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"**Conventional breeding, genome editing and microbial consortia to enhance sustainability and resilience of grapevine cultivation**"

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Summary

In the present PhD thesis, we exploit different complementary approaches for a more sustainable control of grapevine diseases: inheritance of resistance genes, genome-editing to knock-out susceptibility genes and application of plants associated microorganisms to improve plants immunity.

In the second chapter a review article describes in detail the most recent biotechnological approaches for crop protection, including genome editing, cisgenesis, RNAi and epigenetics.

In the third chapter were reported the activities concern the conventional breeding approach. In this section the elite cv. Glera was crossing with resistant hybrids of different geographical origins. First the offsprings were evaluated for resistance loci presence. Then, eight plants carrying resistant genes to powdery and downy mildews were characterized by gas chromatography-mass spectrometry analyses to provide chemical nature of wine aroma.

The fourth chapter described the activities concern the genome-editing application to knock-out susceptibility genes involved in powdery mildew interactions and in general to control the biotrophic pathogenic fungi. In details a novel CRISPR/Cas9 system based on specific recombinase (Cre/LoxP) was developed with the aim of removing the 'entire' T-DNA cassette.

In parallel, a review article about the application of Synthetic Community is presented in Chapter five.

Finally, in Chapter six the exploitation of plant immune system is presented. The study aimed at clarifying the effects of arbuscular mycorrhiza priming on the grapevine growth-defence tradeoff.

CHAPTER 1

General introduction

Grapevine (*Vitis vinifera* L.) is one of most extensively cultivated fruit crops with an essential role in economy of many countries such as Italy, China, France, and USA (FAOSTAT, 2021). Indeed, grapevine is the 21st most valuable cultivated crop worldwide with a global value of 77.1 million tons on 6.9 million of hectares of land (FAOSTAT, 2021; OIV, 2021).

Notwithstanding, a large number of inorganic and synthetic organic pesticides are frequently applied in viticulture. Therefore, the potential hazards posed by their use is currently a cause of public concern, mainly due to the adverse health effects.

In this context, the European policy provided many restrictions trying to balance the necessity to reduce agrochemical products and protect yield and crop quality: Directive 2009/127/CE regards machinery for pesticides applications and Directive 2009/128/EC focuses on sustainable use of pesticides and Regulation 2002/473/EC promotes a reduction of maximum quantity per year of copper fungicides application.

The need to develop an environmentally friendly viticulture models has led to numerous grapevine improvement programs aiming to reduce chemicals input.

The aim of the present PhD thesis is to provide a comprehensive view of alternative strategies for answering to the need of sustainability in grapevine defence against fungal diseases. More in detail, the strategies developed in the present work are aimed to counteract *Erysiphe necator,* the causal agent of powdery mildew, *Plasmopara viticola*, the causal agent of downy mildew, and more in general biotrophic pathogenic fungi. To fulfil this goal the work has been organized setting four main objectives below described.

In Chapter 3, we apply a pyramiding strategy to produce resistant Glera genotype both to powdery and downy mildews. The breeding program is attended by offspring analysis through improved Marker Assisted Selection (MAS) to reduce time and cost analysis. The selected offspring were then evaluated by agronomical and biochemical point of views.
In Chapter 4, we develop a novel CRISPR/Cas9 system based on specific recombinase (Cre/LoxP) to produce grapevine plants without foreign DNA sequences (*non*-GMO), with an unaltered genetic background and with a reduced susceptibility to the biotrophic pathogenic fungi. In specific, we decided to apply genome editing on two gene classes:

Mildew Locus O, selecting *VvMLO6* and *VvMLO7*, and *Non-Expressed Pathogenesis-related Protein 3* (*VvNPR3*).

In Chapter 5 we discuss the possibility to develop more sustainable breeding strategies, exploiting the multiple interactions between plants and the associated microorganisms. This work represents an initial literature review aiming at setting up a new kind of breeding based on the development of Synthetic microbial Communities (SynComs) to increase the agroecosystem resilience and sustainability.

In Chapter 6 we investigate the potential benefits of an inoculum formed by two AM fungal species, with or without a monosaccharide addition, on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. In plants treated with AM the evaluation of gene expression, agronomic traits and metabolites production, revealed a positive impact on plants immunity.

CHAPTER 2

Novel and emerging biotechnological crop protection approaches

Review article

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Novel and emerging biotechnological crop protection approaches

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Abstract

Traditional breeding or Genetically Modified Organisms (GMOs) have for a long time been the sole approaches to effectively cope with biotic and abiotic stresses and implement the quality traits of crops. However, emerging diseases as well as unpredictable climate changes affecting agriculture over the entire globe forces scientists to find alternative solutions required to quickly overcome seasonal crises. In this review, we first focus on cisgenesis and genome editing as challenging biotechnological approaches for breeding crops more tolerant to biotic and abiotic stresses. In addition, we take into consideration a toolbox of new techniques based on applications of RNA interference and epigenome modifications, which can be adopted for improving plant resilience. Recent advances in these biotechnological applications are mainly reported for non-model plants, and woody crops in particular. Indeed, the characterization of RNAi machinery in plants is fundamental to transform available information into biologically or biotechnologically applicable knowledge. Finally, here we discuss how these innovative and environmentally friendly techniques combined with traditional breeding, can sustain a modern agriculture and be of potential contribution to climate change mitigation.

Keywords

New plant breeding techniques, Cisgenesis, Genome editing, RNA interference, Disease resilience, Abiotic stress, DNA methylation, Epigenetics

Introduction

Increasing plant resilience against biotic or abiotic stress and improvement of quality traits to make crops more productive as well as nutritious are focal targets in plant breeding programs. Opposing pressure comes from the increasing virulence of a large number of pests and diseases, caused by insects, fungi, bacteria, viruses and nematodes (Gimenez *et al*., 2018), and legislation limiting the use of agrochemicals (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council). On the other hand, climate changes expand abiotic stress conditions forcing plant breeders to select genotypes resistant to water and thermal stresses to cope with the modification of rainfall patterns and rise in temperatures (Porter *et al*., 2014; Mohanta *et al*., 2017b). These unfavorable constraints are leading to insufficient yield and a strong decrease in quality features (Ebi and Loladze, 2019).

The development of genetically improved varieties of crop plants has long been taking advantage of crossings and mutagenesis to obtain plants with better characteristics in terms of yield and quality features, as well as improved stress resilience traits (Dempewolf *et al*., 2017). Since the 1920s, when introgression of the desired traits from the available germplasm has not been possible, mutagenesis through radiation or chemical agents has been used. Over the last century, genetic engineering and biotechnologies have broadened the toolbox of geneticists and breeders with new instruments and approaches, leading to the creation of genetically modified organisms (GMOs) (Lusser *et al*., 2012). The potential of this approach to obtain improved disease resistance, abiotic stress resistance and nutritionally improved genetically modified crops have been widely demonstrated and discussed, together with the limitations and the concerns associated with the use of GMOs (Low *et al*., 2018; Kumar *et al*., 2020; Van Esse *et al*., 2020; Sabbadini *et al*., 2021).

Thanks to these techniques, the gene pool potentially available to plant breeders has considerably increased, allowing the isolation and transferring of genes to crops from sexually incompatible plant species as well as from other organisms (Carrière *et al*., 2015). Although in 2018 GM crops covered 191.7 million hectares with remarkable benefits (Brookes and Barfoot, 2016; Change, 2018), their use is still associated with strong public concern, which is related to putative risks for human health and environment contamination (Frewer *et al*., 2011; Carzoli *et al*., 2018). Insertion in the crop genome of genes isolated

from genetically distant and/or unrelated organisms (transgenes), which usually includes selectable markers (e.g. resistance to antibiotics), is one of the most criticized aspects by citizens. Over the years, to overcome GM crop limitations, many techniques have been developed up to the latest New Plant Breeding Techniques (NPBTs, e.g. genome editing).

In the last 15 years, Next Generation Sequencing (NGS) technologies fostered a major advancement in crop genomics and contributed to the public availability of many reference crop genomes (Jaillon *et al*., 2007; Xu *et al*., 2011; Sato *et al*., 2012; Verde *et al*., 2017; Linsmith *et al*., 2019). Moreover, high-throughput re-sequencing of hundreds of genotypes allowed researchers to describe the allele diversity of both domesticated and wild plant populations (Morrell *et al*., 2012). In this context, the increased data availability on genome structures deepened the comprehension of plant domestication history, the identification of genes responsible for traits of agrochemical interest and gene functions, promoting the development of NPBTs for overcoming the major GMO laborious and costly regulatory evaluation processes and public concerns. Actually, NPBTs allow a single gene to be transferred, mimicking sexually compatible crosses (cisgenesis) and precise modification of specific DNA sequences (genome editing).

In this review, we summarize the main features, advantages and challenges of various biotechnological approaches, providing examples of applications for the amelioration of plant traits to better cope with biotic and abiotic stresses. The common thread is to describe the recent biotechnological advancements which allow crop traits to be precisely modified and overcome the restrictions imposed on genetically modified products. Therefore, we focused our discussion on cisgenesis and genome editing as the more known techniques, but we also addressed our attention on latest innovative crop breeding technologies, such as RNA interference and epigenome editing. Emphasis is given to non-model plants, such as woody crops, for which the application of biotechnological approaches is not as easy as for herbaceous model plants.

Cisgenesis: approaches and potentials in plant protection

The idea of cisgenesis was first proposed by Shouten in 2006. In its widely accepted definition, the results of cisgenic approaches are crops modified with genes isolated

exclusively from sexually compatible plants, including gene introns and regulative regions, such as promoters and terminators, in their sense orientation (Schouten *et al*., 2006a; Schouten *et al*., 2006b).

Cisgenic strategies

Cisgenic plants may resemble plants derived from traditional breeding and share the same genetic pool with them, since genes of interest are isolated from a species that could be used for traditional crosses and transferred, preserving its "native" form. One of the main drawbacks of gene introgression in a crop genome by classical crosses is that a large number of undesirable associated genes are transmitted along with the gene(s) of interest to the next generation, often negatively influencing many agronomic traits, related to products quality and yield. This phenomenon, defined as linkage drag, is common in introgression breeding and Marker Assisted Selection (MAS) is often adopted to reduce the amount of undesired genes (Hospital, 2005). The use of MAS-complex schemes slows down new cultivar release, which can require decades in the case of woody plants that have long juvenile phases. Cisgenesis allows the linkage drag issue to be overcome by transferring only the desired gene(s) in a single step, preserving all the quality traits selected in the élite cultivars.

The limit of cisgenesis is its suitability only to monogenic traits, although it could also be applied to oligogenic characters: indeed, the technical complexity of the procedure is directly correlated with the number of genes to be transferred. On the other hand, cisgenic plants display greater public and farmers positive consensus compared to transgenic ones (Delwaide *et al*., 2015; Rousselière and Rousselière, 2017; De Steur *et al*., 2019).

Detailed methods and strategies with an interesting success rate for the development of cisgenic plants have been comprehensively reviewed by several authors over the last decade (Schaart *et al*., 2011; Espinoza *et al*., 2013; Holme *et al*., 2013; Cardi, 2016) so these approaches are quite mature for a wide use.

Since its initial application, several strategies have been conceived for cisgenesis (Fig.1), by considering the differences in transformation and regeneration efficiency and length of the breeding cycle, which depend on the selected plant species. The simplest approach consists of the use of vectors where only the gene of interest is cloned in the T-DNA region, transferred to plants through *Agrobacterium*-mediated transformation and then selected by PCR analysis (Fig.1a) (De Vetten *et al*., 2003; Basso *et al*., 2020). Another similar strategy

exploits minimal gene cassettes, made just by promoter, coding sequence and terminator, which are introduced into the plant genome by biolistic transformation (Fig.1b), thus avoiding partial or complete backbone integrations (Vidal *et al*., 2006; Low *et al*., 2018). Nevertheless, these systems require long and expensive PCR screenings, and are suitable only for species with a high transformation efficiency (Vidal *et al*., 2006; Malnoy *et al*., 2010; Petri *et al*., 2011; Low *et al*., 2018). In species where transformation is recalcitrant, the transformation with cisgenic reporter genes or co-transformation with selectable marker genes could greatly simplify the recovery of transformed plants. For example, Myb transcription factors involved in the regulation of anthocyanin biosynthesis were tested in apple (Krens *et al*., 2015) and grapevine (Li *et al*., 2011) as selectable markers for cisgenic plants. The use of exogenous or endogenous reporter genes have been already successfully applied in herbaceous species (Basso *et al*., 2020). However, the possibility of using such reporters is confined to those cases where tissue coloration does not interfere with selection for other traits of interest. In seed propagated crops (e.g. wheat, barley, rice, tomato, etc…) it is possible to use a co-transformation strategy (Fig.1c), crossing them with the parental or original variety and hence exploiting segregation of the selectable marker in the progeny, obtaining plants with the cisgene but without the selectable marker (Holme *et al*., 2012a). For vegetative propagated species with poor transformation efficiencies, a novel developed approach relies on the excision of unwanted DNA sequences after the selection of transformed plants through recombination systems (Fig.1d). In 1991, Dale and Ow used the bacteriophage P1 Cre/Lox recombinase/sites for markers excision in tobacco plants (Dale and Ow, 1991). Since then other alternative systems from *Zygosaccharomyces rouxii (*R/Rs) and *Saccharomyces cerevisiae* (FLP/frt)*,* have been tested (Lyznik *et al*., 1993; Schaart *et al*., 2011). In all these systems, the recombinase expression is usually controlled by chemical or heat shock inducible promoters to avoid a premature excision of the selectable markers (Fig.1d) (Schaart *et al*., 2011; Dalla Costa *et al*., 2016).

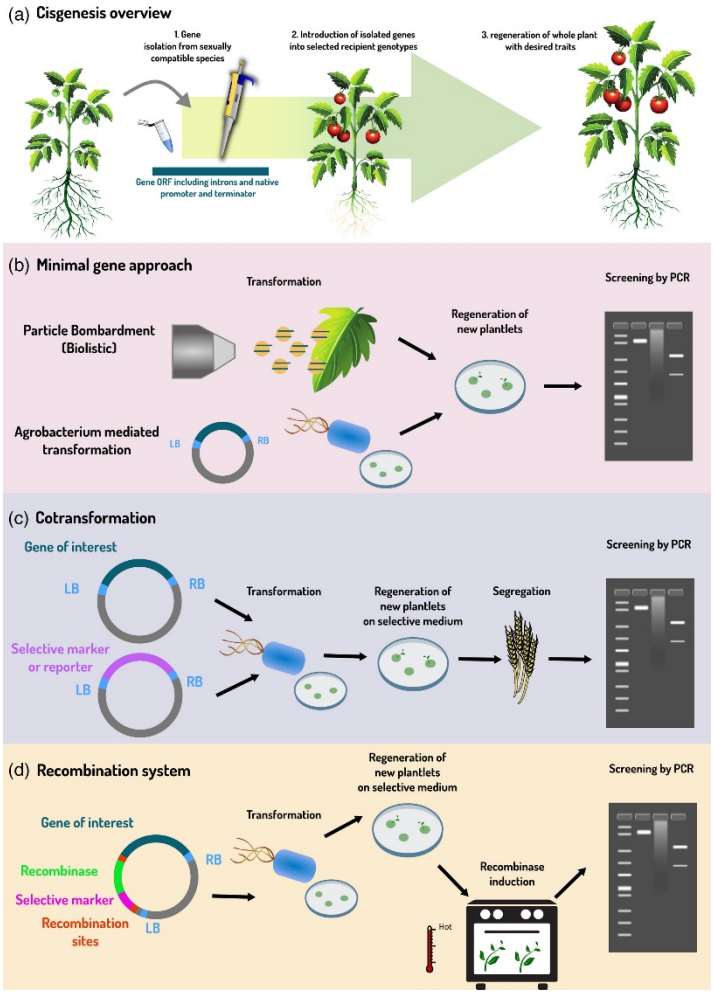

Figure 1 (a) Overview of cisgenic strategies from gene selection to plant phenotyping; (b) minimal gene approach, only the gene of interest is cloned in the TDNA region; (c) co-transformation strategy, the selective marker and gene of interest are introduced by independent transformation events, segregation of the genes allows the selection of cisgenic plants in F1 progeny; (d) excision of unwanted DNA sequences through recombination systems: chemical or physical stimulation induce the excision of DNA fragments flanked by the recombination sites.

Stress tolerant cisgenic crops

Cisgenic approaches were adopted in potato, apple, grapevine, melon, wheat, barley, poplar, rice and strawberry (Gadaleta *et al*., 2008a; Benjamin *et al*., 2009; Han *et al*., 2011; Dhekney *et al*., 2011; Holme *et al*., 2012a; Krens *et al*., 2015; Haverkort *et al*., 2016; Tamang, 2018; Maltseva *et al*., 2018). In most cases the aim was to increase pathogen resistance, although some studies were focused on quality trait improvement.

Haverkort and colleagues pursued a marker free approach to obtain four cisgenic Late Blight (*Phytophthora infestans*) resistant potato varieties, by transferring from one to three

resistance genes (Haverkort *et al*., 2016). In addition, cisgenic apple varieties were developed by introducing the apple scab (*Venturia inaequalis*) resistance gene *Rvi6* in the susceptible cultivar 'Gala' (Schaart *et al*., 2011). In the same work, the authors achieved the removal of the selectable marker gene by inducing the *recombinase R* with dexamethasone. The obtained cisgenic plants were tested in field conditions for three years and showed a stable resistant phenotype (Krens *et al*., 2015). Interestingly, the effectiveness of the same recombinase system was recently also tested in banana, inducing the excision of the green fluorescent protein, used as reporter gene (Kleidon *et al*., 2019).

Several pathogen resistance genes (PR1 variants, *VvTL1*, *VvAlb1*, homologues of *VvAMP1* and *VvAMP2*/defensin, and an orthologue of Snakin-1) have been isolated from species sexually compatible with *Vitis vinifera* and overexpressed in transgenic lines, which are now under evaluation in field conditions (Gray *et al*., 2014). In grapevine, methods using a heat-shock controlled FLP/frt recombination system for selectable marker excision have also been reported (Dalla Costa *et al*., 2016; Dalla Costa *et al*., 2020).

Transgenic lines of melon have been developed overexpressing the glyoxylate aminotransferase *At1* and *At2* genes, conferring resistance to *Pseudoperonospora cubensis*, which causes downy mildew in cucurbits (Benjamin *et al*., 2009). Since the resistance is given by the increased transcription level of these genes, it remains to be assessed whether such an increase can be obtained in cisgenic lines.

In durum wheat, biolistic co-transformation with minimal gene cassettes was used to develop cisgenic lines expressing *1Dy10* HMW glutenin gene, isolated from bread wheat and associated to an improved baking quality. Homozygous cisgenic lines were obtained by segregation at the 4th generation (Gadaleta *et al*., 2008b; Gadaleta *et al*., 2008c). Moreover, cisgenic lines of wheat carrying a class I chitinase gene displayed partial resistance to fungal pathogens (Maltseva *et al*., 2018). Holme *et al*. (2012b) used a barley phytase gene (*HvPAPhy_a*) and the co-transformation strategy to test cisgenic feasibility in barley, obtaining lines with increased phytase activity (Holme *et al*., 2012a).

Cisgenesis has also been applied in rice, to overcome one of the most diffuse and devastating pathogens (*Magnaporthe grisea*), by using a co-transformation strategy to introduce rice blast disease resistance gene *Pi9* into elite rice cultivars (Tamang, 2018).

In addition to stress resistance, cisgenesis is also an effective approach for modifying other crop traits as it has been demonstrated in poplar. Genes from *Populus trichocarpa*

(*PtGA20ox7*, *PtGA2ox2*, *PtRGL1_2*) involved in gibberellin metabolism were transformed in *Populus tremula* x *alba*, showing that negative gibberellic acid regulators determined a slower growth (*PtGA2ox2*) and longer xylem fibers (*PtRGL1_2*), while the positive regulator determined an increased growth rate (*PtGA20ox7*). However, the poplar plants obtained still contained the positive selectable marker and cannot be considered as cisgenic (Han *et al*., 2011).

Intragenic plants, as in the case of cisgenesis, possess only genetic material deriving from sexually compatible species, but the inserted gene is the result of a genetic element isolated from different species (e.g. a gene promoter from one species and a coding sequence from another, both sexually compatible) (Holme *et al*., 2013). An interesting example of this approach comes from the overexpression of cisgenic polygalacturonase inhibitor protein (*FaPGIP*) in strawberry which conferred resistance to grey mold (*Botrytis cinerea*). The overexpression was achieved by cloning the *FaPGIP* coding sequence under the promoter of the strawberry expansin-2 gene and for this reason should be referred to as intragenic (Schaart, 2004).

Genome editing

Genome editing introduces changes in specific target DNA sequences without altering other regions (including the target flanking regions) and with the potential to avoid introduction of foreign DNA. The genome editing is performed using endonucleases which are able to recognize specific DNA sequences. Once the target sequence is recognized, the endonuclease introduces a double strand DNA (dsDNA) break (DSB) and induces subsequent activation of the DNA repair pathway (Manghwar *et al*., 2019). This result can be achieved by exploiting three different classes of enzymes: Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effectors Nucleases (TALENs) and Cas proteins (Zhang *et al*., 2017). Strong efforts have been made by numerous researchers all over the world to improve the Cas-mediated genome editing technology, which became the most used and efficient tool to edit target genomes (Xie and Yang, 2013). The ability of genome editing techniques to help breeders in improving plant resistance against biotic and abiotic stresses is only in its infancy, but some examples are already available and a concise overview of the steps involved in the development of edited plants is presented in Figure 2.

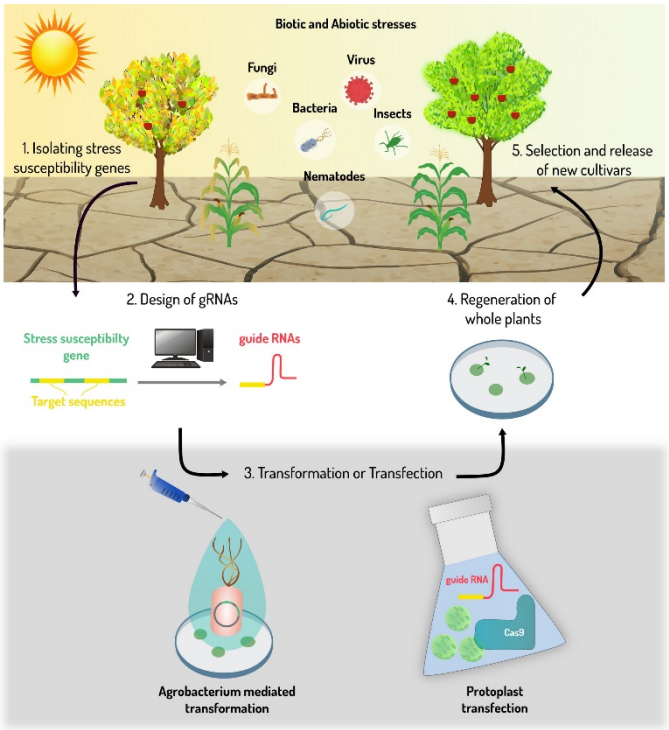

Figure 2 Workflow for the development of genome edited stress-resistant crops: (1) susceptibility genes are isolated and characterized by genetic and functional genomics studies; (2) informatics-aided design of gRNAs for increased specificity and off-target minimization; (3) *Agrobacterium tumefaciens*-mediated transformation of plant tissue cultures or ribonucleoprotein protoplast transfection. (4) Regeneration and selection of transformed plants; (5) testing and selection of transformed lines, release of new varieties.

Focus on CRISPR-Cas: a brief overview

The Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)-Cas systems, discovered as conserved mechanisms against viral invasions in bacteria, require three distinct components: a protein with nuclease activity (e.g. Cas9, Cas12, Cas13 etc.), a single guide RNA (sgRNA) necessary to guide the Cas protein on target sites and a Protospacer Adjacent Motif (PAM), a short sequence upstream of the complementary DNA strand acting as tag of the target site (Fig.3a) (Doudna and Charpentier, 2014). The sgRNA-Cas complex scans the genomic DNA looking for the complementary sequence and, once identified, the Cas protein induces a dsDNA cleavage at a specific position that is determined by the Cas type (Jiang and Doudna, 2017). After DNA cleavage, there are two major pathways of DNA repair in plants: homologous recombination (HR) and nonhomologous end joining (NHEJ), the latter being the most commonly used (Schwartz, 2005; Ran *et al*., 2013). These two repair mechanisms are the basis for exploiting the Cas in NPBTs.

The CRISPR-Cas system shows very versatile features to produce knock-out mutants, to insert a DNA fragment using a donor vector through the HR system, to base edit a target sequence (e.g. substitutions of C to T and/or A to G etc.), to induce mutation in regulatory sequences, and modify the epigenome (Vats *et al*., 2019). Nevertheless, if multiple genes that are closely related have to be targeted (e.g. gene family members, multiple alleles of the same gene) two different strategies are available: i) multiple guide RNAs under the control of a same promoter (polycistronic construct) or multiple guides under the control of their own specific promoter (Xing *et al*., 2014; Tang *et al*., 2016; Cermak *et al*., 2017) and ii) one or a few sgRNAs capable of driving the Cas protein on different genes (Yu *et al*., 2018).

Initial steps through a wide use of CRISPR/Cas system

The first reported genome-editing application using CRISPR/Cas systems in plant was achieved in 2013 using two model organisms: *Arabidopsis thaliana* and *Nicotiana benthamiana* and easily observable reporter genes (Li *et al*., 2013; Mao *et al*., 2013). Over the years, more progress has been made, with several reports in different herbaceous plant species (e.g. tomato, rice, soybean, wheat, etc.) up to the application in woody species (e.g. citrus, apple, grape, etc.) (Ghogare *et al*., 2020). Furthermore, different laboratories are committed in developing new delivery methods for plant systems. Indeed, classically the DNA sequences encoding for Cas and sgRNA(s) have to be delivered into the host plant genome, and to date, different methods have been tested: *Agrobacterium*-mediated transformation, nanoparticle platforms, biolistic transformation and protoplast transfection (Ahmad and Amiji, 2018; Kalinina *et al*., 2020). Even though *Agrobacterium*-mediated transformation is widely used in plants, this method requires integration of T-DNA into the host genome together with selectable marker genes (Dalla Costa *et al*., 2016; Duensing *et al*., 2018). Actually, the integration of selectable markers is an important legislative issue as it can be stably transferred to sexually compatible species and also to other organisms, without reproduction or human intervention, as a consequence of horizontal gene transfer (HGT) (Keese, 2008; Soda *et al*., 2017). Conversely, protoplasts transient transformation and regeneration approach allows the direct delivery of ribonucleoproteins (RNPs) in plant tissues without introducing foreign DNA and GM plant creation (Baltes *et al*., 2015; Cermak *et al*., 2017; Bruetschy, 2019). Recently the *Agrobacterium*-mediated transformation was compared with the RNPs delivery through PEG-mediated protoplasts transfection

approaches in apple and grapevine (Osakabe *et al*., 2018). Although the biolistic method allows the production of transgene-free plants it displays huge limitations in woody plants (Osakabe *et al*., 2018) due to restraint in obtaining the embryogenic tissue, which is then able to regenerate the edited plant (Altpeter *et al*., 2005).

CRISPR technology as a valuable tool to improve crop protection

One of the main tools to enhance plant resistance against fungal and bacterial pathogens relies on targeting susceptible genes (S genes) (Pavan *et al*., 2009) as proven in *Theobroma cacao* and several other species (Fister *et al*., 2018; Langner *et al*., 2018). Susceptibility gene distinctiveness relies on the fact that they are genes that critically facilitate the compatibility between the plant and the pathogen. They are essential for their interaction, especially in the case of biotrophic pathogens. Therefore, mutation or loss of an S gene can limit the ability of the pathogen to cause disease (van Schie and Takken, 2014). An interesting example was given by Paula *et al*. (2016), who introduced a mutation in *Solanum lycopersicum DMR6* gene lowering tomato susceptibility not only to downy mildew but also to *Pseudomonas syringae, Phytophthora capsici* and *Xanthomonas* spp. (Paula de Toledo Thomazella *et al*., 2016). A similar approach was used in apple (*Malus domestica*) to achieve resistance against *Erwinia amylovora* (Pessina *et al*., 2016). Pompili *et al*. (2020) used the Cas9 system to produce an *MdDIPM4* knock-out mutant enhancing plant resistance against the fire blight pathogen. A novelty introduced by this approach is an inducible recombination system (FLP/frt) able to remove almost all the T-DNA insertions after confirming the editing event. CRISPR technology was latterly applied to rice in order to obtain bacterial blight resistant varieties: Cas9-mediated genome editing to introduce mutation in one or multiple susceptible genes, belonging to the sugar transporters SWEET family, was successfully achieved in recent works (Oliva *et al*., 2019; Zeng *et al*., 2020). Finally, another interesting application of CRISPR to counteract biotic stress was provided in tomato. By targeting a microRNA (miRNA) it was demonstrated the possibility to enhance plant immunity against *Fusarium oxysporum* f. sp. *Lycopersici*, the causal agent of tomato wilt disease, enhancing the basal expression of nucleotide-binding site-leucine rich repeat (NBS-LRR) protein (Gao *et al*., 2020).

As for fungal and bacterial pathogens, the CRISPR technology can provide a strategy to generate plants with virus resistance. For instance, it is possible to both directly target viral

replication, by producing GMO plants expressing constitutive Cas protein and gRNA(s) that target viral sequences (Baltes *et al*., 2015; Ji *et al*., 2015) or to generate virus resistant cultivars through modification of plant genes (Kalinina *et al*., 2020 and references therein). Beyond biotic stresses, and despite a limited number of papers, abiotic stresses such as water deficit, high temperature and soil salinity can also be tackled by editing plant genes involved in stress response (Nguyen *et al*., 2018; Zafar *et al*., 2020; Joshi *et al*., 2020). An interesting example was reported in a work where the *OST2/AHA1* locus (which regulates stomata response to abscisic acid) was edited to obtain Arabidopsis with increased stomatal responses upon drought and a consequent lower water loss rate (Osakabe and Osakabe, 2017). In parallel, if not directly applied to achieve drought-resistant crops, CRISPR technology can be exploited to study the function of gene(s) along complex regulatory mechanisms. This was the case of nonexpressor of pathogenesis-related gene 1 (NPR1), a special receptor of salicylic acid (SA), considered as an integral part in systemic acquired resistance (SAR) (Wu *et al*., 2012). Cas9 was used to obtain NPR1 tomato mutants, which showed reduced drought tolerance, demonstrating that, despite its involvement in biotic stress responses, NPR1 is also involved in abiotic stress resilience (Li *et al*., 2019). More recently, the CRISPR activation (CRISPRa) system (Brocken *et al*., 2018) (based on an inactivated version of the nuclease known as dead Cas9 -see next paragraph for more information- fused with a transcription activator) targeting the promoter of ABA-responsive element binding proteins (AREB) was used to study stress-related responses and enhance the drought tolerance in *Arabidopsis* (Roca Paixão *et al*., 2019).

New frontiers in CRISPR/Cas application

Although genome editing has been widely used for editing specific plant genes, several studies relied on the improvement of its efficiency, versatility and specificity (Gleditzsch *et al*., 2019). Indeed, despite many theoretical advantages and potential applications, the genome editing techniques still present one major drawback: Cas proteins can recognize PAM sites in non-target sequences and thus induce DSBs in these sequences, leading to undesirable phenotypes. To mitigate the off-target activities, different bioinformatic approaches were developed and used for computational prediction of Cas activity on specific genomes (Bae *et al*., 2014; Lin and Wong, 2018; Liu *et al*., 2020a). Moreover, development of Cas variants with improved specificity, such as Cas12a and b (Ming *et al*., 2020; Schindele

and Puchta, 2020), eSpCas9 (Slaymaker *et al*., 2016), HiFi-Cas9 (Kleinstiver *et al*., 2016) and HypaCas9 (Ikeda *et al*., 2019) tried to mitigate the off-target activity and these variants have already been applied in plant genome editing strategies.

Beside the improved Cas variants, different authors have been focusing on the implementation of dead Cas9 (dCas9) (a Cas9 where both the nuclease domains have been inactivated) that could be used for several purposes. The simplest one is the ability to interfere with transcription via steric blockage of polymerase without performing endonuclease activity (Brocken *et al*., 2018). Furthermore, the dCas9 system can be engineered by linking it to a transcription activator or repressor. These systems can be applied to species that lack a controllable expression system or to study the overexpression or downregulation of target genes, without changing the genome context or introducing a transgene (Mohanta *et al*., 2017a; Moradpour *et al*., 2020).

The CRISPR-Cas system has also been engineered to perform base-editing. Base-editing is the ability to directly manipulate DNA sequences enabling the conversion of one base pair to another without performing a DSBs (Anzalone *et al*., 2019; Yang *et al*., 2019). A few years ago, Shimatani and colleagues (2017) used CRISPR-Cas9 fused to *Petromyzon marinus* cytidine deaminase *(PmCDA1)* and gRNAs to introduce point mutations in the acetolactate synthase (ALS) gene of rice and tomato, obtaining herbicide resistance (Shimatani *et al*., 2017). Recently, base editing has been improved thanks to the development of prime-editing, which is more efficient than the classic base editing (Anzalone *et al*., 2019; Yang *et al*., 2019). Differently from the classic dCas9, in prime-editing only one nuclease domain is inactivated, generating a DNA nickase enzyme. The latter, combined with a retrotranscriptase enzyme (RT) and a Prime Editing Guide RNA (called pegRNA), can produce both transition and transversion mutations, extending the possibility of common base editing (Fig.3b-c) (Anzalone *et al*., 2019). In a recent article, Plant Prime Editing (PPE) was tested in rice and wheat, giving the first proof of concept in plants. The authors chose six different genes and by evaluating the single base editing efficiencies, confirmed the ability of PPE to produce all kinds of base substitutions (Lin *et al*., 2020).

Lastly, it is worth noting that a new class of CRISPR-Cas systems specifically targets RNA instead of DNA (Abudayyeh *et al*., 2017) and has been successfully used in plants to induce interference toward RNA viruses (Lotterhos *et al*., 2018). Added to this RNA targeting ability of the Cas13, a dCas13 conjugated to a deaminase was also suitable for RNA editing

converting A to G and hence obtaining a system that can be used to edit full-length transcripts with pathogenic mutations (Cox *et al*., 2017). The rapid development of such a powerful and innovative techniques is the basis to achieve increased crop yields, resilient crops to both biotic and abiotic stress and to address consumer's concerns on GMOs approaches as well as nutritional needs (Kumar *et al*., 2020).

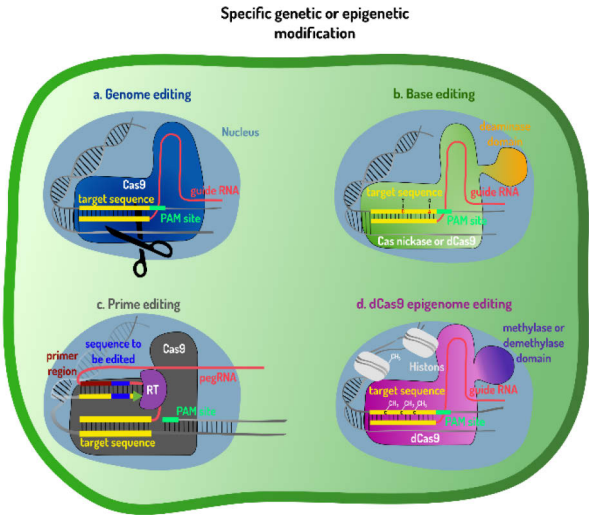

Figure 3 Highly specific genetic and epigenetic modifications by CRISPR-Cas technology: 3a-genome editing; 3b-base editing; 3c-prime editing; 3d-epigenome editing.

Towards new GMO-free approaches: exogenous dsRNA application for crop protection

Small RNAs (sRNAs) and RNA interference (RNAi) have emerged as modulators of gene expression in plant immune responses, pathogen virulence, and communications in plant-microbe interactions. Since the RNAi machinery discovery, many efforts have been made to improve its applicability in plant protection (Cagliari *et al*., 2019; Dalakouras *et al*., 2020). In plants, RNAi is well known as a conserved regulatory strategy playing key roles in endogenous transcript regulation as well as viral defense, resulting in the post transcriptional downregulation of the target RNA sequence(s). The RNAi machinery is triggered by double stranded RNA (dsRNA) molecules that, once produced in the cell, are processed by RNase III DICER-LIKE endonucleases and cleaved into 21-24 nt short interfering RNAs (siRNAs) (Liu *et al*., 2020b). After cleavage, one of the two siRNA strands associates to

ARGONAUTE (AGOs) proteins to form RNA-induced silencing complexes (RISCs) (Poulsen *et al*., 2013; Meister, 2013). Consequently, these RISCs specifically interact with transcripts on sequenced-based complementarity, resulting in mRNA cleavage or translational repression, in a process known as Post Transcriptional Gene Silencing (PTGS) (Fig. 4) (Kim, 2008; Mi *et al*., 2008). Additionally, siRNAs can promote the deposition of repressive chromatin marks in target genomic DNA sequences triggering transcriptional gene silencing (TGS). In plants and invertebrates, siRNAs also have an important function in plant host-pathogen interactions: in the case of viral infections siRNAs are produced in infected cells directly by processing dsRNA molecules derived from the viral genome itself. Interestingly, there is evidence that siRNAs, once produced in a specific cell, are able to move via plasmodesmata reaching the surrounding cells and, through the vascular system, up to distal parts of the plant, inducing the systemic silencing. Both siRNAs short distance and long distance transport mechanisms to the whole plant have been documented and are still under scrutiny (Ham and Lucas, 2017).

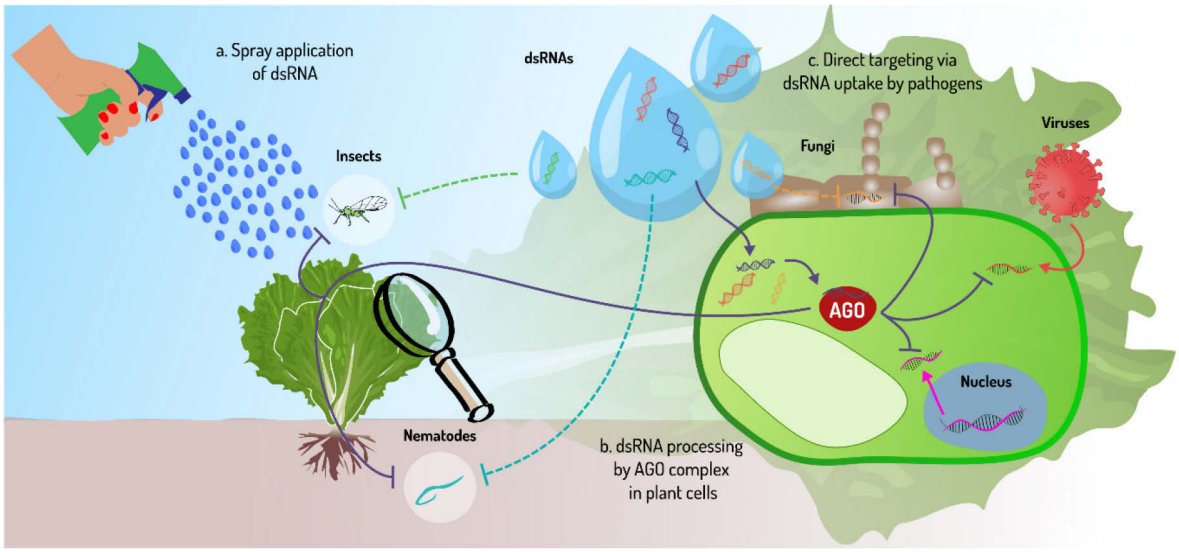

Figure 4 dsRNAs applications in crop protection: (a) dsRNA are sprayed on plants in field conditions; (b) dsRNAs penetrate the plant cells and after being processed by DICER-like nucleases associated with Argonaute protein (AGO) inducing post-transcriptional gene silencing towards pathogens or endogenous genes, continuous lines (—); (c) dsRNA directly enters pathogen cells silencing one or more essential genes, dotted lines (---).

Natural cross-kingdom RNAi and its biotechnological application

The RNAi processes are also pivotal in triggering plant immunity against pests and pathogens, modulating their development and virulence. There are lines of evidence

supporting the observation that sRNAs can be exchanged bidirectionally among the interacting partners (e.g. plant-fungi) inducing gene silencing in each other and leading to a mechanism named as cross-kingdom RNAi (Wang *et al*., 2016a; Cai *et al*., 2018b; Ma *et al*., 2020). The latter is mediated by exosome-like extracellular vesicles able to deliver sRNAs into the interacting organisms, as recently demonstrated in *Arabidopsis-B. cinerea* pathosystem (Cai *et al*., 2018a). In particular, it was demonstrated that plant delivered sRNAs can downregulate the production of pathogen effectors, whereas *Botrytis* is able to deliver sRNAs, which turn off plant defenses. All this evidence indicates that cross-kingdom RNAi can be utilized to control plant diseases caused by pathogens, including fungi, viruses, and pests, such as nematodes and insects and foster the application of RNAi strategy to counteract crop pathogens.

Indeed, beside the fascinating mechanisms of siRNA production and translocation in plants, RNAi also represents a promising sustainable and environmentally friendly tool that can be used against crop pests and pathogens and might represent a good alternative to the application of chemicals. So far, in plants, RNAi has been largely used in functional genomic studies or for inducing resistance against insects in transgenic plants (e.g. in maize against *Diabrotica virgifera virgifera* (Fishilevich *et al*., 2016). *Agrobacterium*-mediated transformation has been applied to express pathogen/pest gene-targeting sRNAs or dsRNA against a selected target. This procedure named as host-induced gene silencing, HIGS, has led to the production of GM crop varieties, not commercialized in Europe (Baulcombe, 2015; Dalakouras *et al*., 2020 and references therein). Alternatively, a virus-induced gene silencing (VIGS) approach can be applied to express designed pathogen-targeting RNAs in plant tissue and circumvent the generation of GMOs (Lee *et al*., 2012; Dommes *et al*., 2019). Indeed, a recent report demonstrated the potentiality of VIGS as a tool for transiently targeting diverse regulatory circuits within a plant and indirectly affecting important agronomic traits, without incorporating transgenic modifications (Torti *et al*., 2021). However, VIGS relies on the use of virus expression vectors, which are themselves pathogenic to the plant and currently the development of a low or non-pathogenic virus expression vector is a major obstacle to the application of VIGS in crops.

The new frontier of RNAi for crop protection

GMO-free RNAi strategies, based on exogenous dsRNA/siRNA direct applications on plants (Dubrovina and Kiselev, 2019) are among the new approaches developed to overcome

plant transformation and its limitations. Some examples of plant endogene modulation by exogenous dsRNAs application are available in the literature. In Arabidopsis, dsRNAs mixed with nanoparticles were adsorbed by plant roots and triggered RNAi against *SHOOT MERISTEMLESS* (*SSTM*) and *WEREWOLF* (*WER*) genes, which are involved in apical meristem and root epidermis regulation (Jiang *et al*., 2014). In another work, the authors suppressed the expression of a *MYB1* gene using crude bacterial extract containing dsRNAs (Lau *et al*., 2015). These studies confirmed the activation of RNAi in plants by dsRNAs adsorption through different tissues and by root soaking in a solution of dsRNAs (Li *et al*., 2015; Dalakouras *et al*., 2016; Dalakouras *et al*., 2018). These results also suggest that dsRNAs direct application could represent an effective disease-control strategy against fungal pathogens in crops. Several articles have indeed reported that the exogenous application *in vitro* or *in vivo* of synthesized long dsRNAs (through bacteria-mediated biosynthesis), hairpin RNAs (hpRNAs) or siRNAs can down-regulate the expression of pest essential genes, thus controlling harmful insects, fungal and viral pathogens. The RNA molecules were successfully applied by using several methods, such as high or low pressure spraying (spray induced gene silencing, SIGS), trunk injection, petiole absorption, soil/root drenching or mechanical inoculation and delivered naked or loaded into carriers (e.g. clay nanosheet, nanoparticles, proteins) to facilitate their uptake and survivability in plant tissues up to 7-8 weeks (Mitter *et al*., 2017; Dubrovina and Kiselev, 2019; Dalakouras *et al*., 2020). In the past few years, reports on plant-mediated delivery of dsRNAs against insects demonstrated the lowering of biological activity and/or increased mortality of aphids, whiteflies, mites, and marmorated sting bugs in tomato and bean crops (Gogoi *et al*., 2017; Ghosh *et al*., 2018). In addition, dsRNAs microinjection in *Euscelidius variegatus,* a natural vector for phytoplasmas, has recently been reported (Abbà *et al*., 2019). In this respect, Dalakouras *et al*. (2018) provided very useful information to improve the plant-mediated dsRNAs efficacy against insects, suggesting the delivery of intact dsRNA, by using specific methods (e.g. petiole adsorption or trunk injection) to avoid the activation of plant RNA processing mechanisms. Indeed, the intact dsRNAs can be translocated by xylem vessels to plant distal tissues, picked up by insects and processed into siRNAs by their own RNAi system, resulting in a more effective response.

Exogenously delivered dsRNAs have been successfully applied in several fungal-plant pathosystems. As for insects, also in fungi, intact dsRNAs are proved to be more efficient in

controlling pathogen development. This was first demonstrated by Koch *et al*. (2016), in which spraying dsRNAs on barley leaves achieved control of *Fusarium graminearum*. In addition, SIGS was effective against several fungal pathogens such as, for example, *Sclerotinia sclerotiorum* in *Brassica napus* (McLoughlin *et al*., 2018), *Fusarium asiaticum* in wheat coleoptiles (Song *et al*., 2018b), *Botrytis cinerea* in several plants (Wang *et al*., 2016a) including grapevine, in both natural and post-harvest condition (Nerva *et al*., 2020).
The exogenous dsRNAs applications for plant gene regulation still require further investigation and development, especially as concerns the necessity to unveil cell regulatory aspects, which are still largely ignored. In detail, some reports showed that the majority of plant endo-genes display a low RNAi susceptibility, depending on the presence of introns, well known to suppress the RNA silencing processes (Christie *et al*., 2011). Similarly, it is worth noting that several technological developments are still needed to achieve the wide diffusion of dsRNAs as protective molecules in crops. First of all, formulations with nanoparticles and/or other synthetic carriers are needed to slow down the rapid dsRNAs degradation, which is a major hurdle in the practical application of SIGS. Secondly, new delivery strategies such as the high-pressure spraying or brush-mediated leaf applications (Dalakouras *et al*., 2016; Dalakouras *et al*., 2018) need to be implemented for effective field applications. Finally, a specific science-based risk assessment procedure for exogenous application of dsRNA have to implemented since the actual evaluation of plant protection products (PPP) is not appropriate to establish the environmental fate and the risk associated to the field application of such products (Mezzetti *et al*., 2020).

Challenges for exogenous dsRNAs application in crop protection

In addition to the above-mentioned formulation issues, it is worth noting that the application of dsRNAs as bio-based pesticides requires a good knowledge of the target organisms. In fact, differences in dsRNAs susceptibility among different organisms and even among genera belonging to the same family have been reported. Specifically, concentrations, length of dsRNA molecules, uptake and recognition pattern by the RNAi machinery can influence the efficacy of the applied treatments.
The total amount of sprayed/supplied dsRNA is one the most variable factors among different reports: effective concentrations from pmol to mg per treated organism were reported (Das and Sherif, 2020 and references therein). This might be one of the most important limiting factors for field applications and implementation, because the amount of

dsRNAs /treatment would affect the price per treatment, discouraging their application in case of high costs. Encapsulation methods would probably reduce this problem protecting from degradation and/or facilitating the entrance of dsRNAs into the target tissues (Dalakouras *et al*., 2020). Together with the concentration, other parameters which show discrepancy in the literature is the optimum length of dsRNAs: lengths from 21bp to more than 1kb were analyzed in several works. In this case all reports highlighted that dsRNAs within a size from 150bp to 500bp are the most efficient in inducing the activation of the RNAi pathway (Das and Sherif, 2020; He *et al*., 2020; He *et al*., 2020; Höfle *et al*., 2020). These results are explained by the nature of RNAi pathway, which requires sequences long enough to be recognized by the molecular machinery but which also need to pass through the cell membrane (and in case of plants and fungi the cell wall) which works as a molecular sieve.

The other important parameters, which represent the most limiting factors at the moment, are the uptake mechanisms of dsRNAs into cells and, once entered, the recognition of specific pattern/sequences by the target RNAi machinery. The dsRNAs uptake mechanism was first described in *C. elegans*, with the description of Systemic RNAi Defective (SID) proteins, which are involved in the acquisition and transportation of dsRNAs and the derived siRNA along the nematode body (Winston *et al*., 2002; Winston *et al*., 2007; Hinas *et al*., 2012). Several SID-like proteins were described in insects with not uniform results: in some insects these proteins are crucial for the activation of a strong RNAi response, whereas in some other cases they seem to be unnecessary (Wytinck *et al*., 2020a and references therein). Another mechanism which has been proposed as one of the preferred routs of entry for dsRNAs is the clathrin mediated endocytosis. Both in insects and fungi it has been demonstrated that endocytosis facilitated the uptake of dsRNAs (Wang *et al*., 2016b; Pinheiro *et al*., 2018; Wytinck *et al*., 2020b) but further studies are needed to clarify the mechanism in more details. Information about adsorption and transportation is fundamental also to understand the onset of resistance mechanisms in pest and pathogens, as already reported for *D. virgifera*, which showed a reduced dsRNAs uptake with an increased resistance to the treatment in just 11 generations (Khajuria *et al*., 2018). Additionally, one of the most important, but poorly understood factor is the recognition of the dsRNAs by the RNAi pathway of the target organism. In this respect, contrasting results have been reported for fungi and insects. In case of fungi, application of dsRNAs to the plant, that will process

them into siRNAs, and which are then adsorbed by the fungus results the most effective strategy (Wang *et al*., 2016b; Song *et al*., 2018a; Nerva *et al*., 2020). These results are consistent with the inability of fungi to activate a secondary siRNA amplification mechanism and the exploitation of the plant machinery to enhance the gene silencing treatment effectiveness. In contrast to fungi, insects display a puzzling variety of responses, which are not always linked to evolutive features and show differences among genera of the same family. For example, as recently reviewed (Dalakouras *et al*., 2020), Coleoptera order are the most susceptible to RNAi, whereas lepidopterans and hemipterans seem recalcitrant to RNAi due to either impaired dsRNAs uptake or to the production of nucleases in their saliva. For this reason GMO approaches relaying on the expression of dsRNAs in chloroplasts, which do not process them into siRNA, displayed a stronger efficacy (Bally *et al*., 2018). Apart from the preference of siRNAs or intact dsRNA delivery treatments, there is also a lack of information about the recognition of preferred nucleotide residues on the dsRNA for their processing into siRNAs by dicer-like enzymes (DCL). Particularly, DCL sequence evolution characteristics appear to be species-dependent (Guan *et al*., 2018; Arraes *et al*., 2020) and can lead to the generation of siRNAs with species-dependent length distribution among different insects (Santos *et al*., 2019). Taken together these data suggest that for an optimal exploitation of dsRNAs as sustainable plant protection strategies, data on formulations (intended as dsRNAs size and concentration), uptakes mechanisms and features of RNAi machinery of target pests/pathogens need to be implemented.

Epigenetic signatures and modifications to improve crop resilience against biotic and abiotic stresses

Both PTGS and TGS are involved in plant immunity and specifically in the control of viral virulence through RNA silencing. However, plants use gene silencing mechanisms, and in particular the RNA-dependent DNA Methylation pathway (RdDM) for regulation of their own gene expression and the transcriptional repression of transposable elements (TEs).
In plants, chromatin can be modified at the level of DNA sequence by DNA methylation at CG, CHG, and CHH (H = A, T or C) contexts through distinct pathways. While METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) are plant enzymes responsible for the maintenance of CG and CHG methylation, respectively, after

DNA replication, CHH methylation is established *de novo* through two pathways. Plant RNA-dependent DNA Methylation pathway (RdDM) involves the biogenesis of small interfering RNAs. ARGONAUTE (AGO) family members target 24-nt siRNAs to corresponding genomic loci, which in turn are methylated in CHH and CHG context *via* DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). DRM2 is responsible for *de novo* DNA methylation of transposons located within euchromatic regions (Yaari *et al*., 2019). A second pathway requires CHROMOMETHYLASE 2 (CMT2) through interaction with DECREASE IN DNA METHYLATION1 (DDM1) in histone H1-enriched chromatic regions (Zemach *et al*., 2013). A family of bifunctional methyl-cytosine glycosylases-apurinic/apyrimidinic lyase actively removes DNA methylation, through a base excision repair mechanism (Penterman *et al*., 2007). DNA methylation may affect gene expression, regulate imprinting and activate transposable elements (TEs) and TE-associated genes, particularly in response to environmental cues (Law and Jacobsen, 2010).

Numerous studies indicate that DNA methylation plays a part in the pathogen-induced immune system and can strongly influence the resistance response in different plant species, as recently reviewed in Tirnaz and Batley (2019). Among these studies, interestingly it has been reported in rice that the epigenetic regulation of PigmS, a gene involved in resistance to rice blast caused by the fungal pathogen *Pyricularia oryzae*, affects plant resistance and indirectly yield. A genome wide methylation analysis demonstrated that the PigmS promoter region contains two tandem miniature transposons MITE1 and MITE2 that are repressed by DNA methylation. Indeed, CHH methylation levels at MITE1 and MITE2 and in particular RdDM-mediated silencing of the MITE-nested PigmS promoter control PigmS expression and consequently resistance to rice blast (Deng *et al*., 2017). Intriguingly, this work on rice highlights the need for a thorough characterization of the RdDM epigenetic pathway and DNA methylation pathway in crops. The double aim of studying the involvement of these pathways in plant pathogen interactions can be to clarify how they regulate the expression of resistance genes and what genes are activated in crops, when exogenous double stranded RNAs are introduced in the plant cell. Answering these questions might pave the way for new strategies both for crop protection management and breeding programs for plant resistance, which can incorporate DNA methylation as a new source of variation.

In the plant cell, along with DNA methylation, other chromatin marks can arrange various chromatin states that epigenetically determine specific transcriptional outputs, thus

influencing both biotic and abiotic plant stress response (Pecinka *et al*., 2020). Nucleosome association to DNA is influenced by many kinds of reversible covalent posttranslational modifications (PTMs e.g. acetylation, methylation, phosphorylation, ubiquitination and many others) of the histone tails, in particular of histone H3 and H4 that are enriched in lysine (K) and arginine (R). In addition to PTMs and the positioning of nucleosomes, DNA accessibility is also affected by the incorporation of histone variants (H2A.Z, H2A.X, H3.1, H3.3) which have different specialized properties and can replace canonical core histones in the nucleosome. The histone code hypothesis postulates that deposition, removal, and recognition of each PTM to histones requires specialized enzymes defined as writers, erasers and readers, respectively (Jenuwein, 2001). Although there is some evidence that histone modifiers and chromatin remodelers can affect the expression of genes involved in the plant immune response, this evidence is limited to a few plant species, such as Arabidopsis and rice (Ramirez-Prado *et al*., 2018). Histone deacetylases (HDACs), acetyltransferases (HATs), methylases, demethylases, ubiquitinases, can act as positive and negative regulators in plant resistance to different stressors. In a recent work, the authors have studied the interactions between the bacterium *Pseudomonas piscium*, from the wheat head microbiome, and the plant pathogenic fungus *Fusarium graminearum*. They have observed that phenazine-1-carboxamide, a compound secreted by the bacteria, influences the activity of a fungal histone acetyltransferase, leading to deregulation of histone acetylation suppression of fungal growth, virulence and mycotoxin biosynthesis. This study highlights a novel mechanism of epigenetic regulation in antagonistic bacterial–fungal interaction that might be potentially useful in crop protection (Chen *et al*., 2018).

Genome editing tools for epigenome modification

Genome-wide mapping of epigenomic marks and epigenetic target identification are currently two major efforts in many important crops. In the future, it is desirable that these efforts will offer breeders new application to increase and manipulate epigenomic variability, for selecting novel crop varieties more resilient to biotic and abiotic stresses. In recent years, different techniques have been developed to modify the epigenome globally or at target sites. In crops, gene silencing and variation in DNA methylation profiles could be achieved by inducing siRNA expression, because DNA methylation deficient mutants, which would be useful to alter the methylome, have not been identified in all crops,

suggesting that they might be lethal (Kawakatsu and Ecker, 2019). At specific genome sites, fusions of epigenome-modifying enzymes to programmable DNA-binding proteins can achieve targeted DNA methylation and diverse histone modifications (Rivenbark *et al*., 2012; Mendenhall *et al*., 2013). Particularly, the genome editing tool CRISPR/deadCas9 can be fused to epigenetic-state modifying enzymes and targeted to genes or cis-regulatory elements (CREs) to modulate plant gene expression. A complete set of plant epigenetic editing tools can be generated by fusing CRISPR-dCas9 system to target modifying enzymes for applications in plant breeding for crop protection. The so-called epigenome editing can be used to re-write an epigenetic mark modifying the endogenous gene expression level of one or several genes (Hilton *et al*., 2015; Miglani *et al*., 2020) (Fig.3d). An example of such an approach was given in Arabidopsis using a dCas9 linked to the histone acetyltransferase *AtHAT1* to improve the transcription of *AREB1*, a gene involved in abscisic acid (ABA) perception (Roca Paixão *et al*., 2019; Miglani *et al*., 2020). The epigenome-edited plant showed enhanced drought resilience and chlorophyll content when compared to controls.
The use of genome editing tools that modify the epigenome at the recombination sites has been proposed as a possible application for manipulating the rate and positions of crossing over (CO), to increase the genetic and epigenetic variation accessible to breeders. In Arabidopsis the disruption of histone 3 di-methylation on lysine 9 (H3K9me2) and non-CG DNA methylation pathways increases meiotic recombination in proximity to the centromeres (Underwood *et al*., 2018). Although the results obtained in a model species suggest that manipulation of epigenetic marks can allow CO position and frequency to be expanded, further studies are needed to determine the effectiveness of similar approaches in different plant species. Strategies for controlling recombination represent novel potential tools to both reveal unexplored epigenetic diversity and control its inheritance, since they have the potential to reduce the time for breeding novel more resilient crops.

Beyond the limits

A main factor limiting the success of NPBTs is plant regeneration after *in vitro* manipulation, particularly for woody plants, being sometimes a cultivar-dependent process. Although the key pathways and molecules have recently been unveiled (Sugimoto *et al*., 2019), the mechanism of regeneration is not fully understood, and technical issues are still present.

Improvements of the regeneration efficiency have been obtained by crop transformation with morphogenic regulators (e.g. *Baby boom* and *Wuschel* genes) which can induce a more efficient meristem differentiation in recalcitrant species (Lowe *et al*., 2016; Maher *et al*., 2020; Yavuz *et al*., 2020). Despite the great potential of such approach, the fact that gene sequences of morphogenic regulators are protected by patents from private companies (Lowe *et al*., 2016; Maher *et al*., 2020) might limit the application of this technological innovation. Hence, it is fundamental to achieve higher regeneration efficiency, opening the way to the minimal gene approach even in recalcitrant woody plant species.

Another limiting factor is the low number of available genes involved in the resistance response with an identified function. Indeed, the identification of resistance genes from landraces and wild crop relatives and their functional genetic validation represents the first steps toward the development of new cisgenic varieties. The importance of these steps was recently reported in several herbaceous and woody plants. In wheat, for example, several genes conferring partial resistance to stem rust have been cloned, including *SR35* (Saintenac *et al*., 2013), *SR33* (Periyannan *et al*., 2013), *SR50* (Mago *et al*., 2015), *SR60* (Chen *et al*., 2020) and *SR55/LR67* (Moore *et al*., 2015). For woody plants, resistance genes *Rpv1* and *Run1* conferring resistance to *Plasmopara viticola* and *Erysiphe necator* have been identified in the wild grapevine relative *Muscadinia rotundifolia* (Feechan *et al*., 2013) and are good candidates on which several research groups are working. In spite of this, the number of genes with a known function is still limited. In parallel, more information on promoters, transcriptional terminators and regulatory elements to control the transcription efficiency has to be addressed because of the high impact on gene of interest expression levels and consequently on the final phenotype (Low *et al*., 2018; Basso *et al*., 2020).

With respect to the CRISPR/Cas DNA editing, RNA editing using Cas13 has the advantage that it is not stable but reversible. This could enable a delicate temporal control over the editing process when editing RNA, both edited and non-edited transcripts can be present simultaneously in the cells, which could enable fine-tuning of the edited transcript amount, whereas DNA editing affects all transcripts. Furthermore, in addition to classic gene knock-out mediated by CRISPR/Cas systems, new approaches were developed to target micro RNA genes (MIR) instead of protein coding ones. By fine-tuning specific MIR genes, the up- or downregulation of derived miRNAs and target mRNAs can be achieved, for controlling either crop different biological responses or phenotypes and, consequently, specific

agronomic traits (Basso *et al*., 2020 and references therein). Similarly, an approach called gene editing-induced gene silencing (Kuscu *et al*., 2017) can be applied to target redundant non-coding RNA sequences that are involved in miRNA/siRNA biogenesis. Once modified, the new RNA molecule will target new sequences, which could be endogenous plant sequences (leading to transcript downregulation) or pathogen vital genes. Contrary to traditional gene editing techniques, gene editing-induced gene silencing could be used to indirectly target pathogenic genes by redirecting the silencing activity of the endogenous RNA interference (RNAi) pathway, supporting a more sustainable crop protection (Zotti *et al*., 2018).

Concluding remarks and future prospects

The NPBTs Era displays the potential to revolutionize the agricultural research field (Pandey *et al*., 2019). Indeed, recent applications and literature data available to date represent only the tip of the iceberg of further discoveries that may change molecular biology. Just as an example, through the combination of DNA and RNA editing systems, the cellular transcriptome can now be manipulated on the transcriptional and posttranscriptional level simultaneously, allowing delicate, and also reversible fine-tuning of gene expression (Schindele *et al*., 2018).

Taking them singularly, they all still present limitations. Pros and cons can be found both in fine tuning each application as well as their application in a wide range of species. For instance, looking at cisgenic strategies, these have been developed and tested for woody and herbaceous crops, but their application still seems far from fulfilling their potential. The lack of efficient tissue culture and regeneration protocols for many crops hinders the range of possible applications. In addition, the identification of candidate genes involved in abiotic and biotic stresses still represents an important limit. For this reason, all NPBTs could greatly benefit from functional genomics, metabolomic and proteomic studies.

Nevertheless, a wide range of different techniques are becoming mature for substituting GMO approaches and supporting traditional breeding, with a realistic possibility of being largely accepted by the international community. Several NPBTs, making small modifications to plant own DNA without introducing foreign genes, do not leave any trace of their application in the improved phenotype. Despite the high impact of such techniques, and because the genome modifications introduced by genome editing are indistinguishable

from those introduced by spontaneous mutations or conventional breeding (Bortesi and Fischer, 2015), to date the debate about considering organisms obtained by NPBTs as non-GMO is still open (Purnhagen *et al*., 2018).

Although NPBTs are powerful tools for basic research and more precise crop improvement, further knowledge, such as the comprehension of the genetic bases of important crop traits, have to be produced for efficiently transferring these tools from the lab to the field. Indeed, NPBTs can pave the way for further understanding of plant-pathogen interaction and different facets of climate change adaptation and for exploiting them for improving food security and nutrition quality.

CONFLICT OF INTEREST STATEMENT

No conflict of interest declared.

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VECTORS ATTRIBUTIONS

Some elements present in the figures were obtained from www.Vecteezy.com

AUTHORS' CONTRIBUTION STATEMENT

GG, LM, LN and WC wrote the introduction. GG wrote the cisgenesis sections. LM and LN wrote the genome editing paragraphs. WC and LN wrote the RNAi strategies sections. SV wrote the epigenetic paragraphs. MFC, CB, GDL and RV commented on the first draft and critically reviewed the final manuscript.

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CHAPTER 3

Introgression of resistance loci to powdery and downy mildews in grapevine cv. Glera

Abstract

Grapevine (*Vitis vinifera* ssp. *vinifera*) is a major fruit crop with high economic importance. *V. vinifera* has usually little genetic resistance against pathogens such as *Erysiphe necator* and *Plasmopara viticola* the causal agents of powdery and downy mildew respectively. Control of these pathogens is based on the use of fungicides, which cause environmental damages and increase production costs. A cost-effective and environmentally friendly alternative to control the disease relies on using resistant varieties. Indeed, the introgression of resistance in grapevine are currently undertaken in many breeding programs, and to ensure the durability of resistance. Although, most *V. vinifera* cultivars are susceptible to powdery and downy mildews, several species belonging to the *Vitaceae* have been considered as resistant.

Taking this into account, we cross pollinated many resistant accessions with the susceptible cv. Glera used to produce Prosecco wines.

During these years, the MAS analysis led to the identification of 4647 plants carrying multiple resistance genes both to powdery and downy mildews. The genetic analysis was coupled with agronomical and biochemical assays to evaluate offsprings aromatic profile.

Keywords

Grapevine, Resistance genes, powdery and downy mildews, Aromatic profile

Introduction

In nature, plants are constantly exposed to attacks from many potential pathogens which exhibit different infection strategies. Unfortunately, physical, and chemical barriers are not sufficient to counteract pathogens attack, for this reason plants have deployed several mechanisms to recognize and respond to interactions. In 2006 Jones and Dangl proposed a model to describe the interactions between plant and pathogens. This model is based on two recognition mechanisms (Jones & Dangl *et al*., 2006). The first and most general plant response (non-specific) is based on the recognition of compounds called elicitors, or Pathogen/Microbial Associated Molecular Patterns (PAMP or MAMP), which are common to many pathogens and infection processes (Schwessinger and Zipfel, 2008). When plant pattern recognition receptors (PRRs) recognize PAMPs the Pattern Triggered Immunity (PTI) is activated and the cell-responses induce the production of reactive oxygen species (ROS) in oxidative burst (Zhang *et al*., 2007) and of salicylic acid, jasmonic acid and ethylene defence hormones (Jones and Dange, 2006, Lee *et al*., 2012, Kachroo and Kachroo, 2007). Nevertheless, many pathogens can overcome this defence line, causing the host induce resistance that is usually specific and based on an adaptive interaction (Jones & Dangl *et al*., 2006). Pathogens release many disease effectors molecules into plant cells (avirulence *Avr* proteins) to enhance microbial fitness and induce Effector Triggered Susceptibility (ETS). Plants are able to perceive pathogens effector proteins through *R*-genes and induce a strong defence response: Effector Triggered Immunity (ETI) (Yuan *et al*., 2021). These *R* genes encode proteins (R proteins) that represent the first line of defense against infection by many biotrophic pathogens (Meyers *et al*., 2005). R gene-mediated recognition of pathogen effectors activate a series of defence signalling cascades and induce pathogenesis-related (PR) gene expression to generate systemic acquired resistance (SAR) with a global and durable resistance in plants (Liu *et al*., 2007). Although the PAMP and PRR are relatively stable and heritable, the components of ETS and ETI are object of diversification and selection due to the continuous co-evolution of plants and pathogens (Bent & Mackey, 2007). Natural selection drives pathogens to avoid ETI either by shedding or diversifying the effectors, or by acquiring additional effectors that suppress the response. Natural selection for plants result in developing new R-genes and proteins to trigger ETI responses.

In vineyard Powdery and Downy Mildews (PM and DM) are the main fungal diseases causing a significant impact in quality and yield features. Management of these mildews on traditional grape genotypes is usually achieved by an intensive application of fungicides leading to National and EU restrictive policies since their risk on human health and their negative environmental impacts (Flemming & Trevors, 1989; Tsakirakis *et al*., 2014; Chen *et al*., 2020). Indeed, Gianessi and colleagues, reported an average yearly of 19.5 kg/ha of active compounds used in vineyard (Gianessi & Williams, 2011); moreover many authors reported an accumulation of cropper based fungicides that impact soil organisms and plants, soil fertility, productivity and damage of non-target organisms (Ballabio *et al*., 2018; Droz *et al*., 2021). Besides, the application of several fungicides can lead to the presence of chemical residues in production process, indeed many authors reported modification yeast activity during fermentation due to the presence of active principles that reduce yeasts growth rate (Calhelha *et al*., 2006; Economou *et al*., 2009; Čuš *et al*., 2010; Russo *et al*., 2019). This scenario has encouraged new management strategies and the development of alternative crop protection.

One way to reduce the application of high amounts of fungicides would be the integration of genetic resistance into grapevine cultivars, therefore several breeding programs have successfully introgressed resistance loci from wild North American and Asian Vitis species into *V. vinifera* resulting in resistant grapevine cultivars (Peressotti *et al*., 2010; Pertot *et al*., 2017; Zendler *et al*., 2020).

PM and DM resistances differ among and within Vitis species (Cadle-Davidson *et al*., 2011) therefore the majority of cultivated grape varieties belonging to *V. vinifera* cultivars showing partial resistance to PM fungus *Erysiphe necator* and to DM fungus *Plasmopara viticola* (Berk. & Curt.) (Dick, 2002; Gessler *et al*., 2011; Di Gaspero *et al*., 2012), on the other hand, North American species pertaining to the genera *V. rupestris, V. riparia, V. aestivalis, V. cinerea* and *V. rotundifolia* developed different mechanisms of resistance due their co-evolution with the pathogens (Ramming *et al*., 2012; Barba *et al*., 2015; Fu *et al*., 2020; Karn *et al*., 2021). Moreover, several grape species native to Central Asia and China are known to represent powdery mildew resistance sources. For instance, in 2013 Riaz and colleagues, investigated the PM resistance in a range of Chinese Vitis species characterizing ten new PM resistant accessions carrying *Ren*1-like local haplotype (Riaz *et al*., 2013). Also in this case, the development of resistant accessions were probably due to the presence of these

mildews in these regions for a long period and the wild grapes could have evolved their resistance genes (Pap *et al*., 2016; Riaz *et al*., 2018).

To dissect the genetic basis of DM and PM resistance, various genotype-phenotype association studies were employed (Di Gaspero *et al*., 2012; Venuti *et al*., 2013; Buonassisi *et al*., 2017) led to the identification of Quantitative Disease Resistance (Poland *et al*., 2009) that has been of interest of plant breeders to provide more durable resistance (Karn *et al*., 2021). In grapevine, up to now, a list of 31 different Quantitative Traits Loci (QTL) is reported associated DM resistance and 13 related to PM resistance (VIVC). In literature are present many works about different mechanisms involved into resistance responses to *P. viticola* infection depending to *Rpv* locus and host genotype (Sevini *et al*., 2002; Merdinoglu *et al*., 2003; Fischer *et al*., 2004; Welter *et al*., 2007; Marguerit *et al*., 2009; Bellin *et al*., 2009; Blasi *et al*., 2011; Casagrande *et al*., 2011; Schwander *et al*., 2012; Di Gaspero *et al*., 2012; Venuti *et al*., 2013; Ochssner *et al*., 2016; Sánchez-Mora *et al*., 2017; Divilov *et al*., 2018; Lin *et al*., 2019; Sapkota *et al*., 2019; Sargolzaei *et al*., 2020; Fu *et al*., 2020; Bhattarai *et al*., 2021) and to *E.necator* infection: *Ren* and *Run* loci (Pauquet *et al*., 2001; Donald *et al*., 2002; Barker *et al*., 2005; Hoffmann *et al*., 2008; Riaz *et al*., 2011, 2013; Blanc *et al*., 2012; Barba *et al*., 2014; Qiu *et al*., 2015; Feechan *et al*., 2015; Pap *et al*., 2016; Zyprian *et al*., 2016; Teh *et al*., 2017; Zendler *et al*., 2020).

One of the most effective ways to increase the effectiveness of Resistance genes is to combine more R-genes from different wild plants. This is due to the assumption that R-genes from different sources have evolved to recognize different pathogens effector proteins, therefore for breaking resistance the pathogens need to mutate more effectors at same times (McDonald & Linde, 2002; Dry *et al*., 2019). In fact, plant resistances can be divided in two categories: quantitative and qualitative. Resistances are quantitative when multiple genes contribute together to the expression of the trait (polygenic resistance). Plants with quantitative resistances display different degrees of susceptibility to the pathogen which depend on the number and strength of the genes possessed by the individual. Polygenic resistances are often durable due to the nature of associated barriers, the contribution of multiple genes and non-specificity against single pathogen strains (Poland *et al.,* 2009). Resistances controlled by one gene are defined as qualitative because each gene is decisive for the ability of the plant to defend itself (monogenic resistance). R-genes typically provide performing qualitative resistances, but they are often limited to a specific pathogen isolate

and characterized by a lack of durability due to the continuous evolution of the pathogen (Gill et al., 2015, Stuthman *et al*., 2007).

Therefore for both downy and powdery mildew, the pyramidization of resistance haplotypes from different grape species is a common practice (Peressotti *et al*., 2010; Venuti *et al*., 2013; Pilet-Nayel *et al*., 2017). Indeed, since the discovery of resistance sources to *P. viticola*, and to *E. necator*, many breeding programs in Austria, Germany, Hungary, France, the USA, Spain and in Italy, have been developed to obtain grapevine hybrids combining more disease resistance genes from the wild grapevine species with the elite varieties (Töpfer *et al*., 2011; Reynolds, 2015; Ibáñez *et al*., 2015). The pyramidization strategy in combination with several backcrossing with vinifera genotypes led to the development of fungi resistant grapes carrying multiple disease resistance genes and a significant percentage (more than 85%) of vinifera in their pedigree (Pedneault & Provost, 2016).

It was made possible through the application of Marker-Assisted Selection (MAS) relied on the development of multiple types of DNA markers such as RFLP, RADP, AFLP, SSR, SNP (Ben-Ari & Lavi, 2012; Stuthman *et al*., 2007). Among them, SSR markers are considered promising due their co-dominant activity, high reproducibility, their abundance, and their applicability in other grapevine breeding programs (Eibach *et al*., 2007; Kalia *et al*., 2011; Nadeem *et al*., 2018).

'Glera' is the main white variety grown in north-eastern Italy, used to produce the world-renowned 'Prosecco' wine that is appreciated all over the world. Thiene and colleagues reported a rapid commercial success is the Prosecco sparkling wine during the years (Thiene *et al*., 2013). Indeed, sparkling wines exports registered a remarkable 234% growth between 2003 and 2015, with international markets strongly contributing to Prosecco's economic performance in particular between 2008 and 2012 (Basso, 2019). Since then, Prosecco has unequivocally become a noticeable product globally. Indeed, the production of Prosecco Controlled and Guaranteed Denomination of Origin is 68.7 million bottles (Thiene *et al*., 2013). In 2005, a qualitative study suggested that motivations to consume sparkling wine are complex and include symbolic function, as well as perceptions of experiential consumption (Charters, 2005). Indeed, the success of this wine is due principally to brand success and the consumers are loyal to the Prosecco appellation (Rossetto & Gastaldello, 2018), moreover the consumers consider a wine with a good aroma profile.

Wine aroma is the result of an extremely complex multi-mixture of numerous volatile substances belonging to different chemical species (Styger *et al*., 2011). It is well-known that the secondary metabolites are responsible for the principal aroma compounds and provide the basis of varietal character. Grapes and wines quality need to be compared with parent sources for metabolites families such as phenolics, terpenes, alcohols etc. to evaluate differences in winemaking process. GC–MS analysis gives detailed specific chemical information about the presence of all components potentially stimulating the olfactory system (Vilanova & Oliveira, 2012).

Given the considerable 'Glera' economic impacts due to the worldwide distribution of Prosecco wine, research efforts are focused on the development of more tolerant Glera to powdery and downy mildews. In the present paper, alternative strategies based on the crossing events using resistant hybrids of different geographical origin, were applied in a 'Glera' vineyard most adapted to Veneto terroir to confer resistance against PM and DM. The susceptible genotype 'Glera' and breeding lines from the different species were crossbred with the aim of creating new grapevine cultivars with higher resistance to downy and powdery mildews and resemble the elite variety with good fruit quality and oenological characteristics.

Materials and methods

Plant material

Different crosses were carried out with 'Glera' and many resistant genotypes reported in Table 1 for 4 years. The crosses were performed at the CREA-VE Grapevine Germplasm Collection in Susegana, Veneto Italy (45°51'07.6"N 12°15'28.6"E).

Table 1 Grapevine controlled crosses between susceptible variety 'Glera' and different resistant genotypes.

Year	ID	Female parent	Male parent
2017	Pop_17_21	Glera	Soreli
2017	Pop_17_22	Glera	Souvignier Gris
2017	Pop_17_23	Glera	Muscaris
2018	Pop_18_24	Glera	Toldi
2018	Pop_18_25	Glera	Cabernet Cantor
2018	Pop_18_26	Glera	Calardis Blanc
2018	Pop_18_27	Glera	SK-00-1/1
2018	Pop_18_28	Glera	SK-00-1/2
2018	Pop_18_31	Toldi	Glera
2019	Pop_19_33	Glera	686
2019	Pop_19_34	Glera	881
2019	Pop_19_35	Glera	Floreal
2019	Pop_19_37	Glera	Voltis
2020	Pop_20_26	Glera	Calardis Blanc
2020	Pop_20_38	Glera	VC531.039
2020	Pop_20_41	SK-00-1/7	Glera
2020	Pop_20_42	Glera	VC156.1017
2020	Pop_20_43	Glera	VC109.033

In May plants that were used as female parent had to be emasculated two month before anthesis (calyptra were removed with anthers in order to prevent auto-pollination) and the ovaries were protected from alien pollen using paper bags as described in Peterlunger *et al*., and Bellin *et al*., (Peterlunger *et al.*, 2003; Bellin *et al.*, 2009). The male genotypes were

used for pollen collection. The pollination was performed when the stigmatic fluid appears on the stigma and so it can support the adhesion of the pollen grains and form the pollen tube. After fruit-set the paper bags were replaced by tulle bags in order to allow the ripening. At the end of growing season, the bunches were harvested at physiological ripeness and then stored in warehouse for 60 days under controlled conditions (18 ± 2°C), RH (40%) and air flow (0.3 m s^{-1}). After dehydration period, the seeds were extracted and treated soaking seeds in 0.5 M H_2O_2 for 24 h followed by a 24 h water soaking as described by Conner et al., (2008). After soaking the seeds were washed with sterile distilled water and stored in petri dishes containing sand and vermiculite. Seeds was stratified at 4° ± 2°C in a dark condition for 90 days. Three months later the seeds were rinsed in 0.5 M H_2O_2 for 30 minutes. In this phase the floating seeds were discarded.

The germination phase was performed in growth chamber. Seeds were put on soaked filter paper with a photoperiod 16/8 h at 26°C. Finally in April the seeds were put in a glasshouse at 20°C until molecular analysis.

DNA extraction

From each plantlet a sample of single unexpanded leaf (1 cm^2 of diameter, about 50 mg of fresh tissue) was collected in 96-well plates. Samples were lyophilized and homogenised by shaking twice for 30 s at a frequency of 30 Hz using two 3-mm diameter steel beads in the TissueLyser II (Qiagen), and maintained at 4 °C.

In 2018 the total DNA was extracted using DNeasy 96-well DNA extraction kits (Qiagen, Hilden, Germany) following manufacturer's protocols.

In 2019, 2020 and 2021 the extraction protocol was modified according to protocol described from Dellaporta *et al.*, 1983 with some exceptions:

The grinded plant material was resuspended in Lysis Buffer (SDS 0,5%, TrisHCl pH 8 200 mM, NaCl 250 mM, EDTA 25 mM) and incubated at 65°C for 15 minutes. To avoid DNA separation, we added a Precipitation Buffer (Potassium Acetate CH_3CO_2K 5M and Acetic Acid CH_3COOH pH 6.5).

For each sample DNA concentration was measured using NanoDrop One Spectrophotometer (Thermo Fisher Scientific). Random samples were tested also for DNA integrity with electrophoresis on 1% agarose gel. DNA was diluted to 10 ng μl^{-1} and used for PCRs.

Multiplex PCR

To genetically characterize the samples through microsatellite markers we set up a multiplex PCR approach. The PCRs were performed using SSR-flanking primer pairs, which included fluorescent labelling at the 5′ ends of the forward primers with different fluorescent dyes (6-FAM, NED, VIC and PET) in order to perform a multiplex PCR (Schwander *et al.*, 2012; Culley *et al.*, 2013). PCR reactions were carried out in a 10 µl volume containing 15 ng of DNA, 150 µM of each dNTP, 0.125 to 0.50 µM of each primer, 1x Buffer Gold and 0,20 U of MyTaq Plant PCR polymerase (MyTaq DNA polymerase, Bioline, Waltham, Massachusetts, USA) and with the following thermal protocol: 94°C for 10 min, followed by 35 cycles at 92°C for 45s, 57°C for 60s, 72°C for 90s, and final elongation of 5 min at 72°C. PCRs were performed in a BioRad PCR System.

SSRs

The resultant PCRs were diluted in H_2O and then 2 µl of diluted PCR products were added to 0.5 µL LIZ 500 size standard and 9,35 µL Formamide (Applied Biosystems) and runned by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystem, Darmstadt, Germany) to separate the amplicons. All PCR fragments were analysed with GeneMapper 4.0 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a specific user annotation to identified peaks. Markers related to resistance loci were utilized to screen the progenies are shown in Table 2 and associated alleles to resistance to *P. viticola* and *E. necator* are reported in Table S1.

Table 2 SSR markers associated with resistance loci and primer sequences.

Resistance loci	Marker associated to loci	Dye	Forward primer	Reverse primer	Marker type
Rpv3	UDV305	6-FAM	TGGTGCAATGGTCATAATTT	GAGGAAAAGAGAAAGCAAAGA	SSRs
	UDV734	NED	TGTGTAATGCAAGGCCAACT	AAGACGTTCAATACCCACATGA	SSRs
	InDel25,941	NED	AAATTATTCTAAATGATATGAGGCATG	TGTCTTGGTCTTTCTTGGTAATGA	InDel
	VMC7F2	6-FAM	AAGAAAGTTTGCAGTTTATGGTG	AAGATGACAATAGCGAGAGAGAA	SSRs
	UDV737	NED	TTTGCATGCGATACCTGAAGC	TCCTGCAGCTGTTGACGATA	SSRs
Rpv12	UDV 350	VIC	TTTTGGGAGTTTCCATGTCC	AAGACACCTTGGGGGATAAAA	SSRs
	UDV 360	NED	TGCTTTACAGGTGACCATCAA	GCAACCAATTGAGGGGATTA	SSRs
Rpv10	GF09-44	VIC	CATCGTTCCTTTCTTACTCGCT	GCTAATGGAGGGTAGTGCTCAA	SSRs
	GF 09-46	6-FAM	GAGAGATTTGAGGGATTGTTGG	ATCCACGTTTGTAGCCTTTTGT	SSRs
	GF 09-47	VIC	CCACATTCTTCCTGCACATAAA	CTGTTGTAAGGGCTCCCAATTA	SSRs
Rpv1/Run1	VMC4f3,1	PET	AAAGCACTATGGTGGGTGTAAA	TAACCAATACATGCATCAAGGA	SSRs
	VMC8g9	6-FAM	AACATTATCAACAACATGGTTTTA	ATATTCATCCTTCCCATCACTA	SSRs
Ren1	SC8-0071-014	PET	CAACCATCAAGCACTCTCCTG	AAGCCTCGCACTCCTCTGTC	SSRs
	Sc47_20	VIC	TGGATCAAATTAGTGCCTGGA	GCCGCTTCAACTCAAAAATC	SSRs
Ren3	GF 15-28	NED	TGCACACAATCACAGAGAGAGA	TGCGGTTAATTTTGACTCCTTC	SSRs
	GF 15-30	6-FAM	TCACAGTATGCAGTAACCTGGC	AAAGGGAAAATGAGCAGTTGAG	SSRs
	Scora760	-	GAAACGGGTGTGAGGCAAAGGTGG	GGCCATTAGGAAATCAACATTAC	SSRs
Ren9	CenGen6	6-FAM	TGGTCAATGATCTCCCCATT	TTCCAATCAAGGTCATGCAA	SSRs

Agronomical traits evaluation

Agronomical traits were scored during harvesting season in 2019 and 2020 on samples belonging to cross population of 2013 and 2014 as reported in Table S2 on the basis of grape descriptors introduced by International Plant Genetic Resources Institute (Ipgri). Fruit samples were collected during few days between August and September, when ripening was completed for most of the individuals. Full ripening was determined based on the measurement of total soluble solids and titratable acidity (Abiri *et al.*, 2020) defined as total soluble solids > 16 °Brix and titratable acidity < 7 g L^{-1} (Alessandrini *et al.*, 2017). Ten bunches were harvested for each plant. Each bunch was weighted to evaluate mass production. Then, two samples of hundred fruits randomly chosen from the clusters were also weighted. Finally, Solid Sugar Content (SSC) was determined through a digital refractometer in °Brix degrees using a two ml of juice sample.

GC-MS samples preparation

Grape samples from the 8 different *Vitis vinifera* offspring's (reported in Table 1) were harvested in 2020 from CREA-VE Grapevine Germoplasm Collection (Susegana, Veneto, Italy). Also, the 4 parental genotypes were collected. The grape samples were kept frozen at -20 °C till the sample preparation.

Briefly, 50 berries were randomly chosen and weighed, pulps were separated from skins and seeds, transferred to a flask containing 50 mg of sodium metabisulphite, homogenised, and centrifuged at 4500 rpm for 10 min. The volume was adjusted to 100 ml with water and the solution was clarified by treatment with 40 mg of pectolytic enzyme Pectazina DC for 4h at room temperature. Skins were extracted with 30 ml of methanol for 4h in the dark. After homogenisation and centrifugation at 4500 rpm for 10 min, the methanol content was reduced under vacuum and the volume adjusted to 100 ml with water. One gram of Polyclar AT was added to the solution in order to reduce the polyphenolic content, then the solution was filtered. One hundred millilitres of juice extract and an equal volume of skin extract were unified, and the solution was added to a 200 µl of 1-heptanol 234,4 mg L^{-1} as an internal standard. After passage, through a 10 g C_{18} Sep-Pak® cartridge (Waters, Milford, Massachusetts, USA), volatile compounds were recovered with 50 ml of dichloromethane and the solution was concentrated at 200 µl before analysis.

The fraction of glycosides was eluted with 30 ml methanol. The solvent was evaporated until dry using a rotary evaporator. The residue was dissolved in 5 ml of citrate-phosphate buffer and 100 mg of AR 2000 enzyme was added to the solution. Reaction was carried out overnight at 40 °C, then the solution was added of 200 µl of 1-decanol 223.2 mg L^{-1} as internal standard and passed through activated 1 g Sep-Pak® C18 cartridge (Waters, Milford, Massachusetts, USA) previously activated. The aglycones liberated by the enzyme were recovered with 6 ml of dichloromethane. Before to perform gas chromatography/mass spectrometry (GC/MS) analysis, the solution was concentrated to 200 µl under slow nitrogen flow. Solvents were purchased from Romil LTD (Cambridge, UK). Standards of 1-heptanol and 1-decanol were purchased from Carlo Erba Reagents (Milan, Italy); pectolytic enzyme Pectazina DC from Dal Cin SpA (Concorezzo, MB, Italy), AR 2000 enzyme, (DSM Food Specialties B.V., Delft, The Netherlands) Polyclar AT from Serva (Serva Electrophoresis GmbH, Heidelberg, Germany), Solid phase extraction was performed with Sep-Pak C_{18} 10 g and 1 g cartridges were purchased from Waters (Milford, MA, US).

Mass Spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) were performed using a 6850-gas chromatograph (Agilent Technologies, Santa Clara, CA, US) equipped with a fused silica HPInnowax polyethylene glycol (PEG) capillary column (30 m x 0.25 mm, 0.25 lm i.d.) (Agilent Technologies, Santa Clara, CA, US) coupled with an HP 5975C mass spectrometer and 7693A automatic liquid sampler injector (Agilent Technologies, Santa Clara, CA, US). Oven temperature program: 40 °C isothermal for 1 min, increase 2°C/ min until 160°C, 3°C/min until 230°C, 230°C isothermal for 15 min. Other experimental conditions: injector temperature 230 °C; carrier gas helium with constant flow rate of 1.2 ml/min; sample volume injected 1 µl; splitless injection mode; transfer line temperature 250°C, quadrupole temperature 150°C, mass range m/z 20–550. The quantification of the identified peaks of volatile compounds were performed using internal standard 1-heptanol and 1-decanol.

Statistical analysis

To analyse the differences in offspring's volatile compound classes (alcohols, aldehydes, terpenes, benzenoids and norisoprenoids), a one-way analysis of variance (ANOVA) was performed in R ver. 4.0.5. For significative F-test (*p*-value) multiple comparisons between means were conducted using Tukey's honestly significant difference (HSD) post-hoc, with an $\alpha = 0.05$. Comparisons between volatile compound classes on each sample were performed using Tukey's test ($\alpha = 211\ 0.05$) on the least-square means. The degrees of freedom were estimated with Kenward-Roger method. The standard deviation (SD) of all means was calculated.

Results

Controlled crosses between Glera and many resistant genotypes

A total of eighteen cross combinations were carried out from 2017 to 2020 and 451 bunches were obtained by crossing events as reported in Table S3. The seeds were checked for their viability and the biggest number of discarded seed were observed for cross between 'Glera' and 'Voltis' in which the 48,5% didn't show viability.

The crosses provided a population of more than 30.000 seeds that were sown in growth chamber, obtaining 11.363 plants that were characterized using molecular marker analysis.

DNA extraction and MAS analysis

For each germinated seedling single young expanded leaf was collected. The collected plants were lyophilized and homogenised and maintained at 4° C.

The number of analysed plants were shown in Table 3. The samples collected during 2018 (Pop_17) were extracted using commercial kit: DNeasy 96-well DNA (Qiagen, Hilden, Germany). From 2019 a new extraction method was validated on Pop_18, Pop_19 and Pop_20. This method allows a significantly reduction in extraction time process bypassing long centrifuge steps. Two different approaches are shown in Fig. 1.

Table 3 Plants analysed from 2018. In table are shown the number of plants analysed through MAS and the number of plants carrying at least two resistant loci. '-' means discarded plants. '*' means population not analysed.

Year	ID	Crossing event	Analysed plants	Retained plants	Loci
2017	Pop_17_21	Glera x Soreli	1062	270	Rpv3-1; Rpv12
	Pop_17_22	Glera x Sauvignier Gris	458	135	Rpv3-1; Ren3-9
	Pop_17_23	Glera x Muscaris	626	208	Rpv10; Ren3
Tot			2146	613	
2018	Pop_18_24	Glera x Toldi	575	147	Rpv12; Ren3-9
	Pop_18_25	Glera x Cabernet Cantor	1494	769	Rpv3-3 or Rpv3-5; Rpv10; Ren3-9
	Pop_18_26	Glera x Calardis Blanc	1059	435	Rpv3-1 or Rpv3-2; Ren3-9
	Pop_18_27	Glera x SK-00-1/1	529	203	Rpv3-1 or Rpv12; Ren3-9
	Pop_18_28	Glera x SK-00-1/2	342	120	Rpv3-1 or Rpv12; Ren3-9
	Pop_18_31	Toldi x Glera	64	24	Rpv12; Ren3-9
Tot			4063	1698	
2019	Pop_19_33	Glera x 686	313	95	Rpv12; Rpv1/Run1
	Pop_19_34	Glera x 881	134	44	Rpv12; Rpv1/Run1
	Pop_19_35	Glera x Floreal	278	105	Rpv1/Run1; Rpv3-1; Ren3-9
	Pop_19_37	Glera x Voltis	13	-	-
Tot			738	244	
2020	Pop_20_26	Glera x Calardis Blanc	*	*	*
	Pop_20_38	Glera x VC531.039	892	625	Rpv3-1; Run1/Ren1
	Pop_20_41	SK-00-1/7 x Glera	542	293	Rpv1 or Rpv3-1; Rpv12; Run1/Ren1; Ren3-9
	Pop_20_42	Glera x VC156.1017	1513	318	Rpv1; Rpv12; Run1/Ren1
	Pop_20_43	Glera x VC109.033	1469	856	Rpv1; Rpv12; Run1/Ren1; Ren 3-9
Tot			4416	2092	

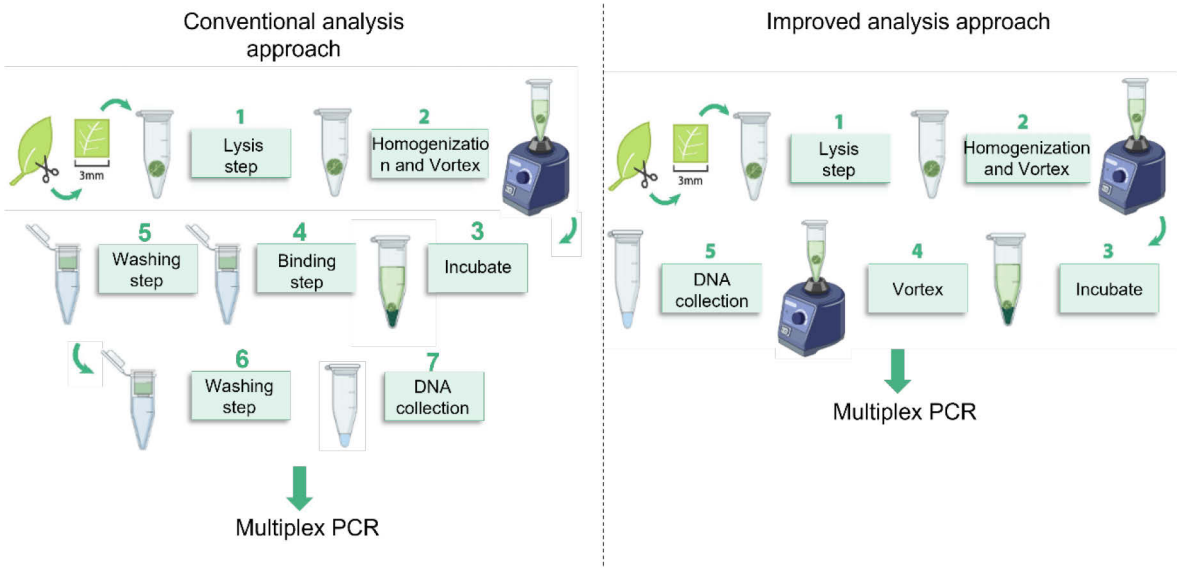


Figure 1 experimental workflow of DNA extraction using conventional analysis approach based on commercial kits, and improved analysis approach bypassing many steps.

Moreover, our approach is based on SSR markers specifically labelled for multiplexing as reported in Table 2. The multiplex PCR allows us to select gene-pyramided offspring for the joint presence of many resistance loci. As reported in Table 3, the offspring were selected for the presence of resistance loci at least one for DM and PM respectively (except for the 5,8 percentage that were selected for two resistant loci to downy mildew) as reported in Fig. 2.

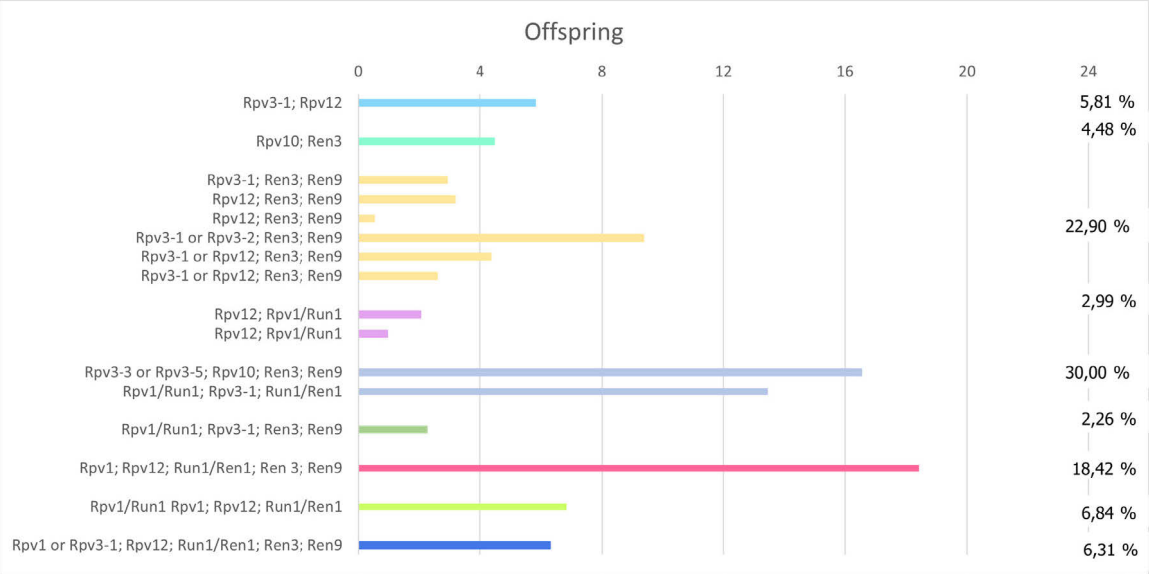


Figure 2 Grouped bar chart showing the percentage of accessions with single resistance locus, combination of two, three, four and five resistance (R) loci. *Rpv*: Resistance to *Plasmopara viticola*; *Run*: Resistance *to Uncinula necator* (from Muscadinia spp.); *Ren*: Resistance to *Erysiphe necator* (from Vitis spp.).

Evaluation of agronomical traits

Among all the individuals of cross population of 2013 and 2014, eight different genotypes were chosen for phenotypic variability evaluation (Fig. 3):

- SR_6-5-1 was obtained by crossing ‘Glera’ x ‘Bronner’ the male parent shows resistance to *Plasmopara viticola* and *Erisyphe necator* through *Ren*3/9, *Rpv*3-3 and *Rpv*10. For this plant molecular markers confirmed the presence of *Rpv*10 locus.
- SR_7-1-7 obtained by crossing ‘Glera’ x ‘Solaris’. The male parent carrying *Rpv*3-3, *Rpv*10 and *Ren*3/9 loci. Among them, molecular markers confirmed the presence of *Rpv*10 locus.
- SR_7-2-2 obtained by crossing ‘Glera’ x ‘Solaris’ molecular markers analysis confirmed the presence of *Rpv*10, *Rpv*3-3 and *Ren*3 loci;
- SR_7-2-6 obtained by crossing ‘Glera’ x ‘Solaris’ molecular markers confirmed the presence of *Rpv*10, *Rpv*3-3 loci
- SR_7-3-8 obtained by crossing ‘Glera’ x ‘Solaris’ molecular markers confirmed the presence of *Rpv*10, *Rpv*3-3 and *Ren*3 loci;

- SR_7-7-8 obtained by crossing ‘Glera’ x ‘Kunleany’ molecular markers confirmed the presence of *Rpv*12;
- SR_7-8-7 obtained by crossing ‘Glera’ x ‘Kunleany’ molecular markers confirmed the presence of *Rpv*12;
- SR_7-8-8 obtained by crossing ‘Glera’ x ‘Kunleany’ molecular markers confirmed the presence of *Rpv*12.

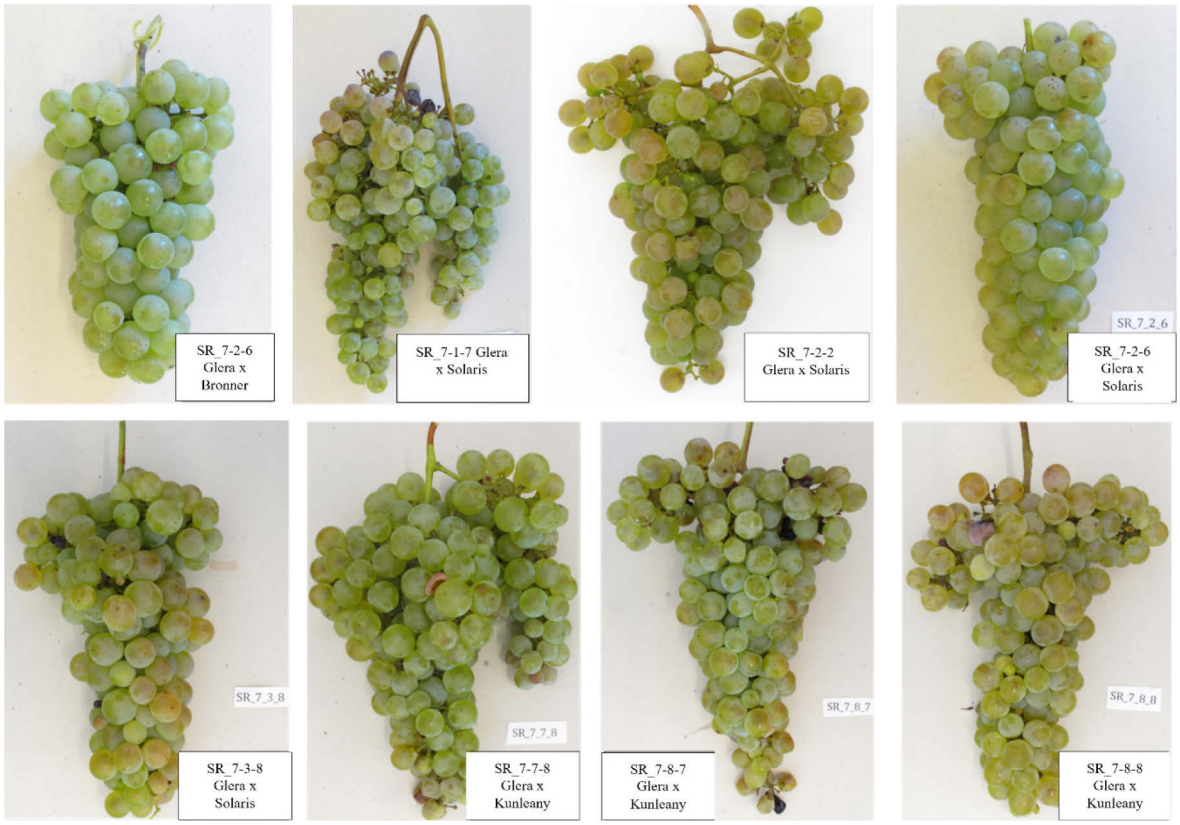


Figure 3 Genotypes used in agronomical traits evaluation and chemical analysis.

Agronomical traits and harvest data were scored during harvesting seasons in 2019 and 2020, chemical analyses were focused on sugars content, acidity and pH values as reported in Fig.4.

The bunches weight presented significant differences compared to Glera genotype and these data are shared with hundred berries weight although the trend were not confirmed, and this is reflected in a difference in total bunches numbers (data non shown).

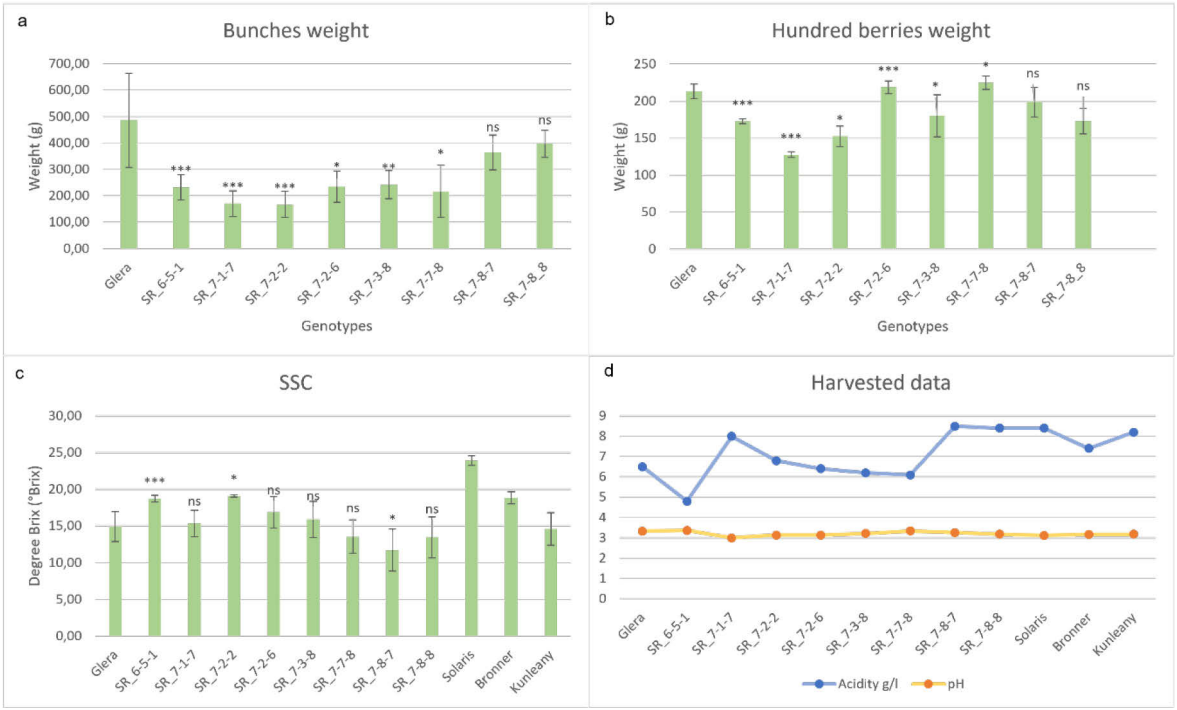
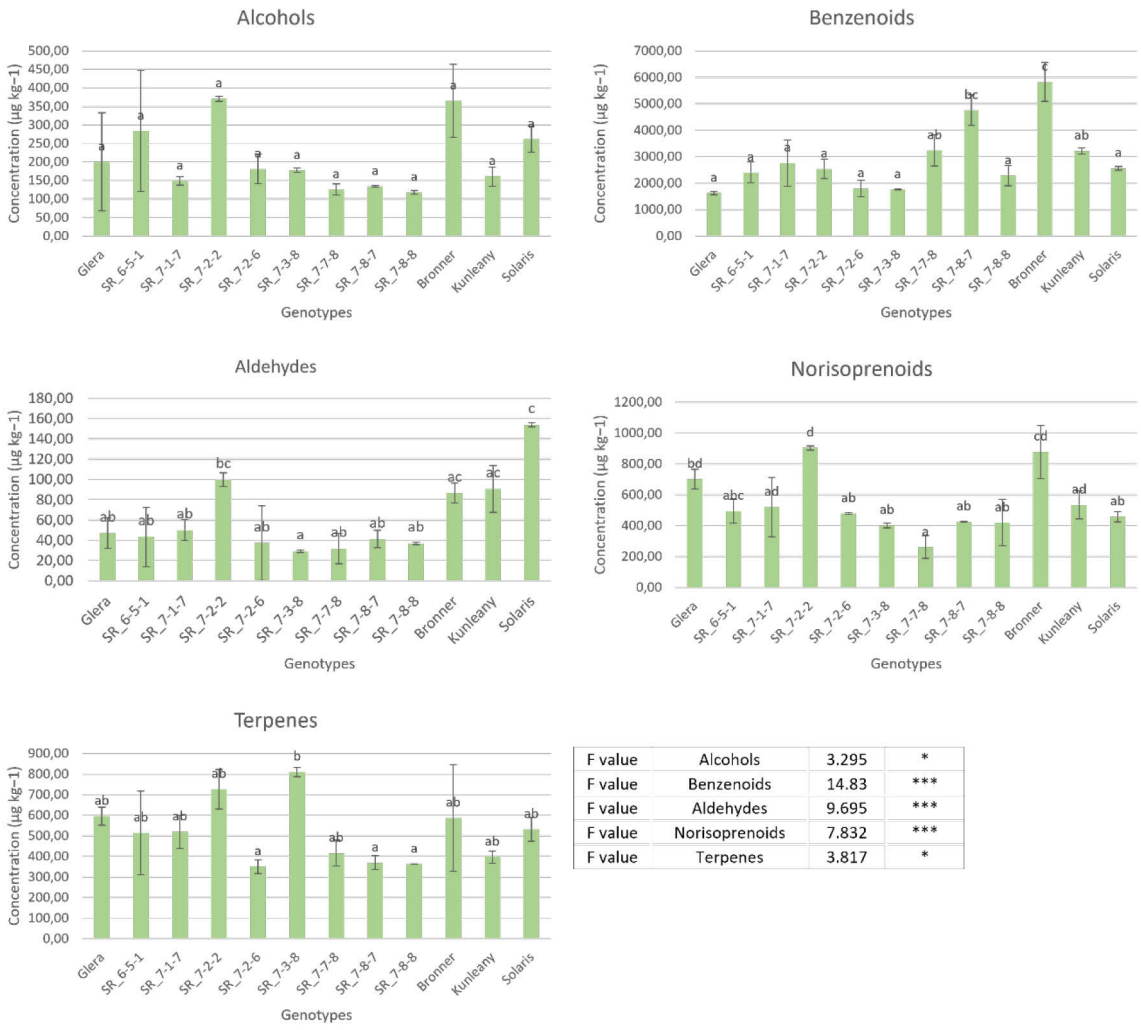


Figure 4 Harvest data collected during 2019 and 2020. Differences in bunches, hundred berries weight, and in Solid Sugar Content were compared using the Student's *t*-test ($P \leq 0.05$).

Aroma compounds identification by GC-MS

The analytical SPE/GC–MS method allowed the correct identification of 89 volatile compounds as reported in Table S5, that were present from 0.4 to 1940 µg Kg^{-1} range. All the volatile compounds identified in the analysed wine samples: Glera, Bronner, Kunleany and Solaris and offsprings: SR_6-5-1; SR_7-1-7; SR_7-2-2; SR_7-2-6; SR_7-3-8; SR_7-7-8; SR_7-8-7 and SR_7-8-8 (Fig. S1), were grouped according to the belonging class: A, alcohols; B, aldehydes; C, terpenes; D, benzenoids; E, norisoprenoids. The data are expressed as means (µg Kg^{-1}) of the GC analyses of triplicate extractions and they correspond to the average of the analysed grapes. Considering the complexity of wine aroma formation and the synergic and antagonistic effects of the involved compounds, a comparison of the subtotal of each chemical classes among grapes was performed, to better characterize the differences as suggested from Toci *et al.*, (Toci *et al.*, 2012).



F value	Alcohols	3.295	*
F value	Benzenoids	14.83	***
F value	Aldehydes	9.695	***
F value	Norisoprenoids	7.832	***
F value	Terpenes	3.817	*

Figure 5 Means of volatile compounds (expressed as µg 1-decanol Kg^{-1} grape) analysed through GC-MS and grouped according to five chemical classes: alcohols; aldehydes; terpenes; benzenoids and norisoprenoids. All data are expressed as mean ± SD. *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering each genotype comparing to female parent 'Glera'. Analysis of variance on the single variables is reported in Table S6.

Discussion

In this paper, we report our results of combining multiple resistance loci to powdery and downy mildew into a single grapevine genome: Glera. We obtained 18 hybrid population from a cross between many hybrids carrying resistance loci to these mildews and Glera, a *Vitis vinifera* genotype.

Molecular plant breeding requires the genotyping of a large number of individuals therefore one purpose of this study was to use marker-assisted selection (MAS) to identify the genotypes carrying resistant loci to PM and DM in the offspring produced by crossing. The development of a simple, rapid and reliable methods for genomic DNA extraction was one objective of this study. To further streamline the selection process, the traditional isolation DNA protocol was modified. The protocol presented here offers a quick, simple and really cost-effective (compared with a purchased kit) approach to large scale DNA extraction for high-throughput genotyping in marker assisted breeding. The extracted DNA is suitable for PCR amplification and then for capillary electrophoresis analysis systems. Previously, many authors used multiplex PCR to simultaneously select resistant loci in a progeny using fluorescent label attached to the forward primers in many species (Merdinoglu *et al.*, 2005; Hayden *et al.*, 2008; Katula-Debreceni *et al.*, 2010). Taking in consideration this strategy, we applied multiplex PCR method to select the valuable genotypes in a single step, saving time, effort, and resources.

Our goal was to prove the presence of the different PM and DM resistant loci in the offspring, to fulfil this purpose we used a set of resistance linked markers to follow the PM and DM resistant genes.

In literature many examples of overcoming resistance barriers were cited both for PM and DM (McDonald & Linde, 2002; Delmotte *et al.*, 2014). For instance, Peressotti *et al*. and Casagrande *et al*. show that the *Rpv*3 gene present in the Bianca variety was defeated by a Czech and an Italian isolate of *P. viticola* (Peressotti *et al.*, 2010; Casagrande *et al.*, 2011).

Due these issues, many authors are agree with the present hypothesis that an alternative environmentally friendly way to control grapevine disease would be to select new genotypes with combinations of resistance loci (Eibach *et al.*, 2007; Schwander *et al.*, 2012; Mundt, 2018; Merdinoglu *et al.*, 2018; Schneider *et al.*, 2019; Zanghelini *et al*., 2019) as reported in

Table S4. Indeed, the resistance conferred by the *Rpv*3-, *Rpv*10- and *Rpv*12-loci to *P. viticola* (Kortekamp *et al.*, 1998; Bellin *et al.*, 2009; Venuti *et al.*, 2013; Bove & Rossi, 2020; Wingerter *et al.*, 2021), and *Ren*1, *Ren*3-, *Ren*9 loci to *E. necator* (Hoffmann *et al.*, 2008; Blanc *et al.*, 2012; Barba *et al.*, 2015; Feechan *et al.*, 2015; Agurto *et al.*, 2017; Zini *et al.*, 2019) have been reported in a great number of previous studies.

Taking this into account, in our breeding programs, we have been able to combine in a single plant multiple resistance loci to PM and DM. The application of molecular markers allows us to select 270 plants carrying two resistance loci to *Plasmopara viticola,* 208 plants carrying a single resistant locus for *Plasmopara viticola* and one for *Erysiphe necator.* An implementation of technique has resulted in selection of plants carrying multiple resistant loci: we obtained 764 plants carrying two resistance loci to *Plasmopara viticola* and one for *Erysiphe necator,* 1064 plant carrying two resistant loci to *E.n.* and one to *P.v.;* and 139 shown two resistance genes to DM and one for PM; 1394 with two resistance loci to *Plasmopara viticola* and *Erysiphe necator* respectively. An additional effort allows to select plants carrying 4 or more resistant loci: in particular we retained 105 plants with two resistant sources to *P.v.* and three to *E.n.,* 1149 plants with three resistant loci to powdery and downy mildews respectively and 318 plants showing three resistance loci to downy mildew and two to powdery mildew. The data illustrated that MAS offers a rapid and accurate method to select hybrid genomes that combine multiple loci in grape. Additionally to genetic point of view, we also evaluated agrochemical variability of resistant plants to predict their quality.

Grape sugar concentration could be defined as a key parameter to predict grape and wine quality, indeed, the final concentrations of sugars and organic acids at ripe stage determine the ethanol to acidity ratio after yeast fermentation, which is a primary factor of wine quality (Bigard *et al.*, 2018). However, in recent years, the sugar concentration has increased in grapes, and it was probably due to climate change scenario (Jones *et al.*, 2005; Koufos *et al.*, 2014). In particular, one of the main features of berry development is the accumulation of hexose sugars (i.e. glucose and fructose), which begins at véraison and continues throughout the ripening process; their accumulation in grape berries significantly impact crop yield and economical value. In literature is reported a variation in sugar concentration according to environment, cultivation practices and variety (Dai *et al.*, 2011; Duchêne *et al.*, 2012; Bigard *et al.*, 2018).

Eight different plants, obtained from previous crossing events and carried more resistance loci, were evaluated for solid sugar content. In all samples sugar content ranging from the parentals as reported in Fig 3. Among them the plant SR_6-5-1 shown a significant difference with female genotypes, resulting in higher °Brix, according to male parent Table S7.

Grape and wine aroma development is a complex process due the presence of numerous volatile organic compounds. Understanding the chemical nature of wine aroma demands the determination of different odour-active compounds both in free volatile and non-volatile forms (glycoside aroma compounds). The hydrolysis of glycoside compounds during winemaking releases volatile that could replace or enhance the aromatic wine pattern (Tamborra *et al.*, 2004; Toci *et al.*, 2012). Glera, is semi-aromatic grape variety and contains significant amounts of glycoside precursors that were investigated through GC-MS.

In studied genotypes alcohols and benzenoides were the most abundant groups with a concentration of 20% and 49% respectively, followed by terpenes, aldehydes and norisoprenoids. Among analysed classes, only a few aroma compounds have been directly linked to specific flavours and aromas as suggested from Ebeler and colleagues (Ebeler *et al.*, 2008). About alcohol profiles it has been reported that the composition of amino acids depends on the grape variety; therefore, these volatile compounds are related to the grapes used (Li *et al.*, 2008) in fact in our samples the subtotal concentration for alcohols varied from 117,4 μg kg^{-1} in sample SR_7-8-8 to a maximum amount of 370,6 μg kg^{-1} in SR_7-2-2. Higher alcohols have largely been studied in wines due to their potential impact on the sensory profile as reported from Del Barrio-Galán et al., (Del Barrio-Galán *et al.*, 2021). In addition, total higher alcohol concentration below 300 mg L^{-1} could contribute positively to the aromatic complexity of wines, and higher concentrations generate unpleasant aromas such as alcoholic, chemical and fusel notes (Gómez-Míguez *et al.*, 2007; Del Barrio-Galán *et al.*, 2021). Higher concentrations of monoterpenes such as linalool, nerol and geraniol and C_{13} norisoprenoids such as β-damascone and vomifoliol were detected in the sparkling wines (Strauss *et al.*, 1987). The norisoprenoids aroma is related to flowery, sweet and fruity notes, while β-ionone supplies an aroma of violets. They are considered to be a quality factor and typical for each variety, as they supply an agreeable scent of tobacco, fruits, tea, etc. as previously reported (Gómez-Míguez *et al.*, 2007). The C_{13}-norisoprenoid pattern was composed by 3-hydroxy-β-damascone, 3-oxo-α-ionol, vomifoliol, and, in smaller

concentrations, 3-hydroxy-7,8-dihydro-β-ionol (Wirth *et al.*, 2001; Boido *et al.*, 2003). As suggested from Boido and colleagues, the presence of 3-hydroxy-α-damascone could be an index of a possible remarkable copresence of the acetylenic damascenone precursor (3-hydroxy-7,8-didehydro-α-ionol), more efficient than the 3-hydroxy-β-damascone to generate the potent rose-hay flavorant (Boido *et al.*, 2003).

Conclusion

The latest breakthroughs achieved in this study lead to development of cv Glera carrying polygenic downy and powdery mildews resistances. Indeed, the presence of at least two resistance loci aims in improved result in term of pathogens control. Moreover, resistance gene pyramiding has been essentially based on MAS. The improvements in MAS analysis led to an accelerated selection process allow us to evaluate a higher plants number.

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Supporting Tables and Figures

Table S4 Genotypes used in breeding program and relative marker alleles. Alleles associated to the QTL identified in resistant genotypes are underlined. Alleles expressed in base pairs (bp).

Parental plants	Resistance loci	Markers associated to the loci	Parental alleles	Reference for the loci
Soreli	Rpv3-1	UDV305;	299/356	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Venuti *et al.*, 2013
		UDV737;	282/297	
		VMC7f2;	200/211	
	Rpv12	UDV350;	302/308	
		UDV360;	201/208	
Souvignier Gris	Rpv3-1	UDV305;	299/356	Di Gaspero *et al.*, 2012; Akkurt *et al.*, 2007; Zyprian *et al.*, 2016; Zendler *et al.*, 2017
		VMC7f2;	200/211	
	Ren3-2	VMC7f2;	200/211	
		UDV737;	282/299	
	Ren3	Scora760;	-	
	Ren3-9	CenGen6;	274/283	
		GF15-28;	342/373	
Muscaris	Rpv10	GF09-46;	388/410	Schwander *et al.*, 2012; Zyprian *et al.*, 2016
		GF09-47;	292/297	
	Ren3	GF15-28;	342/373	
		GF15-30	424/445	
Toldi	Rpv3-4	UDV305;	328/361	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Venuti *et al.*, 2013; Zyprian *et al.*, 2016; Zendler *et al.*, 2017
		VMC7f2;	194/200	
		UDV737;	300/303	
	Rpv12	UDV350;	null/308	
		UDV360;	183/208	
	Ren3-9	CenGen6;	271/283	
		GF15-28;	342/376	
		GF15-30;	445/461	
Cabernet Cantor	Rpv3-3	UDV305;	null/322	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Schwander *et al.*, 2012; Zyprian *et al.*, 2016; Zendler *et al.*, 2017
		UDV737;	274/313	
		VMC7f2;	200/202	
	Rpv3-5	UDV305;	null/322	
		UDV737;	274/313	
		VMC7f2;	200/202	
	Rpv10	GF09-44;	232/240	
		GF09-46;	402/410	
		GF09-47;	288/297	
	Ren3-9	CenGen6;	281/283	
		GF15-28;	342/364	
		GF15-30;	null/455	

Parental plants	Resistance loci	Markers associated to the loci	Parental alleles	Reference for the loci
Calardis Blanc	Rpv3-1	VMC7f2;	200/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Zyprian *et al.*, 2016
		UDV305;	null/299	
		UDV734;	238/242	
		UDV737;	282/299	
	Rpv3-2	VMC7f2;	200/211	
		UDV734;	238/242	
		UDV737;	282/299	
	Ren3-9	GF15-28;	342/373	
		GF15-30;	445/461	
SK-00-1/1	Rpv3-1	VMC7f2;	198/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Venuti *et al.*, 2013; Zyprian *et al.*, 2016; Zendler *et al.*, 2017
		UDV737;	282/null	
	Rpv12	UDV350;	302/308	
		UDV360;	201/208	
	Ren3-9	CenGen6;	270/283	
		GF15-28;	342/363	
		GF15-30;	445/454	
SK-00-1/2	Rpv3-1	VMC7f2;	198/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Venuti *et al.*, 2013; Zyprian *et al.*, 2016; Zendler *et al.*, 2017
		UDV305;	null/299	
		UDV737;	282/null	
	Rpv12	UDV350;	302/308	
		UDV360;	201/208	
	Ren3-9	GF15-28;	342/373	
		GF15-30;	445/454	
Floreal	Rpv3-1	VMC7f2;	200/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Barker *et al.*, 2005; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
		UDV734;	230/237	
		UDV737;	282/297	
	Rpv1/Run1	VMC4f3.1;	190/192	
		VMC8g9;	157/163	
	Ren3-9	CenGen6;	268/283	
		GF15-28;	342/364	
		GF15-30;	415/445	
6-8-6	Rpv12	UDV350;	308/322	Venuti *et al.*, 2013; Barker *et al.*, 2005
		UDV360;	208/211	
	Rpv1/Run1	VMC4f3.1;	174/192	
		VMC8g9;	157/183	

Parental plants	Resistance loci	Markers associated to the loci	Parental alleles	Reference for the loci
8-8-1	Rpv12	UDV350;	308/322	Venuti *et al.*, 2013; Barker *et al.*, 2005
		UDV360;	208/211	
	Rpv1/Run1	VMC4f3.1;	174/192	
		VMC8g9;	157/196	
Voltis	Rpv3-1	VMC7f2;	200/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Barker *et al.*, 2005; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
		UDV734;	230/237	
		UDV737;	282/297	
	Rpv1/Run1	VMC4f3.1;	190/192	
		VMC8g9;	157/163	
	Ren3-9	CenGen6;	268/283	
		GF15-28;	342/364	
		GF15-30;	415/445	
VC531.039	Rpv3-1	VMC7f2;	211/null	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Barker *et al.*, 2005; Venuti *et al.*, 2013; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
		UDV734;	230/237	
		UDV737;	282/297	
	Rpv12	UDV350;	306/308	
		UDV360;	197/208	
	Rpv1/Run1	VMC4f3.1;	174/192	
		VMC8g9;	157/183	
	Ren1	SC8-0071-014;	147/203	
		Sc47_20;	202/206	
SK-00-1/7	Rpv3-1	VMC7f2;	198/211	Di Gaspero *et al.*, 2012; van Heerden *et al.*, 2014; Barker *et al.*, 2005; Hoffmann *et al.*, 2008; Venuti *et al.*, 2013; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
		UDV734;	237/347	
		UDV737;	282/295	
	Rpv3-3	UDV305;	null/322	
		UDV737;	274/313	
		VMC7f2;	200/202	
	Rpv12	UDV350;	308/322	
		UDV360;	208/211	
	Rpv1/Run1	VMC4f3.1;	176/192	
		VMC8g9;	157/167	
	Ren3-9	CenGen6;	268/283	
		GF15-28;	342/364	
		GF15-30;	445/454	
VC156.1017	Rpv12	UDV350;	302/308	Barker *et al.*, 2005; Venuti *et al.*, 2013; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
	Rpv1/Run1	VMC4f3.1;	174/192	
		VMC8g9;	157/null	
VC109.033	Rpv12	UDV350;	302/308	Barker *et al.*, 2005; Venuti *et al.*, 2013; Zendler *et al.*, 2017; Zyprian *et al.*, 2016
	Rpv1/Run1	VMC4f3.1;	182/192	
		VMC8g9;	157/185	
	Ren3-9	GF15-28;	342/373	
		CenGen6	268/283	

Table S5 Grape controlled crosses in 2013 and 2014

Seed Parent	Pollen Donor	Crossing Year	Resistance loci	Analysed Plants	Retained plants
Glera	Bronner	2013	Rpv10,Ren3	51	26
Glera	Solaris	2013	Rpv10,Ren3	7	5
Glera	Kunleany	2014	Rpv12	133	47

Table S6 Results of grape controlled crosses between the susceptible variety Glera and many resistant genotypes. The '-' means not analysed.

Seed Parent	Pollen Donor	Crossed bunches	Ripened bunches	Collected berries	Collected seeds	Stored seeds	Seeds per berry	Discarded seeds %
Glera	Muscaris	41	35	1437	1694	1029	1,18	39,3
Glera	Soreli	50	42	2256	2774	2118	1,23	23,6
Glera	Souvignier Gris	37	31	1022	1118	760	1,09	32,0
Glera	Calardis Blanc	15	13	1932	2140	1744	1,11	18,5
Glera	SK-00-1/1	20	18	1925	1990	1509	1,03	24,2
Glera	SK-00-1/2	18	16	1346	1433	1082	1,06	24,5
Glera	Toldi	14	13	1097	1104	841	1,01	23,8
Toldi	Glera	7	5	404	792	766	1,96	3,3
Glera	Cabernet Cantor	25	23	663	2683	1983	4,05	26,1
Glera	686	22	14	515	624	563	1,21	9,8
Glera	881	19	6	274	336	276	1,23	17,9
Glera	Voltis	25	5	63	66	34	1,05	48,5
Glera	Floreal	25	12	542	659	599	1,22	9,1
Glera	Calardis Blanc	22	5	482	583	583	1,21	-
Glera	VC531.039	12	12	1938	3014	3014	1,56	-
SK-00-1/7	Glera	17	17	3006	5440	5440	1,81	-
Glera	VC156.1017	48	23	2111	3052	3052	1,45	-
Glera	VC109.033	60	45	3627	4979	4979	1,37	-

Table S4 Resistance factors to downy and powdery mildew identified in grapevine.

Factor	Controlled disease	Origin of resistance	Chromosome	Reference
Rpv1	Downy mildew	*V. rotundifolia*	12	Merdinoglu *et al.*, 2003
Rpv2	Downy mildew	*V. rotundifolia*	18	Wiedemann-Merdinoglu *et al.*, 2006
Rpv3	Downy mildew	*V. rupestris*	18	Bellin *et al.*, 2009; Welter *et al.*, 2007
Rpv4	Downy mildew	*American Vitis*	4	Welter *et al.*, 2007
Rpv5	Downy mildew	*V. riparia*	9	Marguerit *et al.*, 2009
Rpv6	Downy mildew	*V. riparia*	12	Marguerit *et al.*, 2009
Rpv7	Downy mildew	*American Vitis*	7	Bellin *et al.*, 2009
Rpv8	Downy mildew	*V. amurensis*	14	Blasi *et al.*, 2011
Rpv9	Downy mildew	*V. riparia*	7	Moreira *et al.*, 2011
Rpv10	Downy mildew	*V. amurensis*	9	Schwander *et al.*, 2012
Rpv11	Downy mildew	*American Vitis*	5	Fischer *et al.*, 2004
Rpv12	Downy mildew	*V. amurensis*	14	Venuti *et al.*, 2013
Rpv13	Downy mildew	*V. riparia*	12	Moreira *et al.*, 2011
Rpv14	Downy mildew	*V. cinerea*	5	Ochssner *et al.*, 2016
Rpv15	Downy mildew	*V. piasezkii*	18	Pap *et al.*,
Rpv16	Downy mildew	-	-	Pap *et al.*,
Rpv17	Downy mildew	-	8	Divilov *et al.*, 2018
Rpv18	Downy mildew	-	11	Divilov *et al.*, 2018
Rpv19	Downy mildew	*V. rupestris*	14	Divilov *et al.*, 2018
Rpv20	Downy mildew	-	6	Divilov *et al.*, 2018
Rpv21	Downy mildew	-	7	Divilov *et al.*, 2018
Rpv22	Downy mildew	*V. amurensis*	2	Fu *et al.*, 2020
Rpv23	Downy mildew	*V. amurensis*	15	Fu *et al.*, 2020
Rpv24	Downy mildew	*V. amurensis*	18	Fu *et al.*, 2020
Rpv25	Downy mildew	*V. amurensis*	15	Lin *et al.*, 2019
Rpv26	Downy mildew	*V. amurensis*	15	Lin *et al.*, 2019
Rpv27	Downy mildew	*V. aestivalis*	18	Sapkota *et al.*, 2019
Rpv28	Downy mildew	*V. rupestris*	10	Bhattara *et al.*, 2020
Rpv29	Downy mildew	*V. vinifera*	14	Sargolzaei *et al.*, 2020
Rpv30	Downy mildew	*V. vinifera*	3	Sargolzaei *et al.*, 2020
Rpv31	Downy mildew	*V. vinifera*	16	Sargolzaei *et al.*, 2020
Run1	Powdery mildew	*V. rotundifolia*	12	Pauquet *et al.*, 2001
Run 2.1	Powdery mildew	*V. rotundifolia*	18	Riaz *et al.*, 2011
Run 2.2	Powdery mildew	*V. rotundifolia*	18	Riaz *et al.*, 2011
Ren1	Powdery mildew	*V. vinifera*	13	Hoffmann *et al.*, 2008
Ren2	Powdery mildew	*V. cinerea*	14	Dalbó *et al.*, 2000
Ren3	Powdery mildew	*American Vitis*	15	Welter *et al.*, 2007
Ren4	Powdery mildew	*V. romanetii*	18	Riaz *et al.*, 2011
Ren5	Powdery mildew	*V. rotundifolia*	14	Blanc *et al.*, 2012
Ren6	Powdery mildew	*V. piasezkii*	9	Pap *et al.*, 2016
Ren7	Powdery mildew	*V. piasezkii*	19	Pap *et al.*, 2016
Ren8	Powdery mildew	*American Vitis*	18	Zyprian *et al.*, 2016
Ren9	Powdery mildew	-	15	Zendler *et al.*, 2017
Ren10	Powdery mildew	-	2	Teh *et al.*, 2017
Ren11	Powdery mildew	-	-	Cadle-Davidson *et al.*,

Table S5 Chemical compounds obtained through GC-MS.

No.	Compound	RI	Formula	Concentration ($\mu g\ Kg^{-1}$)											
	(A) Alcohols			SR_6-5-1	SR_7-1-7	SR_7-2-2	SR_7-2-6	SR_7-3-8	SR_7-7-8	SR_7-8-7	SR_7-8-8	Glera	Kuleany	Solaris	Bronner
A1	2-Pentanol	7,23	$C_5H_{12}O$	1,56 ± 0,41	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
A2	1-Butanol	8,06	$C_4H_{10}O$	32,28 ± 7,82	8,42 ± 2,13	43,02 ± 3,14	24,10 ± 1,84	6,73 ± 1,02	9,47 ± 2,51	8,76 ± 1,14	5,93 ± 0,71	6,55 ± 6,18	4,00 ± 0,09	33,17 ± 3,81	20,40 ± 1,22
A3	3-Methyl-1-butanol	10,7	$C_5H_{12}O$	24,93 ± 3,00	23,89 ± 2,52	43,91 ± 1,33	18,99 ± 4,78	17,75 ± 2,86	15,25 ± 1,96	20,28 ± 0,60	16,40 ± 2,67	11,01 ± 0,13	18,48 ± 1,82	39,40 ± 3,21	161,19 ± 7,58
A4	3-Methyl-3-buten-1-ol	12,74	$C_5H_{10}O$	29,55 ± 10,34	18,32 ± 1,48	32,72 ± 1,55	21,11 ± 5,15	11,45 ± 0,91	24,40 ± 3,18	24,29 ± 0,63	13,69 ± 0,27	17,73 ± 0,33	26,14 ± 1,79	26,57 ± 2,40	19,17 ± 15,53
A5	1-Pentanol	15,24	$C_5H_{12}O$	4,49 ± 3,96	8,25 ± 2,73	6,21 ± 6,18	6,23 ± 0,60	5,86 ± 1,46	4,99 ± 0,35	6,02 ± 0,62	6,12 ± 0,28	6,25 ± 0,54	5,60 ± 0,27	6,49 ± 1,19	7,48 ± 0,83
A6	2-Methyl-2-buten-1-ol	16,5	$C_5H_{10}O$	64,89 ± 29,64	38,59 ± 3,14	143,64 ± 2,42	48,51 ± 16,82	89,02 ± 5,21	25,62 ± 3,36	36,01 ± 0,58	29,49 ± 4,05	30,59 ± 0,10	nd	94,72 ± 9,22	nd
A7	1-Hexanol	18,24	$C_6H_{14}O$	28,39 ± 7,73	10,08 ± 7,75	39,32 ± 0,19	21,68 ± 0,47	12,76 ± 0,44	14,00 ± 2,88	12,81 ± 1,75	15,77 ± 1,22	15,80 ± 1,22	18,65 ± 2,37	23,01 ± 2,05	37,21 ± 0,30
A8	Trans-3-Hexen-1-ol	18,8	$C_6H_{12}O$	0,50 ± 0,15	3,65 ± 2,55	1,13 ± 0,08	0,70 ± 0,36	0,45 ± 0,01	0,53 ± 0,07	2,64 ± 2,33	0,38 ± 0,05	0,40 ± 0,01	0,44 ± 0,11	0,61 ± 0,11	0,98 ± 0,07
A9	Cis-3-Hexen-1-ol	19,911	$C_6H_{12}O$	14,89 ± 10,25	13,13 ± 5,13	19,50 ± 0,85	21,77 ± 16,89	16,69 ± 5,93	13,90 ± 3,66	5,99 ± 0,24	10,03 ± 3,25	12,61 ± 0,01	14,40 ± 1,21	8,70 ± 1,11	8,06 ± 0,52
A10	4-Methyl-3-penten-1-ol	20,22	$C_6H_{12}O$	3,42 ± 0,28	3,81 ± 1,19	3,49 ± 0,02	2,61 ± 0,11	2,69 ± 0,13	3,99 ± 0,23	3,56 ± 0,01	3,05 ± 0,92	3,32 ± 0,06	4,19 ± 0,01	3,02 ± 0,50	4,06 ± 0,13
A11	2-Butoxyethanol	20,73	$C_6H_{14}O_2$	59,15 ± 57,88	3,21 ± 0,38	4,51 ± 0,82	2,46 ± 0,03	2,58 ± 0,41	2,28 ± 0,35	3,24 ± 0,13	3,07 ± 1,05	86,76 ± 1,00	4,68 ± 0,59	4,29 ± 0,23	49,34 ± 46,37
A12	2-Hexen-1-ol	21,26	$C_6H_{12}O$	11,09 ± 4,16	10,31 ± 0,59	19,07 ± 1,11	8,19 ± 1,97	6,11 ± 0,70	4,73 ± 0,08	2,78 ± 0,37	8,05 ± 0,03	4,25 ± 0,55	8,22 ± 1,51	12,09 ± 1,10	10,54 ± 0,35
A13	1-Octen-3-ol	23,92	$C_8H_{16}O$	1,22 ± 0,13	1,14 ± 0,11	4,97 ± 2,12	0,85 ± 0,01	0,64 ± 0,03	1,61 ± 0,73	1,35 ± 0,03	0,84 ± 0,02	1,92 ± 1,43	2,71 ± 1,01	3,15 ± 0,96	2,99 ± 1,06
A14	Butanoic acid-3-hydroxy-methyl ester	25,33	$C_5H_{10}O_3$	2,49 ± 0,23	nd	2,06 ± 0,32	nd	nd	nd	nd	nd	nd	nd	nd	nd
A15	2-Ethylhexanol	26,19	$C_8H_{18}O$	1,18 ± 0,04	1,49 ± 0,11	1,42 ± 0,17	0,71 ± 0,09	0,95 ± 0,28	0,69 ± 0,07	1,05 ± 0,04	0,96 ± 0,02	1,01 ± 0,07	1,07 ± 0,03	1,42 ± 0,60	0,74 ± 0,14
A16	1-Octanol	29,95	$C_8H_{18}O$	3,69 ± 0,78	4,52 ± 0,75	5,68 ± 0,08	3,23 ± 0,06	3,84 ± 0,07	3,23 ± 0,45	3,04 ± 0,48	3,61 ± 0,55	2,14 ± 0,01	3,06 ± 0,47	5,26 ± 0,90	4,42 ± 0,35
A17	2-Methyl-1-propanol	6,26	$C_4H_{10}O$	nd	nd	nd	nd	nd	1,07 ± 1,07	2,72 ± 2,72	nd	nd	20,27 ± 6,23	nd	nd
A18	3-Methyl-2-buten-1-ol	16,47	$C_5H_{10}O$	nd	nd	nd	nd	nd	nd	nd	nd	nd	29,06 ± 1,65	nd	38,56 ± 0,05
	Tot			**283,73**	**148,80**	**370,67**	**181,13**	**177,52**	**125,76**	**134,55**	**117,40**	**200,34**	**160,97**	**261,90**	**365,12**
	(B) Aldehydes														
B1	Hexanal	6,18	$C_6H_{12}O$	7,63 ± 1,06	8,71 ± 4,57	22,40 ± 0,89	10,48 ± 7,08	9,88 ± 1,21	4,85 ± 3,14	8,82 ± 4,83	8,62 ± 1,02	11,57 ± 4,00	19,80 ± 8,28	37,78 ± 0,52	28,98 ± 0,74
B2	2-Hexenal	11,1	$C_6H_{10}O$	27,29 ± 21,02	32,54 ± 4,18	69,52 ± 1,99	25,52 ± 18,91	14,14 ± 0,14	22,17 ± 6,42	25,05 ± 1,55	22,65 ± 0,13	26,79 ± 4,94	63,08 ± 7,53	107,02 ± 0,84	48,31 ± 4,69
B3	Geranial	39,04	$C_{10}H_{16}O$	1,28 ± 1,28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
B4	5-methyl-2-furancarboxaldehyde	30,47	C_6H_6O	1,79 ± 0,16	2,54 ± 0,80	1,71 ± 0,02	1,11 ± 0,11	1,21 ± 0,01	0,78 ± 0,27	1,57 ± 0,06	1,22 ± 0,38	1,36 ± 0,39	1,43 ± 0,03	3,08 ± 0,19	1,76 ± 0,32
B5	1H-Pyrrole-2-carboxaldehyde	53,7	C_5H_5NO	5,19 ± 0,68	6,16 ± 0,47	6,07 ± 1,76		3,93 ± 2,06	3,92 ± 0,76	5,56 ± 0,33	4,47 ± 0,33	6,29 ± 2,50	5,05 ± 0,58	6,09 ± 0,13	6,34 ± 0,73
B6	Furfural	24,53	$C_5H_4O_2$	nd	nd	nd	nd	nd	nd	nd	nd	0,92 ± 0,31	1,25 ± 0,09	nd	1,12 ± 0,43
	Tot			**43,19**	**49,94**	**99,70**	**37,11**	**29,15**	**31,72**	**41,00**	**36,97**	**46,93**	**90,61**	**153,98**	**86,51**
	(C) Terpenes														
C1	Trans-Linalool oxide	22,86	$C_{10}H_{18}O_2$	2,75 ± 1,48	3,36 ± 0,67	4,44 ± 0,70	1,80 ± 0,89	1,26 ± 0,33	0,57 ± 0,09	1,71 ± 0,06	2,44 ± 0,63	1,22 ± 0,03	4,40 ± 0,33	8,62 ± 0,47	18,10 ± 1,81
C2	Cis-Linalool oxide	24,19	$C_{10}H_{18}O_2$	3,12 ± 2,03	5,16 ± 0,62	2,45 ± 0,51	2,20 ± 1,35	0,81 ± 0,11	0,72 ± 0,10	3,65 ± 0,29	3,24 ± 0,16	0,43 ± 0,03	6,07 ± 0,18	4,90 ± 0,57	16,56 ± 1,70
C3	Linalool	29,5	$C_{10}H_{18}O$	4,05 ± 2,82	1,51 ± 0,52	2,81 ± 0,04	3,58 ± 2,68	21,59 ± 0,44	1,27 ± 0,23	0,65 ± 0,15	0,91 ± 0,02	5,94 ± 0,66	0,83 ± 0,19	7,13 ± 2,39	0,87 ± 0,20
C4	α-Terpineol	37,3	$C_{10}H_{18}O$	15,82 ± 13,14	3,69 ± 0,59	33,85 ± 3,70	10,59 ± 8,37	8,79 ± 1,29	2,89 ± 0,77	5,92 ± 0,09	2,30 ± 0,95	3,23 ± 0,57	4,82 ± 0,31	8,51 ± 0,34	2,36 ± 1,55
C5	Trans-Linalool oxide pyranoid	39,58	$C_{10}H_{18}O_2$	3,69 ± 1,73	8,44 ± 0,64	3,59 ± 0,38	2,55 ± 0,87	3,09 ± 0,01	0,91 ± 0,05	3,05 ± 0,23	7,14 ± 2,26	2,42 ± 0,21	16,89 ± 2,26	15,28 ± 1,30	65,15 ± 27,06
C6	Cis-Linalool oxide pyranoid	41,19	$C_{10}H_{18}O_2$	9,01 ± 6,70	5,48 ± 0,48	1,85 ± 0,27	3,90 ± 2,98	2,19 ± 0,33	1,63 ± 0,66	5,70 ± 1,48	4,44 ± 0,03	10,15 ± 2,64	17,38 ± 1,73	5,82 ± 0,56	139,02 ± 56,50
C7	Nerol	43,06	$C_{10}H_{18}O$	10,54 ± 6,63	11,80 ± 2,17	3,76 ± 0,10	9,78 ± 7,09	5,95 ± 0,01	5,13 ± 1,08	3,88 ± 0,85	6,83 ± 2,48	8,76 ± 3,48	4,40 ± 0,15	7,05 ± 0,41	10,47 ± 1,30
C8	Geraniol	45,67	$C_{10}H_{18}O$	113,06 ± 26,71	77,74 ± 16,27	95,19 ± 4,82	100,25 ± 14,32	77,49 ± 0,69	47,05 ± 6,03	53,76 ± 10,22	89,31 ± 35,33	75,02 ± 9,77	40,35 ± 0,75	74,33 ± 10,97	21,86 ± 2,79
C9	Diendiol 1	50,91	$C_{12}H_{30}O_3$	1,92 ± 0,40	3,08 ± 0,85	8,20 ± 1,26	2,65 ± 0,25	2,26 ± 0,30	nd	nd	2,01 ± 1,67	0,70 ± 0,03	nd	48,74 ± 9,48	27,83 ± 6,06
C10	Trans-hydroxy-linalool	65,39	$C_{10}H_{18}O_2$	27,69 ± 19,90	48,28 ± 3,66	25,65 ± 2,32	21,59 ± 14,87	26,91 ± 1,58	14,08 ± 1,72	17,75 ± 1,91	33,42 ± 8,66	28,37 ± 3,62	41,39 ± 1,39	24,27 ± 0,61	56,19 ± 20,74
C11	8-Hydroxygeraniol	66,78	$C_{10}H_{18}O_2$	62,20 ± 39,03	70,26 ± 22,01	60,85 ± 0,05	45,90 ± 18,07	80,21 ± 2,71	79,13 ± 14,62	58,22 ± 0,46	38,18 ± 0,77	112,03 ± 11,96	44,49 ± 1,54	22,10 ± 1,12	24,31 ± 8,70
C12	Cis-hydroxy-linalool	66,78	$C_{10}H_{18}O$	157,47 ± 90,21	135,85 ± 40,19	127,16 ± 11,46	37,90 ± 10,19	466,36 ± 0,15	103,99 ± 0,95	74,08 ± 3,64	93,91 ± 38,24	201,14 ± 20,36	109,84 ± 4,61	85,18 ± 7,08	95,72 ± 31,47
C13	Geranic acid	67,54	$C_{10}H_{16}O_2$	40,31 ± 15,98	104,50 ± 62,18	57,55 ± 1,85	30,02 ± 3,38	43,17 ± 5,09	39,57 ± 7,20	28,96 ± 1,46	42,51 ± 22,27	104,61 ± 10,14	15,59 ± 0,99	29,37 ± 2,71	91,77 ± 26,14
C14	7-hydroxy-alpha-terpineol	73,17	$C_{10}H_{18}O$	63,33 ± 57,14	40,50 ± 5,09	269,64 ± 11,35	77,95 ± 71,66	71,75 ± 8,79	118,27 ± 12,68	90,41 ± 6,94	36,45 ± 13,68	41,72 ± 0,17	76,53 ± 10,82	161,29 ± 11,13	16,96 ± 0,45
C15	Lilac alcohol	44,31	$C_{10}H_{18}O_2$	nd	nd	nd	nd	nd	0,81 ± 0,14	1,11 ± 0,33	nd	nd	nd	nd	nd
C16	exo-2-Hydroxycineole	45,72	$C_{10}H_{18}O_2$	nd	nd	nd	nd	nd	nd	20,34 ± 0,74	nd	nd	13,43 0,78	29,02 ± 0,61	nd
	Tot			**514,97**	**519,67**	**696,98**	**350,68**	**811,83**	**416,02**	**369,18**	**363,08**	**595,75**	**396,40**	**531,63**	**587,15**
	(D) Benzenoids														
D1	Benzaldehyde	27,29	C_7H_6O	13,37 ± 0,51	17,56 ± 5,16	13,84 ± 0,95	9,16 ± 2,89	14,53 ± 1,73	10,06 ± 1,49	17,49 ± 2,67	11,83 ± 1,69	13,39 ± 1,02	12,23 ± 2,27	14,20 ± 3,52	16,79 ± 0,18
D2	Benzeneacetaldehyde	34,12	C_8H_8O	25,14 ± 7,30	8,97 ± 4,18	22,23 ± 1,90	2,94 ± 1,06	12,68 ± 10,61	16,82 ± 3,92	11,11 ± 2,62	2,54 ± 0,66	4,74 ± 1,79	17,93 ± 4,79	11,49 ± 2,95	46,22 ± 20,53
D3	3-Hydroxybenzaldehyde	35,72	$C_7H_6O_2$	4,82 ± 0,21	6,77 ± 1,53	9,59 ± 3,33	3,12 ± 0,56	7,59 ± 0,71	3,54 ± 0,34	4,16 ± 1,01	6,22 ± 2,03	8,47 ± 3,06	1,94 ± 0,02	6,52 ± 0,84	8,25 ± 3,74
D4	Methyl salicylate	40,9	$C_8H_8O_3$	7,97 ± 0,72	32,18 ± 2,08	9,23 ± 0,37	6,00 ± 1,15	11,17 ± 0,09	10,49 ± 0,50	32,73 ± 1,24	24,33 ± 0,07	17,91 ± 6,19	102,77 ± 13,27	11,83 ± 0,68	17,89 ± 2,03
D5	Benzene methanol	43,55	$C_7H_{10}O$	1,54 ± 0,06	1,99 ± 0,29	1,83 ± 0,12	0,89 ± 0,02	1,17 ± 0,19	1,13 ± 0,19	2,71 ± 0,16	1,13 ± 0,01	1,86 ± 0,52	3,39 ± 0,21	6,23 ± 0,81	3,98 ± 0,12
D6	2-Methoxyphenol	45,86	$C_7H_8O_2$	10,46 ± 2,62	15,95 ± 2,41	nd	nd	nd	nd	28,08 ± 7,94	11,80 ± 2,66	5,57 ± 2,63	17,17 ± 5,06	34,22 ± 9,18	11,69 ± 5,17
D7	Benzyl Alcohol	46,7	C_7H_8O	707,95 ± 22,39	888,32 ± 257,95	686,12 ± 48,47	558,53 ± 105,55	677,63 ± 32,23	1133,72 ± 257,69	1441,64 ± 45,00	687,07 ± 239,95	491,28 ± 34,47	896,97 ± 90,88	872,19 ± 33,62	1942,35 ± 96,55
D8	2-Phenylethanol	48,36	$C_8H_{10}O$	537,65 ± 125,63	363,85 ± 78,52	524,49 ± 5,60	425,05 ± 59,50	397,38 ± 15,06	367,66 ± 33,03	271,71 ± 10,55	277,54 ± 68,89	230,42 ± 9,25	317,77 ± 15,35	544,39 ± 9,49	301,85 ± 1,43
D9	3-Methylbenzyl alcohol	51,35	$C_8H_{10}O$	2,34 ± 0,45	3,57 ± 0,69	3,35 ± 1,08	2,09 ± 0,27	2,60 ± 0,04	2,62 ± 0,34	3,78 ± 0,22	2,13 ± 0,08	2,42 ± 0,07	2,55 ± 0,52	3,36 ± 0,26	3,07 ± 1,10
D10	Phenol	53,26	C_6H_6O	9,41 ± 5,43	8,62 ± 4,49	6,01 ± 0,79	2,91 ± 0,27	1,76 ± 0,24	3,63 ± 0,53	8,41 ± 0,06	21,22 ± 14,49	0,25 ± 0,35	6,88 ± 0,49	4,45 ± 0,84	7,14 ± 1,16
D11	o-Cresol	56,86	C_7H_8O	2,13 ± 0,09	0,89 ± 0,14	nd	1,97 ± 0,13	nd	nd	2,10 ± 0,72	nd	2,59 ± 0,89	2,92 ± 0,51	2,54 ± 0,44	1,80 ± 0,00
D12	2-Phenoxyethanol	59,29	$C_8H_{10}O_2$	3,13 ± 0,07	3,65 ± 0,72	4,57 ± 0,66	2,77 ± 0,34	3,11 ± 0,33	4,48 ± 0,34	4,84 ± 0,46	6,13 ± 2,91	5,10 ± 2,18	9,00 ± 1,41	1,81 ± 0,94	3,98 ± 0,16
D13	Eugenol	60,47	$C_{10}H_{12}O_2$	8,82 ± 0,01	58,99 ± 38,92	12,09 ± 0,13	9,13 ± 0,35	3,61 ± 0,82	13,39 ± 1,02	46,18 ± 1,45	41,48 ± 27,54	14,28 ± 3,40	13,37 ± 0,82	4,29 ± 0,22	31,32 ± 0,80
D14	2-Methoxy-4-vinylphenol	61,79	$C_9H_{10}O_2$	301,17 ± 119,08	456,08 ± 177,07	240,94 ± 9,84	182,17 ± 32,77	152,78 ± 1,25	436,64 ± 123,67	717,77 ± 63,38	298,69 ± 59,44	121,10 ± 14,57	643,54 ± 29,23	298,23 ± 16,54	1123,48 ± 123,20
D15	Methyl gentisate	63,51	$C_8H_8O_4$	38,38 ± 23,74	44,22 ± 20,71	87,75 ± 19,12	24,71 ± 17,18	60,87 ± 12,98	70,35 ± 6,05	364,78 ± 22,46	49,68 ± 9,94	154,48 ± 34,04	nd	nd	nd
D16	2,6-Dimethoxyphenol	64,7	$C_8H_{10}O_3$	7,85 ± 0,43	14,86 ± 3,08	9,95 ± 0,47	5,77 ± 1,20	4,41 ± 1,59	7,12 ± 0,85	18,20 ± 3,92	5,76 ± 1,69	8,25 ± -0,32	15,11 ± 5,30	8,00 ± 0,16	20,67 ± 6,70
D17	2,3-Dihydrobenzofuran	69,27	C_8H_8O	175,85 ± 83,84	100,51 ± 53,94	117,09 ± 20,18	53,10 ± 2,90	39,89 ± 6,01	56,17 ± 10,44	596,49 ± 124,57	61,78 ± 5,33	70,66 ± 23,81	248,07 ± 92,34	148,73 ± 24,91	214,54 ± 8,42
D18	Vanillin	74,08	$C_8H_8O_3$	23,56 ± 8,13	20,15 ± 1,21	6,01 ± 1,50	11,75 ± 3,37	9,73 ± 0,26	18,26 ± 0,53	23,25 ± 4,43	14,93 ± 1,05	10,59 ± 1,95	36,61 ± 0,15	18,54 ± 0,85	45,74 ± 16,57
D19	Methyl vanillate	75,38	$C_9H_{10}O_4$	18,47 ± 10,02	19,78 ± 5,62	11,46 ± 0,43	31,57 ± 0,34	25,84 ± 1,97	10,15 ± 1,87	19,94 ± 8,70	18,40 ± 4,31	4,63 ± 2,32	62,92 ± 9,70	98,14 ± 33,22	471,28 ± 134,84
D20	Acetovanillone	76,22	$C_9H_{10}O_3$	43,57 ± 8,40	52,78 ± 31,71	12,77 ± 2,96	48,20 ± 1,02	34,64 ± 1,68	306,33 ± 27,39	54,28 ± 15,52	145,19 ± 14,15	91,73 ± 0,15	124,17 ± 37,30	64,50 ± 24,26	179,13 ± 27,24
D21	Salicyl alcohol	76,93	$C_7H_8O_2$	10,30 ± 5,62	52,30 ± 50,46	93,23 ± 2,20	5,34 ± 4,03	18,01 ± 1,14	7,86 ± 0,87	26,48 ± 8,80	29,44 ± 11,18	12,24 ± 5,86	9,09 ± 6,17	16,52 ± 2,67	17,08 ± 0,39
D22	4-Hydroxy-3-methoxybenzyl alcohol	80,81	$C_8H_{10}O_3$	35,58 ± 3,94	40,03 ± 0,28	17,79 ± 9,70	40,61 ± 15,61	37,90 ± 18,93	47,74 ± 19,70	51,10 ± 5,31	27,04 ± 5,82	13,66 ± 1,09	63,03 ± 18,81	42,84 ± 7,84	86,42 ± 4,44
D23	Benzeneethanol, 2-hydroxy	81,63	$C_8H_{10}O$	23,57 ± 10,11	27,93 ± 10,59	47,26 ± 3,45	51,87 ± 4,35	17,57 ± 0,97	nd	nd	nd	17,59 ± 5,96	22,05 ± 2,83	30,16 ± 5,76	53,15 ± 10,00
D24	Homovanillyl alcohol	81,8	$C_9H_{12}O_3$	98,48 ± 41,40	122,75 ± 6,38	37,41 ± 16,45	73,95 ± 32,28	45,33 ± 13,84	258,92 ± 12,31	175,73 ± 7,43	313,21 ± 83,29	46,66 ± 7,11	206,78 ± 16,90	35,05 ± 3,34	112,09 ± 11,67
D25	(3,4,5-Trimethoxyphenyl)methanol	82,97	$C_{10}H_{14}O_4$	14,20 ± 5,66	47,70 ± 19,56	60,57 ± 15,08	81,18 ± 18,79	21,68 ± 10,19	45,70 ± 7,52	61,47 ± 4,90	23,63 ± 3,27	17,67 ± 3,05	38,55 ± 8,32	nd	22,20 ± 1,78
D26	Syringaldehyde	84,22	$C_9H_{10}O_4$	9,00 ± 1,60	20,63 ± 4,07	39,16 ± 15,88	nd	nd	nd	nd	20,88 ± 8,86	8,14 ± 3,25	nd	nd	nd
D27	Benzeneacetic acid, 4-hydroxy-3-methoxy	85,17	$C_{10}H_{12}O$	56,82 ± 46,76	21,43 ± 0,39	nd	nd	15,26 ± 3,28	92,39 ± 1,40	232,26 ± 66,83	14,86 ± 3,04	76,17 ± 2,01	89,70 ± 11,43	54,95 ± 11,35	96,73 ± 23,41
D28	Methyl 2,5-dihydroxybenzoate	85,73	$C_8H_8O_4$	117,74 ± 70,44	97,76 ± 14,23	236,78 ± 89,30	nd	48,56 ± 30,02	93,93 ± 0,14	255,09 ± 39,14	45,21 ± 11,43	47,65 ± 25,60	60,93 ± 3,99	181,15 ± 9,39	821,11 ± 271,97
D29	4-Hydroxybenzoic acid	85,99	$C_7H_6O_3$	41,93 ± 10,49	96,76 ± 5,42	117,40 ± 16,23	nd	55,21 ± 11,90	77,00 ± 11,07	70,15 ± 11,81	42,24 ± 6,68	nd	nd	nd	nd
D30	3,4,5-Trimethoxyphenol	87,75	$C_9H_{12}O_4$	56,67 ± 6,55	96,95 ± 43,80	104,24 ± 0,39	nd	25,05 ± 3,39	139,19 ± 49,75	140,83 ± 7,18	62,18 ± 21,47	88,37 ± 22,56	79,84 ± 25,80	7,07 ± 0,35	126,64 ± 35,95
D31	4-Methylphenol	53,4	C_7H_8O	nd	3,67 ± 2,29	nd	nd	nd	nd	nd	1,41 ± 0,15	nd	nd	nd	nd
D32	Benzeneacetic acid, alpha-hydroxy	74	$C_8H_8O_3$	nd	8,17 ± 5,02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
D33	3-hydroxy benzenemethanol	85,1	$C_7H_8O_2$	nd	8,93 ± 5,22	nd	nd	nd	nd	nd	8,24 ± 2,01	34,85 ± 30,76	nd	nd	3,07 ± 1,08
D34	2-Ethylphenol	77	$C_8H_{10}O$	nd	nd	2,01 ± 0,68	nd	nd	nd	nd	nd	nd	nd	nd	nd
D35	Benzenemethanol, 4-(1-methylethyl)	57,52	$C_{11}H_{14}O_2$	nd	nd	6,63 ± 0,17	3,48 ± 3,48	nd	nd	2,54 ± 1,46	nd	nd	nd	nd	nd
D36	4-Ethylphenol	53,34	$C_8H_{10}O$	nd	nd	nd	1,02 ± 0,15	nd	nd	nd	nd	nd	nd	nd	nd
D37	2-Methoxybenzyl alcohol	60,22	$C_8H_{10}O_2$	nd	nd	nd	1,78 ± 0,54	nd	nd	nd	nd	nd	nd	nd	nd
D38	1,6-Hexanediol	59,99	$C_6H_{14}O_2$	nd	nd	nd	nd	nd	nd	10,84 ± 0,48	nd	nd	nd	nd	nd
D39	4'-Hydroxy-3'-methylacetophenone	61,03	$C_9H_{10}O_2$	nd	nd	nd	nd	nd	nd	33,52 ± 1,29	2,82 ± 0,21	nd	nd	nd	nd
D40	3,4-Dimethoxyphenyl	80,07	$C_{10}H_{12}NO_2$	nd	nd	nd	nd	nd	nd	24,95 ± 0,71	nd	nd	nd	nd	nd
D41	Methyl 2,3-dihydroxybenzoate	63,48	$C_8H_8O_4$	nd	nd	nd	nd	nd	nd	nd	nd	nd	42,64 ± 10,00	38,47 ± 9,40	36,57 ± 2,16
D42	Benzenepropanol, 4-(hydroxymethyl)-alpha-methyl	87,89	$C_{11}H_{16}O2$	nd	nd	nd	nd	nd	nd	nd	nd	nd	72,12 ± 27,58	nd	nd
D43	m-Cresol	56,86	C_7H_8O	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3,29 ± 0,18
D44	4-Hydroxy-3-methoxybenzyl alcohol	80,79	$C_8H_{10}O_3$	nd	nd	nd	51,87 ± 4,35	nd	nd	51,10 5,31	nd	nd	nd	nd	nd
	Tot			**2407,88**	**2764,68**	**2541,80**	**1692,95**	**1745,96**	**3235,32**	**4805,71**	**2279,03**	**1622,71**	**3220,03**	**2559,88**	**5829,52**
	(E) Norisoprenoids														
E1	3-Hydroxy-beta-damascone	73,48	$C_{13}H_{20}O_2$	69,03 ± 11,16	74,75 ± 9,21	83,55 ± 4,16	46,73 ± 3,78	54,44 ± 5,47	54,55 ± 6,97	74,34 ± 3,85	51,55 ± 9,30	42,72 ± 1,23	89,59 ± 9,64	56,30 ± 2,14	94,95 ± 5,64
E2	3-Oxo-alpha-ionol	76,1	$C_{13}H_{20}O_2$	73,01 ± 10,23	67,60 ± 16,42	176,03 ± 8,86	167,63 ± 2,14	72,21 ± 2,24	37,82 ± 9,24	17,06 ± 0,20	63,69 ± 10,87	186,01 ± 1,66	64,41 ± 12,00	45,74 ± 1,24	267,81 ± 84,57
E3	3,9-dihydroxy megastigma-5-ene	77,18	C19H30O8	81,19 ± 17,61	33,86 ± 12,26	33,88 ± 1,29	58,26 ± 7,71	89,98 ± 9,40	59,97 ± 13,30	134,13 ± 15,44	30,45 ± 6,91	19,05 ± 2,25	58,28 ± 9,15	46,35 ± 2,97	82,31 ± 1,17
E4	3-hydroxy-7,8-didehydro-β-ionol	79,78	$C_{13}H_{20}O_2$	60,34 ± 6,11	69,69 ± 4,35	66,30 ± 7,24	51,70 ± 4,18	60,99 ± 9,50	46,40 ± 9,51	77,89 ± 2,07	46,75 ± 5,20	41,34 ± 1,24	68,05 ± 8,47	48,78 ± 6,54	106,98 ± 14,50
E5	Vomifoliol	90	$C_{13}H_{20}O_3$	210,88 ± 88,06	500,92 ± 126,23	543,79 ± 3,29	154,61 ± 13,67	124,31 ± 18,58	62,83 ± 13,57	122,33 ± 17,79	227,79 ± 109,55	413,64 ± 38,14	254,15 ± 25,29	260,41 ± 22,69	326,86 ± 58,07
	Tot			**494,45**	**746,81**	**903,55**	**478,92**	**401,94**	**261,56**	**425,75**	**420,24**	**702,77**	**534,48**	**457,58**	**878,91**

Table S6 Analysis of variance (ANOVA) outcomes of chemical compounds. Different letters within each column indicate differences according to Tukey HSD test ($P \leq 0.05$).

Compound	Genotypes	mean_conc		conc_sd	
Alcohols	Glera	200,35	±	132,44	a
Alcohols	SR_6-5-1	283,75	±	163,13	a
Alcohols	SR_7-1-7	148,80	±	11,17	a
Alcohols	SR_7-2-2	370,70	±	6,79	a
Alcohols	SR_7-2-6	181,10	±	39,74	a
Alcohols	SR_7-3-8	177,80	±	5,66	a
Alcohols	SR_7-7-8	125,75	±	15,06	a
Alcohols	SR_7-8-7	134,55	±	1,77	a
Alcohols	SR_7-8-8	118,05	±	5,02	a
Alcohols	Bronner	365,10	±	98,71	a
Alcohols	Kunleany	160,95	±	25,95	a
Alcohols	Solaris	261,90	±	35,36	a
Aldehydes	Glera	46,93	±	15,21	ab
Aldehydes	SR_6-5-1	43,20	±	29,27	ab
Aldehydes	SR_7-1-7	49,95	±	10,54	ab
Aldehydes	SR_7-2-2	99,75	±	6,58	bc
Aldehydes	SR_7-2-6	37,10	±	36,91	ab
Aldehydes	SR_7-3-8	29,15	±	1,06	a
Aldehydes	SR_7-7-8	31,70	±	14,99	ab
Aldehydes	SR_7-8-7	41,00	±	8,63	ab
Aldehydes	SR_7-8-8	36,95	±	1,20	ab
Aldehydes	Bronner	86,50	±	9,76	ac
Aldehydes	Kunleany	90,60	±	23,06	ac
Aldehydes	Solaris	154,00	±	1,98	c
Benzenoids	Glera	1622,70	±	59,11	a
Benzenoids	SR_6-5-1	2407,90	±	405,74	a
Benzenoids	SR_7-1-7	2764,70	±	870,31	a
Benzenoids	SR_7-2-2	2539,20	±	369,53	a
Benzenoids	SR_7-2-6	1803,85	±	316,85	a
Benzenoids	SR_7-3-8	1759,20	±	14,71	a
Benzenoids	SR_7-7-8	3240,10	±	587,61	ab
Benzenoids	SR_7-8-7	4754,60	±	576,57	bc
Benzenoids	SR_7-8-8	2279,00	±	381,41	a
Benzenoids	Bronner	5829,50	±	731,57	c
Benzenoids	Kunleany	3220,00	±	121,20	ab
Benzenoids	Solaris	2559,85	±	65,69	a
Norisoprenoids	Glera	702,75	±	63,00	bd
Norisoprenoids	SR_6-5-1	494,45	±	77,99	abc
Norisoprenoids	SR_7-1-7	520,25	±	191,27	ad
Norisoprenoids	SR_7-2-2	903,55	±	14,07	d
Norisoprenoids	SR_7-2-6	478,90	±	5,94	ab
Norisoprenoids	SR_7-3-8	401,95	±	15,49	ab
Norisoprenoids	SR_7-7-8	261,60	±	74,39	a
Norisoprenoids	SR_7-8-7	425,75	±	0,49	ab
Norisoprenoids	SR_7-8-8	420,25	±	150,26	ab
Norisoprenoids	Bronner	878,45	±	172,32	cd
Norisoprenoids	Kunleany	534,45	±	91,29	ad
Norisoprenoids	Solaris	457,60	±	31,82	ab
Terpenes	Glera	595,75	±	43,49	ab
Terpenes	SR_6-5-1	514,95	±	202,73	ab
Terpenes	SR_7-1-7	519,65	±	80,26	ab
Terpenes	SR_7-2-2	726,45	±	96,10	ab
Terpenes	SR_7-2-6	350,65	±	33,16	a
Terpenes	SR_7-3-8	810,85	±	22,27	b
Terpenes	SR_7-7-8	416,05	±	62,86	ab
Terpenes	SR_7-8-7	369,20	±	33,66	a
Terpenes	SR_7-8-8	363,05	±	0,64	a
Terpenes	Bronner	587,15	±	259,44	ab
Terpenes	Kunleany	396,40	±	28,60	ab
Terpenes	Solaris	531,60	±	58,41	ab

Table S7 Differences in Solid Sugar Content were compared using the Student's *t*-test ($P \leq 0.05$) among offspring and male parent.

Sample	p-value	
SR_6-5-1	0,665656	ns
SR_7-1-7	5,3E-05	***
SR_7-2-2	0,002321	**
SR_7-2-6	0,000326	***
SR_7-3-8	0,000164	***
SR_7-7-8	0,413818	ns
SR_7-8-7	0,088898	ns
SR_7-8-8	0,45199	ns

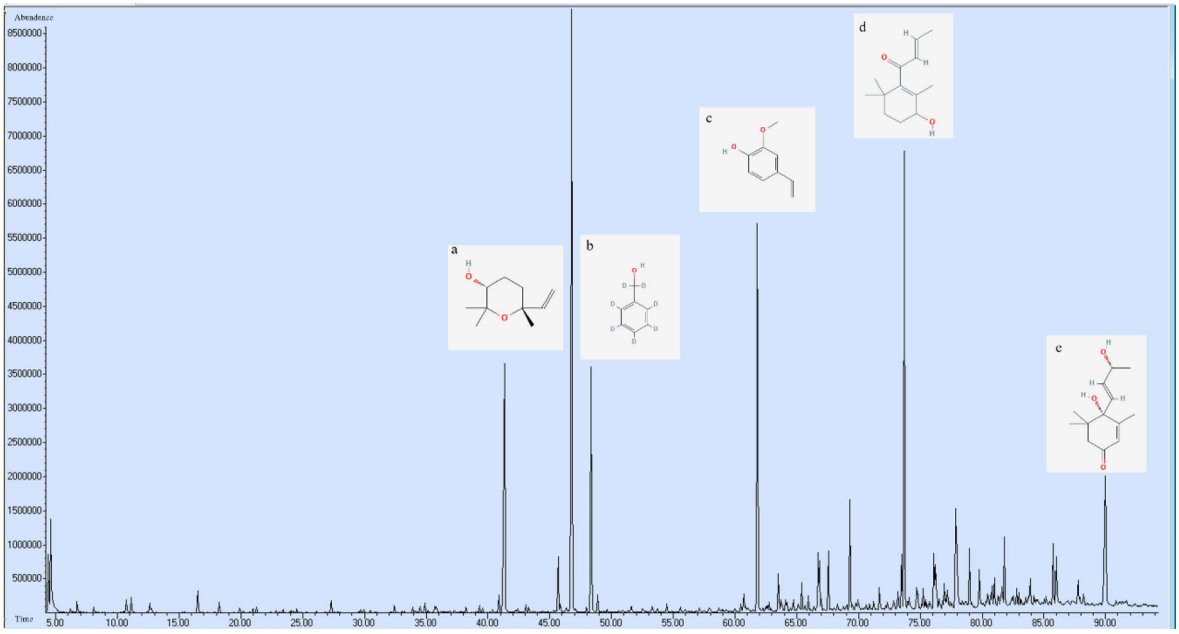

Figure S5 Chromatogram analysis for sample SR_7-1-7. In details are shown a) cis-linalool oxide pyranoid, b) benzyl alcohol, c) 2-methoxy-4-vinylphenol, d) 3-hydroxy-β-damascone, e) vomifoliol.

CHAPTER 4

Genome editing towards resilient and sustainable viticulture

Abstract

Pesticides are used in great quantity in viticulture to cope the spread of fungal diseases. As an alternative to this hazardous practice, susceptibility genes could be inactivated. This led to control pathogen infections. Target editing of host disease- susceptibility genes represent a durable alternative in crops breeding. Here we report improvements in grapevine through CRISPR/Cas9-target modification of the susceptibility genes: *VvMLO7*, *VvMLO6* and *VvNPR3* via *Agrobacterium tumefaciens.* Moreover, to avoid one of drawbacks linked to Agrobacterium-mediated transformation, hence the insertion of unrelated transgene, we exploit an inducible excision system based on Cre/LoxP recombinase technology.

Keywords

Grapevine, Genome editing, Susceptibility genes, T-DNA free, Cre/LoxP recombination

Introduction

Among the most advanced technologies of genetic engineering (New Plant Breeding Technologies, NPBTs), gene editing via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) has emerged as an effective tool for gene functional analysis in plants. It can directly introduce mutations into the plant genome by operating through specific guide RNA (designed to target a specific genomic sequence) and the Cas9 protein (which cleaves the specific site within the target gene) CRISPR/Cas9 system has emerged as a powerful strategy to precisely and quickly insert the desired traits into a plant genome, with the aim of facing both biotic and abiotic stresses as well as improving other important agronomic traits (as reported in Chapter 1).
In the last few years, the application of genome-editing technologies has been increased. Editing technologies, in specific CRISPR/Cas9, have been applied to various crops such as apple (Pompili *et al.*, 2020), pear (Malabarba *et al.*, 2021), citrus (Jia *et al.*, 2017; Peng *et al.*, 2017; Huang *et al.*, 2020), lemon (Catalano *et al.*, 2021), grapefruit (Jia *et al.*, 2021), kiwi (Wang *et al.*, 2018b; De Mori *et al.*, 2020), banana (Tripathi *et al.*, 2019; Kaur *et al.*, 2020; Wu *et al.*, 2020) and grape (Dalla Costa *et al.*, 2020; Wan *et al.*, 2020; Ren *et al.*, 2021) in order to first give a proof of concept about their applicability, than to enhance crop resistance against biotic/abiotic stresses and to enhance fruit qualities (Pimentel & Fortes, 2020; Sattar *et al.*, 2021).

From the first application of CRISPR/Cas9 more advances have been made with several reports focused on the improvement of its efficiency, versatility and specificity as comprehensively reported from many authors (Gleditzsch *et al.*, 2019; Giudice *et al.*, 2021; Li *et al.*, 2021). Among these studies interestingly it has been reported numerous efforts to knockout multiple genes simultaneously (Hsieh-Feng & Yang, 2020), for instance, Yu et colleagues reported how a single gRNA seed is capable to guide CRISPR/Cas9 in knocking out three homologous member of Arabidopsis Ribosomal Protein Large subunits 10, involved in protein translation and plant response to viral infection and abiotic stresses (Yu *et al.*, 2018). Moreover, multiplex CRISPR/Cas9 gene editing can also be simultaneously achieved using different gRNAs to edit a single gene and enhance editing efficiency (Minkenberg *et al.*, 2019; Armario Najera *et al.*, 2019). For instance the multiplex gRNAs expression system could be used to generate multiple mutations in target sites (Xing *et al.*,

2014; Ma *et al.*, 2015; Cermak *et al.*, 2017) as demonstrated in a recent work, Santillán Martínez et colleagues investigated the applicability of four gRNAs to induce the knockout of *SlPMR4* gene and consequently obtained tomato plant with enhanced resistance to powdery mildew (Santillán Martínez *et al.*, 2020). In parallel, many researchers focused their attention on the efficient expression of gRNAs. Usually, the CRISPR/Cas system is formed by two components: Cas nuclease transcript and gRNA(s). These components are controlled individually by different promoters, typically the Cas9 is expressed from an RNA Polymerase II promoter, while gRNAs are expressed from RNA Polymerase III promoter (Lowder *et al.*, 2016). However, Pol III promoters are not well characterized in non-model organisms, making it difficult to find heterologous promoters for CRISPR/Cas editing among different plant species (Tang *et al.*, 2016; Hsieh-Feng & Yang, 2020). On this issue Tang and colleagues used a Single Transcriptional Unit (STU) CRISPR-Cas9 system in rice. In their work both Cas9, gRNAs, and a self-cleaving hammerhead ribozyme are controlled by Pol II promoter. The authors compared the efficiency of Pol II and Pol III promoters to transcribe gRNAs. Interestingly NHEJ-induced mutations were similar between the STU system and classical expression of Cas9 and sgRNAs from separate promoters (Tang *et al.*, 2016). The Pol II promoters in gRNAs processing lead to some advantages such as temporal control of gRNAs activity, and no limits in CRISPR target sequences linked to classical Pol III promoters such as U6 and U3 sequences (Gao & Zhao, 2014; Gao *et al.*, 2015).

Although the CRISPR/Cas9 potential is immense (Chen *et al.*, 2019; Xu *et al.*, 2019; Biswas *et al.*, 2021; Miladinovic *et al.*, 2021), its applicability is hampered by legislation. In fact, in 2018, the Court of Justice of the European Union (CJEU) declares that organisms obtained by new techniques of directed mutagenesis are classified as Genetically Modified Organisms (GMOs). Nevertheless, the CJEU does not considered that many products of directed mutagenesis would be indistinguishable from those resulting from natural, spontaneous mutations (Eriksson *et al.*, 2019; El-Mounadi *et al.*, 2020; Schmidt *et al.*, 2020). In addition to these regulatory issues, in woody plant species, the most used method to delivery CRISPR/Cas9 component into host cell, still rely on *Agrobacterium tumefaciens*-mediated transformation (Sandhya *et al.*, 2020). This method includes the drawback of T-DNA integration into plant genome (Gelvin, 2003, 2017; Singer, 2018) and lead to the production of edited plants containing exogenous DNA including the presence of marker genes necessary to conferring resistance to selective chemicals component such as hygromycin or

kanamycin. In case of plant species propagated by seeds such as tomato, the elimination of T-DNA cassette could be obtained by co-transformation with different transformation vectors and segregation of marker genes from the gene of interest in the progeny but in vegetatively propagated plants and trees that are characterized by long juvenile periods this is not feasible. A possibility to avoid the presence of exogenous DNA is offered by Site Specific Recombinase (SSR) such as Cre/LoxP from bacteriophage P1, R/Rs from *Zygosaccharomyces rouxii* or FLP/FRT from *Saccharomyces cerevisiae* (Corneille *et al.*, 2001; Wang *et al.*, 2005; Ballester *et al.*, 2007; Würdig *et al.*, 2015). All these methods are based on specific sites with a short region of sequence identity, and a specific recombinase (Cre, R and FLP). When the recombinases recognize and bind the short (30-40 nt) target DNA sequences (LoxP, Rs or FRT) they break DNA at specific, staggered positions in the top and bottom strands (Craig, 1988; Van Duyne, 2001). The recombination event can be result in insertion, translocation, inversion, and deletion (Zhou *et al.*, 2021). These two components, recombination sites, and recombinase enzyme, are sufficient to perform recombination reaction and induce marker gene removal or entire T-DNA cassette removal. So, these approaches for transgene elimination can be used to remove the entire T-DNA cassette containing CRISPR/Cas9 machinery, selection marker and foreign DNA. The use of site-specific recombination technology in plant genome manipulation has been demonstrated to remove unwanted DNA (Zhang *et al.*, 2003; Cuellar *et al.*, 2006; Abdallah *et al.*, 2015); in particular recombinase excision approaches have been validated in apple and poplars (Fladung & Becker, 2010; Timerbaev *et al.*, 2019). Few studies have been carried out to obtain free genome-edited plants using these systems; for instance, Pompili et colleagues, demonstrated the feasibility of FLP/FRT recombination system in apple edited lines (Pompili *et al.*, 2020).

Taking into account the previous works about genome editing using multiple gRNAs, the efficiency of Polymerase II promoters (Cermak *et al.*, 2017), and the specific excision system (Hoff *et al.*, 2001) the objective of our work is to exploit a CRISPR/Cas9 gene editing approach to knockout two *Mildew Locus O* (MLO) genes and one *Non Expressed Protein 3* (NPR3) which have a role in susceptibility to powdery mildew and as defence responses respectively (Fu *et al.*, 2012; Pessina *et al.*, 2016; Fister *et al.*, 2018). For instance in 2016, Pessina et, al., demonstrated how the simultaneous knockdown of *VvMLO6* and *VvMLO7* in grapevine reduced susceptibility to powdery mildew (Pessina *et al.*, 2016). About NPR3

gene no previously studies were performed in grapevine and his role is partially known but seems to be a components of the SA (Salicylic Acid) signal transduction pathway, acting as a repressor of NPR1 (Dong, 2004; Zhang *et al.*, 2006; Fu *et al.*, 2012; Fister *et al.*, 2018). More in detail, NPR3 acts as transcriptional repressors to repress SA-responsive genes in the absence of pathogen(s) infection whereas NPR1 works as a transcriptional activator of SA-responsive genes (Liu *et al.*, 2020). Indeed, Shi et colleagues demonstrated an increase resistance to *Phytophthora capsici* in *Theobroma cacao* when knocking-down *TcNPR3* which prevent *TcNPR1* degradation hence inducing accumulation of SA and pathogens related (PR) genes (Shi *et al.*, 2013).

In addition to the gene editing strategies, a heat-shock inducible Cre/LoxP recombination system was implemented to attempt the entire T-DNA removal.

Materials and Methods

Embryogenic calli

Cultivars of Chardonnay, Pinot Noir, Glera (*V.vinifera*), 110 Richter (*V. berlandieri* x *V. rupestris*) and Selektion Oppenheim 4 (*V. berlandieri* x *V. riparia*) were used for embryogenic calli obtainment. In spring months (April-May depending on the climatic conditions and phenological stage), flower clusters were collected in vineyard located in Rauscedo (PN) (Vivai Cooperativi Rauscedo - VCR) retaining only the basal half of inflorescence (Fig. 1).

The pollen developmental stage was evaluate as previously described (Gribaudo *et al.*, 2004a; Gambino *et al.*, 2007). Once collected and checked the pollen stage, flowers were sterilized for 15 minutes with sodium hypochlorite (1,5% available chlorine) solution containing 100 µl L^{-1} of Tween 20 followed by several washing steps with sterile distilled water. Flowers were kept at 4°C for 2 days. A second sterilization step was performed two days after collection. For Chardonnay, Pinot Noir and Glera both ovaries and anthers were collected, in 110 Richter and SO4 only anthers were collected due to the absence of fully developed ovaries (Table S3). Ovaries and anthers (including the filaments) were excised and placed on the starter callus induction medium PIV: (Franks *et al.*, 1998; Gambino *et al.*, 2007, 2021; Dhekney *et al.*, 2019) containing Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6% sucrose, 0,3% Gelrite, 4,5 µM 2,4 D and 8,9 µM BAP, pH 5.8. For all cultivars tested, the explants numbers vary from a minimum of 4000 to a maximum of 15000 anthers/ovaries as reported in Table 1. The cultures were kept in dark at 25°C.

Three months from callus induction, embryogenic calli were visually selected and transferred on proliferation media C1 (Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6 % sucrose, 0,5% Gelrite, 5 µM 2,4 D and 1 µM BAP, pH 5.8) with the exception for 110 Richter that were transferred on RUP medium: Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 3% sucrose, 0,8% Bactoagar, 5 µM IBA, pH 5.7. The embryogenic calli were maintained and multiplied by monthly transfer on fresh medium.

Grapevine guideRNAs design

Total DNA was extracted from two genotypes: Chardonnay and Sangiovese using NORGEN Plant/Fungi DNA isolation kit (Norgen Biotek Corp, Canada) according to manufacturer's protocol. Two MLO genes, MLO7 and MLO6 (VIT_ 13s0019g04060 and VIT_ 13s0019g04070) were amplified using oligonucleotide primers derived from grape *MLO* gene sequences available in the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For NPR3 the grapevine sequence was the homologue of the Arabidopsis gene described by (Fu *et al.*, 2012). The NPR3 gene sequence was evaluated performing a BLAST analysis against PN40024 and the resulting homologue was used (VIT_10s0042g01250). The specific primers were designed to amplifying the genomic region of three selected genes: *VvMLO7*, *VvMLO6* and *VvNPR3*. The PCR products were purified from agarose gel using Zymoclean Gel DNA recovery kit and sequenced at BioFab Research srl, Italy. The sequenced DNAs were aligned to *Vitis vinifera* PN40024 genome and SNPs on target sites were evaluated.

gRNAs on target sites were evaluated using on-line tool CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/) (Lei *et al.*, 2014). Two guideRNAs for each MLO gene, and three for NPR3 gene were designed and an OFF-target analysis were performed using CRISPOR (Concordet & Haeussler, 2018) the list of guides were reported in Table S1.

Binary vectors design and optimization of an excisable system

The binary vectors were conceived and assembled by us starting from pDIRECT_22c vector (16.058 bp) containing plant codon-optimized Cas9 from *Streptococcus pyogenes* controlled by 35S CaMV promoter and *Neomycin phosphotransfare II* (*NPTII*) marker gene (Cermak *et al.*, 2017). Interestingly, the selected system allow the polycistronic gRNAs to be processed by Csy4 protein that will release single gRNA (Haurwitz *et al.*, 2010). In order to develop a cisgenic-like approach we decided to clone in the selected plasmid a recombinase system based on Cre/LoxP system. To insert the lox site near to the LB border we custom designed a synthetic sequence containing SacII-LB-lox-RsrII in pUC57-Amp (produced by Genewiz). To insert such sequence in the entry vector digestion using RsrII (CpoI) and SacII enzymes was performed, followed by dephosphorylated with alkaline phosphatase (Thermo

Fisher Scientific, MA, USA) to prevent self-ligation. Similarly, the SacII-LB-lox-RsrII in pUC57-Amp (786 bp) was digested with CpoI and, in a second step with SacII, for 2 and 4 hours respectively and cloned into CpoI-SacII digested pDIRECT_22c. A second plasmid carrying the Cre enzyme and Heat Shock Promoter (HSP-CreINTRON-Ca35PolyA-loxP) was obtained by Genewiz, digested using MssI and BglI and inserted into the resulting vector pDIRECT_22c-LB-LoxP using T4 DNA ligase (Anza, Thermo Fisher Scientific, USA) in an overnight reaction at 4°C.

The vector was assembled using multiple gRNAs for each gene. In the Golden Gate assembly, the PCR products of gRNAs (obtained following the procedure described by (Cermak *et al.*, 2017) were cloned into the previously obtained plasmid. Promoter for gRNAs expression were obtained from pMOD_B2103 (Cermak *et al.*, 2017), containing the *Cestrum Yellow Leaf Curling Virus* CmYLCV promoter (Stavolone *et al.*, 2003); The Golden Gate reaction was performed as described from (Lampropoulos *et al.*, 2013): the resultant DNA were used in a final digestion-ligation step using Anza T4 DNA ligase, LguI and BsaI restriction enzymes (Thermo Fisher) 5 ng of each fragments containing a fragment of gRNA, and 50 ng of transformation backbone pDIRECT_22c-Cre-LoxP-LB-LoxP in 20 µl final volume. The ligation step was performed in 10 cycles (37°C 5 min + 16°C 10 min) + 37°C 15 min + 80°C 5 min + 4°C hold.

A graphical illustration was provided in Figure S1.

Transformation in JM-109 and *Agrobacterium tumefaciens* GV3101 cells

Transformation of the ligation products occurred in chemically competent JM-109 *E. coli* cells through a heat shock protocol. Transformed cells were planted on LB medium supplied with kanamycin 50 mg L^{-1}. The resultant colonies were verified by PCR assay using primers described in Table S2. Plasmids were isolated using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA) following manufacturer's instructions and then sequenced at BioFab Research srl, Italy. Plasmids were transformed into competent cells of GV3101 *Agrobacterium tumefaciens* using electroporator at 2,5 kW. Agrobacterium was cultured for 3 hours at 28°C with 210 rpm in LB medium and then plated on LB agar containing rifampicin (25mg L^{-1}) and kanamycin (50mg L^{-1}).

The presence of gRNAs was confirmed by PCR assays (primer list for Golden Gate assembly was reported in Table S1). Furthermore, all constructs were confirmed by Sanger sequencing.

Gene transfer experiment

Two weeks before the transformation event, embryogenic calli were transferred on GS1CA medium (Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6 % sucrose, 0,42 % Gelrite, 0,25 % Active Coal, 1 µM BAP, 10 µM NOA, 20 µM IAA, pH 5.8) as previously reported (Gribaudo *et al.*, 2004a).

Embryogenic calli of Chardonnay, Pinot Noir, 110 Richter and Selektion Oppenheim 4 (SO4) were used in *Agrobacterium tumefaciens* (*A.t.*)-mediated transformation. Several experiments were carried out using *A.t.* strain GV3101 for all constructs (Berres *et al.*, 1992). The vector containing GV3101 *Agrobacterium tumefaciens* were sub-cultured in MG/L medium: Mannitol 0,5%, L-Glutamic acid 0,1%, KH_2PO_4 150, NaCl 0,5%, Tryptone 0,5%, Yeast extract 0,25%, $MgSO_4$ * $7H_2O$ 0,1g, Biotin 0,15g, pH 7; to reach a final concentration of 0.8 (OD_{600}). Cell suspension was centrifuged at 4500 g for 10 minutes and resuspend in LCM medium: Nitsch and Nitsch basal salt, Murashige and Skooge vitamins, 6 % sucrose, pH 5.8, supplemented with 100 µM acetosyringone. The resuspended culture was kept at 28°C and 210 rpm for 3 hours.

Embryogenic calli were co-cultured with 3 ml of GV3101 for 10 minutes. Excess of bacterial culture was removed using a cell strainer and embryogenic calli were transferred on petri dishes containing filter paper soaked with GS1CA plus 100 µM acetosyringone. The co-culture was kept a 22°C for 48 hours, then embryogenic calli were washed in LCM medium supplemented with Cefotaxime 600 mg L^{-1}. Excess bacterial culture was removed using a cell strainer and then embryogenic calli were dried on sterile filter paper. The culture was transferred on GS1CA media supplemented by cefotaxime 450 mg/l and maintained for 9 days at 26°C.

After 9 day the embryogenic culture were subcultured monthly on GS1CA medium supplemented with 450 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin for 4 months; after that, embryogenic calli were transferred monthly on GS1CA medium supplied with 100 mg L^{-1} kanamycin.

Transformations were performed in duplicate: from GT-1 to GT-4 for knocking-out MLO6 and MLO7 genes in two genetic accessions: Chardonnay and Microvine (Chaïb *et al.*, 2010; Torregrosa *et al.*, 2019); for knocking-out NPR3 gene GT-5 was performed in Chardonnay, GT-6 was performed in 110R and GT-7 was performed in SO4. For all the transformation events two negative control transformation were performed using *Agrobacterium tumefaciens*: one with a strain containing the genome-editing construct but without gRNAs and one using *Agrobacterium tumefaciens* without any T-DNA.

Embryo's regeneration

After 6 to 9 months (depending on the genotype), the more developed embryos at torpedo stage, were transferred on MS-SH media (Murashige and Skooge basal salt, Murashige and Skooge vitamins, 1,5% sucrose, 0,4%, BAP 10 µM, Gelrite pH 5.6). Two months later the embryos were transferred on half strength MS basal salts, containing indole-3-butyric acid (IBA) 2,5 µM and naphthaleneacetic acid (NAA) 2,5 µM. Ten days after the rooted embryos were transferred on MS 1/2 without phytohormones with the following conditions 26 °C, 200 µmol s^{-1} m^{-2} of photosynthetic photon flux density and a 16/8 h day/night photoperiod.

Screening

Regenerated embryos were screened by PCR assay to detect the presence of T-DNA cassette. For each plant, genomic DNA was extracted from a single leaf using the Genomic DNA Isolation Kit NORGEN (Norgen Biotech Corp), quantified on NanoDrop One Spectrophotometer (Thermo Fisher Scientific) and diluted to 10 ng μl^{-1}. The DNA was used for PCRs using DreamTaq Green DNA polymerase 5X (Thermo Fisher Scientific) and primers TC320F, TC089R; CRE130 F, CRE713 R; CRE455 and Cas484 listed in Table S2.

T-DNA removal

Plants displaying the presence of both Cas and Cre sequences in the genomic DNA were micropropagated on MS ½ medium without regulators. For each line 3 plants were maintained. The heat-shock induction for T-DNA removal was carried on 2 replicates in Magenta box (Sigma-Aldrich) (70 mL $vessel^{-1}$).

Different conditions of induction were evaluated using different temperatures, time exposition and number of treatments as described previously (Herzog *et al.*, 2012; Dalla Costa *et al.*, 2016, 2020; Pompili *et al.*, 2020). Twenty explants of Chardonnay carrying the backbone pDIRECT_22c-Cre-LoxP-LB-LoxP were propagated and tested at 42°C for 6 hours. Two incubation treatments were performed with 48 h interval between consecutive incubations.

After the incubations plants were recovered in growth chamber at 26 °C for further growth and DNA assays.

Validation of T-DNA remotion

T-DNA insertions were quantifying using two housekeeping genes *VvACT* and *VvUBI* and target gene *Cas9* (Table S3). qPCR reactions were carried out in a final volume of 10 µL using the SYBR® Green protocol (Bio-Rad Laboratories Inc., USA) and 5 ng of DNA templates. The standard curves were built by means of 6 decreasing concentrations for calibration plasmid in a serial dilution of 1:10. Plasmids and plant samples were analysed in duplicate. Nuclease-free water was used as negative control. Reactions were run in a Bio-Rad CFX96 instrument (Bio-Rad Laboratories Inc., USA) using the following conditions: an initial denaturation phase at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Each amplification was followed by melting curve analysis (65-95°C) with a heating rate of 0.5°C every 5 s (melting curves are reported in Fig. S1). All reactions were performed with at least two technical replicates.

Identification of T-DNA genomic insertion site

Two regenerated Chardonnay plants were used to assess the editing efficiency. Genomic DNAs were extracted using Genomic DNA Isolation Kit NORGEN (Norgen Biotech Corp) and used for PCR amplification using primers: *VvMLO6* F, *VvMLO6* R, *VvMLO7* F and *VvMLO7* R reported in Table S2. PCR products were purifying using Zyppy™ DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA) following manufacturer's instructions and then sequenced at BioFab Research srl, Italy.

Results

Embryogenic calli

Collected inflorescence (Tab.S3) from all the selected genotypes developed callogenesis responses 45 days after incubation in induction media as reported in Fig 2. Five months after collection we observed different responses: anthers and/or ovaries with no embryogenic competence, dark and compact calli, non-embryogenic watery calli (Fig. 2b) and granular white/yellow calli (Fig. 2a).

Figure 6 Collected inflorescences of Chardonnay and 110 Richter.

First Pro Embryogenic Masses (PEM) were observed from Chardonnay anthers 7 weeks after culture, showing friable and granular yellow appearance. Embryogenic cultures were obtained from Chardonnay and Pinot Noir explants and from 110 Richter and SO4 anthers. Although, Sabbadini *et al*., (2019) described embryogenic callus formation from Glera genotype, in this work no somatic embryos were observed. This was probably due to an inexact inflorescence development stage and to an abundance of non-embryogenic watery calli formation that could prevent granular calli development.

For long-term cultures maintenance of *V. vinifera* embryogenic calli, the C1 medium was reported by many authors (Torregrosa; Martinelli *et al.,* 2001). To induce embryos formation and at same time keeping embryogenic the culture of 110 Richter and SO4, we tested RUP medium (supplemented with IBA 5 µM) that allowed embryogenic calli propagation as reported by Martinelli in 1993 (Martinelli *et al.*, 1993).

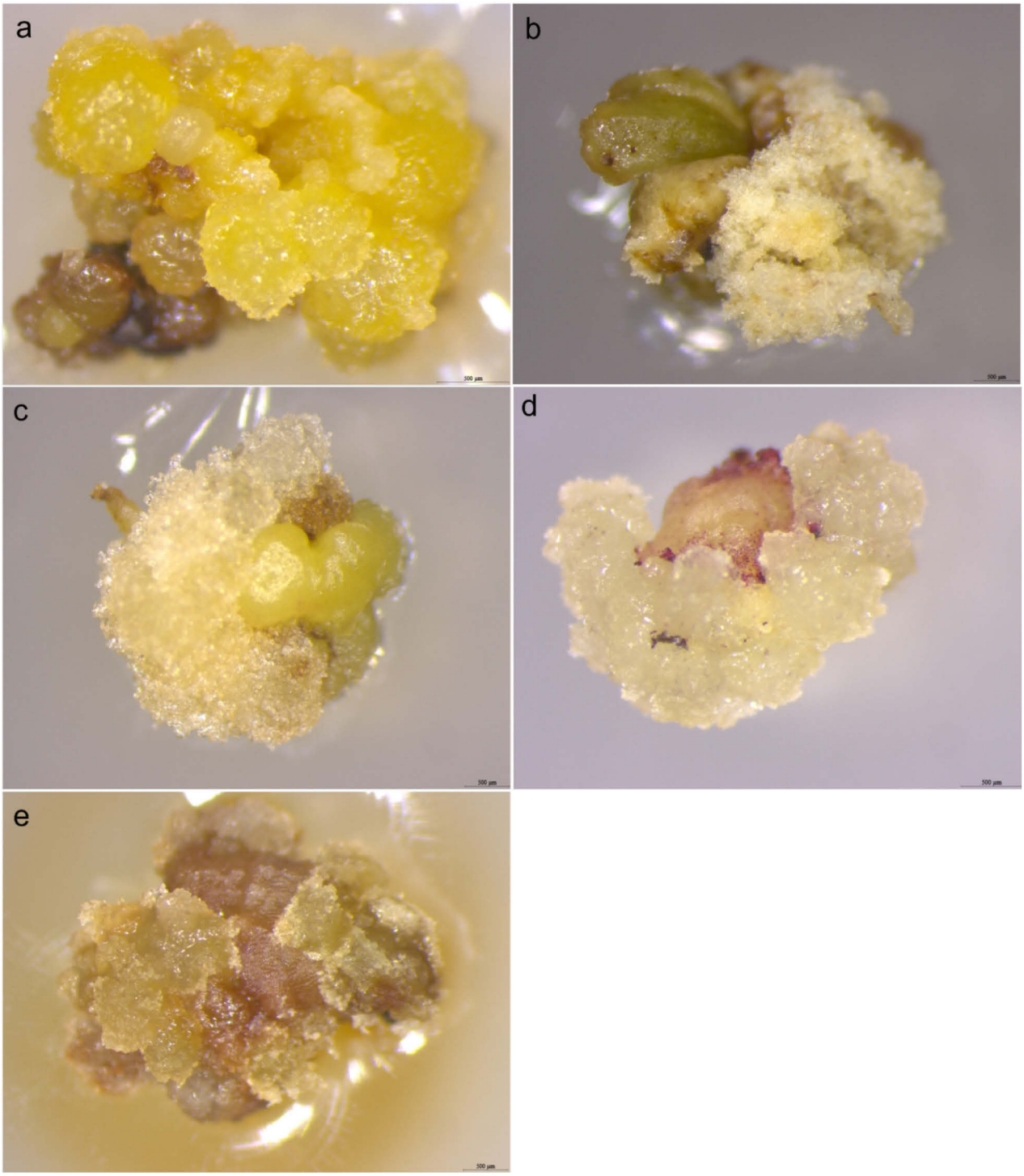

Figure 2 Callogenesis responses in ovaries and anthers 45 days after collecting in 5 grapevine genotypes. a) Chardonnay calli (granular type); b) early callogenesis responses in Glera anther; c) Pinot Noir pre-embryogenic masses; d) formation of de-differentiation cells from 110R anther and e) browning masses from anther in SO4 rootstock.

Binary vector assembling

The transformation vector pDIRECT_22c-Cre-LoxP-LB-LoxP was derived from three vectors: i) pDIRECT_22c, ii) SacII-LB-lox-RsrII in pUC57-Amp and iii) HSP-CreINTRON-Ca35PolyA-loxP in pUC57-Amp (Fig. S2).

For each cloning step a target sequencing were performed. Resultant vector contains: Cas9 controlled by P2A peptide (Szymczak *et al.*, 2004), two LoxP sites near to left and right borders derived from SacII-LB-lox-RsrII in pUC57-Amp vector and HSP-CreINTRON-Ca35PolyA-loxP respectively, a Cre enzyme and an Heat Shock Promoter (HSP Gmhsp17.5-E from *Glicine maxima*) from HSP-CreINTRON-Ca35PolyA-loxP, *nptII* marker gene and specific guide RNAs for target genes (Fig. S2). A specific grapevine intron was inserted into the Cre gene in order to prevent its expression in *E. coli* and *A. tumefaciens* during cloning and/or transformation processes as previously reported by many authors (Zuo *et al.*, 2001; Joubès *et al.*, 2004; Peng *et al.*, 2015).

Here we target respectively two *Mildew Locus O* genes: (MLO7 and MLO6) and one gene belong to *Nonexpresses PR* gene (NPR3). Guide RNAs were chosen among data output from CRISPR-P and CRISPOR considering GC content, out-of-frame scores, and lower mismatch evaluation among *Vitis vinifera* PN40024 genome and taking into account the mismatch position, in fact mismatches in seed region are less tolerated leading to an inhibition of Cas activity (Hsu *et al.*, 2013). Moreover, guideRNAs were selected based on their localization point in order to create a premature stop codon and/or a large deletion. Among the output of CRISPR-P and CRISPOR in order to select the *in-silico* best performing guides we considered the following parameters: a high on-score percentage more to 0.6, GC content around 50 % and lower off-score minor to 0.3. After this analysis we selected guideRNAs 13 and 25, located and exon 6 and intron region in *VvMLO7* gene; guideRNAs 28 and 7 for

VvMLO6 gene, located in intron and the fourth exon respectively; finally, *VvNPR3* guides 2, 29 and 40 targeting exon 2 and exon 1 as reported in Fig.3.

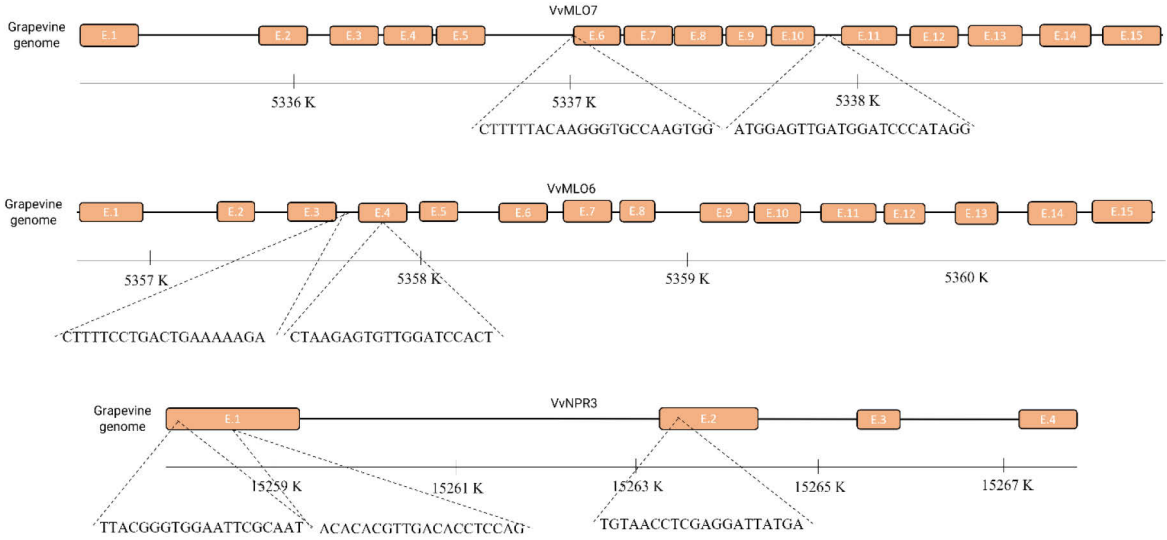

Figure 3 Target genes with selected gRNAs. In orange boxes are shown exon regions for each target gene.

Generally, in a multiple target gene strategy, each gRNA is processed from an independent Pol III promoters such as U6 or U3. However, this strategy led to more cumbersome constructs compared to a polycistronic message produced by Pol II promoters in addition repeated use of U6 or U3 promoters in the same construct may cause variation in gRNA expression level as demonstrate in rice and Arabidopsis (Ma *et al.*, 2015; Hassan *et al.*, 2021). To overcome these limits, we used the enzyme CRISPR-associated RNA endoribonuclease Csy4 from *Pseudomonas aeruginosa* (Tsai *et al.*, 2014). In fact as demonstrate by (Qi *et al.*, 2012) the expression of Csy4 with gRNAs led to a size reduction of the transcript. A schematic representation of T-DNA cassette is reported in Fig.4.

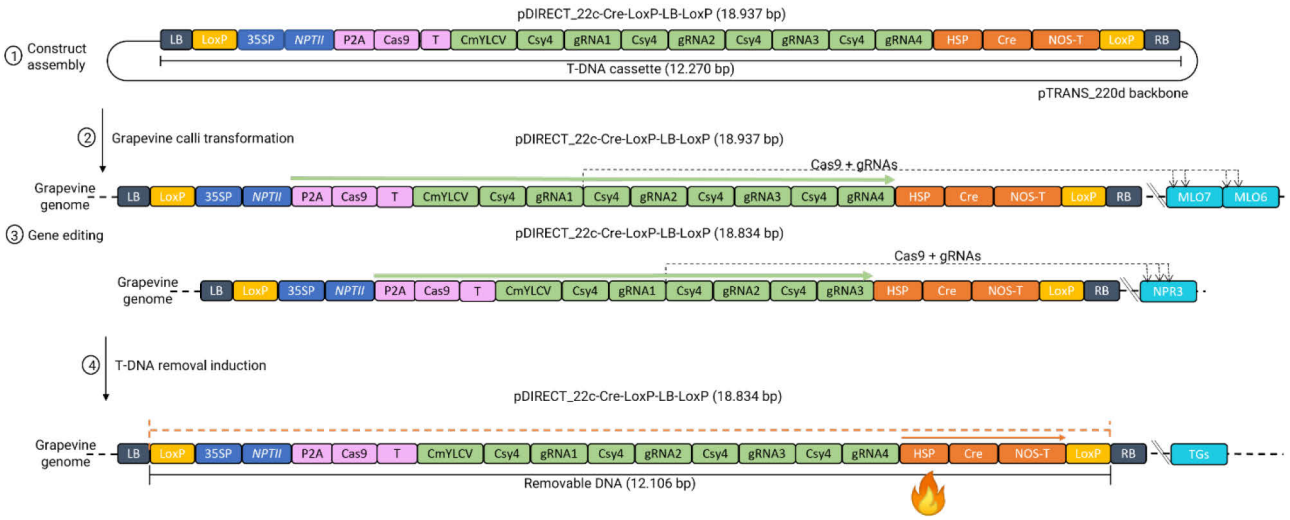

Figure 4 Schematic representation of pDIRECT_22c-Cre-LoxP-LB-LoxP carrying specific gRNAs. The binary vector is produced by cloning the T-DNA cassette into from pDIRECT_22c performing two different digestion/ligation steps. The T-DNA cassette contains: kanamycin resistance *NPTII* (blue rectangles) necessary for plant selection; the specific gRNAs controlled by *CmYLCV* Pol II promoter (green rectangles); the Cas9 enzyme (rose boxes) and specific recombination system Cre/LoxP (orange and yellow boxes). In Figure are illustrated the four phases in grapevine embryogenic calli transformation: 1) construct assembly; 2) grapevine calli transformation mediated *Agrobacterium tumefaciens*; 3) genome editing events on specific genes and 4) T-DNA remotion induction through activation of HSP.

Embryogenic calli transformations

Embryogenic calli of Chardonnay, Microvine 04C023V0006 (derived from a cross between "Grenache" and the original L1 mutant Microvine) were used in *Agrobacterium*-mediated transformation carrying the construct to knock-out MLO6 and 7; to knock-out NPR3 gene, three independent transformations were performed using Chardonnay, 110R and SO4 embryogenic calli. For each genotype a transformation using *Agrobacterium tumefaciens* GV3101 without any plasmid were performed in order to evaluate antibiotic selection (Fig.5). In addition to that, other transformations were performed using *Agrobacterium tumefaciens* carrying pDIRECT_22c-Cre-LoxP-LB-LoxP without specific guideRNAs. These transformations were designed to investigate unwanted Cas9 activities.

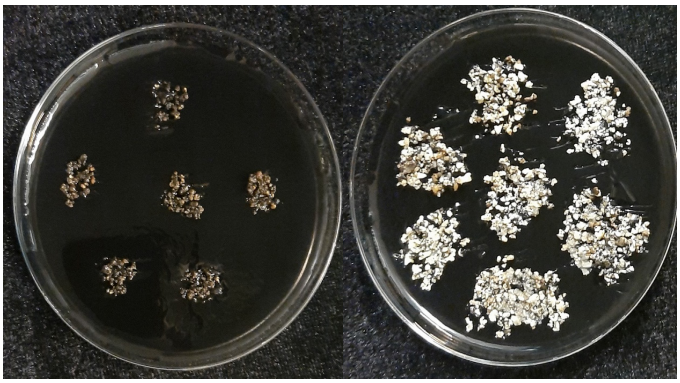

Figure 5 Embryogenic calli of Chardonnay 3 months after transformation. On the left embryogenic calli transformed using *Agrobacterium tumefaciens* GV3101 without CRISPR/Cas9 system; on the right embryogenic calli infected with pDIRECT_22c-Cre-LoxP-LB-LoxP carrying specific gRNAs.

Transformed plants

In total 7 plants of Chardonnay and none of Microvine were obtained by *A. tumefaciens* gene transfers GT-1, GT-3, GT-2 and GT-4 after 13 months. Among them 3 Chardonnay plants carrying two guideRNAs for *VvMLO7* and two guideRNAs for *VvMLO6* (Fig. S3) (the other plants were obtained from pDIRECT_22c-Cre-LoxP-LB-LoxP without specific guideRNAs) (Fig. S4).

Exogenous T-DNA integrations ware checked by PCR assays from regenerated plants using TC320F, TC089R; CRE; and Cas484 specific primers listed in Table S2.

T-DNA removal

To optimize T-DNA removal conditions in grapevine regenerated plants, an experiment was set-up to evaluate effective activation of Heat Shock promoter and consequently induce T-DNA cassette removal. Biological replicates of Chardonnay line n° 2 (obtained from GT-1 transformation without specific gRNAs) were used in this assay. Twenty nodal explants were put on MS ½ medium without regulators. These explants were treated at 42°C for 6 hours following by recovery step at 26°C, 200 µmol s^{-1} m^{-2} of photosynthetic photon flux density and a 16/8 h day/night photoperiod in growth chamber for 48 hours. A second treatment were performed following the same parameters. To check the level of exogenous DNA elimination, a single leave for each plant were used for DNA extraction. The T-DNA elements such as Cre enzyme and Cas9 were quantified by PCR assay. Then the plants were subject to a qRT-PCR to verify the expression variation induced by treatments.

The first treatment showed a low reduction in Cas9 copies level as reported in Fig. 6 compared to not treated plant.

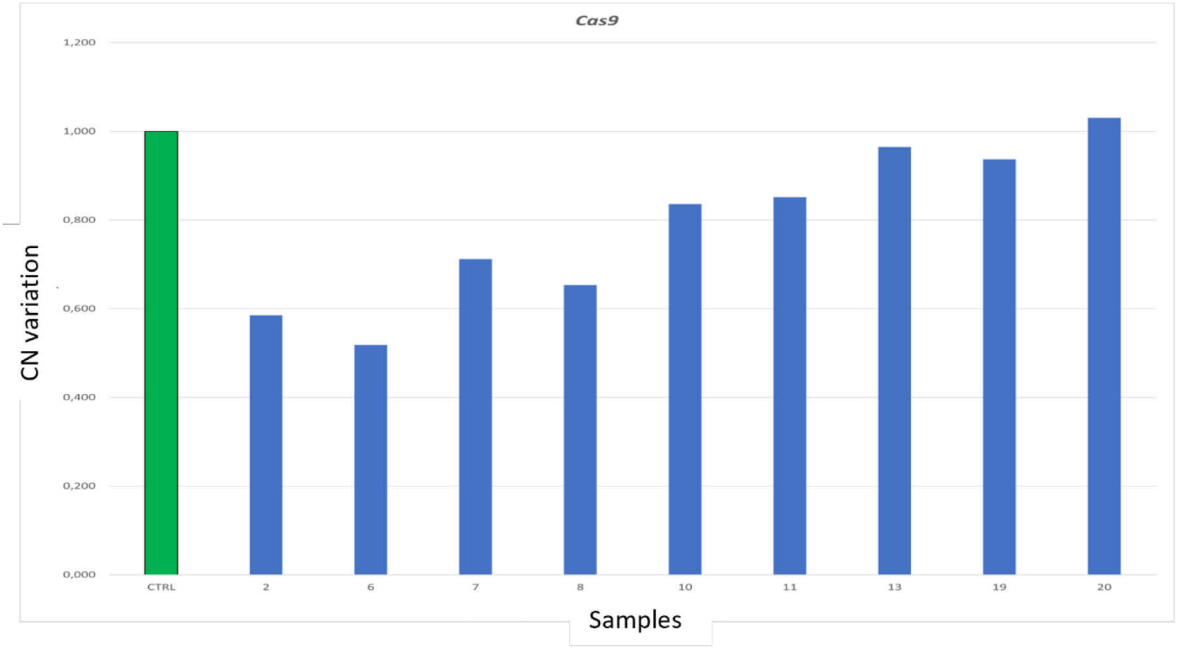

Figure 6 Cas9 CN variation after two heat-shock treatments.

Editing detection

The *VvMLO* (both MLO6 and MLO7) target regions were screened in two Chardonnay regenerated plants using Sanger sequencing. Editing results show a -1 and +2 and -9 and +1 InDels respectively.

Discussion

The first objective of this work was to assess the applicability of innovative genome editing technology CRISPR/Cas9 in grapevine in order to enhance plant protection against powdery mildew and in general to induce resistance against a broad-spectrum of pathogens through SA accumulation and induction of Pathogenesis-related (PR) genes. Therefore, we tested the exploitability of genome editing constructs able to remove the entire T-DNA cassette from edited plants, leaving minimal exogenous trace into plant genome.

Many reports are focused on initiation of somatic embryogenic cultures from elite grapevine genotypes, however nowadays, researchers are interested in embryogenic development of different genotypes to explore and to expand genetic transformation projects. Here we report a media composition based on NN medium with 2,4-D and BAP regulators. This medium seems to be suitable for all tested genotypes excepts for Glera cultivar. Probably in Glera explants the physiological phases (stage III) is not suitable, in addition we observed that anthers are smaller when compared to other cultivars and difficult to handle. To overcome this limitations, we also tested the culture of whole flowers however no embryogenic masses were produced due to an overgrowth of non-embryogenic watery calli as reported in Fig. 1b. Usually, both the genotype and the explant type had a significant effect on the differentiation of somatic embryos as reported in many studies, in fact we observed a highest percentage of embryogenic calli from Chardonnay anthers and ovaries compared to the other genotypes (Vidal *et al.*, 2008; Rama *et al.*, 2009). The physiological stage of collected explants (both ovaries and anthers) was considered crucial in embryogenic culture development (Gribaudo *et al.*, 2004b) in fact we observed an higher calli production from Chardonnay explants collected in a specific window of 3 days in each year. These days correspond to the developmental stage III as previously reported (Gribaudo *et al.*, 2004b). Although anthers are the most used explant for somatic embryogenesis induction, in case of Pinot Noir the first report on embryogenic calli obtainment shown no differences between the two explant types (Kikkert *et al.*, 2005). In accordance with this, we observed the same calli efficiency development in both explant types for this genotype. The obtained friable and granular calli were then used to perform *Agrobacterium*-mediated transformation carrying a specific excisable CRISPR/Cas system. However, this strategy leads to production of transgenic plants that are considered GMO and must undergo both environmental and food and feed

risk assessments (Kawall *et al.*, 2020). This have induced scientists to asked for a change in current legislation (Eriksson *et al.*, 2018). In other countries such as USA, Australia, and Brazil the genome edited products that do not contain foreign DNA, will not be subject to additional regulatory oversight and risk assessment as required for GMO (Eriksson *et al.*, 2019).

Io our study we focused on the possibility to remove T-DNA cassette, and consequently all foreign DNA, from edited grapevine by using Cre/LoxP system controlled by an inducible heat-shock promoter. Few works were carried out considering the possibility to obtain woody plant species without exogenous DNA (Petri *et al.*, 2012; Righetti *et al.*, 2014; Peng *et al.*, 2015; Timerbaev *et al.*, 2019; Pompili *et al.*, 2020; Dalla Costa *et al.*, 2020). All of them are based on specific recombinase systems: R/RS, Flp/FRT and Cre/LoxP.

In this work, we tested heat treatment on twenty biological replicates of Chardonnay carrying only T-DNA cassette without specific gRNAs to evaluate the possibility to remove an entire transformation cassette. Among 20 plants under thermic treatment, none of them shown a complete T-DNA remotion, nevertheless we observed a reduction in Cas9 copy number in several plantlets. Probably, the heat treatments at 42°C for 6 hours are not sufficient to induce Cre recombinase activation; in addition grapevine seems to be more resistant to heat treatment than other woody plant such as apple (Würdig *et al.*, 2013). Further improvement of the excisable system would be possible applying two or more heat treatments without recovery phase and increasing incubation hours from 6 to 8. The latter approach was already tested suggesting that 8 hours of heat-treatment is harmful for many grapevine genotypes (Dalla Costa *et al.*, 2016).

In parallel, we focused our effort on system efficiency developing a flexible T-DNA cassette in order to maximize Cas9 activity (Uslu *et al.*, 2021), prevent unwanted cleavage (Zou *et al.*, 2021) and induce disruption of target genes. The multiplex strategy to target more genes at once has been the focus for many works (Ma *et al.*, 2015; Armario Najera *et al.*, 2019; Abdelrahman *et al.*, 2021). Here we decided to use multiple guide RNAs for each *VvMLO* gene and *VvNPR3* gene. The first two regenerated plants for genome editing on *VvMLO* displayed both mutations that can produce truncated mRNA. This confirms that the new developed system is working efficiently.

In previous studies aiming at grape genome editing, expression of gRNAs were controlled by *AtU6* promoters (Wang *et al.*, 2018a). Recently, plant species-specific Pol III promoters could contribute to increased sgRNA levels as demonstrated in cotton (Long *et al.*, 2018) and soybean (Sun *et al.*, 2015); among them, new grapevine native promoters were discovery: *VvU3* and *VvU6* (Ren *et al.*, 2021), however, all these promoters are processed by Pol III. Furthermore, higher genome editing efficiencies have been observed using Pol II promotes instead to Pol III as reported from many authors (Mikami *et al.*, 2017). Cermak *et al.*, (2017) reported the application of Csy4 and tRNA in multiplex genome editing construct assembly. Both Csy4 and tRNA are processing using *CmYLCV* promoter as a Pol II. The Pol II promoter seems to be almost twice as effective in inducing mutation as gRNAs controlled by single Pol III promoters (Cermak *et al.*, 2017; Mikami *et al.*, 2017). In addition, the use of Csy4 ribonuclease allow to expressed multiple gRNAs from a single *CmYLCV* promoter, overcame the specificity of a G nucleotide at the 5' end of the gRNAs had to *AtU6* promoter (used in dicot plants). Here we took advantage from these recent improvements in genome editing architecture to develop a CRISPR/Cas9 system able to target multiple genes (or the same genes in different points) from a single promotes avoiding the development of a cumbersome construct that could be led to a more difficult integration event.

Conclusion

In conclusion, we applied the CRISPR/Cas9 technology to reduce susceptibility to powdery mildew and in general to biotrophic fungi. We developed the construct based on specific recombinase system in order to produce 'clean' edited grapevine, without exogenous DNA. The T-DNA removal could protect plants from any effects due to the presence of the exogenous endonuclease.

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Supporting Tables and Figures

Table S7 Primer sequences for in Golden Gate assembly in pDIRECT_22c-Cre-LoxP-LB-LoxP vector.

Primer name	Sequence (5'-3')
CmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC
Csy4-B_M6_gRNA7	TCGTCTCCCAACACTCTTAGCTGCCTATACGGCAGTGAACCTG
orep-C_M6_gRNA7	TCGTCTCAGTTGGATCCACTGTTTTAGAGCTAGAAATAGC
oCsy-D_M6_gRNA28	TCGTCTCAAGTCAGGAAAAGCTGCCTATACGGCAGTGAAC
orep-C_M6_gRNA28	TCGTCTCAGACTGAAAAAGAGTTTTAGAGCTAGAAATAGC
oCsy-E	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC
Csy4-B_M7_gRNA13	TCGTCTCCCCTTGTAAAAAGCTGCCTATACGGCAGTGAACCTG
orep-C_M7_gRNA13	TCGTCTCAAAGGGTGCCAAGGTTTTAGAGCTAGAAATAGC
oCsy-D_M7_gRNA13	TCGTCTCACCTTGTAAAAAGCTGCCTATACGGCAGTGAAC
oCsy-D_M7_gRNA25	TCGTCTCACATCAACTCCATCTGCCTATACGGCAGTGAAC
orep-C_M7_gRNA25	TCGTCTCAGATGGATCCCATGTTTTAGAGCTAGAAATAGC
oCsy4-B_NPR3g2	TGGTCTCCCTCGAGGTTACACTGCCTATACGGCAGTGAACCTG
orep-C_NPR3g2	TGGTCTCACGAGGATTATGAGTTTTAGAGCTAGAAATAGC
Csy4-B_NPR3g29	TGGTCTCATTCCACCCGTAACTGCCTATACGGCAGTGAAC
orep-C_NPR3g29	TGGTCTCAGGAATTCGCAATGTTTTAGAGCTAGAAATAGC
Csy4-B_NPR3g40	TGGTCTCAGTCAACGTGTGTCTGCCTATACGGCAGTGAAC
orep-C_NPR3g40	TGGTCTCATGACACCTCCAGGTTTTAGAGCTAGAAATAGC

Table S8 Sequence of primers used for the PCR-based screening of transformant grapevine.

Primer name	Sequence (5'-3')
TC320 F	CTAGAAGTAGTCAAGGCGGC
TC089 R	GGAACCCTAATTCCCTTATCTGG
LoxP F	ACTTCGTATAGCATACATTATACGAA
pD22c_1155 F	TCCAATTCACTGTTCCTTGCA
pD22c_2039 R	ATGGCCGCTTTTCTGGATTC
pD22c_11123 F	TAATCGCCTTGCAGCACATC
pD22c_11388 R	CAAATGGACGAACGGATAAACC
CRE130 F	AATGCTTCTGTCCGTTTGCC
CRE713 F	CTGTTTTGCCGGGTCAGAAA
CRE455 F	TACCTCGCGTATCCCCTTCG
CRE455 R	GCCTGTTTTGCACGTTCACC
Cas9_484 F	CCAAACGAGAAGGTGCTCCC
Cas9_484 R	TTGAGAGCCTTCCCCAACCA
VvMLO6 F	CAAGAAAGCTGTGTGGTGGC
VvMLO6 R	TACTTCAGTGCCGCATGTGT
VvMLO7 F	TTGGCTTGTGTTGTGAAGAGC
VvMLO7 R	TCCTCCCAAGCCTTCCATCT

Table S9 Sequence of primers used for the qRT-PCR.

Primer name	Sequence (5'-3')
VvACT F	TGCTGCTCCTCAAATGCTCA
VvACT R	CTAGGAAACACTGCCCTGGG
VvUBI F	AGCAAATCTCCCTCCGCATC
VvUBI R	GTGAGACGACAGAAAGGGCA
Cas9 F	TCCAAACGAGAAGGTGCTCC
Cas9 R	AAGCAGGCTTCCTCATTCCC

Table S10 Collected inflorescences for three years.

Genotype	Year	Anthers	Ovaries
Chardonnay	2019	4000	1000
Glera	2019	2160	540
Pinot Noir	2019	1440	360
Chardonnay	2020	8000	2000
Glera	2020	12000	3000
SO4	2020	6000	-
110 Richter	2020	10000	-
Chardonnay	2021	4400	1100
Pinot noir	2021	11200	2800
SO4	2021	6500	-

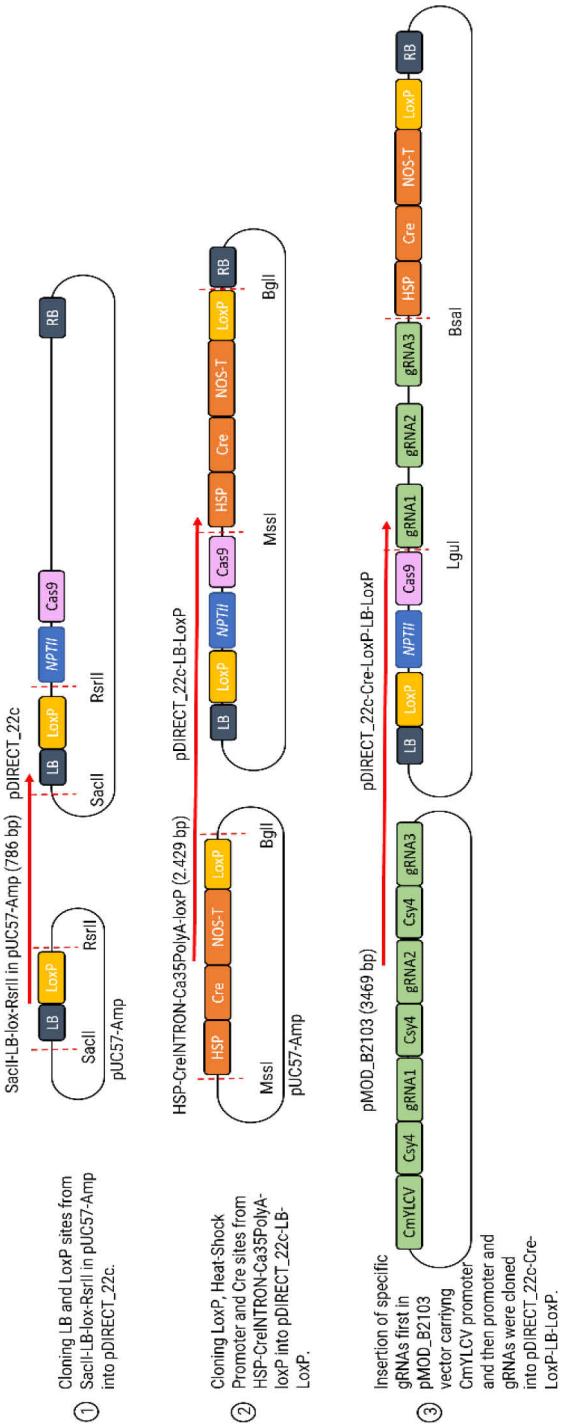

Figure S7 Workflow in genome editing constructs design.

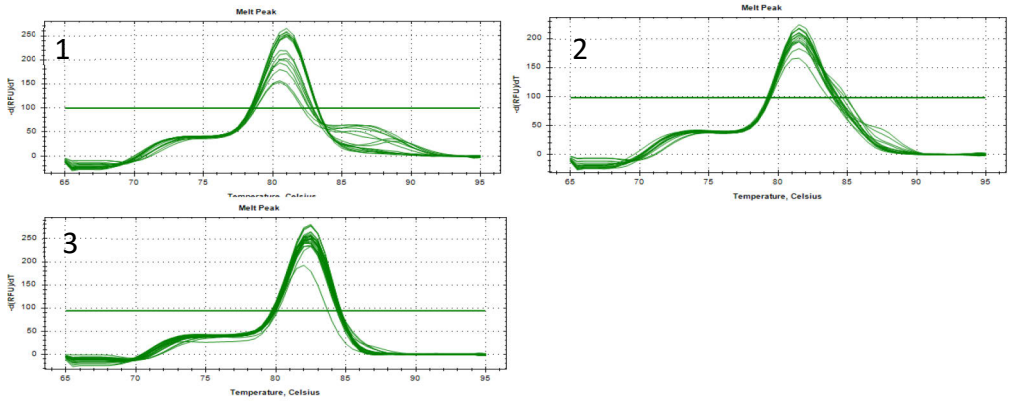

Figure S2 Melting curves for qRT-PCR: 1) *VvACT,* 2) *VvUBI,* 3) *Cas9.*

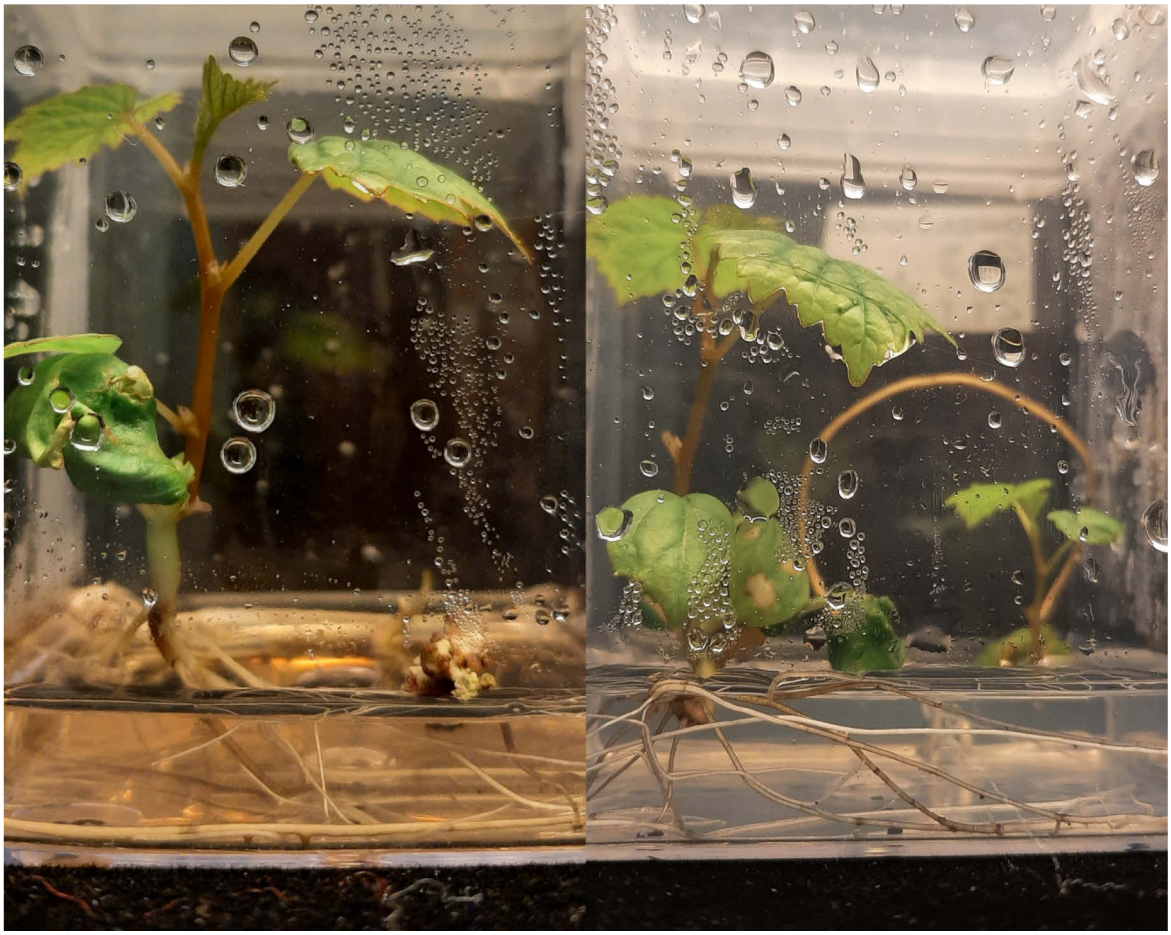

Figure S3 Positive regenerated Chardonnay plants from embryogenic calli after transformation with DIRECT_22c-Cre-LoxP-LB-LoxP carrying *VvMLO* gRNAs.

Figure S4 Positive regenerated Chardonnay plant from embryogenic calli after transformation with DIRECT_22c-Cre-LoxP-LB-LoxP without specific gRNAs.

CHAPTER 5

Improving ecological plant-microbiome interactions as mitigators of anthropocentric breeding

Improving ecological plant-microbiome interactions as mitigators of anthropocentric breeding

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Abstract

Over the years, traditional plants breeding and domestication processes have allowed the selection of more productive genotypes and of more suitable crop lines to contrast climate change. Notwithstanding these advancements, the impact of breeding on the ecology of plant-microbiome interactions, as well as the exploitation of beneficial interactions to develop crop with improved performance and more adapted to climate change scenario, have not still adequately considered. This is particularly true for woody plants where breeding programs are more complicated. This perspective will discuss the possibility to develop more sustainable breeding strategies, based on the multiple interactions between plants and the associated microorganisms (*i.e.*, beneficial soil microorganisms), with a particular focus on the status of breeding strategies in woody plants.

Keywords

Actinomycete, Climate change, Holo-omics approaches, Microbial breeding, Mycorrhizal symbiosis, Priming state, Soil microorganisms, SynCom

Introduction

Plant domestication is the outcome of a selection process that leads to increased adaptation of plants to cultivation and utilization by humans-being (Gepts, 2003). This process is grounded on the selection and modification of wild plant species, and it is aimed to achieve useful characteristics for human requirements. The increasing yield is one of the most important goal reached by humans during the domestication process and it has allowed a continuous and constant food supply (Fasoulas, 1973). However, plants do not have unlimited quantity of energy and the allocation of limited carbon sources is consequently influenced by a growth-defence trade-off (Karasov *et al*., 2017). This phenomenon is based on the concept that the limited carbon sources produced by photosynthesis are allocated toward growth or defence processes in order to maximize the plant's adaptation strategies and fitness costs in diverse environments (Huot *et al*., 2014). Indeed, if plants focus their energy mainly on growing, they automatically have less ability to deal with different kind of stresses such as pathogen infections or harsh environmental conditions. Furthermore, domesticated plants are kept in safe in a cotton wool, constantly fertilized, irrigated, and protected by anthropic inputs, so they are characterized by less ability to interact with the surrounding environment than the wild parents. This negative trend is called "domestication syndrome" where intensively domesticated plants have lost their ability to survive on their own away from the care of humans (Chen *et al*., 2015). The modern agriculture in now entering in a green revolution and so it is necessary finding out new sustainable strategies for plants management without an excessive use of external inputs (Pimentel, 1996). Nowadays plants are no longer considered as standalone entities, while it has been shown how plant-associated microorganism are essential for improving its wellness and the sustainability of agricultural system (Vandenkoornhuyse *et al*., 2015; Singh *et al*., 2020a). It is against this background that we decided to examine in depth the critical issues of the classic plants breeding, its effects on the ecology of plant-microbiome interactions and the possibility to develop a new kind of it in accordance with the principles of agricultural sustainable development and the optimization of beneficial interactions. Breeding programs are more complicated and longer for woody plants compared to herbaceous ones and so we have drawn our efforts on them. In summary, more sustainable breeding strategies, based on the multiple interactions between plants and the associated microorganisms (*i.e.*, beneficial soil microorganisms), with a particular focus on the status of breeding in woody plants.

Considering the ongoing climate change, in the next future, it is essential to obtain an agricultural system more self-sufficient and sustainable thus reducing the need of external inputs that can negatively influence the ecological equilibrium of agricultural ecosystems.

Side effects of the "anthropocentric breeding"

Plant breeding, characterized by an anthropogenic point of view, has led to low self-support production systems with an increased need for external inputs such as substantial fertilization plans and a large use of pesticides (Matson *et al*., 1997). This process caused a dramatic reduction of plant genetic diversity leading to a significant impact of pathogens and pests on plant productivity and consequently an excessive use of chemical inputs to protect them. Thus, the main side effect of plant domestication is the loss of human neglected traits which are very important for wild plants wellness (Gepts, 2003). The incessant plant selection of genomic traits and the considerable number of inputs needed to sustain the selected genotypes, negatively influence the interactions between plants and beneficial organisms and microorganisms (Likar *et al*., 2017). There is evidence in literature showing that anthropocentric breeding has profoundly altered the interactions between plants, insects, and their natural enemies. It has been demonstrated that domestication process led to lower levels of volatile emissions during a pest attack as compared to wild relatives, which in turn might affect the attraction of natural enemies of pests and pathogens (Chen *et al*., 2015). Additionally, breeding processes reduced the microbial biodiversity and functioning associated to plant tissues and organs in agricultural systems hampering the essential interactions that make wild species more resilient to biotic and abiotic stresses (Fig. 1) (Pérez-Jaramillo *et al*., 2016).

The selected crop cultivars might have lost some of the traits needed to recruit host-specific microbiota as compared to their wild relatives. For instance, it was shown that long-term nitrogen fertilization resulted in the recruitment of less-functional rhizobacteria in leguminous species, providing fewer benefits to the host (Weese *et al*., 2015). Additionally, the abundance and composition of the rhizosphere and root-associated microbiota are influenced by several factors, including host's species and genotype. Recent studies have in fact shown that root exudates are essential for plants to assemble a functional microbiome and that changes in plant genetics derived from breeding programs result in different root

exudates composition undermining microbiome assembling and functioning (Pérez-Jaramillo *et al*., 2016; Zhalnina *et al*., 2018). Chaluvadi and Bennetzen (2018) have demonstrated that there are species-associated differences in the below-ground microbiome associated to wild and domesticated *Setaria*, highlighting how crop domestication can play an important role in selecting prokaryotes present in the rhizosphere and root compartment (Chaluvadi and Bennetzen, 2018). There is also evidence about the impact of plant breeding on the assembly of rhizosphere fungal communities that seem to be strongly influenced by host genotype (Leff *et al*., 2017). Additionally, root exudates, such as flavonoids or strigolactones, play key roles in symbiotic relationships such as between plants and rhizobia or mycorrhizal fungi (Wang *et al*., 2012) and, consequently, changes in their composition might limit or negatively influence these positive interactions. In this regard, Martìn-Robes stated that colonization by mycorrhizal fungi is lower while infection rate by nematodes is higher in the roots of plants that grow in soils previously cultivated by domesticated plants, concluding that domesticated plants are characterized by lower mycorrhizal colonization and higher nematode infection rates than their wild progenitors (Martín-Robles *et al*., 2020). Furthermore, the rhizosphere and root-associated microbiome is not only able to help plants survive and growth, but also offers a further level of genetic variability that was little considered as a strategy by breeders until now, having so far exploited the mainly the host plant variability to develop improved crops (Sergio Eduardo Contreras-Liza ED1 - Ibrokhim Y. Abdurakhmonov, 2021). Plants and all associated microorganisms are now considered as a unique organism named “holobiont” (Vandenkoornhuyse *et al*., 2015), and the term “hologenome” is used to indicate the entire set of genomes within the holobiont (Zilber-Rosenberg and Rosenberg, 2008). Considering this premise, a new kind of breeding is required to protect and improve the interactions between plants and the natural interacting microbiomes.

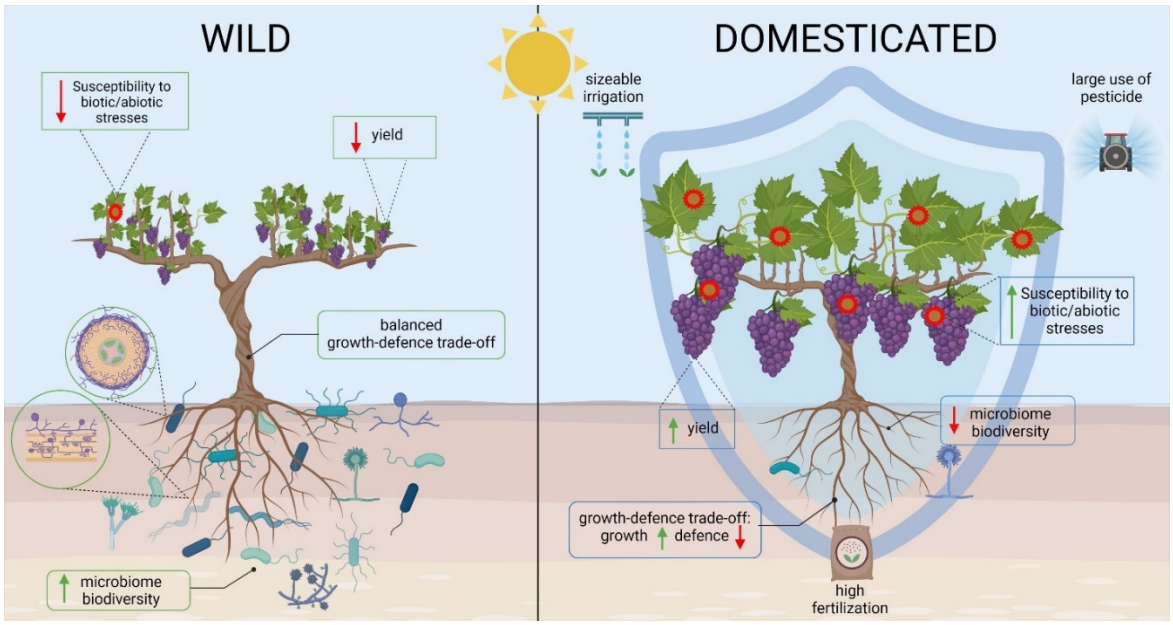

Figure 1 Comparison between wild and domesticated plant species. On the left, wild relative displays a better adaptation to environmental stresses (both biotic and abiotic), a balanced growth-defence trad-off, a rich associated-microbiome but a low yield. On the right side, domesticated plant shows an improved productivity but a reduction of both stress resilience and ability to recruit plant-associated microbes. To cope with the increased susceptibility, human practices such as irrigation, fertilization and pesticide application are needed.

Critical point of woody plants breeding

Despite breeding programs aim to produce resilient cultivars to diseases and environmental stresses, many traits are regulated by several genes (*e.g.*, polygenic resistance) and, for this reason, hardly transmissible to the progeny in a single crossing (Stuthman *et al*., 2007). In this scenario, the monogenic resistance, exploited by classical breeding programs, is highly effective in moving single gene traits, but it is easily suppressed by the pathogen along time (Nelson *et al*., 2018; Fonseca and Mysore, 2019). For woody plants breeding programs there are several further limitations, such as linkage drag, long times needed for backcrossing and high heterozygosity degrees, hampering the development of resilient genotypes and raising the costs (Harshman *et al*., 2016; Limera *et al*., 2017; Ricci *et al*., 2020). Additionally, the obtained resilient/resistant genotype is often associated to modifications which could be detrimental from the commercial point of view, such as an altered phenotype or a different aromatic profile (Krishnan and Jez, 2018) which is often less accepted by the consumers.
For example, grapevine is characterized by different cultivars which are associated to the production of many aromas and therefore different commercial wines. The products of

grapevine breeding programs produce new individuals with different characteristics from the parental cultivars that need to be registered as new varieties and in consequence the necessity of market acceptance (Eibach and Töpfer, 2015; Carbonell-Bejerano *et al*., 2019). Thus, breeding programs on woody species result in laborious and time-consuming processes which can be quite easily overcame by pathogens and that in parallel negatively affect qualitative traits of fruits (Fig. 2). On the other hand, plants that are used as row matter in industrial processes, such as aspen, present fewer limitation than fruit crops, due to a major acceptance of the product that will not enter in the food chain. These plants are subject of breeding to improve important industrial characteristics, such as cellulose accumulation or biomass production (Aravanopoulos, 2010).

Application of biotechnological approaches in breeding processes has recently led to development of New Plant Breeding Technologies (NPBTs) that are able to modify specific target DNA sequences without altering other regions and without the need of long backcrossing stages (Massel *et al*., 2021; Giudice *et al*., 2021; Biswas *et al*., 2021). These techniques, and in particular the genome-editing one, promise to overcome the limits imposed by traditional breeding both in terms of time-consuming and costs. Although NPBTs-derived products are accepted in several countries, many restrictions remain, especially in Europe (Zhang *et al*., 2020) due to environmental risks and for long and expensive risk assessment trials for feed and food commodities (Kawall *et al*., 2020).

Plants share their habitat with many microorganisms including bacteria, fungi and viruses and their biodiversity depends by many factors. These microorganisms may constitute an important target to enhance plant features, such as productivity and/or resilience to environmental stresses thus reducing chemical inputs (Andreolli *et al*., 2016; Cai *et al*., 2017; Nigris *et al*., 2018; Leung *et al*., 2020; Palmieri *et al*., 2020; Silva-Valderrama *et al*., 2021). Additionally, the use of microorganisms to protect plants may overcome the limits associated to both classical breeding and NPBTs. Microbiological products could in fact be more sustainable for three major reasons: i) the final products will not contain different characteristics (such as altered phenotypic or aromatic features); ii) the original genotype is preserved and therefore the product will not undergo specific safety assessments (*e.g.*, those for genetically modified organisms); iii) the development of a microbiological product is less expensive and time consuming if compared to breeding programs especially for woody plants.

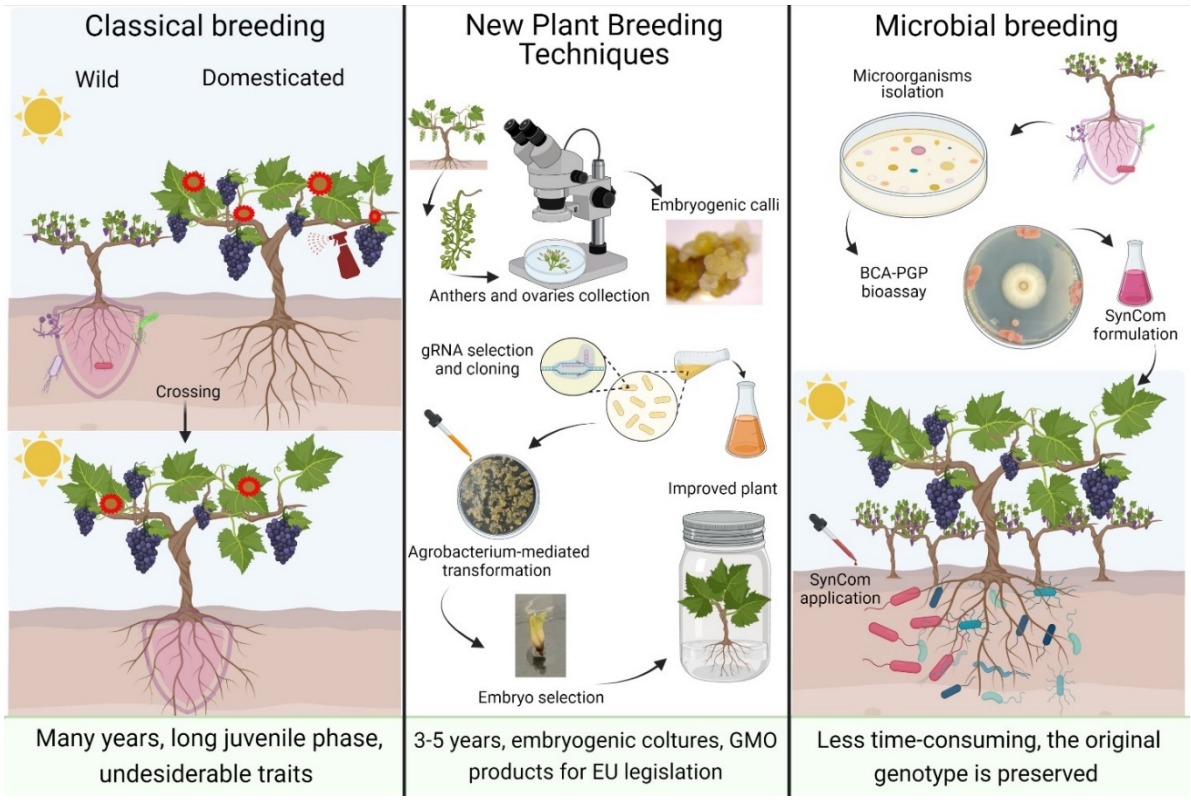

Figure 2: overview of the classical breeding, new plant breeding techniques and the microbial breeding approaches. On the left, classical breeding relies on the possibility to transfer traits (*e.g.*, related to biotic or abiotic stress resilience) from wild sexually compatible species to domesticated species of high economically importance through crossing. The main limitation in such approach, especially for woody species, is the presence of juvenile stages, which increase the time for back-crossing, and the transmission of undesired traits (linkage drag). In the middle, new plant breeding techniques allow to reduce the time needed by conventional breeding, but they still present limitations such as the need of specific tissues and/or cellular types (*e.g.*, embryogenic calli) and several limitations related to GMO regulations. On the right, microbial breeding approach can overcome the limitation of both the previously techniques preserving the original genotype and reducing development times and costs of a synthetic community (SynCom).

A new kind of breeding

Research is now focusing its attention on the interactions among plants and their microbiomes as alternative to the selection of specific plant trait such as high yield or genetic resistances to pathogens (Wei & Jousset, 2017; Singh *et al.*, 2020a,b). Looking for a breeding approach that pays attention for understanding and improving plant-microbe interactions might be the right way for the future green-revolution of agriculture. It also represents a promising tool to restore the growth-defence trade-off balance of domesticated plants achieving an agricultural system able to survive with as little as possible external inputs (Nerva *et al*., 2021). Thus, analyzing plant microbiomes and trying to select and identify

genomes of the microorganisms able to improve the plant growth or the plant resilience to any kind of stress should be the way forward. In fact, the identification and characterization of microorganisms activities, allowed us to influence the plant trade-off features (Bastías *et al*., 2021). Cultivars selected for quality and/or quantity yield characteristics are usually more sensitive to biotic and abiotic stresses: using microorganisms able to place the plants in the so called "priming state" it is possible to restore the natural trade-off equilibrium attaining more resilient cultivars (Van Wees *et al*., 2008; Alagna *et al*., 2020; Mannino *et al*., 2020). Indeed, plants have a defence system that can be enhanced and alerted using biological and chemical priming thus making them "ready for the battle" (Westman *et al*., 2019). Priming (or acclimatization) is a complex phenomenon that consists in preconditioning the plant immune system and abiotic defences, so that responses to stress result quicker, stronger, and more effective (Lohani *et al*., 2020). In addition to enabling a state of priming, many of these associated microorganisms can also perform a direct antagonism towards several pathogens acting as plant allies (Wan *et al*., 2008; Loqman *et al*., 2009). On the other hand, since most of resistant cultivars obtained by conventional breeding have showed less quality and quantity yield capacity, the possibility to exploit plant growth-promoting (PGP) microorganisms could partially restore the original features (Fadiji and Babalola, 2020). Remarkably, 'resistant' cultivars are characterized by specific resistances toward target pathogens, but they do not make provisions for the rest of biotic and abiotic stresses. A rich microbiomes, on the contrary, guarantee a broad-spectrum tolerance toward several pathogens contributing to phenotypic plasticity and adaptability of the plants to the changing environment (Hacquard *et al*., 2017; Carrión *et al*., 2019). In fact, results collected from several studies have shown that specific microbiota members can modulate plant immunity processes through bidirectional microbiota–root–shoot mechanisms which play crucial functions for plant health (Berendsen *et al*., 2018a; Hou *et al*., 2020). Furthermore, microbiome-plant interactions allow to modulate the allocation of carbon plant resources. Indeed, the modulation of plant growth and defence can be conferred by microbiota which play a role in rapidly orchestrating resource investment into the two processes according to the stress encountered (Hou *et al*., 2021). This microbiome mediated dynamism in the allocation of plant resources is very important also considering the ongoing climate change and the resulting increased incidence of abiotic stresses that will threaten agriculture (Fig. 3).In order to highlight the importance of managing woody plant trade-off, it is useful looking up to grapevine as a model plant for several reasons: it has a well

annotated genome and transcriptome, numerous plant-microbe interaction studies were reported and the importance of several associations has been already highlighted (Velasco *et al*., 2007; Pinto *et al*., 2014; Pacifico *et al*., 2019). Furthermore, grapevine is usually grafted, and the choice of the rootstock genotype also have an important role in shaping microbes inhabiting the rhizosphere. Indeed rootstocks differ from each other by specific growth-defence trade-off features and for the ability to recruit different microbial consortia (Marasco *et al*., 2018). Modulating the interactions between rootstocks and their own associated microbe, growth and defence features could be managed, making grapevine more suitable to sustainable practices.

In addition to the selection and characterization of specific microorganisms, the study and the optimization of the interactions between plants and soil microorganisms might be also considered as a new breeding approach that can simultaneously promote crop productivity and environmental sustainability. Particularly, it could be very useful to study and unearth the ability of crops to assemble useful and healthy microbial communities. Several studies have already shown that diverse plant species are able to recruit specific microorganisms, establishing active interkingdom interactions that could be perceived as a "cross talk" (Berendsen *et al*., 2012; Zancarini *et al*., 2021). Berendsen and colleagues have demonstrated that plants can recruit beneficial bacteria upon pathogen infections, particularly disease resistance-inducing and growth-promoting ones (Berendsen *et al*., 2018b). In addition, the "cry out for help" concept has been proposed, considering root exudates as an adaptive mechanism by which stressed plants assemble health-promoting soil microbiomes (Rolfe *et al*., 2019). Considering this, a comprehensive understanding of mechanisms governing this selection of microbial community by the plant will provide new strategies to improve the future of agriculture. To reach this fundamental goal, a new breeding approach more focused to protect and improve the interactions between plants and the associated microbial communities is required, and the application of novel approaches, such as the exploitation of Synthetic Communities (SynCom)and omic-tools, seems a good way to get it. In this perspective, both mentioned possibilities will be analyzed and discussed.

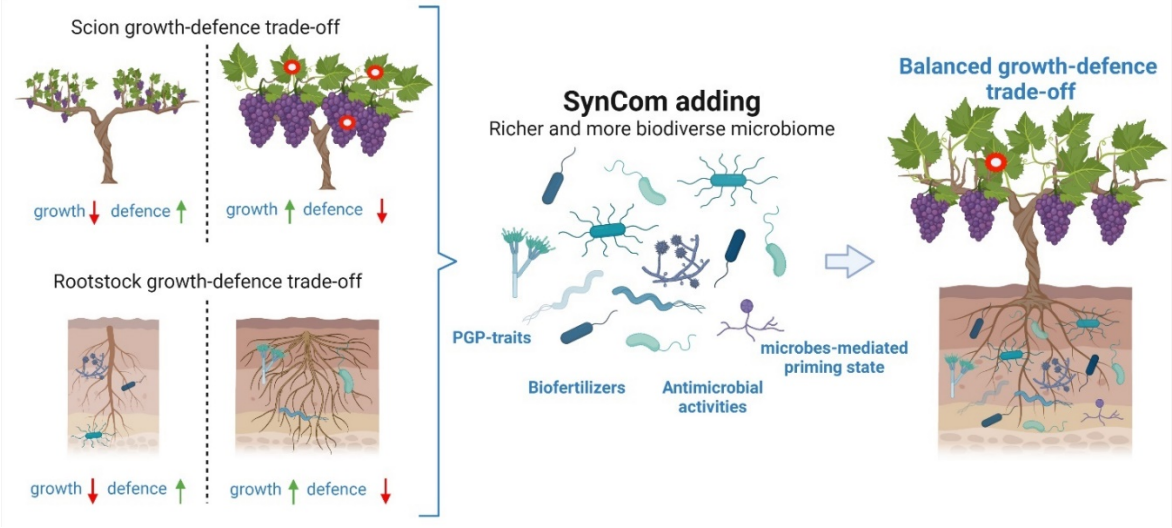

Figure 3 Schematic representation of SynCom adding traits for balancing the growth-defence trade-off in grafted crops. Depicting different (culturable) microbial populations, associated to diverse environments, can allow the development of SynCom which can in turn modulate the growth-defence trade-off, leading to more resilient plants showing balanced growth-defence features.

Actinomycetes: promising allies to manage plant trade-off and to support more holistic breeding programs

Many studies looking for beneficial plant-associated bacteria have been done in the last few years and among these Actinomycetes have shown a considerable number of positive effects on plants fitness and health (Van der Meij *et al*., 2017). They displayed great potential to improve the future of sustainable agriculture supporting plants during their growth and during the interaction with the surrounding environment (Bhatti *et al*., 2017). Actinomycetes interact with plants as free-living non-obligated symbiotic bacteria, they have been found mainly in the soil but they are able to colonize also roots and plant tissues, living as endophyte or establishing mutualistic relationships (Olanrewaju and Babalola, 2019). They are an ecologically divergent group which is able to occupy a huge range of environmental niches thanks to its peculiar capacity to live in wide range of temperatures and pH (Millán-Aguiñaga *et al*., 2019) and which showed a filamentous morphology helping them to cleave the rhizosphere soil particles easier than others bacteria. Moreover, they are able to produce many bioactive compounds with antibiotic, antifungal or insecticidal activities or acting for the catabolism of complex molecules, such as lignocellulose, achieving a nutritional advantages respect to the other soil habitants (Barka *et al*., 2016).

Regarding the allocation of plant carbon sources, Actinomycetes seem to be a promising tool to manage plant growth-defence trade-off since they are able to improve both the growth (Chukwuneme *et al*., 2020) and the resilience of plants to several stresses (Segaran *et al*., 2017). Actinomycetes can be considered as biofertilizers thanks to their PGP-traits and so they can be exploited to improve yield of those genotypes characterized by low productivity. They are characterized by phosphate solubilization activity (Chukwuneme *et al*., 2020), nitrogen fixation (Bhatti *et al*., 2017), production of indole-3-acetic-acid (IAA), siderophores and are able to prevent ethylene accumulation by the degradation of 1-aminocyclopropane-1-carboxylic acid (ACC) (Nimnoi *et al*., 2010; Sathya *et al*., 2017). With regards to biotic stresses, several studies have shown the Actinomycetes ability to inhibit the growth of different pathogens and to control their incidence and severity (Loqman *et al*., 2009; Gangwar *et al*., 2012). In this respect, they can act through both a direct antagonism towards pathogens (Wan *et al*., 2008; Loqman *et al*., 2009) and by activating a state of priming in the plants (Kamal *et al*., 2014; Mhlongo *et al*., 2018). Finally, agricultural productivity is increasingly affected worldwide because of climate change-induced abiotic stresses and Actinomycetes can work as mitigators of this negative trend. Indeed they have the ability to enhance the plant capacity to face up with abiotic stresses such as drought and salinity (Grover *et al*., 2016; Chukwuneme *et al*., 2020). The use of Actinomycetes in agriculture is a very new topic and, for this reason, so far there are not studies in literature about their ability to form a stable association with the plant once they have been inoculated in *in vivo* condition. However, it is possible find out several works about the efficiency of *in vivo* applications of Actinomycetes bio-inoculants, both as biofertilizers and bio-control agents (Abdallah *et al*., 2013; Soltanzadeh *et al*., 2016). Thus, considering the ability of Actinomycetes to support plants in several ways, it is clear that agriculture needs to develop modern breeding programs that respect and improve the close relationship between them and plants. Furthermore, it has been reported that these bacteria can enter in a close association also with AM fungi, giving an additional reason to contemporaneously analyze the potentiality of both Actinomycetes and AMF (Kamal *et al*., 2014). Modern breeding programs should in fact not risk losing these fundamental plant's traits.

Arbuscular mycorrhizal symbiosis responsiveness as a trait for breeding

Among beneficial root-associated microorganisms, arbuscular mycorrhizal fungi (AMF) are the most important bio-fertilizers. These symbiotic fungi colonize plant roots of several crop species, including woody plants such as grapevine and poplar, and help their host in the uptake of water and nutrients, by receiving in turn carbon compounds (Bucher *et al*., 2014). Lipids were recently demonstrated to be an additional source of organic carbon delivered to the AM fungus during symbiosis in addition to carbohydrates (Luginbuehl *et al*., 2017). In addition, they are thought to be exported out of the root cells across the periarbuscular membrane for the use by the fungus (Bravo *et al*., 2017). Additionally to an improved nutrition, mainly related to an improvement in phosphate (Pi) uptake that particularly occur in limiting nutrient conditions, several papers have described the impact of AM fungi on plant tolerance under abiotic stresses such as drought, salinity, and cold conditions (Balestrini *et al*., 2017). AM associations are broadly present in cultivated soil from diverse environments, where they form symbiosis with the roots of major crop species, and their potential to improve crop productivity is an opportunity for plant breeding that should be more exploited. In addition, it is worth noting that developing crops with higher P-use efficiency is an important goal for breeders (Jeong *et al*., 2015). However, notwithstanding the AM symbiosis advantages, these associations are not generally directly considered in plant breeding (Jacott *et al*., 2017). However, this is generally addressed mainly considering root traits in diverse genotypes without considering the interactions with soil microorganisms. Breeding programs are more devoted to target traits correlated to shoot architecture and yield, while root-related traits have been so far omitted (Chen *et al*., 2018). As recently suggested (Pozo *et al*., 2021), breeding approaches to improve the results from beneficial plant-fungus interactions should be obtained through the selection of traits of both symbionts (*i.e.*, the plant and the fungus) involved in the optimization of association establishment and functioning. Thus, the capacity to benefit from fungal symbiosis should be evaluated in breeding programs. It will be important that future breeding strategies takes in account the interaction of root traits with symbiosis-related traits, with the aim to achieve optimal production also reducing application of fertilizers (mainly P-based products). However, it is always more evident the importance to identify optimal genotype combinations to obtain the best results in term of plant performance and resilience. An increasing number of studies report that AM responsiveness varies among plant accessions (Ramírez-Flores *et al*., 2020; Berger and Gutjahr, 2021). An important point that should be developed is related to the characterization of additional host genotypes, including landrace

and wild-relative whose diversity should be more explored (Sawers *et al*., 2018). The evaluation of mycorrhizal dependency in diverse plant species accessions has been performed since a long time. A comparison among varieties of wheat generated before and after 1990 suggested that the oldest varieties were more responsive to AM colonization than those obtained later (Hetrick *et al*., 1992), suggesting that plant breeding under high nutrient conditions has selected wheat lines with an increased phosphorous demand contrary to the capacity to form AM interactions. However, the impact of breeding on symbiosis effectiveness is still debate. (Sawers *et al*., 2008) suggested that plant breeding selected for a reduction in dependence on AM symbiosis and not for a loss of compatibility, leading to modern cultivars that have reduced but still retained the ability to form AM symbioses. An important point that should be more addressed by future studies is how domestication have influenced the root exudate composition and, consequently, the interactions in the rhizosphere (Iannucci *et al*., 2017). Looking at the root exudate composition in ten wheat genotypes, it has been demonstrated that the composition of the rhizosphere metabolites can be dependent by the genotypes of the domestication groups, suggesting that domestication and breeding have had major effects on root exudates in the rhizosphere.

Genotype dependent plant reactions to AMF colonization have been demonstrated on biomass, while less is known when it comes to genotypic variation for AMF-mediated disease resistance (Hohmann and Messmer, 2017). It has been in fact proposed to include disease resistance as a trait for mycorrhizal responsiveness and that, to observe differences in the efficiency, genotype selection needs to occur in environments that do not suppress the plant–microbe interaction (Hohmann and Messmer, 2017).

As for the classical breeding, also novel breeding protocols evaluating a genotype responsiveness to AMF colonization could takes advantage from the development of protocols for the high-throughput phenotyping platforms, allowing to test many plants contemporaneously. The combination with high-throughput genotyping systems already led to the identification of quantitative trait loci (QTLs) linked to host benefit, supporting the feasibility of breeding crops to maximize profit from symbiosis with AMF (Zuccaro and Langen, 2020). QTLs with a role in colonization have been reported in several crops, although have been mainly defined in herbaceous ones (Lehnert *et al*., 2017; Zuccaro and Langen, 2020).

A relevant bottleneck that should be considered in field studies is the lack of appropriate AMF free controls when an exogenous AM fungal inoculum is applied to soil, rendering

difficult the evaluation of the efficiency of the AM symbiosis in agriculture. Additionally, we have seen above that some plant and AMF combinations are more productive than others, and the nutrient status of soils also affects the species composition of AMF and the success for the symbiotic interaction, complicating the real application of these beneficial microorganisms (Jacott *et al*., 2017). For these reasons, in parallel to the development of breeding protocols that also consider potential to form AM symbiosis as a priority trait, a successful strategy could be to maintain and improve the soil AMF potential with the use of soil managements with a low impact on soil microbial communities (Rillig *et al*., 2016). This could be also a good strategy for woody plants for which breeding programs are very complicated and longer with respect to herbaceous crops.

Considering that the non-symbiotic growth and spore production of the AMF *Rhizophagus irregularis* was reported in the presence of an external supply of certain fatty acids, *i.e.*, myristates (Sugiura *et al*., 2020), an application useful for agriculture could be the developing of crop plants for myristate production with the aim to have AM fungi-friendly plants and varieties (Rillig *et al*., 2020). Additionally, the application of myristates could enhance the AM fungal biomass *in loco*, leading to a reduction in an external inoculation. This should particularly useful could be directly applicable in agriculture, where often AM fungal abundance is suppressed by a range of agricultural management practices (Rillig *et al*., 2016).

Synthetic communities to reach the optimal microbe selection

Microbial communities can provide several benefits to their host as analyzed in the previous paragraphs. However, their effectiveness has been proven to be often inconsistent (Ownley Bonnie H. *et al*., 2003) and one of the main causes has been linked to the host plant and to the insufficient knowledge about host-microbiota interactions (Yang *et al*., 2018; Rodriguez *et al*., 2019) . For this reason, within the plant microbiome research field, unearthing the functional relationships between plants and their microbial partners is the next step for effectively using the microbiome to improve plant fitness and for adopting breeding programs focused on the holobiont (Wissuwa *et al*., 2008). Natural microbial communities and the interactions between them and their host plant are known to be very complex and variable resulting thus difficult to study and precisely define. This complexity derives from the large number of microbes inhabiting the environment coupled to the often unknown

functions for most of them and from the uncharacterized interactions occurring among one another (Curtis *et al*., 2002). Furthermore, these communities are very dynamic and subjected to many changes due to several causes such as environmental conditions(Waldrop and Firestone, 2006) which make studying and comprehending natural microbial communities a really complex process.

The production of Synthetic microbial Community approach seems to be a promising technique to exclude confounding environmental effects and to reduce the complexity of natural systems (Großkopf and Soyer, 2014). It can be described as a comprehensible system of reduced complexity that keep key features of natural ones and that is amenable to modelling (Bodenhausen *et al*., 2014). The establishment of a SynCom is grounded on previous collected knowledge of the overall composition of the plant microbiome needed to formulate a "core microbiota" (Toju *et al*., 2018) (Fig. 4). Thanks to multi-omics and bioinformatics technologies it is now possible to identify core microorganisms that are expected to mediate interactions between plants and native microbiomes. In this line it is possible defining the impact that specific taxa have on the recruiting of others that in turn influence diverse physiological and ecosystem functions (Toju *et al*., 2018).

Such microorganisms are fundamental since they have naturally evolved in close cooperation with a specific plant's genotype and phenotype and so they can be considered in a breeding program grounded on the improvement of the holobiont (Simonin *et al*., 2020). Carlström and co-workers (2019) showed how community assembly is subject to priority effects and, additionally, they indicated that specific strains have the greatest potential to affect community structure as keystone species (Carlström *et al*., 2019).

Once the keystone strain collection and the molecular identification is achieved, these microorganisms need to be tested for their antagonistic activity towards different pathogens, for their PGP-traits, and for keeping out the possibility that they have a reciprocal inhibition or any human or animal pathogen feature. Bacteria showing great and useful traits can be used to establish a SynCom being tested in controlled gnotobiotic system and by which subsequently try to improve the plant growth and wellness in a natural environment (Mazzola and Freilich, 2016).

For example, Zhuang and colleagues (2021), have adopted high-efficiency top-down approaches based on high-throughput technology and synthetic community approaches to find PGPR in garlic rhizosphere (Zhuang *et al*., 2021). They have found out that *Pseudomonas* was a key PGPR in the rhizosphere of garlic and, subsequently, SynCom with

six *Pseudomonas* strains isolated from the garlic rhizosphere was constructed, showing the ability to promote plant growth.

Additionally, through the SynCom exploitation, it is possible highlighting the role of plant immune system in the assemblage of a protective microbiome (Bodenhausen *et al*., 2014). Recent studies have demonstrated that plants can recruit beneficial bacteria upon pathogen infections, specifically disease resistance-inducing and growth-promoting ones (Dudenhöffer *et al*., 2016; Berendsen *et al*., 2018a).

In conclusion, SynCom seem to be a very important approach for capitalizing associated microorganisms, increasing the agroecosystem resilience and finally driving new breeding programs. However, since SynCom development and application a very current topic, further study are needed to improve the knowledge about them and to better understand how stable and efficient they could be in natural environments.

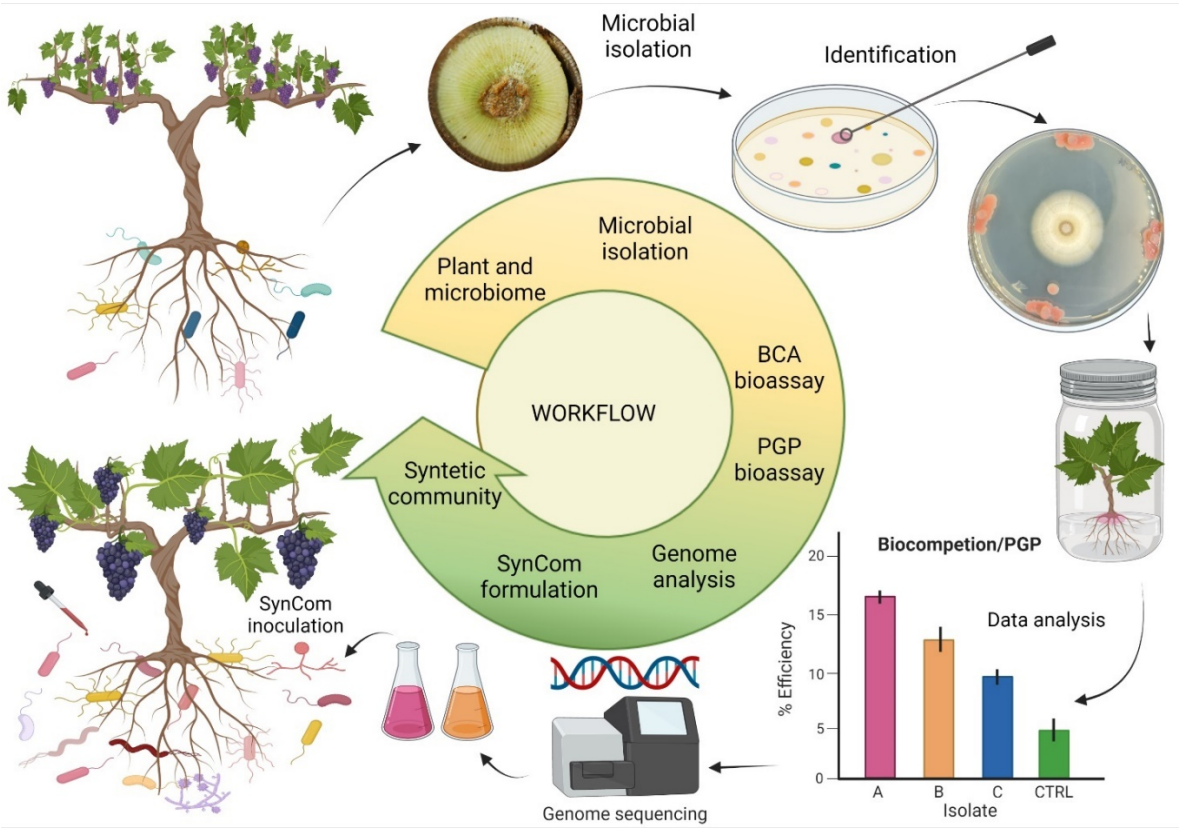

Figure 4 Workflow for the development of a SynCom. Starting from a specific environment and/or wild well-adapted plant population, the first step is to isolate and identify the culturable microbial endophytes. Thus, the identification and selection of potentially beneficial microbes occur through several *in-vitro* tests (*e.g.*, biocompetition against phytopathogens and assessment of plant growth-promoting traits). Finally, prior to SynCom formulation, it is highly desirable to perform genomes sequencing of the best performing microbes (at least for bacteria) to have a clear picture of the biosynthetic pathways present in their genomes and to avoid the selection of isolates which can potentially produce metabolites with detrimental effects on animals and humans.

Holo-omics approaches to unearth plant-microbiome interactions and to improve SynCom efficiency

In the previous paragraph it has been highlighted how knowledge on plant-microbiome interaction, the recruitment plant ability and SynCom approach can be enhanced by omic-tools, enabling thus the development of protocols for a new kind of breeding based on the management of the holobiont (Xu *et al*., 2021). Interestingly, a work the potential role of epigenomic for realizing a breeding program based on the identification of beneficial microbial communities has been recently showed (Corbin *et al*., 2020). Association between individual changes in DNA methylation (novel epialleles) and changes in phenotype (novel microbial community composition and functions) have been determined using epigenome wide association studies. Moreover, pairing host-centered omic-tools, such as transcriptomics, metabolomics, epigenomics, and proteomics, in combination with the more commonly used microbial-focused techniques, such as amplicon sequencing, shotgun metagenomic, metatranscriptomics, and exometabolomics seems to be very promising approaches to achieve a more integrated knowledge on plant microbiome function (Xu *et al*., 2021). Regarding this holistic approach, Nyholm *et al*. (2020) coined the term "holo-omics" to describe such experiments that integrate data across multiple omics levels from both host and microbiota domains. In literature it is possible to find a great number of works grounded on this current approach (Nyholm *et al*., 2020; Deng *et al*., 2021). Recently, Castrillo *et al*. (2017), have explored the relationship between Phosphate Starvation Response (PSR) and microbiome composition and functionality in *Arabidopsis* (Castrillo *et al*., 2017). With this holo-omics (16S and host RNA-seq) design, they have demonstrated that the plant root microbiome directly connects phosphate stress response and plant immune system, and that gene controlling PSR contribute to assembly of root microbiome. Additionally, microbial communities of PSR mutants were distinct from those of wild type and SynCom inoculation enhanced the activity of a master regulator of PSR (PHR1) under limited phosphate conditions, confirming that PHR1 directly regulates a functionally relevant set of plant-microbe recognition genes.

As it has seen since the beginning of this perspective, the impact of plant domestication on microbiome assembly has now been clearly demonstrated. However, insights into microbiomes of wild plant relatives and native habitats could contribute to reinstate or enrich

for microorganisms with beneficial effects on plant growth, development, and health (Pérez-Jaramillo *et al*., 2018). Once identified and characterized these microorganisms, inoculating them in agricultural soil systems might be a very promising tool to restore and improve a beneficial microbial community that has been damaged due to long time of anthropocentric breeding (Gopal *et al.*, 2013; Klein *et al.*, 2012).

Acknowledgement

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Figure legends

Figure 1. Comparison between wild and domesticated plant species. On the left, wild relative displays a better adaptation to environmental stresses (both biotic and abiotic), a balanced growth-defence trad-off, a rich associated-microbiome but a low yield. On the right side, domesticated plant shows an improved productivity but a reduction of both stress resilience and ability to recruit plant-associated microbes. To cope with the increased susceptibility, human practices such as irrigation, fertilization and pesticide application are needed.

Figure 2. Overview of the classical breeding, new plant breeding techniques and the microbial breeding approaches. On the left, classical breeding relies on the possibility to transfer traits (*e.g.*, related to biotic or abiotic stress resilience) from wild sexually compatible species to domesticated species of high economically importance through crossing. The main limitation in such approach, especially for woody species, is the presence of juvenile stages, which increase the time for back-crossing, and the transmission of undesired traits (linkage drag). In the middle, new plant breeding techniques allow to reduce the time needed by conventional breeding, but they still present limitations such as the need of specific tissues and/or cellular types (*e.g.*, embryogenic calli) and several limitations related to GMO regulations. On the right, microbial breeding approach can overcome the limitation of both the previously techniques preserving the original genotype and reducing development times and costs of a synthetic community (SynCom).

Figure 3. Schematic representation of SynCom adding traits for balancing the growth-defence trade-off in grafted crops. Depicting different (culturable) microbial populations, associated to diverse environments, can allow the development of SynCom which can in turn modulate the growth-defence trade-off, leading to more resilient plants showing balanced growth-defence features.

Figure 4. Workflow for the development of a SynCom. Starting from a specific environment and/or wild well-adapted plant population, the first step is to isolate and identify the culturable microbial endophytes. Thus, the identification and selection of potentially beneficial microbes occur through several *in-vitro* tests (*e.g.*, biocompetition against phytopathogens and assessment of plant growth-promoting traits). Finally, prior to SynCom formulation, it is highly desirable to perform genomes sequencing of the best performing

microbes (at least for bacteria) to have a clear picture of the biosynthetic pathways present in their genomes and to avoid the selection of isolates which can potentially produce metabolites with detrimental effects on animals and humans.

CHAPTER 6

Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs

Original Paper

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Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs

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Abstract

It is well known that AM symbiosis provides several ecosystem services leading to plant adaptation in different environmental conditions and positively affects physiological and production features. Although beneficial effects from grapevine and AM fungi interactions have been reported, the impact on growth-defence tradeoffs features has still to be elucidated. In this study, the potential benefits of an inoculum formed by two AM fungal species, with or without a monosaccharide addition, were evaluated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. Inoculated and non-inoculated plants were maintained in potted vineyard substrate under greenhouse conditions for three months. Here, agronomic features were combined with biochemical and molecular techniques to assess the influence of the different treatments. Despite the opposite behaviour of the two selected rootstocks, in AM samples the evaluation of gene expression, agronomic traits and metabolites production, revealed an involvement of the whole root microbiome in the growth-defence tradeoffs balancing. Noteworthy, we showed that rootstock genotypes and treatments shaped the root-associated microbes, stimulating plant growth and defence pathways. Progresses in this field would open new perspectives, enabling the application of AMF or their inducers to achieve a more sustainable agriculture also in light of the ongoing climate change.

Keywords

AM fungi, trade-off, plant priming, stress tolerance, N, growth-defence balance

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

Author contributions

WC, RMB and LN designed the experimental system. LN, GQ, GG, LM, NB, LL, RP, MGV, MS, FG, RMB and WC conducted the wet lab experiments and performed data elaboration. LN, GQ, RMB and WC performed RT-qPCR analyses. LN, GG and WC performed the microbiome data analysis of root endophytes. LN, RMB and WC wrote the first draft of the manuscript. All the authors carefully revised the final version.

Introduction

Grapevine is one of the most cultivated crop worldwide since its great economic importance resulting from grape and wine production, and commercialization (Chitarra *et al*. 2017). For this reason, over the years viticulture industry has selected several cultivars showing different traits (*i.e.*, flavour, yields, colour) influenced by geology, soil-scape and climate features, driving some major wine peculiarities (Priori *et al*. 2019). These components, and their interactions, concur to define the *terroir* of a particular environment (Resolution OIV/VITI 333/2010). Besides scion variety features, rootstocks are able to strongly affect scion performances by means of water transport, biochemical and molecular processes, impacting the whole plant functions and its response to biotic/abiotic stress factors (Chitarra *et al*. 2017). In the last decade, research on scion/rootstock interactions strongly increased, aiming to develop more sustainable practices against pests and ameliorating plant adaptability to the ongoing climate change (Lovisolo *et al*. 2016; Warschefsky *et al*. 2016; Zombardo *et al*. 2020). Key drivers influencing defence features and adaptive traits are thought to be the microbial communities residing in plant tissues. To date, several studies reported evidence about their influence on physiological performances (*e.g.*, production of flavours, hormones, VOCs) in many plants, including grapevine, where residing microbiota contribute to defining the *microbial terroir* (Gilbert *et al*. 2014).

According to the Intergovernmental Panel on Climate Change (IPCC 2014), an increase in the global surface temperature is expected over the next years, affecting crop production as a consequence of the predicted occurrence of biotic and abiotic stresses (Mittler and Blumwald 2010). To achieve resilience to stress, numerous efforts have been done over the years, such as the adoption of specific breeding programs and genetic engineering approaches (Cushman and Bohnert 2000). Researchers have been focusing just recently their attention on the exploitation of 'native' plant defence mechanisms (e.g. hormone signalling, plant immunity activation) against biotic and abiotic stressful factors (Feys and Parker 2000; Jones and Dangl 2006; Hirayama and Shinozaki 2007). The triggering of these responses can occur using chemical treatments (Balestrini *et al*. 2018), root-associated microorganisms and RNA interference technologies (Alagna *et al*. 2020), leading plants in a state of alertness - 'Primed state' or 'Priming' – and enabling them to respond more quickly and robustly in case of the exposure to a stress (Beckers and Conrath 2007).

Among soil beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) establish symbioses with the majority of land plants showing an important role in providing nutrients, particularly phosphate and N, but also water and other elements to the host plant (Jacott *et al*. 2017; Balestrini and Lumini 2018). Mycorrhizal symbiosis is able to influence plant growth and productivity and enhance the tolerance to biotic and abiotic stresses as demonstrated in many crops (Balestrini and Lumini 2018; Balestrini *et al*. 2018; Alagna *et al*. 2020). In addition, AM fungi are able to increase aggregation of soil surrounding roots, improving soil matrix stability and physicochemical characteristics (Uroz *et al*. 2019). Grapevine roots are naturally colonized by native AM fungi with a great impact on growth, yield, quality and development performances (Deal *et al*. 1972; Karagiannidis *et al*. 1995; Linderman and Davis 2001; Trouvelot *et al*. 2015). Thanks to the application of metagenomics approaches to soil and roots, new insights about the AMF living in symbiosis with grapevine have been discovered (Balestrini *et al*. 2010; Holland *et al*. 2014; Balestrini and Lumini 2018).

Rootstocks-mediated adaptation to a specific environment is based on the growth-defence trade-offs-mediated mechanisms (Chitarra *et al*. 2017). Trade-off phenomenon was firstly observed in forestry plants-insect interaction studies and is based on the idea that the limited carbon resources produced by photosynthesis are allocated toward growth or defence processes in order to maximize the adaptation strategies and fitness costs in diverse environments (Huot *et al*. 2014; Chitarra *et al*. 2017; Züst and Agrawal 2017). Stresses impair plant growth, redirecting energy and carbon sources toward defence, reducing growth and reproduction performances (Bandau *et al*. 2015; Züst and Agrawal 2017). Recently, it was suggested that through a meta-analysis, that the increased plant resistance promoted by *Epichloë* fungal endophytes does not compromise plant growth, eliminating the trade-off between growth and defence (Bastías *et al*. 2021). A role in tradeoffs balance has been demonstrated also for AM symbioses, improving nutrient uptake, disease tolerance and abiotic stress resilience (Jacott *et al*. 2017).

In this study, we aimed to evaluate if AM fungi and rootstocks can concomitantly contribute to fine-tuning growth-defence tradeoffs features in grapevine, thus enabling plants to trigger earlier and enhanced defence responses against a potential stressor. The use of specific molecules that can promote the AM fungal colonization have been proposed to improve mycorrhizal inoculum applications under practical field condition (Bedini *et al*. 2018). In

this context, an affordable strategy is the application at low doses of oligosaccharides (*i.e.*, glucose, fructose, and xylose) that have a stimulant effect on AM symbiosis colonization (Lucic and Mercy 2014 - Patent application EP2982241A1). These compounds, initially called as elicitors, in relation to the impact on plant defense, can promote mycorrhizal performances and, for this reason, the term "inducer" was proposed (Bedini *et al*. 2018). In this work, the impact of an inoculum formed by two AMF species (*Funneliformis mosseae* and *Rhizophagus irregularis*), already reported among the species present in vineyards (Berruti *et al*. 2018), with or without the addition of a monosaccharide (D-glucose) at low dose (the so called inducer), has been evaluated on young grapevine cuttings cv. Glera grafted onto 1103 Paulsen and SO4 rootstocks, well known to trigger an opposite growth-defence behaviour in the scion. The effect of the several treatments on the root-associate microbiota has been also evaluated, to verify the response mediated by the AM and its recruited mycorrhizosphere.

Materials and methods

Biological materials and experimental set-up

Two hundred one year-old dormant vines of 'Glera' cultivar grafted onto 1103 Paulsen (1103P) and SO4 rootstocks certified as 'virus free' were purchased from an Italian vine nursery (Vivai Cooperativi Rauscedo, Italy; http://www.vivairauscedo.com). Vine roots were washed with tap water and cut to about 4 cm before plantation in 2 L pot containers filled with not sterilized substrate mixture of vineyard soil/*Sphagnum* peat (8:2, v:v) to better simulate the field conditions. The substrate composition was a sandy-loam soil (pH 7.8; available P 10.4 mg kg^{-1}; organic matter 1.80 %; cation exchange capacity 20.11 mew 100 g^{-1}).

Grapevine cuttings were inoculated with AMF mixed inoculum (INOQ GmbH, Germany, 238,5 Million propagule per kg inoculum) at planting time by placing it in the hole and in contact with the roots following the manufacturer's instructions. Mycorrhizal inoculum, a powder based mycorrhizal root fragment (Advantage Grade II, 2016 - INOQ GmbH) contained 50% *Rhizoglomus irregulare* (syn. *Rhizophagus irregularis*; 450 million propagules per Kg) and 50 % *Funneliformis mosseae* (27 million propagules per Kg). The fungal lines were produced *ex vitro*, on *Zea mays* and *Plantago lanceolata* (sand/vermiculite, v/v). Both AMF inoculum and D-glucose at low dose (i.e., the Inducer) were prepared by Louis Mercy (INOQ GmbH; patent EP2982241A1). The containers were prepared according to treatments as follow: i) 25 plants for each rootstock as uninoculated control plants (C); ii) 25 plants for each rootstock inoculated with 50 mg/L of AMF mixed inoculum (M); iii) 25 plants for each rootstock inoculated with 50 mg/L of AMF mixed inoculum + inducer (M+I); iv) 25 plants for each rootstock amended with 50 mg/L of inducer to stimulate the exploitation of native AMF symbiosis (I). Daily watered grapevine plants were kept under partially climate-controlled greenhouse, under natural light and photoperiod conditions for three months.

After three months, at the end of the experiment, engraftment, growth index and chlorophyll content were recorded. Leaf and root samples for molecular and biochemical analysis were collected from at least three randomly selected plants and immediately stored at -80°C. A

part of the root apparatus was used to estimate the level of mycorrhiza formation as described (Balestrini *et al*. 2017).

Morphological observations in the colonized fragments of thin roots allowed to identify the presence of the typical structures of the symbiosis, regardless of the thesis. However, the patchy level of colonization, and the quality of the root segments after the staining, made morphological quantification difficult, and therefore the AMF presence has been assessed by molecular analyses (see below).

Growth index, engraftment, and chlorophyll content

At the end of the experiment, phenological stages were recorded and classified according to Biologische Bundesanstalt, bundessortenamt und CHemische industrie (BBCH) scale (from 00 to 12, from dormancy to 9 or more leaves unfolded, respectively). BBCH scales have been developed for many crops, including grapevine, and it is based on a decimal code system that identify the growth stage (Lancashire *et al*. 1991), engraftment % (i.e. rooting %) were visually determined for each plant and treatment. Chlorophyll content was determined using a portable chlorophyll meter SPAD (Konica Minolta 502 Plus). Readings were collected from the second or third leaf from the top on at least three leaves per plant on five randomly selected vines for each experimental condition (Chitarra *et al*. 2016).

Targeted metabolite analyses

Contents of *trans*-resveratrol, viniferin and abscisic acid (ABA) were quantified on at least three biological replicates per condition according to the protocol previously described (Pagliarani *et al*. 2019, 2020; Mannino *et al*. 2020). Leaves and roots from two randomly selected plants were pooled to form a biological replicate, immediately frozen in liquid nitrogen, freeze-dried and stored at -80°C until use. Briefly, about 100 mg of freeze-dried sample (leaf or root) were transferred with 1 mL of methanol:water (1:1 v/v) acidified with 0.1 % (v/v) of formic acid in an ultrasonic bath for 1 h. Samples were centrifuged for 2 min at 4°C and 23.477 *g*, and the supernatant was analysed by high-performance liquid chromatography (HPLC). Original standards of resveratrol (purity ≥ 99 %), viniferin (purity ≥ 95 %) and ABA (purity ≥ 98.5%, Sigma-Aldrich) were used for the identification by comparing retention time and UV spectra. The quantification was made by external calibration method. The HPLC apparatus was an Agilent 1220 Infinity LC system (Agilent R, Waldbronn, Germany) model G4290B equipped with gradient pump, auto-sampler, and

column oven set at 30°C. A 170 Diode Array Detector (Gilson, Middleton, WI, United States) set at 265 nm (ABA and IAA) and 280 nm (for stilbenes) was used as detector. A Nucleodur C18 analytical column (250x4.6 mm i.d., 5 µm, Macherey Nagel) was used. The mobile phases consisted in water acidified with formic acid 0.1% (A) and acetonitrile (B), at a flow rate of 0.500 mL min^{-1} in gradient mode, 0-6 min: from 10 to 30 % of B, 6-16 min: from 30 % to 100 % B, 16-21 min: 100% B. Twenty µL was injected for each sample.

Total N, soluble carbohydrate content in leaf and net nitrate uptake in root

The Kjeldahl method was performed according to method 981.10 of the AOAC International (2016), using VELP Scientifica DKL 20 Automatic Kjeldahl Digestion Unit and UDK 159 Automatic Kjeldahl Distillation and Titration System. Approximately 0.2 g of leaf raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one catalyst tablets (3.47 g K_2SO_4 + 0.003 Se, VELP Scientifica, Italy) in a heat block (DK Heating Digester, VELP Scientifica, Italy) at 300°C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization with NaOH (30%) and subsequently distilled in a current of steam. The distillate was collected in 25 mL of H_3BO_3 (1%) and titrated with HCl 0.1 M. The amount of total N in the raw materials were calculated.

Leaf soluble carbohydrate content was quantified (Chitarra *et al*. 2018).

At the end of the experiment, white non-lignified roots (0.5 – 1 g) were collected from four randomly selected plants for each treatment and rootstock. Root samples were washed in 0.5 mmol L^{-1} $CaSO_4$ for 15 min, then transferred to a 20 mL aerated uptake solution containing 0.5 mmol L^{-1} $Ca(NO_3)_2$ and 0.5 mmol L^{-1} $CaSO_4$. Net uptake of NO_3^- was measured removing samples of uptake solution (aliquot of 200 µL) for its determination every 2 min for 10 min(Tomasi *et al*. 2015). The aliquots were carefully mixed with 800 µL of salicylic acid (5% w/v in concentrated H_2SO_4) and incubated for 20 min at room temperature following the addition of 19 ml of 2 mol L^{-1} NaOH. After cooling, nitrate concentration was measured at the absorbance of 410 nm (Shimadzu UV Visible Spectrophotometer UVmini-1240. Kyoto, Japan) and the net nitrate uptake was expressed as µmol (g FW h^{-1}).

RNA isolation and RT-qPCR

Expression changes of target transcripts were profiled on root and leaf samples (three independent biological replicate for each treatment) by quantitative real-time PCR (RT-

qPCR) (Chitarra *et al*. 2018). Total RNA was isolated from the same lyophilized samples (leaves and roots) used for HPLC-DAD analysis and cDNA synthesis was performed as previously reported (Chitarra *et al*. 2016). The absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using *VvUBI* specific primers of grapevine. RT-qPCR reactions were carried out in a final volume of 15 µL containing 7.5 µL of Rotor-Gene™ SYBR® Green Master Mix (Qiagen), 1 µL of 3 µM specific primers and 1:10 of diluted cDNA. Reactions were run in the Rotor Gene apparatus (Qiagen) using the following program: 10 min preincubation at 95°C, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. Each amplification was followed by melting curve analysis (60–94°C) with a heating rate of 0.5°C every 15 s. All reactions were performed with at least two technical replicates. The comparative threshold cycle method was used to calculate relative expression levels using plant (elongation factors, actin and ubiquitin, *VvEF* and *VvUBI* for root and *VvACT* and *VvEF* for leaf tissue) reference genes. While *R. irregularis* and *F. mosseae* elongation factors (*RiEF1*, *FmEF,* respectively) were used to normalized the expression of the AMF phosphate transporter (*PT*) genes. Oligonucleotide sequences are listed in Supplementary Table 1. Gene expression data were calculated as expression ratio (Relative Quantity, RQ) to Control 1103P plants (C 1103P).

Root DNA isolation and sequencing

Root samples were lyophilized prior to DNA extraction. About 30 to 40 mg of freeze-dried and homogenized material were used to extract total DNA following manufacturer instruction of plant/fungi DNA isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) as previously reported (Nerva *et al*. 2019). Total DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA), and DNA integrity was inspected running the extracted samples on a 1% agarose electrophoretic gel. Before sending DNA to sequencing a further quantification was performed using a Qubit 4 Fluorometer (Thermo Fisher Scientific,Waltham, MA, USA).

To inhibit plant material amplification, we added a mixture of peptide nucleotide acid (PNA) blockers oligos (Kaneka Eurogentec S.A., Belgium) targeted at plant mitochondrial and chloroplast 16S rRNA genes (mitochondrial and plastidial) and plant 5.8S nuclear rRNA. Mitochondrial sequence was derived from (Lundberg *et al*. 2013) with a 1bp mismatch, mitochondrial sequence was derived from (Cregger *et al*. 2018). PNA was custom-designed

for *V. vinifera* (VvpPNA: GGCTCAACCCTGGACAG; Vv-ITS-PNA: CGAGGGCACGCCTGCCTGG; Vv-mPNA: GGCAAGTGTTCTTCGGA). Thermal cycler conditions were maintained as suggested by the Illumina protocol as previously reported (Nerva *et al*. 2019).

Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

Rhizoplane metaphylogenomic analyses, taxonomic distributions

A first strict quality control on raw data was performed with PrinSeq v0.20.4 (Schmieder and Edwards 2011) and then processed with Qiime2 (Bolyen *et al*. 2019). A previously reported and specific pipeline was used for fungal analysis: retained reads were used to identify the start and stop sites for the ITS region using the hidden Markov models (HMMs) (Rivers *et al*. 2018), created for fungi and 17 other groups of eukaryotes, which enable the selection of ITS-containing sequences. Briefly, the software allows to distinguish true sequences from sequencing errors, filtering out reads with errors or reads without ITS sequences. To distinguish true sequences from those containing errors, sequences have been sorted by abundance and then clustered in a greedy fashion at a threshold percentage of identity (97%). Trimmed sequences were analyzed with DADA2 (Callahan *et al*. 2016) and sequence variants were taxonomically classified through the UNITE (Abarenkov *et al*. 2010) database (we selected the reference database built on a dynamic use of clustering thresholds). For graphic representation, only genera with an average relative abundance higher than the settled threshold (1%) were retained.

A 16S specific pipeline was used for bacteria: quality filtering was performed with DADA2 which is able to perform chimera removal, error-correction and sequence variant calling with reads truncated at 260 bp and displaying a quality score above 20. Feature sequences were summarized and annotated using the RDP classifier (Cole *et al*. 2014) trained to the full length 16S database retrieved from the curated SILVA database (v132) (Quast *et al*. 2012).

Statistics

Metagenome analyses were performed using R version 3.6.3 (2020-02-29). Fungal and bacterial data were imported and filtered with Phyloseq package (version 1.28.0) (McMurdie

and Holmes 2013), keeping only the operational taxonomic units (OTUs) with a relative abundance above 0.01 in at least a single sample. Differential abundance of taxa due to the effects of rootstock-treatment interaction was then tested using DESeq2 (version 1.24.0) (Love *et al*. 2014) package.

For phenotypic, biochemical and RT-qPCR data, when ANOVA indicated that for either Rootstock (R, 1103P and SO4), Inducer (I, NI) and Myc inoculum (M, Myc and NMyc) factors or their interaction was significant, mean separation was performed according to Tukey's HSD test at a probability level of $P \leq 0.05$. ANOVA and Tukey's HSD test were also used to analyze the treatments effects for each rootstock individually. The standard deviation (SD) or error (SE) of all means were calculated.

Results

Growth, primary metabolism and N uptake and accumulation

The impact of an AM inoculum, an inducer and a combination of both was evaluated on growth parameters (both rooting % and growth stages coded by BBCH scale) in two grapevine rootstock genotypes (R, 1103P and SO4). Four conditions for each genotype were considered: C, not inoculated plants; I, plants treated with the inducer (I); M, AM-inoculated plants; M+I, AM-inoculated plants + inducer.

Results showed a similar impact of the three treatments on the cutting growth parameters (Fig. 1, Table S2), independently from the genotype. Particularly, in SO4 genotype both the rooting % and the BBCH values were higher in treated plants with respect to the control (Fig. 1a,b). Chlorophyll Content Index (CCI) has been evaluated at the end of the experiment, showing no strong differences among the genotypes and treatments (Fig. 1c), although it was significantly influenced by root colonization (M), the inducer (I) and the M x I interaction in both rootstock genotypes.

Treatments generally led to slightly lower values of carbohydrates content in leaves with the exception of M, and only R and I factors significantly influenced this measurement (Fig. 1d). In detail, for each rootstock I and M+I plants showed significant lower levels of carbohydrates (Fig. 1d).

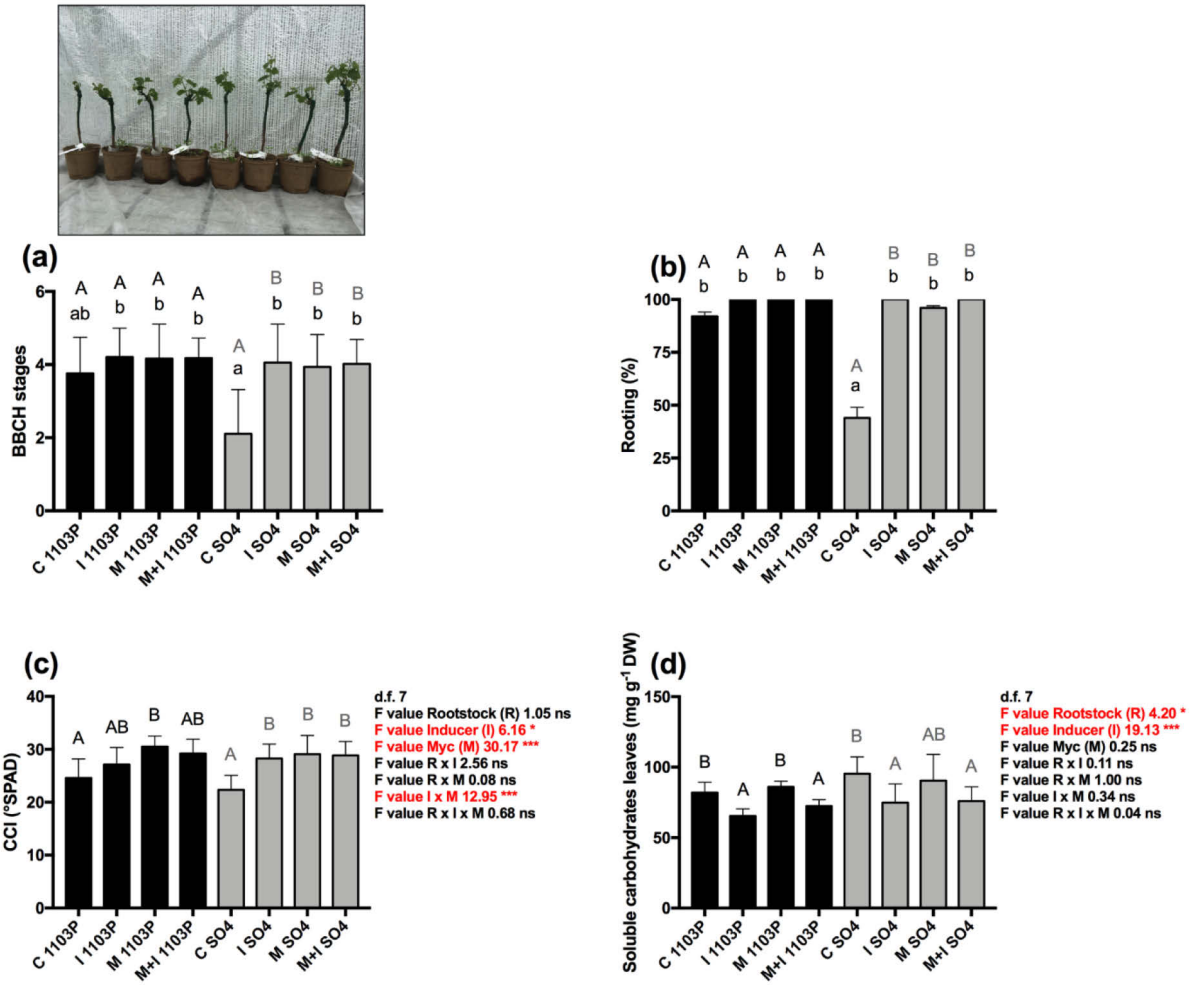

Figure 1 Growth-related traits and metabolites. **a** Growth index according to BBCH scale recorded for each treatment at the end of the experiment ($n = 25$). Upper picture showed an overview of the cuttings' development in response to the treatments at the end of the experiment. **b** Rooting % of cuttings at the end of the experiment ($n = 25$). **c** Chlorophyll Content Index (CCI) measured at the end of the experiment ($n = 25$). **d** Quantification of soluble carbohydrates contents in leaves at the end of the experiment ($n = 4$). All data are expressed as mean ± SD. ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks

Net Nitrate uptake (NNU) was evaluated (Fig. 2a Table S2), showing that it was significantly affected by M factors and the interaction M x I with lower values in treated samples for both genotypes, particularly in M SO4 plants with respect to C SO4 ones (Fig. 2a).

As for the CCI, only slight differences in total N content in leaves were evident among genotypes and treatments, although was significantly affected by the M factor and the M x I interactions (Fig. 2b).

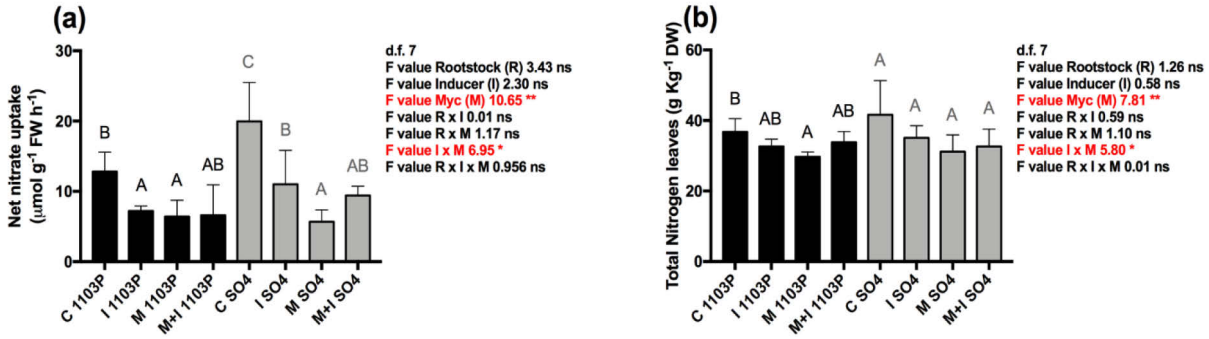

Figure 2 Net nitrate uptake in roots and total N in leaves. a In vivo Net nitrate uptake. b Total N in leaves (g kg^{-1} DW). All data are expressed as mean ± SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks

ABA Content and the Expression of ABA-related Genes

To complete the physiological characterization of the two genotypes in response to treatments, the concentration of ABA was quantified in roots and leaves (Fig. 3, Table S2). ABA levels showed a complex scenario in roots where all treatments led to higher ABA levels with respect to the control with the greater significant increase recorded in M SO4. Statistical analyses showed that factors influencing its level were R and M, alone or in the interactions with I (R x I, M x I, R x M x I) (Fig. 3a). ABA content in leaves was under the detection limit among the treatments (data not shown).

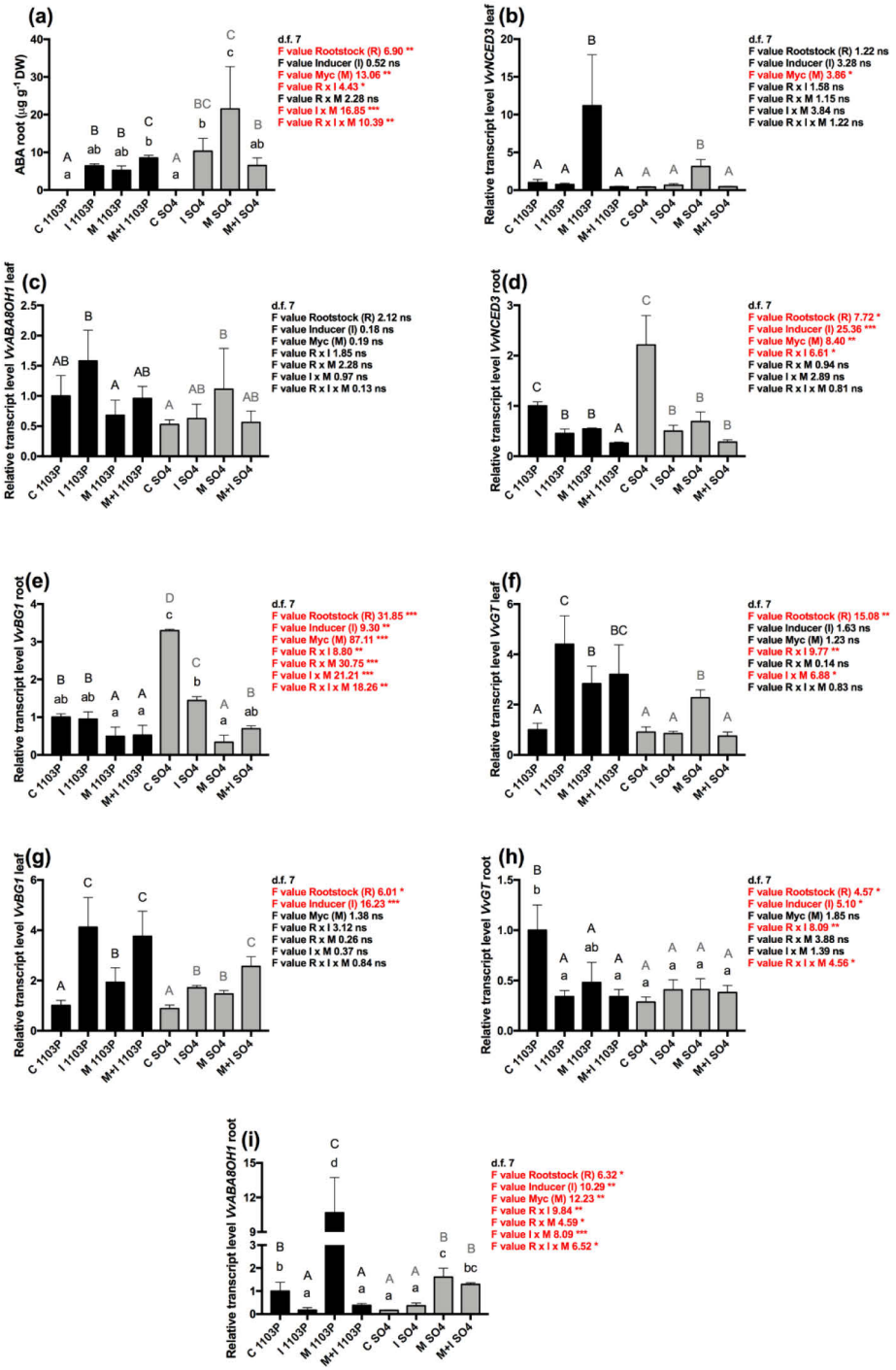

Figure 3 Expression changes of ABA-related genes and metabolite quantification in both root and leaf tissues. **a** ABA content in roots. **b** *VvNCED3* in leaf. **c** *VvABA8OH1* in leaf. **d** *VvNCED3* in root. **e** *VvBG1* in root. **f** *VvGT* in leaf. **g** *VvBG1* in leaf. **h** *VvGT* in root. **i** *VvABA8OH1* in root. All data are expressed as mean ± SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

To better understand the role of ABA in our system, the expression of ABA-related genes was analyzed in both leaves and roots. Relative expression of: i) a gene encoding for a 9-cis-epoxycarotenoid dioxygenase potentially involved in ABA biosynthesis (*VvNCED3,* VIT_19s0093g00550 previously reported as *VvNCED1*); ii) a gene coding for an enzyme involved in conversion of ABA to 8′-hydroxy ABA (*VvABA8OH1*); iii) a β-glucosidase (BG) involved in free ABA biosynthesis *via* hydrolysis of ABA glucose ester to release the ABA active form (*VvBG1*; Jia *et al*. 2016); iv) a gene encoding an ABA glucosyltransferase (*VvGT*; Sun *et al*. 2010) were evaluated in leaves and roots. In leaves, *VvNCED3* expression was not affected by rootstock genotype whereas M samples showed significantly higher expression levels with respect to the other samples (Fig. 3b). No significant difference was detected for *VvABA8OH1* expression in leaves although 1103P generally showed higher values with respect to SO4 (Fig. 3c). By contrast, *VvNCED3* expression in roots was influenced by R, M and I factors as well as by R x I interaction, and values for each rootstock genotype were lower in all treatments when compared to C plants (Fig. 3d). Similar to that observed in leaves, M+I treatment led to the significant lowest *VvNCED3* transcripts level in root samples (Fig. 3d). Two pathways promote free ABA accumulation: (1) NCED-mediated *de novo* synthesis (Qin and Zeevaart 1999) and (2) BG-mediated hydroxylation (Lee *et al*. 2006). Looking at *VvBG1* gene, its expression was significantly influenced by R and I in leaves, while the presence of the AMF was not significantly relevant. In roots all the factors and interactions, significantly affected *VvBG1* expression level, with the highest level in C SO4 samples (Fig. 3e,g). Finally, *VvGT* showed a trend similar to *VvBG1* in leaves where its expression was significantly influenced by R, I and I x M with the exception of SO4 samples where its expression was significantly higher only in M SO4 with respect to C SO4 (Fig. 3f). Conversely, in roots *VvGT* transcript levels were significantly lower in all the conditions with respect to the C 1103P plants (Fig. 3h).

Although *VvABA8OH1*, coding for an enzyme involved in ABA conversion, was not significantly regulated among genotypes and treatments in leaves, it results to be affected by all the considered factors and interactions in roots (Fig. 3i) where it appeared significantly upregulated in M 1103P, M SO4 and M+I SO4 plants with respect to their C (Fig. 3i). It is worth noting the low expression in I root samples, suggesting that the inducer may affect ABA catabolism independently from the genotype and the presence of the AM inoculum.

Defense

Stilbenes are the main defense-related metabolites synthesized in grapevine. In this study *trans*-resveratrol and viniferin levels were measured in leaves among the several conditions tested (Fig. 4, Table S2). Particularly, resveratrol was only affected by the MxI interaction, showing in parallel significantly higher levels in I and M plants, independently from genotype, with respect to M+I and C plants (Fig. 4a). Viniferin, which was not detectable in C plants, was affected by the M x I interaction and by the I factor alone. I, M and M+I treated plants presented in fact significantly higher values of viniferin than C plants in both rootstocks (Fig. 4b). To correlate biochemical data with molecular responses, expression levels of genes coding for two stilbene synthases (*VvSTS1* and *VvSTS48*) were assessed. Results showed that in both rootstocks *VvSTS1* was upregulated mainly in M 1103P whereas in SO4 plants was observed an upregulation in both I and M with respect to the other treatments (Fig. 4c). *VvSTS48* expression was influenced by all the factors and their interactions, with the highest expression value in leaves of I-treated SO4 plants (Fig. 4d). Looking independently at each rootstock, in 1103P only I and M induced significant overexpression of *VvSTS48* while in SO4 plants all the treatments showed enhanced gene expression compared to their controls (Fig. 4d).

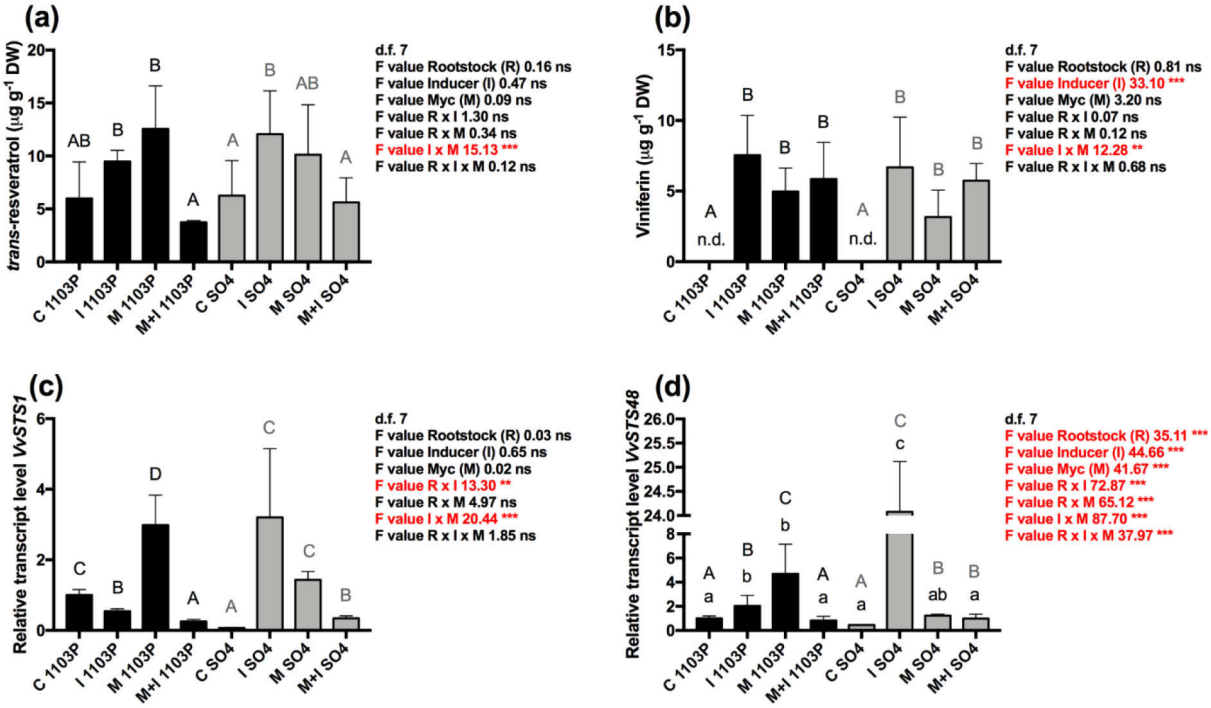

Figure 4 Expression changes of stilbenes-related genes and metabolites quantification in leaf tissues. **a** *trans*-resveratrol quantification. **b** Viniferin quantification. **c** *VvSTS1* gene expression changes. **d** *VvSTS48* gene expression changes. All data are expressed as mean ± SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks

RT-qPCR was also applied to detect the expression levels of several target genes as markers of diverse defense response pathways (Fig. S1, Table S2). Two genes were studied both in leaves and roots (a sugar transporter, *VvSPT13* and a class III chitinases, *VvChitIII*), three genes only in leaves (a callose synthase, *VvCAS2*; a lipoxygenase *VvLOX,* and the Enhanced Disease Susceptibility 1, *VvEDS1*) (Fig. S1a-g). Expression of all the considered genes were influenced by I factor, while influence by M was more variable, suggesting a different impact of the treatments on plant metabolism. Among these genes, *VvSTP13*, encoding a sugar transporter, in leaves of both rootstocks was significantly upregulated in all treatments with respect to their C plants (Fig. S1a) while in root only M-treated plants showed significantly higher expression values (Fig. S1). *VvChitIII* showed a different pattern in leaves and roots. In leaves, *VvChitIII* transcript was significantly induced in M- and M+I-treated plants (Fig.

S1c) while in roots an upregulation was observed only in M-treated ones (Fig. S1d). *VvCAS2*, coding for a callose synthase (Santi *et al*. 2013), showed a downregulation in all the treatments, while *VvLOX* gene, encoding a lipoxygenase involved in the jasmonic acid biosynthesis, was upregulated in all the treatments: among them, the lowest value was observed in M SO4 plants (similar to the C 1103P leaves), suggesting a different response to symbioses in the two genotypes (Fig. S1e-f). *VvEDS1*, selected as marker of Systemic Acquired Responses (SAR) mediated by Salicylic Acid (SA), was influenced by I and M, showing an upregulation trend in I-treated leaves. Conversely, this gene was downregulated in M-treated plants (Fig. S1g).

Rhizoplane metaphylogenomic analyses

Bacterial community was analyzed at both order and genus level: the number of retained sequences after chimera removal and taxonomical assignment was always above 35,000 (detailed results of sequencing are reported in Table S3). Shannon index diversity indicated that the only significant difference was observed for the I SO4 samples which show higher index values (Table S4). No significant differences were observed among samples comparing the Shannon index on the fungal community (Table S5). Similar to Shannon index, non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrixes showed that the bacterial community (Fig. 5a) is more affected by treatments than the fungal one (Fig. S2).

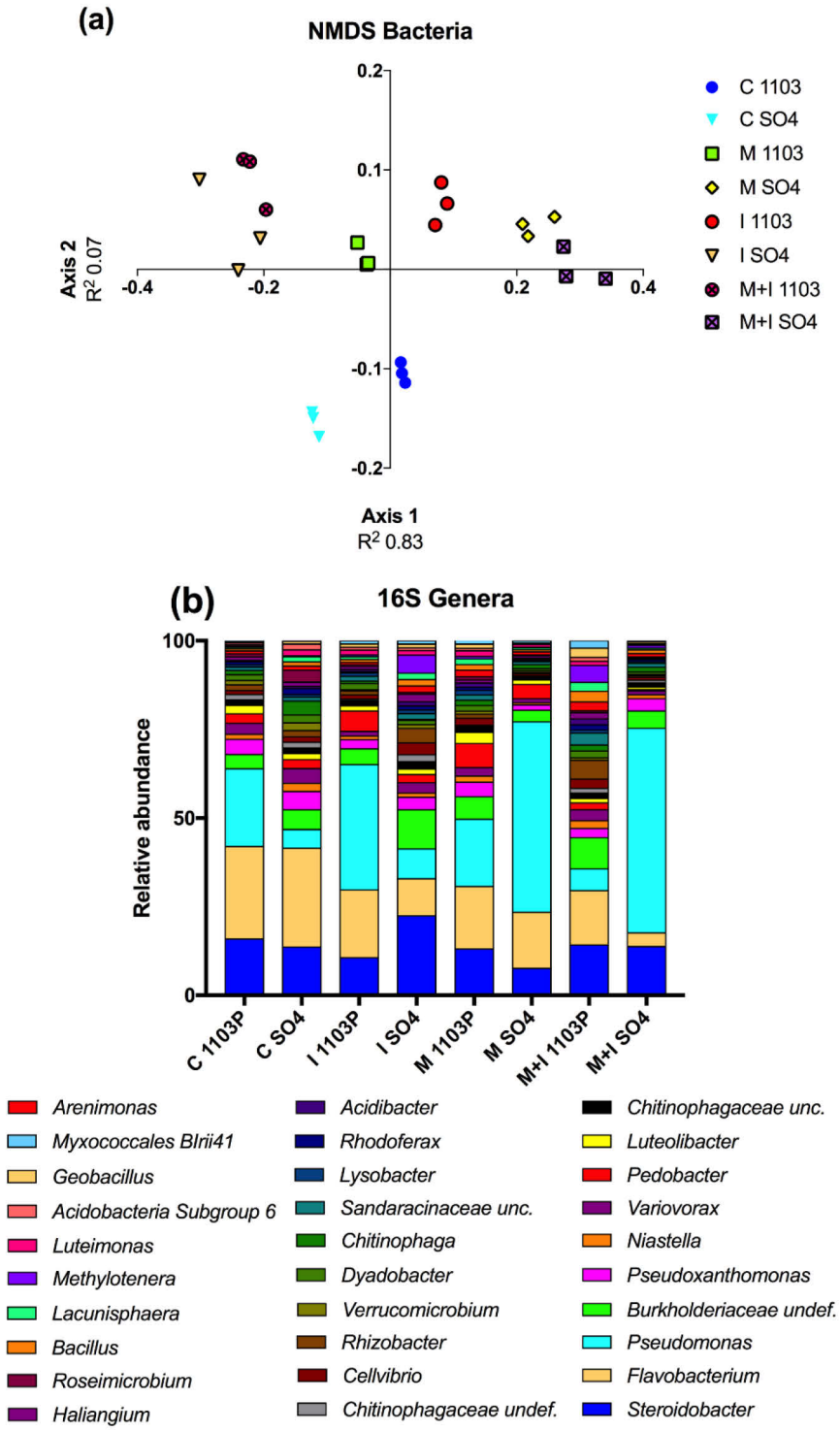

Figure 5 Distinct root-associated bacteria community composition among treatments. NMDS algorithm based on Bray–Curtis distances matrixes was used to reduce into a bi-dimensional scaling data obtained for bacteria community (**a**). Relative abundance of bacterial genera (**b**) among treatments. Only genera representing at least the 1% over the total number of classified amplicons were retained ($n = 3$). C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

The bacteria community composition for each sample type at order and genus levels are reported in Table S6. Statistical results of pairwise comparisons among genera are reported in Table S7. To simplify, results are described for the orders and genera that represent at least the 1% of the bacterial community (Fig. 5b). Comparison of the bacterial community between the two rootstocks (1103P *vs* SO4) revealed that 1103P has a significant higher relative abundance of *Pseudomonas* species whereas SO4 has a significant higher relative abundance of *Bacillus* species. Among the bacterial genera, which display significant differences among the treatments, M 1103P vines stimulated the presence of *Bacillus* species but repressed the interaction with *Pseudomonas* ones. In parallel, when comparing treatments on SO4 rootstock, a positive interaction between the mycorrhizal inoculation and the *Pseudomonas* abundance was observed, whereas the inducer treatment showed a negative impact on *Flavobacterium* abundance.

The fungal community composition for each sample type at order and genus levels are reported in Table S6. Statistics of the pairwise comparisons among genera are reported in Table S8. Results for the fungal orders and genera that represent at least the 1% of the fungal community are reported in Fig. S3. Focusing on AMF, results confirm the presence of *Rhizophagus* and *Funneliformis* in inoculated plants. However, AMF were detected also in the I-treated plants (Fig. 6a). Despite the presence of AMF associated to these roots, gene expression analysis on fungal PT genes showed the presence of *RiPT* and *FmPT* transcripts only in M-inoculated plants. Surprisingly, absent or low expression levels were detected in I-treated plants (Fig. 6b,c; Table S2). Indeed, fungal *PT* genes were expressed in a different way in the two genotypes, suggesting a different symbiosis efficiency of the two rootstocks. This finding was further confirmed by a plant PT gene (*VvPT1-3*), which expression level was mainly affected by R and M factors, and by 'R x I' interaction. It was up-regulated in 1103P roots, independently by treatment, with respect to C 1103P and strongly up-regulated in M SO4 ones (Fig. 6d, Table S2).

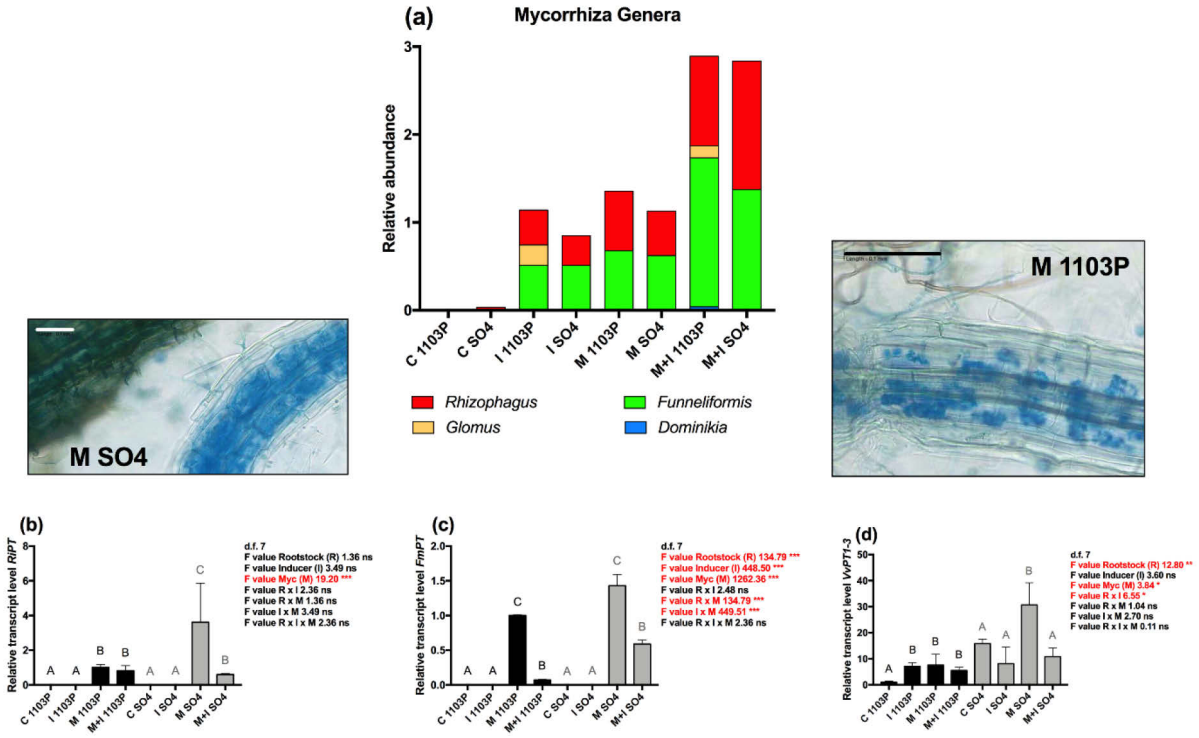

Figure 6 Mycorrhiza genera and expression changes of plant and fungus phosphate transporter (PT) genes as markers of functional symbioses. **a** Relative abundances of mycorrhiza genera ($n = 3$). **b** *RiPT*. **c** *FmPT*. **d** *VvPT1-3*. Gene expression data are expressed as mean ± SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks. Insets: microscope images of typical AM symbioses structures in 1103P and SO4 M-colonized roots

Comparing the fungal composition in C, 24 genera with significant differences of relative abundance were observed. Among the analyzed genera, *Clonostachys* displayed a significant negative correlation with all the treatment in both rootstock genotypes. Focusing on significant genera, usually involved in pathogenic interaction, such as *Fusarium*, *Rhizoctonia* and *Ilyonectria* (Fig. S4), the concomitant use of mycorrhizal inocula with the inducer brought to a significant reduction of *Ilyonectria* in both rootstocks. Conversely, *Fusarium* abundance was stimulated in all treatments except for the inoculation with AMF in the 1103P rootstock. Finally, *Rhizoctonia* genus was positively influenced by the inducer, but only in the SO4 rootstock.

Discussion

Treatments and genotypes differently shape the root-associated bacterial and fungal communities

The importance of root-associated microbes was extensively demonstrated in several crops including grapevine, with the potential to exploit biocontrol strategies that rely on the beneficial traits of plant growth-promoting microorganisms (PGPBs) naturally associated with plants (Verbon and Liberman 2016; Marasco *et al*. 2018; Yu *et al*. 2019). Among them, AMF and their impacts on diverse plant species, including economically important crops, have been largely studied highlighting the importance of this relationship that can positively affect both growth and defense traits (Jacott *et al*. 2017). However, despite these advantages, grapevine breeders normally focus their attention more on phenotypic or metabolic peculiarities rather than on the improvement of the interactions with root-associated microbes (Marín *et al*. 2021).

Grapevine roots are commonly colonized by different AMF taxa depending on the considered environment, season and soil management making them relevant in defining the 'microbial terroir' of a specific grape cultivar (Massa *et al*. 2020). Svenningsen *et al*. (2018) reported that AMF ecosystem services might be suppressed by some bacterial groups belonging to Acidobacteria, Actinobacteria, Firmicutes, Chitinophagaceae, and Proteobacteria. Our results showed an inverse correlation between the presence of some of these bacteria (*i.e.*, Acidobacteria, genus *Vicinamibacter* and Actinobacteria genus *Gaiella*) and AMF "functionality", although ITS sequencing showed a similar level in term AMF abundance. It is also necessary to consider that, ITS was used in the present work as universal fungal marker (Schoch *et al*. 2012; Lindahl *et al*. 2013) to better define the overall fungal population despite ribosomal large subunit (LSU) region consistently shows greater utility for taxonomic resolution for AMF (Xue *et al*. 2019a). Despite the latter approach can give better results, it has rarely been used in environmental studies of AMF because of sequencing and bioinformatics challenges (Delavaux *et al*. 2021). Similarly, for a better description of the AMF population, it was recently reported that, the use of AMF specific primers, coupled to nested PCR, can greatly help in better define the AMF population (Suzuki *et al*. 2020).

Additionally, results obtained from the microbiome analysis confirm that the response of microbial communities to the different treatments are genotype dependent (Marasco *et al*. 2018). This is particularly clear for the bacterial community, where the addition of the mycorrhizal inoculum promoted the *Pseudomonas* genus in 1103P and the *Bacillus* genus in SO4. It is important to remind that both these genera were largely investigated in grapevine because of their ability to protect vine plants against several fungal pathogens. *Pseudomonas* genus was studied for its ability to impair *Botrytis*, *Neofusiccocum*, *Ilyonectria*, *Aspergillus*, *Phaeomoniella* and *Phaeoacremonium* genera, which are all well-known grape fungal pathogens (Andreolli *et al*. 2019; Niem *et al*. 2020). On the other hand, *Bacillus* species were studied for their ability to reduce the impact of black foot disease (mainly due to infection by *Cylindrocarpon* and *Ilyonectria* species) and downy mildew on grapes (Zhang *et al*. 2017; Russi *et al*. 2020). These studies well fit with our data where we observed the lower *Ilyonectria* abundance in M+I 1103P and concomitantly the higher abundance of *Bacillus* species. Looking at the fungi, all the treatments promoted the presence of different AMF species, suggesting the recruitment of native AM fungal communities by the I-treated roots, independently from the rootstock genotypes. In detail, it is worth noting a higher diversity in AMF colonization in I 1103P with respect to I SO4 plants, independently from the presence of the AMF inoculum, confirming a diverse recruitment pattern for the two genotypes. Interestingly, *Clonostachys* genus negatively correlated with all the treatments. This genus was extensively studied for its promising exploitation as biological control agents against soil and root pathogens (Nygren *et al*. 2018; Sun *et al*. 2020). Considering that in all treatments the *Rhizophagus* genus was more abundant than in C, we can confirm that a mutual exclusion between *Clonostachys* and *Rhizophagus* genera is present. Although a full explanation for this reciprocally inhibitory interaction is still missing, the complex microbial community modulation mediated by the AM fungi could impair the ability of *Clonostachys* to endophytically colonize the host plant (Ravnskov *et al*. 2006; Akyol *et al*. 2018; Xue *et al*. 2019b). These findings, in accordance with the increase in defense-related metabolites and the expression data on defense-related genes, well fit with the concept of mycorrhizal-induce resistance (MIR) (Cameron *et al*. 2013) as a cumulative effect of direct and indirect (i.e. mediated by mycorrhizosphere associated microorganisms) defense responses. Recently, Emmett *et al*. (2021) also demonstrated that a conserved community is associated to AMF extraradical hyphae, suggesting an influence on the plant-fungal symbiosis.

AM fungi and root-associated microbes balance rootstocks growth traits showing a different pattern of functional symbioses

The impact of the different treatments on two different rootstock genotypes was evaluated. The selected rootstocks (i.e. 1103P and SO4) were well characterized at both agronomic and molecular level (Chitarra *et al*. 2017), showing opposite growth and defense attitudes. Among rootstock features, fine root development and density, imparting vigor to the scion, varied considerably with an impact on water and nutrient uptake as well as on the interaction with soil microorganisms. AM colonization showed that SO4 consistently presented higher levels of root colonization, together with Kober 5BB and Ruggieri 140, with respect to the others (Chitarra *et al*. 2017). This is in agreement with previous works (Bavaresco and Fogher 1996; Bavaresco *et al*. 2000), who showed a variation in the range of AM-colonized grape rootstocks among genotypes, which could be considered the main factor driving AM recruitment. However, functional symbiosis was strongly influenced also by scion requirements, soil fertility and soil pH (Bavaresco and Fogher 1996; Bavaresco *et al*. 2000). Here, both rooting and growth parameters, and partially the CCI, clearly showed a compensation effect in the less vigorous SO4 with respect to 1103P, reaching similar values in all the treatments. A role could be attributed to AMF particularly in SO4. To attest this hypothesis, considering that high-affinity PTs in AM have been characterized and it has clearly been demonstrated that plants possess a symbiotic Pi uptake pathway (Berruti *et al*. 2016), AM fungal PT genes (*RiPT* and *FmPT*) have been tested showing a highly expression in M SO4 for both, and also in M+I SO4 for *FmPT*. Similarly, the plant gene *VvPT1-3*, homolog of mycorrhiza-inducible inorganic phosphate transporters such as *LePT4* and *OsPT11* (Balestrini *et al*. 2017), was significantly up-regulated in M SO4. The positive effects exerted by AM symbiosis in growth and physiological features were largely documented in several plants (*e.g.*, Chitarra *et al*. 2016; Balestrini *et al*. 2020). Surprisingly, although the ITS sequencing showed a certain abundance of AM genera in both I and M+I, the inducer seemed to lower the expression of plant and fungal genes generally involved in symbiosis functioning. This should be related to presence of bacteria reported to diminish AMF functionality (Svenningsen *et al*. 2018). As well, an impact of the inducer on the number of fine roots, which are those colonized by AMF, cannot be excluded also considering that IAA was not detectable in I samples. Looking at the whole microbial

community, in addition to a selection based on the rootstock genotype, it is worth noting that I treatment (particularly I SO4) was able to significantly increase diversity of the microbiota (Table S4). Samples treated with the inducer showed higher bacterial diversity hosting many groups of PGPBs such as *Burkholderiaceae* that might be linked to potassium (K) and phosphorous (P) solubilization and availability (Gu *et al*. 2020); *Pseudomonas* and *Bacillus* spp. able to produce siderophores, auxin, cytokinins and characterized as phosphate-solubilizing bacteria (Saad *et al*. 2020; Subrahmanyam *et al*. 2020) (Table S7). These findings could explain the bacteria-mediated growth effects in I treatments particularly for the SO4 genotype. By contrast, the whole fungal diversity was not significantly affected among the treatments.

Nitrogen (N) is an essential element for all grapevine processes and N transporters were found among the genes upregulated by both a single AMF and a mixed bacterial-fungal inoculum through transcriptomics in grapevine roots (Balestrini *et al*. 2017). However, although AMF may positively influence plant N compound uptake and transport (Balestrini *et al*. 2020), negative, neutral or positive AMF effects on N nutrition has been reported (Bücking and Kafle 2015). Due to the fact that several nitrate transporters were found to be regulated by an AMF inoculum (Balestrini *et al*. 2017), the attention was mainly focused on nitrate uptake. Lower values of nitrate uptake with respect to controls were observed among all treatments, independently from the considered genotypes. Furthermore, any relevant effect on N accumulation in leaves was observed, suggesting that a positive correlation between N content and growth is not relevant in our system or likely due to a biomass dilution effect since the higher growth index recorded particularly in SO4-treated plants. AMF have been reported to show NH_4^+ preference to be assimilated in extraradical mycelium and translocated to plant roots after completion of the GS-GOGAT cycle (Balestrini *et al*. 2020). In this respect, to the plants side the lower NNU observed in M inoculated plants suggest a role of AMF in regulating root N uptake strategies helping plants in acquire N.

The plant hormone ABA is a chemical signal involved in the plant response to various abiotic environmental factors, but it can also play a role in interactions with phytopathogens by modulating tissue colonization depending on microorganism type, site and time of infection (Ton *et al*. 2009). An impact of ABA on AMF colonization has been also reported at diverse colonization stages (Bedini *et al*. 2018). A role for ABA in the mechanisms by which AM

symbiosis influences stomata conductance under drought stress was also suggested (Chitarra *et al*. 2016). Here, ABA levels were affected by both the genotype and the AMF inoculum. A significant effect of the M treatment was found on the expression of a key gene involved in the ABA synthesis in leaves *(VvNCED3*), showing a positive correlation with the ABA levels in roots. Our result is in accordance with the fact that ABA produced in leaves is then translocated in roots where it might act as a signal to promote root growth (McAdam *et al*. 2016). AMF presence led to higher ABA content in M SO4 roots, despite the fact that generally SO4 rootstock was reported to have a low endogenous content (Chitarra *et al*. 2017), suggesting a potential enhanced tolerance to abiotic stresses in M SO4. As already reported by (Ferrero *et al*. 2018), the relationship between biosynthetic and catabolic processes may be complex and diverse in the different plant organs. Our results showed a different expression pattern of most of the considered genes involved in ABA synthesis and catabolism in leaves and roots. A gene coding for an ABA 8'-hydroxylase (*VvABA8OH1*), belonging to the CYP707A gene family and with a primary role in ABA catabolism, showed an opposite trend in M and I root apparatus, in agreement with the ABA root accumulation. Overall, obtained data are in accordance with that reported by Martín-Rodríguez *et al*. (2016) showing that both ABA biosynthesis and catabolism are finely tuned in AM-colonized roots. Although with the activation of different mechanisms depending on the treatment, an impact on ABA homeostasis can be suggested particularly in SO4 genotype.

AM symbiosis triggers defence-related transcripts and metabolites more in 1103P than in SO4 rootstock

Plants finely tune the immune system to control both pathogen infection and beneficial microorganism accommodation. Soil bacteria and fungi play a double role in promoting growth and defense response, helping in maintaining the homeostasis in the whole microbial communities associated to the roots through the Induced Systemic Resistance (ISR) pathways (Liu *et al*. 2020). In grapevine, stilbenes are phytoalexins with proved antifungal activities (Chalal *et al*. 2014). Here, resveratrol content was higher in I and M leaves with respect to untreated controls, while viniferin, that is highly toxic for grape foliar pathogens such as downy and powdery mildew (Chitarra *et al*. 2017), has a similar trend in all the treatments while it was not detected in C plants. These patterns clearly highlight a stimulating effect mediated by root-associated microbes (native or inoculated), with

differences that might be related to the diverse microbiome composition. Among the genes involved in stilbene synthesis, *VvSTS48*, coding for a stilbene synthase reported as induced by downy mildew infection, showed the highest expression value in I SO4 plants, suggesting a different modulation among treatments and genotypes.

Carbohydrate metabolism is also involved in plant defense responses against foliar pathogens (Sanmartín *et al*. 2020). In tomato, AM symbiosis was reported to be involved in *Botrytis cinerea* resistance through the mycorrhiza-induced resistance (MIR) mediated by callose accumulation. A tomato callose synthase gene (*PMR4*) was in fact upregulated by mycorrhization mainly upon biotic infection (Sanmartín *et al*. 2020). In the present study, attention has been focused on the homolog grape gene *VvCAS2*. Conversely to that previously observed, *VvCAS2* showed a downregulation trend in all the treatments with respect to control plants. These findings suggest a primary role in microbe-mediate stimulating of defense responses against biotic factors in grape. Since a correlation between MIR and sugar signaling pathway was reported (Sanmartín *et al*. 2020), the expression of a grapevine sugar transporter gene (*VvSTP13*), homolog to the *Arabidopsis STP13*, involved in intracellular glucose uptake and in *B. cinerea* resistance, was followed in leaves and roots. Although total soluble carbohydrates were not affected by treatments in leaves, *VvSTP13* expression showed an upregulation trend in all the treatments, particularly in both I sample and M 1103P leaves, suggesting an effect of AMF inoculum in the susceptible genotype. Looking at the roots, *VvSTP13* upregulation trend was observed mainly in mycorrhizal roots, in agreement with the fact that expression of genes from the STP family was revealed in arbuscule-containing cells of *Medicago truncatula* (Hennion *et al*. 2019). The same trend observed for *VvSTP13* was also found for a gene coding for a class III chitinase (*VvChitIII*). Class III chitinases have been already reported to be markers of functional symbioses (Balestrini *et al*. 2017), being localized in arbuscule-containing cells (Hogekamp *et al*. 2011). Finally, the expression of two target genes (*VvLOX* and *VvEDS1*), respectively involved in ISR mediated by jasmonate and SAR mediated by salicylic acid, although differently modulated by the inducer and AM fungi, confirmed the role of the whole microbiome on the plant immunity system in the scion of both rootstock genotypes (Cameron *et al*. 2013).

Conclusion

Overall, our results allowed to provide new insights into growth-defense tradeoffs responses in a model fruit crop (Fig. 7). Although molecular mechanisms at the basis of plant priming are still matter of debate, several hypotheses have been proposed. In this study, a finely tune regulation of growth and defence traits have been highlighted considering three main influencing factors, *i.e.,* the plant genotype, an AM inoculum and an oligosaccharide described as involved in AMF colonization induction. The attention has been focused on two rootstocks characterised by opposite trade-offs. Growth traits have been improved mainly in the low vigour genotype (SO4) by all the treatments probably through the activation of diverse pathways by the root associated microbes. It is worth noting that all the treatments shaped the microbial communities associated to the roots in both the genotypes. Looking at the defence response, a positive impact on immunity system has been revealed both by the AMF inoculum and the oligosaccharide, although with the activation of different pathways. Results suggest that AM symbiosis triggers a mycorrhiza-induced resistance (MIR) also in a model woody plant such as grapevine.

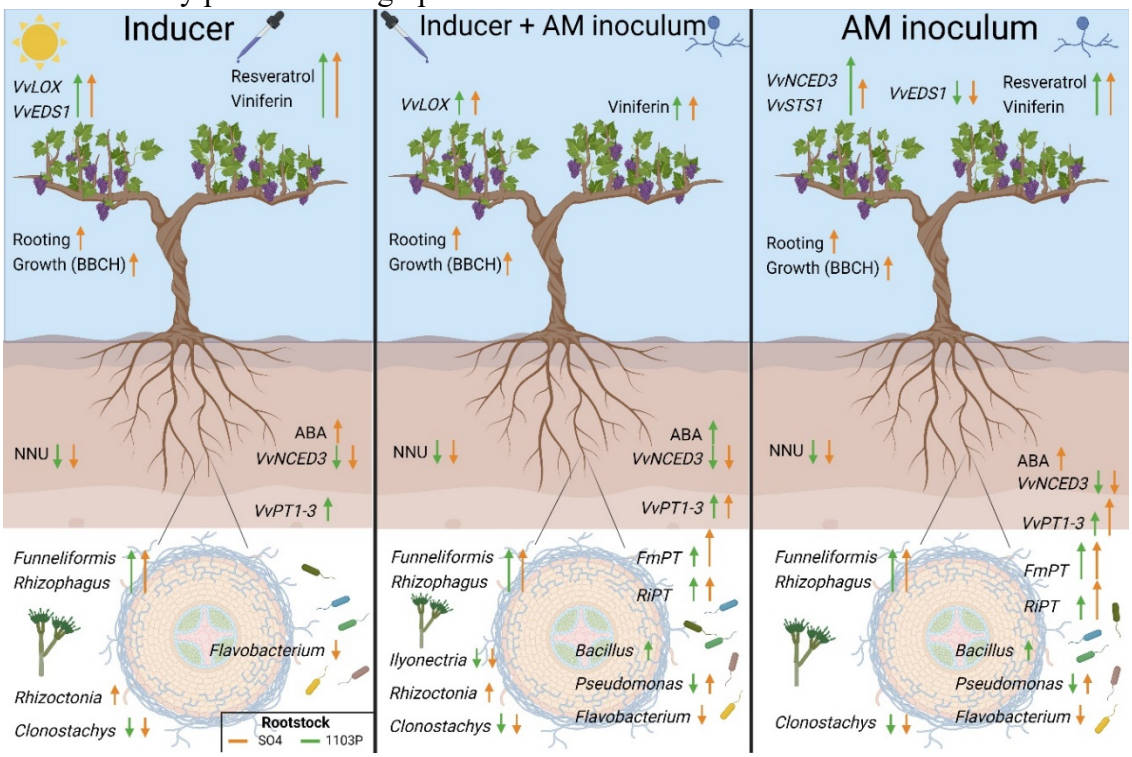

Figure 7 Overview of phenotypic, biochemical and molecular changes induced by the treatments. Green arrows indicate responses in 1103 Paulsen (1103P) rootstock whereas orange ones are referred to SO4 genotype. Upward arrows indicate an increase whereas downward arrows represent a decrease in content of metabolites or gene relative expression or relative abundance of microbial taxa with respect to control (C) plants. NNU, net nitrate uptake; ABA, abscisic acid.

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Supporting informations

All supporting informations are available online:

Nerva, L., Giudice, G., Quiroga, G. *et al.* Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs. *Biol Fertil Soils* **58,** 17–34 (2022). https://doi.org/10.1007/s00374-021-01607-8

CHAPTER 7

General conclusions and future perspectives

Considering both the vineyard system as the most treated crop, and the European policies about the reduction of chemical inputs, the development of new strategies to control pests and diseases is a mandatory.

This research aims to provide a solution to reduce chemical applications in vineyards through the state-of-the-art technologies.

In Chapter 3, we investigate the current used approach to obtain more resistant grapevine genotypes to *Plasmopara viticola* and *Erysiphe necator*. We crossed cv. Glera with many genotypes carrying few resistant genes, indeed the pyramidization of resistance genes helps to strengthen the resistance durability. The crossing event is followed by the selection of offspring through MAS. Then, the plants carrying resistance genes were evaluated for agronomical traits and aromatic profile.

In Chapter 4, we present the development of a CRISPR/Cas9 based on site specific recombinase Cre/LoxP gene editing system mediated by *Agrobacterium tumefaciens* for the production of grapevine cultivars with reduced susceptibility to *Erysiphe necator* and, in general, to biotrophic fungi.

In fact, the inactivation of a susceptibility genes, required for compatible plant-pathogen interaction, could be considered as a revolutionary tool to breed grapevine resistant genotypes, since it is more durable compared to resistance gene-based strategies described in Chapter 3. Moreover, in order to remove the exogenous DNA introduced in the genome through *Agrobacterium tumefaciens*, the Cre/LoxP site-specific recombination system was exploited. The T-DNA removal from edited plants with reduced susceptibility to the disease, also allows to protect them from any effects due to the presence of an exogenous endonuclease and, simultaneously, to add other traits of interest by subsequent gene transfer rounds using the same selection marker gene. This methodology could represent a promising alternative strategy to the classical breeding, especially for those woody plant species which require long maturation and crossing times.

The Chapter 6 aims to investigate the potential benefits of two AM fungal species inoculum with or without a monosaccharide addition. Their effects were evaluated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. Interestingly, for both rootstocks, the

monosaccharide fostered the recruitment of native AMF present in the soil. Moreover, the microbes recruitment led to stilbenes accumulation inducing antifungal activities. These findings suggest that also in grapevine the mycorrhiza induced resistance could improve plant protection.

All the strategies presented in this thesis provide practical solutions for the pathogens control in a sustainable manner, however there are limits that need to be addressed, including time-consuming processes and host specialization in conventional breeding approach, genotype - dependent regeneration efficiency in somatic embryogenesis and evaluation of new AMF or their inducers to achieve a more sustainable agriculture.

General Appendices

APPENDIX I: Poster and oral presentations

Poster presentation at: 6th International Horticulture Research Conference 4th October 2019, Venice, Italy.

This work was awarded 'Third Best Poster'.

Bioinformatic approaches to identify target-specific guide RNAs for CRISPRCas9 genome editing

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Vitis vinifera L. is the most cultivated species in the world for wines, raisins, juices and sparkling productions. The majority of cultivated grapevines are highly susceptible to several pathogens and, *Erysiphe necator*, the causal agent of powdery mildew, is one of the most devastating. To manage this pathogen many fungicides applications are required, making the viticulture one of the agricultural activities with the greatest chemical input. The genetic improvement of the grapevine gains a great benefit from New Plant Breeding Technologies, which resemble traditional breeding but with shorter times and lower impact on the cultivar's genome. In order to reduce the fungus susceptibility our approach will be the knocking out two different members of MLO gene family. Three different editing events will be performed in order to evaluate the activity of each gene, and the synergic effect of their knock-out. This purpose will be achieved by CRISPR/Cas technology applied to Glera, Pinot Nero, Sangiovese, three of the most cultivated Italian varieties. Even if this technique let us to introduce an

InDel in very specific target sequences, it could manage off target activities in different regions of genomic DNA which have a similar sequence. To reduce this effect, we use different tools to design guides, predict on-target editing efficiency and minimize unspecific events. Each software was used to calculate gRNAs for *VviMLO6* and *VviMLO7*, using as a reference the PN40024, the near-homozygous line sequenced Pinot. We analysed SNPs calling for our varieties, we considered the specificity for each gene, as final criteria for guides selection, as well as the absence of mismatches with genomic DNA. Finally, in order to obtain transformed plants, we induced dedifferentiated embryogenic calli from ovaries and anthers. Due to a short temporal availability of optimal development stage of flowers in field, we developed a strategy to obtain flowers during throughout the year from winter cuttings. These materials will be used to perform *A.tumefaciens*-mediated stable transformations.

Poster presentation at: SIGA-SEI-SIBV-SIPAV Web Workshop "Young Scientists for Plant Health" 16 December, 2020

New plant breeding technologies toward a more sustainable viticulture

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genome editing, CRISPR/Cas9, Cre-loxP system, embryogenic calli, grapevine

European grapevine cultivars are highly susceptible to many pathogens that are managed through large pesticide use. Nevertheless, the European policies promote pesticide use reduction and new environmentally friendly methods for a more sustainable agriculture. In this framework, grapevine genetic improvement could benefit from New Plant Breeding Technologies. In order to reduce fungal susceptibility, we will produce knock-out plants from embryogenic calli using CRISPR/Cas9 technology. Studies in barley reported the acquisition of powdery mildew resistance by knocking out susceptibility genes belonging to the MLO (*Mildew Locus O*) family. In this study, our approach takes advantage from CRISPR/Cas9 technology to perform a multiple knockout of MLO genes. Among the 17 *VvMLOs* reported in grapevine we designed constructs to target *VvMLO6* and *VvMLO7*. Golden Gate assembly was used to produce three different constructs (containing two

guideRNAs for each gene) to knocking-out the targets singularly or by producing a double mutant. Usually, the genetic engineering techniques, mediated by A. tumefaciens, involve the insertion of exogenous selectable marker genes. These markers are required for selection of transgenic plants, but they are undesirable to be retained in commercial transgenic plants due to possible toxicity or allergenicity to humans and potential environmental hazard. To overcome these limits, we opted for a "clean" editing strategy developing an inducible excision system. This approach is based on a recombinase technology involving the Cre-loxP system from the P1 bacteriophage under a heat-shock inducible promoter to be activated once the editing event(s) will be confirmed. Obtainment of embryogenic calli is one of the main bottlenecks for application of CRISPR/Cas9: for two seasons, we collected inflorescences from Chardonnay, Glera, Microvine, Pinot Noir, Sangiovese cultivars and two rootstocks, 110 Richter and SO4, cultured and maintained in vitro up to embryo development and then used to perform *Agrobacterium tumefaciens* GV3101 mediated transformation.

Oral presentation at: III Giornate Scientifiche SOI "I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana" 22-23 June 2021

Sustainable viticulture through NPBTs for biotic and abiotic stress management

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Key words: cisgenesis, genome editing, biotic and abiotic stress, Cre-lox, grapevine

New plant breeding techniques (NPBTs) aim to overcome traditional breeding limits for plant improvement to biotic and abiotic stresses satisfying the European Policies requirements that promote chemical input reduction and new environmental-friendly methods for a more sustainable agriculture. We decide to apply genome editing (via CRISPR/Cas9) focusing on susceptibility genes to control powdery mildew: we chosen to knock-out two genes belonging to MLO (*Mildew Locus O*) family: *VvMLO7* and *VvMLO6*. In parallel we used the same approach to cope with abiotic stresses, in specific drought, performing a knock-out of four genes, two belonging to GST (*Glutathione S-Transferase*)

and two to PME (*Pectin Methyl Esterase*) gene families. Previous studies demonstrated a better drought tolerance in knock-out mutant for both these two gene families.
In parallel to genome editing, we also applied cisgenesis to move the resistance locus *RPV*3-1 (Resistance to *Plasmopara viticola*)(†) into economically important cultivars. This locus is formed by two different genes that were inserted (with native promoters and terminators) individually and in combination to evaluate their effects.
One of the drawbacks linked to classical *Agrobacterium tumefaciens* mediated transformation is the insertion of unrelated selectable marker genes (e.g., antibiotic resistance). These markers are required for transgenic plants selection, but undesirable to be retained in commercial plants due to possible toxicity or allergenicity to humans and animals, in addition to their potential hazards for the environment. To overcome these limits, we developed a "clean" transformation strategy using an inducible excision system based on a recombinase technology from the P1 bacteriophage. The Cre-lox system is controlled by a heat-shock inducible promoter that will be activated once the transformation event(s) will be confirmed. Embryogenic calli of Chardonnay, Glera, Microvine, Pinot Noir, Sangiovese, were used in stable transformation with *A. tumefaciens* GV3101 carrying the genome editing construct with the MLO-guideRNAs (two for each gene) and the cisgenic construct carrying the two *RPV*3-1 genes. Embryogenic calli of rootstocks 110 Richter and SO4 were transformed with genom editing construct carrying GST and PME guideRNAs in two independent transformations. Regenerated embryos from all the transformation events are now under evaluation.

(†) In cooperation with the Institut of Applied Genomics, Udine.

Poster presentation at: “II Convegno AISSA #UNDER40” July 1-2 2021, Sassari, Italy

NPBTs for sustainable viticulture management to biotic and abiotic stress

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transgene (e.g., antibiotic resistance). These markers are required for transgenic plants selection, but undesirable to be retained in commercial plants due to possible toxicity or allergenicity to humans and animals, in addition to their potential hazards for the environment. To overcome these limits, we exploit an inducible excision system based on a Cre-lox recombinase technology controlled by a heat-shock inducible promoter that will be activated once the transformation event(s) will be confirmed. Embryogenic calli of Chardonnay, Glera, Microvine, Pinot Noir, Sangiovese, were used in stable transformation with A. tumefaciens carrying the genome editing construct with the MLO-guideRNAs and the cisgenic construct carrying the two *RPV*3-1 genes. Embryogenic calli of rootstocks 110 Richter and SO4 were transformed with genome editing construct carrying GST and PME guideRNAs in two independent transformations. Regenerated embryos from all the transformation events are now under evaluation.

Oral and poster presentation at: "2nd PlantED Conference: Plant genome editing: the wide range of applications" September 20-22 2021, Lecce Italy

This work won the 'Best Poster Award' during poster competition.

NPBTs for sustainable viticulture management to biotic and abiotic stress

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APPENDIX II: Scientific contributions

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