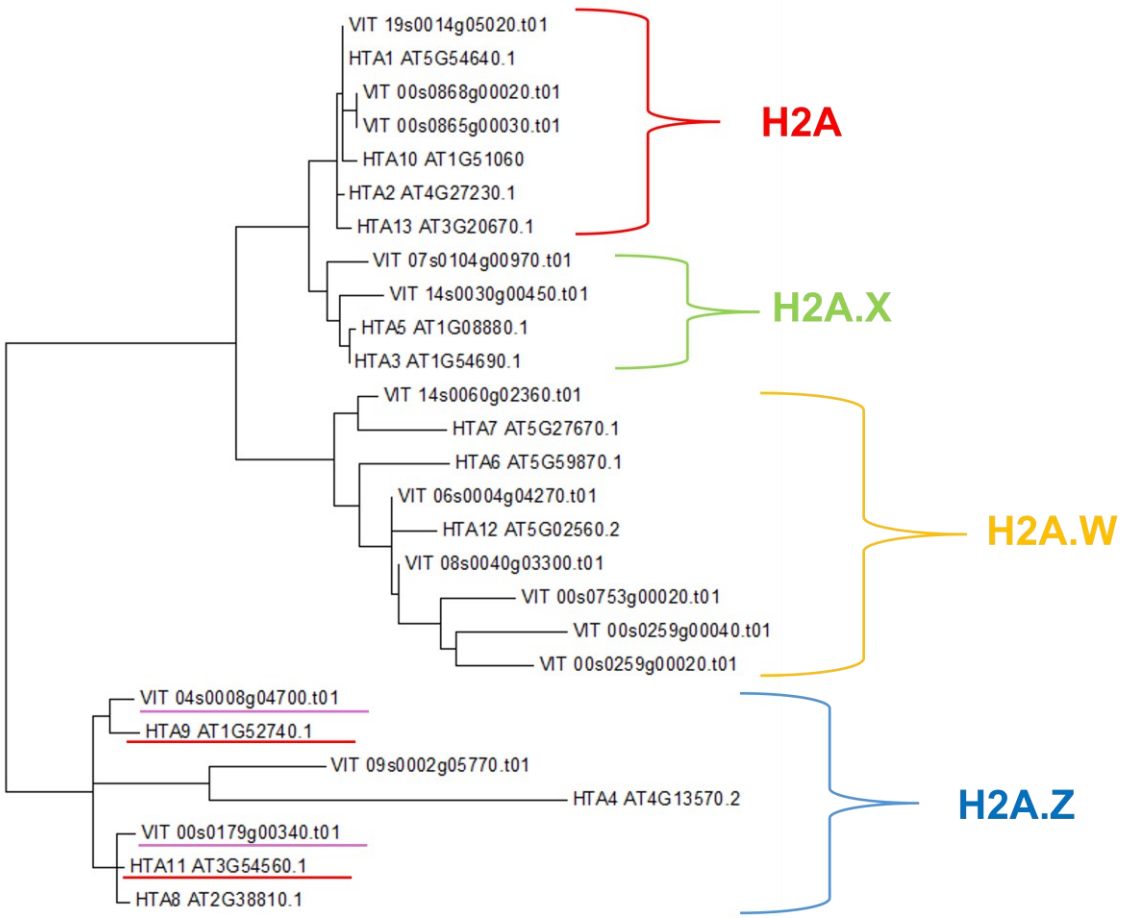

Figure 1 Phylogenetic tree of histone H2A variants from *V. vinifera* and *A. thaliana*. Grapevine genes are indicated with the prefix Vit, whereas Arabidopsis genes are labeled with the prefix HAT. The analysis highlights the clustering of H2A variants into distinct groups and the identification of the three *V. vinifera* homologs belonging to the H2A.Z clade.

Two independent transformation strategies were then employed to obtain *VvH2A.Z*-edited grapevine plants. The first attempt, using the pDIRECT_22c construct (the same employed for *VvDNMT2*), did not result in the regeneration of any plantlets. Conversely, when the same construct was modified by adding the GRF4–GIF1 complex, it successfully led to the regeneration of 53 plants, seven of which were confirmed as transformed (Cas9-positive). To date, five of these lines have been analyzed to evaluate the editing efficiency at both *VvH2A.Z* target loci and one only for VIT_204s0008g04700. All the analyzed lines exhibited editing events in both target genes, although with variable efficiencies and different mutation types (Table 1).

Table 1. Summary of regenerated grapevine lines obtained after CRISPR/Cas9 editing of the two *H2A.Z* homologs (VIT_200s0179g00340 and VIT_204s0008g04700). The table reports the regenerated and transformed lines, the editing efficiency for each event, and the type of mutations identified. Lines carrying distinct editing outcomes (e.g., small in-frame deletions, frameshift insertions, or minimal/no editing) are indicated, and those selected for subsequent structural protein predictions are highlighted in the results section.

	VIT_200s0179g00340		VIT_204s0008g04700	
	% editing	indel	% editing	indel
H2A.Z_6	not sequenced		17 12	+ 1 bp - 3 bp
H2A.Z_12	72	+ 1 bp	5	- 3 bp
H2A.Z_33	75	- 3 bp	69	+ 2 bp
H2A.Z_34	52	- 3 bp	70	+ 2 bp
H2A.Z_35	49	- 3 bp	76	+ 2 bp
H2A.Z_36	75	- 3 bp	80	+ 2 bp
H2A.Z_53	not sequenced		not sequenced	

Targeted editing of the two *H2A.Z* homologs, VIT_200s0179g00340 and VIT_204s0008g04700, generated several regenerated lines with distinct mutation patterns (Table 1). For *VIT_200s0179g00340* line #12 carried a 1-bp insertion. Structural analysis of the corresponding protein models revealed that the insertion in line 12 induced a frameshift starting from amino acid 73, leading to a complete alteration of the C-terminal region, whereas the first 72 residues remained identical to the wild type (Figure 2-B). In contrast, lines #33, #34, #35, and #36 all carried the same 3-bp deletion, resulting in the loss of a single glutamic acid at position 69. This small in-frame deletion did not substantially disrupt the overall protein structure compared to the wild type (Figure 2-C). For the second homolog, *VIT_204s0008g04700*, a different editing profile was observed. Lines #33, #34, #35, and #36 carried a 2-bp insertion, which introduced a frameshift that truncated the protein from 134 to 68 amino acids, with only the first 47 residues conserved with respect to the wild type (Figure 2-E). Such a drastic truncation is predicted to impair protein functionality. Conversely, line #12 showed almost no detectable editing (~5%), whereas line #6 exhibited two distinct minor editing events, indicating heterogeneity at the target locus. In future work, the editing profiles of lines H2A.Z_41 and H2A.Z_53 will be sequenced and characterized, and protein structure predictions will be performed for both *H2A.Z* homologs. For line #6, structural predictions will instead be carried out only for *VIT_200s0179g00340*, given the editing patterns identified.

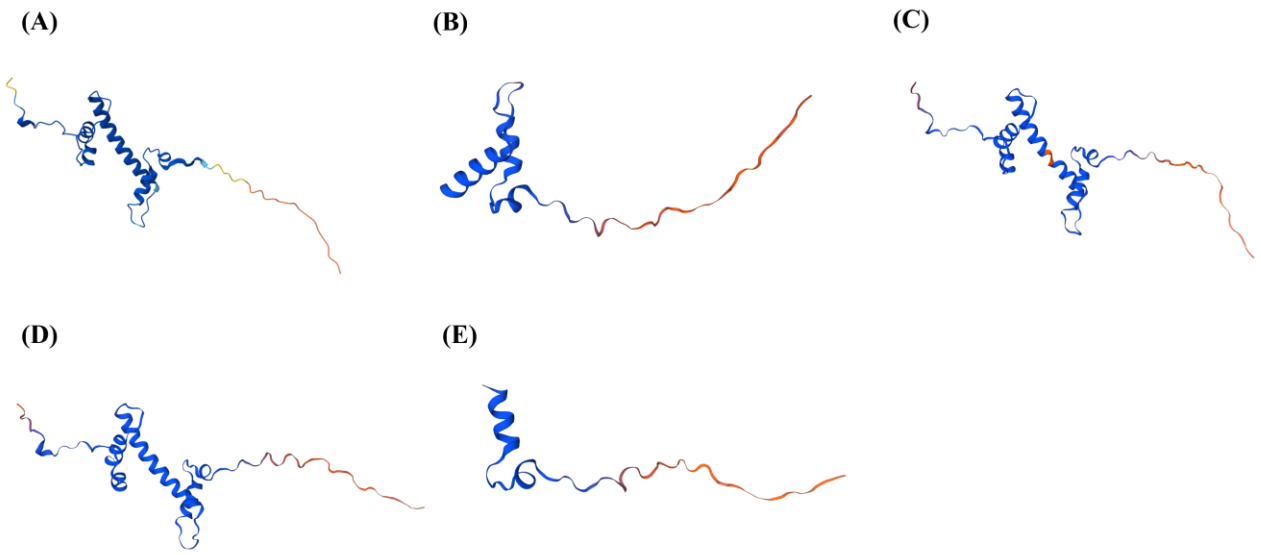

Figure 2. Predicted structures of grapevine H2A.Z proteins after CRISPR/Cas9 editing. (A) Wild-type VIT_200s0179g00340; (B) line #12 with a 1-bp insertion causing a frameshift from amino acid 73; (C) line #33 (representative of lines #33–36) with a 3-bp deletion leading to the loss of Glu69. (D) Wild-type VIT_204s0008g04700; (E) lines #33–36 with a 2-bp insertion causing a frameshift and truncation of the protein from 134 to 68 amino acids.

For VvDNMT2, 27 independent transgenic lines were regenerated, all of which tested positive for transformation and editing. However, each regenerated line exhibited chimerism, a condition in which edited and non-edited cells coexist within the same individual, resulting in genetic mosaicism. Among these, three lines displaying the highest editing efficiency (RMT#12, RMT#18, and RMT#19) were selected and successfully acclimatized in the greenhouse for further studies. Importantly, in vitro, no pleiotropic effects were observed in the regenerated lines edited for either VvH2A.Z or VvDNMT2. In all three selected VvDNMT2-edited lines, the predominant editing outcomes consisted of 36- and 38-bp deletions corresponding to the genomic region excised by the two sgRNAs. The −36 bp deletion represented approximately 50% of the alleles in RMT#12 and RMT#18, and about 60% in RMT#19, whereas the −38 bp deletion accounted for ~20% of the alleles in all three lines. These deletions removed the sequence between the two cleavage sites, disrupting the open reading frame and thereby preventing the production of a functional VvDNMT2 protein.

Susceptibility of VvDNMT2-*edited lines to* Plasmopara viticola

To evaluate whether silencing of *VvDNMT2* affected grapevine resistance to *P. viticola*, a controlled infection assay was performed on three independent edited lines (RMT#12, RMT#18, and RMT#19) together with a wild-type control. Non-inoculated leaves did not show any symptom of infection,

confirming the absence of pre-existing contamination. The evaluation of disease severity after six days, according to OIV descriptor 452-1, revealed a reduction in sporulation for the edited lines compared to WT plants. In particular, WT leaves were assigned an average score of 4.8, indicating widespread sporulation, while RMT#12, RMT#18, and RMT#19 showed mean values of 3.3, 3.2, and 2.8, respectively (Figure 3).

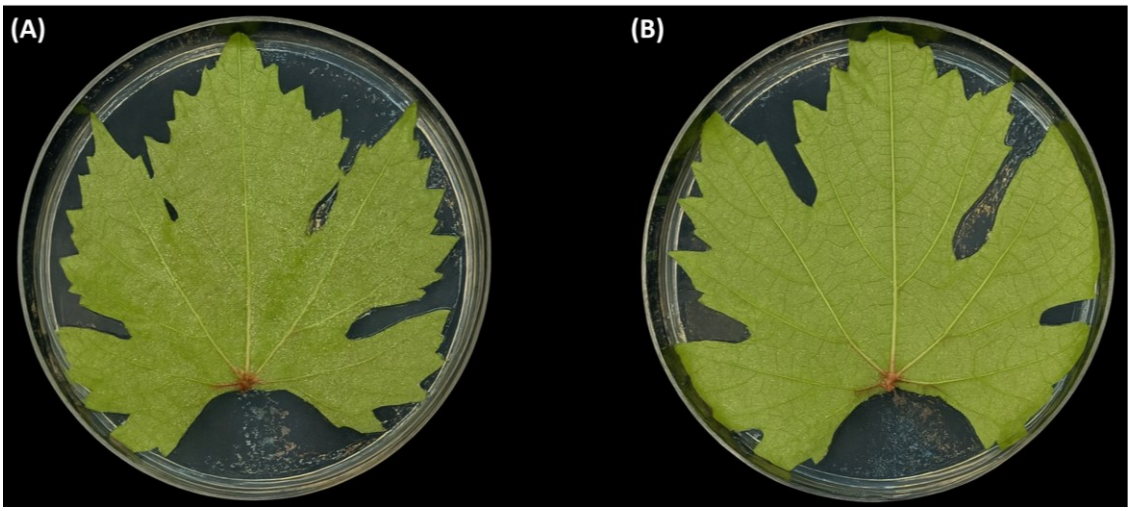

Figure 3. Representative symptoms of downy mildew infection on grapevine leaves. (A) Wild-type (WT) leaf showing extensive sporulation of *Plasmopara viticola* on the abaxial surface. (B) Leaf from a VvDNMT2-edited line (RMT) displaying reduced sporulation compared to WT. Images were taken six days post-inoculation during the controlled infection assay.

Discussion and Conclusions

CRISPR/Cas9 has rapidly emerged as a powerful strategy to investigate the role of epiregulators in plant development and stress responses. In this study, we applied this approach to target two major epigenetic regulators in grapevine, the histone variant *VvH2A.Z* and the tRNA methyltransferase *VvDNMT2*, demonstrating the feasibility of genome editing for functional epigenetics in a perennial woody species.

Our phylogenetic analysis, performed with grapevine and *A. thaliana* orthologs, allowed us to classify the different H2A variants and to identify three genes encoding H2A.Z. Based on this classification, two of them (VIT_200s0179g00340 and VIT_204s0008g04700) were selected as editing targets to avoid a complete knockout of *VvH2A.Z*, which could trigger severe pleiotropic effects, as observed in Arabidopsis hta9 hta11 double mutants (March-Díaz et al., 2007; Foroozani et al., 2022). Using CRISPR/Cas9, we obtained multiple edited lines, confirming that *VvH2A.Z* can be effectively

targeted in grapevine. Interestingly, while in the first transformation attempt using the conventional construct pDIRECT_22C we did not regenerate any plants, the introduction of the GRF4–GIF1 complex to the pDIRECT_22C backbone, enabled successful regeneration of eight transformed lines. This finding suggests that GRF4–GIF1 may alleviate regeneration bottlenecks in grapevine and supports previous evidence of its positive effect on morphogenesis and regeneration efficiency (Debernardi et al., 2020). Furthermore, the difficulty in regenerating complete knockout lines for VvH2A.Z suggests a possible involvement of this histone variant in the regeneration process.

Given that grapevine encodes three *H2A.Z* paralogs, the editing of two of them is expected to reduce, rather than abolish, the overall pool of functional H2A.Z. This partial depletion may impair nucleosome dynamics and decrease the responsiveness of stress- and development-associated genes, thereby attenuating, but not eliminating, the epigenetic regulatory functions of *H2A.Z*. In this context, our edited lines may display altered transcriptional plasticity, potentially leading to differential regulation of defence responses under various biotic and abiotic stresses. Indeed, studies in Arabidopsis and crops have shown that H2A.Z eviction facilitates activation of stress-inducible genes under drought and heat (Kumar and Wigge, 2010; Sura et al., 2017; Xu et al., 2016), while its loss in immune pathways can compromise defence against necrotrophs such as Sclerotinia sclerotiorum (Cai et al., 2021). These findings suggest that our partially edited grapevine lines could serve as an informative model to dissect how histone variant dosage shapes chromatin-mediated adaptation to environmental challenges.

Parallel experiments targeting *VvDNMT2* also yielded promising results, with 27 transgenic lines regenerated, all of which carried editing events. However, each regenerated line exhibited chimerism, a condition in which edited and non-edited cells coexist within the same individual, resulting in genetic mosaics. Consequently, different sectors of a plant may carry distinct genotypes, complicating the evaluation of editing efficiency and the fixation of mutations. Although chimerism represents a technical limitation, strategies such as additional regeneration cycles, selection of fully edited sectors, or the use of morphogenetic enhancers may help reduce its impact in future experiments. Despite this constraint, preliminary susceptibility assays revealed that *VvDNMT2* (RMT#12, RMT#18 and RMT#19) lines displayed reduced sporulation of *P. viticola* compared to WT, suggesting an enhanced tolerance to downy mildew. Interestingly, similar findings were reported for chimeric *VviEDR1*-edited grapevine lines, which showed no growth defects and maintained durable resistance to powdery mildew across multiple years (Yu et al., 2024).

Although chimeric mutations are often regarded as suboptimal and discarded due to their imperfect editing patterns (Charrier et al., 2019; Ishizaki, 2016), the stability of resistance phenotypes observed

in such lines indicates that chimerism can sometimes provide a balanced outcome, retaining growth capacity while enhancing disease resistance. In this context, our results on VvDNMT2 support the idea that partially edited plants can display enhanced resilience, underscoring the potential value of chimeric outcomes in perennial crop improvement. Importantly, the persistence of editing-induced chimerism in long-lived species such as grapevine, apple, and pear raises key considerations for trait stability (Malabarba et al., 2020). Chimerism arises in T0 regenerants from asynchronous editing across cell lineages; while typically unstable in annuals and lost through sexual reproduction, it can persist for years in clonally propagated perennials. In grapevine, for instance, chimeric VviEDR1-edited lines maintained powdery mildew resistance without growth penalties over multiple vegetative cycles (Yu et al., 2024). Similar cases of stable phenotypes despite underlying mosaicism have been reported in apple and pear (Malabarba et al., 2020). Collectively, these findings indicate that chimerism, rather than being a transient or undesirable by-product, can represent a stable and beneficial state in vegetatively propagated perennials, ensuring durable trait expression without compromising vigor. Furthermore, these results align with previous reports in *Arabidopsis*, where *DNMT2* mutants showed altered protein synthesis and proteomic shifts affecting stress and defense-related pathways, including responses mediated by jasmonic acid, reactive oxygen species, and pathogen defense (Wadhwa et al., 2023). Thus, CRISPR-based editing of *VvDNMT2* may provide a new route to dissect the role of RNA methylation in grapevine immunity and its integration with canonical defense pathways.

Overall, our findings demonstrate that CRISPR/Cas9 can be successfully employed to study epigenetic regulators in grapevine, notwithstanding the risk of chimerism and recalcitrance to transformation. The ability to generate targeted knockouts of H2A.Z and tRNA methyltransferases opens the way to explore how chromatin variants and RNA modifications influence grapevine development and resilience. Future work should focus on refining regeneration protocols to reduce chimerism and on expanding functional analyses to additional epigenetic regulators. These efforts will be critical to fully exploit genome editing as a tool to unravel the complex interplay between epigenetics, immunity, and adaptation in perennial crops.

Supplementary

Supplementary Table S1. List of the gRNAs used in this study. For each gRNA, the PAM site is underlined.

Target Gene	sgRNA	Gene ID	Localization
VvDNMT2	AAGGGTTTGTGCAATCCCCAAGG	VIT_204s0008g05060	Exon
VvDNMT2	CGAGTCCTCGAATTCTACAGTGG	VIT_204s0008g05060	Exon
VvH2A.Z	CCTGGAGTATCTGACAGCAGAGG	VIT_200s0179g00340	Exon
VvH2A.Z	CCACCGACTTCTGAAGTCAAGGG	VIT_204s0008g04700	Exon

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CHAPTER 6 | Abroad period at the University of British Columbia

Six months of the third year of my PhD were dedicated to a research stay at the University of British Columbia (UBC) in Vancouver, where I was hosted in the laboratory of Professor Todesco. During this period, I joined a project investigating the genetic regulation of the branching trait in sunflower (*Helianthus annuus*), a model chosen for its contrasting phenotypes in axillary bud development. The work involved the characterization of candidate regulatory genes and promoter regions, aiming to better understand the molecular basis of branching. My activities in Vancouver allowed me to gain experience with a wide range of molecular and functional approaches. RNA extractions were carried out from axillary buds and leaves, followed by quantitative PCR analyses to evaluate gene expression differences between sunflower genotypes. Cloning of candidate genes and promoter regions for functional assays in heterologous systems were performed, and their activity were tested through reporter constructs. In parallel, transgenic lines were generated and analyzed to investigate the potential role of auxin-related regulators in branching. Importantly, this research stay provided hands-on training in single-cell RNA sequencing (scRNA-seq), from protoplast isolation to library preparation. This approach enabled the production of high-resolution transcriptomic data and offered valuable insight into cellular heterogeneity and gene regulation in complex tissues. Although the project focused on a developmental trait in sunflower rather than on plant immunity in grapevine, it substantially broadened the methodological expertise acquired during the PhD and introduced innovative skills relevant for future research. In particular, single-cell transcriptomics represents a promising tool for grapevine studies, with the potential to clarify how specific cell types perceive and respond to microbial signals during interactions with both pathogens and beneficial symbionts. Beyond the technical training, the experience of working in an international and collaborative environment was highly valuable for scientific development, providing new perspectives and fostering opportunities for future collaborations. Overall, this period abroad complemented the central work of the thesis, contributing additional skills and knowledge applicable to the study of grapevine resilience and sustainability.

CHAPTER 7 | CONCLUSIONS

Grapevine is a woody crop of major cultural and economic importance, one of the most environmentally demanding due to its high susceptibility to fungal pathogens and the consequent reliance on intensive fungicide applications. Developing innovative strategies to enhance grapevine resilience and sustainability is therefore an urgent priority in the context of climate change, environmental concerns, and stricter regulations on agrochemical use. This thesis, entitled *"Innovative strategies to enhance grapevine sustainability and resilience against stresses"*, explored the potential of CRISPR/Cas9 as a versatile tool to study plant immunity and epigenetic regulation, ultimately aiming to generate knowledge and resources that may contribute to a more sustainable viticulture. The research was organized into two complementary parts. In the first part, CRISPR-mediated knockout of *NPR3*, a negative regulator of systemic acquired resistance (SAR), demonstrated that targeted editing can enhance disease tolerance against both powdery and downy mildew. Importantly, this work extended beyond pathogen resilience, as it also investigated the impact of immune reprogramming on beneficial interactions. Edited lines displayed improved accommodation of the fungus, promoting arbuscule development and the establishment of a functional symbiosis, while strengthening plant defense without compromising AMF associations. The second part of the thesis addressed the epigenetic dimension of grapevine defense. Using CRISPR-based approaches, key regulators of chromatin dynamics and RNA methylation were targeted, including the histone variant *VvH2A.Z* and the tRNA methyltransferase *VvDNMT2*. Phylogenetic analyses enabled the classification of H2A variants in grapevine and the identification of specific H2A.Z genes for functional studies. Although initial attempts did not yield regenerants, adding a GRF4–GIF1 complex to our construct successfully enhanced plant regeneration, underscoring both the technical challenges and possible solutions in grapevine transformation. Edited lines provided preliminary evidence that disruption of these epigenetic regulators influences plant responses to pathogens, with *VvDNMT2* lines showing chimeric editing but also indications of increasing tolerance to downy mildew. Taken together, these two parts converge on a common conclusion: CRISPR/Cas9 is not only a powerful tool to dissect complex regulatory networks of immunity and epigenetics, but also a promising avenue to develop innovative strategies for grapevine improvement. By simultaneously targeting defense regulators and epigenetic factors, this thesis illustrates how immunity enhancement and chromatin-mediated regulation can be studied and potentially harnessed in a perennial crop. In conclusion, the work presented here contributes to a broader vision of sustainable viticulture in which genetic and epigenetic innovations are integrated with ecological approaches. CRISPR technology has proven to be both a functional genomics platform and a potential breeding strategy, bridging fundamental discoveries with applied outcomes.

Future research will need to consolidate these preliminary findings, extend them to field conditions, and further explore the potential of epigenome editing and epibreeding. By combining molecular precision with ecological awareness, grapevine breeding can move towards a new paradigm of sustainability and resilience, better aligned with environmental challenges and societal expectations.

PUBBLICATIONS, CONFERENCES, TRAINING AND PROJECTS

PUBBLICATIONS:

- Moffa, L. *, Mannino, G. *, **Bevilacqua**, I. *, Gambino, G., Perrone, I., Pagliarani, C., Bertea, C. M., Spada, A., Narduzzo, A., Zizzamia, E., Velasco, R., Chitarra, W., Nerva, L. (2025). CRISPR/Cas9-driven double modification of grapevine MLO6-7 imparts powdery mildew resistance, while editing of NPR3 augments powdery and downy mildew tolerance. *The Plant Journal*, *122*(2), e17204.

- Pereira, J. P., **Bevilacqua**, I., Santos, R. B., Varotto, S., Chitarra, W., Nerva, L., & Figueiredo, A. (2025). Epigenetic regulation and beyond in grapevine-pathogen interactions: a biotechnological perspective. *Physiologia Plantarum*, *177*(2), e70216.

- Secchi, F., **Bevilacqua**, I., Agliassa, C., Maghrebi, M., Cavalletto, S., Morabito, C., Lembo, S., & Vigani, G. (2023). Alkaline soil primes the recovery from drought in Populus nigra plants through physiological and chemical adjustments. *Plant Physiology and Biochemistry*, *201*, 107838.

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CONFERENCE PAPER:

- Nerva, L., Sandrini, M., Spada, A., Paradiso, G., Cometto, A., **Bevilacqua**, I., Moffa, L., Balestrini, R., Chitarra, W. (2023, August). Preserving the grape-associated microbiota to improve the hologenome plasticity and adaptability to climate change. In XIII International Conference on Grapevine Breeding, Genetics and Management 1385 (pp. 103-110).

SCIENTIFIC CONGRESS COMUNICATIONS – ORAL PRESENTATIONS:

- **Bevilacqua**, I., Loredana, M., Varotto, S., Walter, C., & Luca, N. (2023). HARNESSING GENOME EDITING TO ELUCIDATE THE ROLE OF HISTONE VARIANT H2A. Z IN GRAPEVINE. In *Proceedings of the LXVI SIGA Annual Congress*.

- **Bevilacqua**, I., Moffa, L., Narduzzo, A., Varotto, S., Perrone, I., Gambino, G., ... & Nerva, L. (2024). Exploring the impact of NPR3 gene silencing on grapevine defense responses and arbuscular mycorrhizal symbiosis establishment. In *Proceedings of the LXVII SIGA Annual Congress*. Società Italiana di Genetica Agraria.

- **Bevilacqua**, I., Moffa, L., Varotto, S., Balestrini, R., Chitarra, W., Nerva, L., (2024). Exploring the impact of NPR3 gene silencing on the interaction between grapevine and mycorrhizal fungi through genome editing. Open International Conference on Grapevine Physiology and Biotechnology (Open-GPB2024).

SCIENTIFIC CONGRESS COMUNICATIONS – POSTER PRESENTATIONS:

- Nerva, L., Sandrini, M., Spada, A., Cometto, A., **Bevilacqua**, I., Paradiso, G., ... & Chitarra, W. (2023). Unlocking the potential of the hologenome: development of microbial breeding approaches to face the domestication syndrome and improve resilience against climate change. In *Proceedings of the LXVI SIGA Annual Congress*. Società Italiana di Genetica Agraria.

- Moffa, L., Nerva, L., **Bevilacqua**, I., Narduzzo, A., Perrone, I., Pagliarani, C., ... & Chitarra, W. (2024). Advancements in in vitro culture techniques and genetic transformation for grapevine improvement. In *In Vitro Culture of Woody Crops: problem solving by new approaches*.

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SCIENTIFIC TRAINING INHERENT TO PhD COURSE:

- WORKSHOP: Cropinno 2023, Introduction to (Crop) Epigenetics

- Seminars at UBC:

  The Matrix Has You: The Importance of Accurate, Efficient and Open Methods Upstream of Single Cell Count Matrices; Featured Speaker: Dr. Rob Patro

  Organelle Biogenesis and Function in Plants; Hosted by: Dr. Xin Li and Dr. Liwen Jiang

  MSL TRAINEE TALKS: Trainee Talk 1, Michael Easson– Bohlmann lab: Advancing Bioengineered Production of Montbretin A-Explorations in Enzyme Engineering.