Euphytica (2022) 218:5
https://doi.org/10.1007/s10681-021-02952-3

Molecular characterization of the *Rpv3* locus towards the development of KASP markers for downy mildew resistance in grapevine (*Vitis* spp.)

Andriele Wairich · Jaiana Malabarba · Vanessa Buffon · Diogo D. Porto · Roberto Togawa · Luís F. Revers

Received: 25 March 2021 / Accepted: 16 November 2021 / Published online: 10 December 2021

Abstract *Plasmopara viticola* is the oomycete that causes downy mildew in grapevines. Varying levels of resistance to *P. viticola* across grape cultivars allowed quantitative trait loci to be identified. The *Rpv3* locus is located at chromosome 18, in a region enriched in TIR-NBS-LRR genes, and the phenotype associated is a high hypersensitive response. In this work, we aimed to identify candidate genes associated with resistance to downy mildew on the *Rpv3* locus and to evaluate their transcriptional profiles in a susceptible and a resistant grapevine cultivar after challenging with *P. viticola*. Candidate genes were identified by in silico functional enrichment tests. Many predicted genes associated with resistance to diseases were found at the *Rpv3* locus. In total, seventeen genes were evaluated by RT-qPCR. Differences in the steady-state expression of these genes were observed between the two cultivars. Four genes were found to be expressed only in Villard Blanc, suggesting their association to the hypersensitivity reaction. Aiming to assist marker assisted-selection for downy mildew resistance, we show the efficient use of a set of SSR markers. Furthermore, from on a set of forty-one *Rpv3*-located SNPs, whose segregation was tested in the populations studied, the two segregating markers, Rpv3_15 and Rpv3_33, were considered efficient for downy mildew resistance identification. This study presents a genomic characterization of the *Rpv3* locus, confirms its involvement in resistance against *P. viticola* infection and presents promising biotechnological tools for the selection of young resistant individuals.

Keywords Downy mildew resistance · *Plasmopara viticola* · RT-qPCR · *Rpv3* locus · *V. vinifera* · Marker-assisted selection

Andriele Wairich, Jaiana Malabarba authors contributed equally to this work

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10681-021-02952-3.

A. Wairich · J. Malabarba
Graduate Program in Cell and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

A. Wairich · J. Malabarba · V. Buffon · L. F. Revers (✉)
Laboratory of Plant Molecular Genetics, Embrapa Uva e Vinho, Rua Livramento, 515,
P.O. Box 130, CEP, Bento Gonçalves 95701-008, Brazil
e-mail: luis.revers@embrapa.br

D. D. Porto
Embrapa Semiárido, Petrolina, PE, Brazil

R. Togawa
Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil

Introduction

Fungal diseases are one of the main constraints for grape production, both quantitatively and qualitatively (Saifert et al. 2018). In humid subtropical areas, as southern Brazil, the most prevalent fungal pathogens responsible for diseases in grapevines aerial parts are *Elsinoë ampelina*, *Plasmopara viticola,* and *Uncinula necator,* agents that cause, respectively, the anthracnose, downy mildew and powdery mildew diseases (IBRAVIN 2012). *P. viticola* (Berk. Et Curt.) Berl. et de Toni. is a major biotrophic oomycete belonging to the Peronosporaceae (Chromalveolata), known to be a large family of phytopathogens (Grenville-Briggs and Van West 2005). Grapevine infection by this oomycete starts by the zoospores infiltration via the stomata with the development of a tubular network of hyphae culminating in haustoria proliferation in the leaf mesophyll (Fröbel and Zyprian 2019). After entering the grapevine tissues, it colonizes leaf, petioles, shoots, berries and seeds by obtaining nutrients from the infected plant cells with the help of specialized fan-shaped hyphae, that seem to be necessary to overcome physical barriers in plant tissues (Fröbel and Zyprian 2019). The haustoria also allows the exchange of signals involved in the compatibility infection establishment, allowing the pathogen to growth intercellularly, retrieve nutrients and also playing a role in developing beneath appressoria and arise from penetration pegs (Polesani et al. 2010; Fröbel and Zyprian 2019). *Vitis vinifera* cultivars are, in general, susceptible to downy mildew infection, which can cause total production loss by the destruction of inflorescences and/or fruits and by premature defoliation (Erwin and Ribeiro 1996).

In attempting to reduce losses, the intense application of multiple fungicides becomes necessary during each new growth cycle (Gisi et al., 2007). Even after managed, *P. viticola* infection may still trigger future crop damages, due to the poor formation of the branches and consequent plant weakened development (Tessmann and Vida 2005; Garrido, L. da R. Sônego 2007). In addition, the disease control measures increase production costs, pose a risk factor for human health and generate environmental impacts in the application areas, such as contamination of soil and groundwater with chemical residues (Brun et al. 2003; Cus et al. 2010). Besides, there are some *P. viticola* strains with resistance to certain fungicides, which leads to a decrease in the efficiency of disease control (Blum et al. 2010; Wang et al. 2013; Kassemeyer et al. 2015; Santos et al. 2020).

Plant defence responses to pathogens are triggered after the recognition of exogenous chemical signatures by the host which initiate pattern-triggered immunity (PTI) (Jones and Dangl 2006). Thereafter, PTI is followed by the immune response signal transduction mechanisms resulting in a significant reprogramming of the plant cell metabolism, which involves changes in gene expression activity, and PTI is able to prevent non-adapted pathogens from successfully colonizing the plant and causing disease. (Díez-Navajas et al. 2008; Pinto et al. 2012; Bigeard et al. 2015; Eisenmann et al. 2019). This response triggered by plants does not inhibit colonization of the pathogen, but it limits the extent of propagation since the action of disease resistance proteins (R proteins) accelerates and amplifies the innate basal response process. This happens when plants can recognize the pathogen's effectors via R proteins initiating the effector-triggered immunity (ETI). Plants that are not able to detect these effectors are susceptible to the infecting pathogen, resulting in effector-triggered susceptibility (ETS) (Belkhadir et al. 2004; Flor, 1971; Bigeard et al. 2015). In this context, ETI is an accelerated and amplified PTI response, leading to disease resistance and, in general, a hypersensitive cell death response at the infection site (Jones and Dangl 2006).

The downy mildew resistance mechanism includes cell wall strengthening, production of antimicrobial compounds such as phytoalexins, the assembly of complex ETI responses such as the hypersensitivity reaction (HR), which is the programmed death of the cells around the infected region, blocking the pathogen progression and the pathogenesis-related (PR) protein synthesis (Polesani et al. 2010, Brili et al. 2018). North American grapevine species (*V. riparia, V. cinera, V. labrusca, V. rupestris, V. berlandieri, V. lincecumii* and *Muscadinia rotundifolia*) and Asian species (*V. amurensis*) present variable levels of resistance to *P. viticola* attack (Merdinoglu et al 2003; Kortekamp and Zyprian 2003; Unger et al. 2007; Díez-Navajas et al. 2008, Fröbel et al. 2019b). More than 200 RGA (R-gene analogs) resistance genes were identified in grapevine species employing genetic and genomic approaches. (Velasco et al. 2007, Jaillon et al 2007, Canaguier et al. 2017, Liang et al 2019, Di Gaspero and Cipriani 2003). Many of these genes are located in

genomic regions associated with resistance to *P. viticola* in wild *Vitis* species and many have orthologous genes in *Arabidopsis thaliana* that regulate pathways of defence against pathogens (Sargolzaei et al. 2020). Furthermore, the variable levels of resistance to *P. viticola* presented by North American species allowed for the identification of R genes and quantitative trait loci (QTLs) as reported by Merdinoglu et al. (2003), Fischer et al. (2004), Welter et al. (2007), Revers et al. (2010), Blasi et al. (2011), Moreira et al. (2011) and Schwander et al. (2012). Thirty-one QTLs were detected with effect on downy mildew resistance in grapevines, as *Rpv1* (Merdinoglu et al. 2003), *Rpv2* and *Rpv3* (Fischer et al 2004; Welter et al 2007; Bellin et al. 2009), *Rpv5* (Marguerit et al. 2009), *Rpv8* (Blasi et al. 2011), *Rpv10* (Schwander et al. 2012), *Rpv11* (Salmaso et al. 2008; Schwander et al. 2012) and *Rpv12* (Venuti et al. 2013). In a more recent report, the discovery of another three loci conferring resistance against *P. viticola* in *V. vinifera* Georgian germplasm, *Rpv29, Rpv30* and *Rpv31,* allowed the identification of potential target genes for grapevine breeding against *P. viticola* based on the possibility to perform crosses with cultivated varieties that present good agronomic traits (Sargolzaei et al. 2020).

The *Rpv3* locus is located in the linkage group (LG) 18 and was identified by different research groups as being associated with a strong HR against downy mildew in resistant individuals (Fischer et al. 2004; Welter et al. 2007; Bellin et al. 2009; Revers et al. 2010; Schwander et al. 2012; Eisenmann et al. 2019; Possamai et al. 2020). This locus accounts for up to 75% of the phenotypic variation, and more than 30 R genes encoding TIR-NBS-LRR proteins and LRR-kinase-type receptor proteins are located within the *Rpv3* region (Velasco et al. 2007; Bellin et al. 2009). The *Rpv3* is also co-localized with the *VviAGL11* locus (AGAMOUS-LIKE 11 (Costantini et al. 2008)), a major QTL for seedlessness, which is a quantitative complex trait (Ocarez et al. 2020). Currently it is known that the *Rpv3* locus contains at least three different haplotypes (*Rpv3-1, Rpv3-2* and *Rpv3-3*) in segregating populations for downy mildew resistance (Welter et al., 2007; Di Gaspero et al., 2012; Zyprian et al., 2016). The mediated resistance of *Rpv3–1,* which was identified in the cultivar Villard Blanc, is associated with a defence mechanism that triggers synthesis of fungi-toxic stilbenes and HR, resulting in inhibition of pathogen growth and development (Eisenmann et al. 2019). In addition, the transient co-expression of TIR-NB-LRR pair genes, from *Rpv3* locus, in *V. vinifera* leaves activated the HR induced by the pathogen and the sporulation was reduced when compared with leaves from non-transformed plants (Foria et al. 2020).

The TIR-NBS-LRR proteins are related to gene-to-gene resistance response, or the zigzag model (Jones and Dangl 2006). This mechanism is based on the presence of the proteins encoded by the R genes that act in the recognition of specific race effectors, the Avr proteins (Takken et al. 2006; Díez-Navajas et al. 2008; Dry et al. 2010). Recently, a secretome of *P. viticola* was predicted by transcriptome sequencing analysis and 51 RxLR effector candidates were identified (Xiang et al 2016) and, in addition *P. viticola* genome contains a total of 1,301 putative secreted proteins, including 100 putative RXLR effectors and 90 crinkling and necrosis-inducing (CRN) effectors (Yin et al 2017). TIR-NBS-LRR proteins are responsible for the recognition of pathogen-associated molecular patterns (PAMPs), being triggered by effector action/Avr proteins perturbations (Takken et al. 2006). These proteins are composed of three major domains: 1) an amino-terminal variable domain (coiled-coil (CC) or a homologous domain Toll/Interleukin -1- Receptor (TIR), 2) a central nucleotide-binding site (NBS), and 3) leucine-rich repeats (LRR), which is responsible for the recognition of pathogen effectors (Takken et al. 2006). When TIR-NBS-LRR levels increase, they trigger the induction of a rapid defence response that is characterized by calcium and iron fluxes, extracellular oxidative burst, and transcriptional reprogramming in the infected sites and in the peripheral regions, culminating with the HR, ceasing the growth of the pathogen (Belkhadir et al. 2004; Jones and Takemoto 2004; Greenberg and Vinatzer 2003). Therefore, these proteins are determinants of the specificity of the immune response (Takken et al. 2006; Díez-Navajas et al. 2008; Dry et al. 2010).

We chose the cultivar Cabernet Sauvignon because it is one of the most prestigious *V. vinifera* varieties being cultivated in all producing regions because of its capacity to maintain aromas and flavours, independently of the region where it is cultivated (Figueira 2013). However, this variety is sensitive to a series of biotic stresses, for example, to the attack of grapevine pathogens such as *P. viticola* and *U. necator* (Figueira

2013). Contrasting to 'Cabernet Sauvignon', the downy mildew resistant cultivar selected for this study, Villard Blanc, also known as Seyve Villard 12,375, is a member of the Seyve Villard family (INRA et al. 2013). 'Villard Blanc' is a complex hybrid composed by accesses of six *Vitis* species: *V. aestivalis, V. berlandieri, V. cinera, V. lincecummi, V. rupestris* and *V. vinifera* (INRA et al. 2013). This cultivar presents a strong HR that does not allow the spread of infection beyond the infected cell, which may delay the growth of the pathogen in many interactions, particularly those involving haustoria parasites (Jones and Dangl 2006).

In this work we aimed to identify candidate genes associated with resistance to downy mildew at the *Rpv3* locus in grapevine and evaluate their transcriptional profiles in the susceptible cultivar, Cabernet Sauvignon, and in the resistant cultivar, Villard Blanc, after challenging with *P. viticola*. Moreover, we used SSR markers and the KASP ™ genotyping assay to identify single nucleotide polymorphisms (SNPs) for the evaluation of the biotechnological applicability of candidate markers, located within the *Rpv3* locus and associated to the resistance phenotype.

Materials and methods

Plant material

P. viticola challenge assay prior to gene expression analysis

Leaves from the cultivar Isabel (*V. labrusca*) naturally infected with downy mildew were harvested from a vineyard in the district of Faria Lemos (29°06′13,09 '' South and 51°36′26,10 '' West—362 m altitude) in Bento Gonçalves – RS, Brazil. This vineyard was subjected to no phytosanitary treatments. The leaves were humidified and stored in a humid chamber at 25 °C and in the absence of light, until sporangia formation. For *P. viticola* challenge assay, 20 young branches of 'Cabernet Sauvignon' and 20 young branches of 'Villard Blanc' were harvested in the vineyards of Embrapa Uva e Vinho headquarters (29°09′48'' South and 51°31′53.95″ West – 640 m altitude) and settledon a Styrofoam box. For obtaining optimum *P. viticola* sporangia growth to be used in the sporulation assay a dark and humid incubation chamber was created to ensure high humidity conditions due to the necessity of free water for the production and release of sporangia, formation of zoospores, and subsequent infection of healthy tissues (Tessmann and Vida 2005). Furthermore, to guarantee the assay reproducibility the temperature for fungal growth was adjusted at 25°C for sporangia formation in up to eight hours, following (Ribeiro 2001). Upon the visual sporulation on the majority of the leaves on the humid dark chamber, leaves were rinsed for sporangia capture on a 250 mL Beaker with around 150 mL of water. A suspension containing 3×10^5 sporangia mL^{-1} of *P. viticola* was sprayed onto the abaxial surface. This experiment was carried out in biological triplicates, under controlled environmental conditions (temperature of 25 ± 2 °C and 100% relative humidity). Ten leaves per biological replicate were sampled at 0 (time which was used as a control) 6, 12, 24, 48 and 72 h after inoculation (HAI). These leaves were immediately frozen in liquid nitrogen and stored at −80 °C until processing. The effectiveness of the challenge assay for gene expression analysis was confirmed by verifying its ability to induce the expression of Stilbene Synthase (VIT_16s0100g00990) and β-1,3-glucanase (PR3, VIT_08s0007g06060)—data not shown.

Evaluation of P. viticola resistant genotypes

Ninety-four grapevine genotypes from a cross between 'Villard Blanc' X 'Crimson Seedless' (SF-VB population) and segregating for downy mildew resistance were used for SSR markers evaluation. For the Kompetitive Allele Specific PCR (KASP) markers, two hundred genotypes from a self-fertilized population VB ('Villard Blanc' X 'Villard Blanc') were used. Moreover, 'Villard Blanc' leaves were used for resistance control evaluation and leaves from 'Cabernet Sauvignon' were used as a positive control of the infection. For phenotyping their resistance to *P. viticola*, sixteen leaf discs of 1 cm in diameter were detached from each genotype and a suspension containing 3×10^5 sporangia mL^{-1} of *P. viticola* was sprayed onto the abaxial surface of the discs. Next, they were incubated in Petri dishes with one layer of filter paper under humid conditions, at 25 °C and medium light ($\sim$ 150 μmol m^{-2} s^{-1}). The phenotypic classification of downy mildew resistance was determined according to the descriptor OIV—452

(Anonymous 1983), in which the most susceptible genotypes are classified as 1 and the most resistant, as 9. Genotypes are considered resistant if scored 7 to 9. Disease progression was monitored for nine days. From five days post-inoculation (DPI) onwards, photographs from the dishes were taken daily until the ninth day and a score was given for each genotype by the end of the experiment (9 DPI). These experiments were done in two distinct years.

P. viticola resistance-related candidate genes selection

For candidate genes selection, Fisher's test was performed using the software Blast2Go (B2G) (Götz et al. 2008). All genes located at the distal portion of chromosome 18, corresponding to the *Rpv3* locus QTL (Di Gaspero et al. 2007; Welter et al. 2007 and Revers et al. 2010), were evaluated for enrichment in functional annotation terms (Gene Ontology—GO) related to resistance, taking the entire grapevine gene set as a reference. RT-qPCR direct and reverse primers specific for each candidate gene were designed manually, limiting the size of amplicons to the maximum of 200 base pairs. The candidate genes were named according to their position at the *Rpv3* locus in the 12Xv0 version of the grape genome (Jaillon et al. 2007).

Amplification of Rpv3 candidate genes

To characterize the 23 candidate genes selected, conventional PCR reactions based on genomic DNA were performed to verify their presence in the genome of 'Cabernet Sauvignon' and 'Villard Blanc', as well as to confirm the size of the amplicons of each gene, based on the grapevine genome from cultivar Pinot Noir (PN40024). Each specific primer used is described on Supplementary Table 1.

Plant RNA extraction

Total RNA was isolated from grapevine leaves by LiCl precipitation using the Zeng and Yang (2002) protocol scaled to 2 mL volumes. Each sample extraction was performed in triplicate and final volumes were pooled before the LiCl precipitation step. Genomic DNA was removed using the TURBO DNA-free Kit (Ambion, Foster City) according to the manufacturer's protocol. RNA integrity and quantity were monitored by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

RT-qPCR analysis

Complementary DNAs were synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City) according to the manufacturer's instructions. The gene-specific primers were designed using the OligoAnalyzer 3.1 tool (IDT, http://www.idtdna.com) with the standard settings of 0.2 µM of oligo concentration, 1.5 mM of $MgCl_2$ and 0.2 mM of dNTP (Supplementary Table 1). RT-qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City). SYBR Green (Invitrogen, Carlsbad) was used to monitor dsDNA synthesis. Each biological sample was analyzed in technical quadruplicates. Cycling consisted of one step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and finished by a dissociation curve between 60 °C and 95 °C. The specificity of PCR amplification was assessed by the presence of a single peak in melting curves, visualization of single amplification products of the expected size in 3% ethidium bromide-stained agarose gel electrophoresis and sequencing of the amplicons. Primer efficiency was calculated using LinRegPCR software (version 11.0) (Ruijter et al. 2009). Mean relative gene expression was calculated by the (Pfaffl 2001) method employing *ACTIN* (GenBank EC969944) as a reference gene (Reid et al. 2006) and samples harvested at the onset of treatments (0 HAI) were used as a calibrator. All data are represented as averages of three biological replicates and four technical replicates. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test ($p < 0.05$), due to comparison of treatments with a control, in which the 0 HAI time-point of each cultivar was used as a control for comparison to the following time-points of the same cultivar samples.

SSR markers evaluation

Each individual progeny DNA sample from the 'Villard Blanc' X 'Crimson Seedless' cross was purified as described by Lefort and Douglas (1999)

and used in PCR amplifications for each locus as described by Revers et al. (2010). The SSR markers P2_VVAGL11 (Mejía et al. 2011; F- 5' TGTACAC CAATACGGGTTTCAT 3' and R- 5' GTTTGCTGG ATTTCCGATGT 3'), P3_VVAGL11 (Mejía et al. 2011; F- 5' CTCCCTTTCCCTCTCCCTCT3' and R- 5' AAACGCGTATCCCAATGAAG 3'), VMC7F2 (GenBank BV005171; F- 5' AAGAAAGTTTGCAGT TTATGGTG 3' and R- 5' AGATGACAATAGCGA GAGAGAA 3') and VVIN16 (GenBank BV140662; F- 5' ACCTCTATAAGATCCTAACCTG 3' and R- 5' AAGGGAGTGTGACTGATATTTC 3') UDV108 (F- 5' TGTAGGGTTCCAAAGTTCAGG and R 5'CTTTTTATATGTGGTGGAGC 3') were used. The amplicons were resolved in 6% polyacrylamide gel and stained with silver nitrate as described by Creste et al. (2001). Deviations between the observed and expected genotype segregations and the possible associations between the phenotypes and the alleles evaluated were tested by concordance and chi-square independence (χ^2).

SNP markers evaluation

The forty-one SNP-type markers tested in this work were chosen due to their position on the Rpv3 QTL and based on the GrapeReSeq_Illumina_20K chip (https://urgi.versailles.inra.fr/Species/Vitis/GrapeReSeq_Illumina_20K). Leaf discs from the two hundred genotypes from the self-fertilized population VB were shipped to LGC Genomics (http://www.lgcgroup.com, England) to perform the KASP ™ genotyping technique.

DNA amplicon sequencing

All the DNA amplicons from RT-qPCR were sequenced and analyzed in an ABI Prism® 310 Genetic Analyser (Applied Biosystems, Foster City) using standard sequencing protocols described in Falavigna et al. (2014). Sequence analysis was carried out with DNA Sequencing Analysis Software v5 (Applied Biosystems, Foster City) and MEGA7 software (http://www.megasoftware.net/home). Sequences were compared to the grapevine reference ('Pinot Noir' PN40024) genome 12Xv1.

Results

Identification of resistance genes on Rpv3 *locus associated with downy mildew defence response*

For better exploring the *Rpv3* locus (LG18) we aimed to identify and test possible candidate genes for downy mildew resistance. By performing a Fisher's test, we observed that the genomic region located in the *Rpv3* locus, based on the reference genome PN40024, is enriched with genes associated with defence responses, programmed cell death, signal transduction, immune system processes and responses to stress (Supplementary Table 2). About 70 genes were found at the *Rpv3* locus, of which 40 had at least one of the TIR-NBS-LRR domains, while eight genes had all three domains. From this set of 40 predicted candidate genes, 23 were selected for RT-qPCR evaluation (Supplementary Table 2), 8 of them for having the 3 TIR-NBS-LRR domains and 15 for having more GO terms associated with programmed cell death and defence responses, besides being located close to the seedlessness selection molecular marker VMC7F2 (Cabezas et al. 2006) and having at least one of the TIR-NBS-LRR domains. The location of the 23 genes on the *Rpv3* locus selected for the accomplishment of the present work is demonstrated in Fig. 1.

Amplification of Rpv3 candidate genes

To characterize the candidate genes, conventional PCR reactions based on genomic DNA were performed to verify their presence in the genome of both cultivars, as well as to confirm the size of the amplification product of each gene, based on the grapevine genome from cultivar Pinot Noir (PN40024). Of the 23 candidate selected genes, 21 yielded the predicted product after amplification of 'Cabernet Sauvignon' DNA samples. On the other hand, 'Villard Blanc' was positive for only 16 candidate genes under the conditions tested (Supplementary Fig. 1). This allowed us to confirm the presence of most candidate genes in the genomes of both cultivars. A few genes presented differences in the amplification product sizes between cultivars. The gene loci, specific primers and expected amplification products are described in Supplementary Table 1.

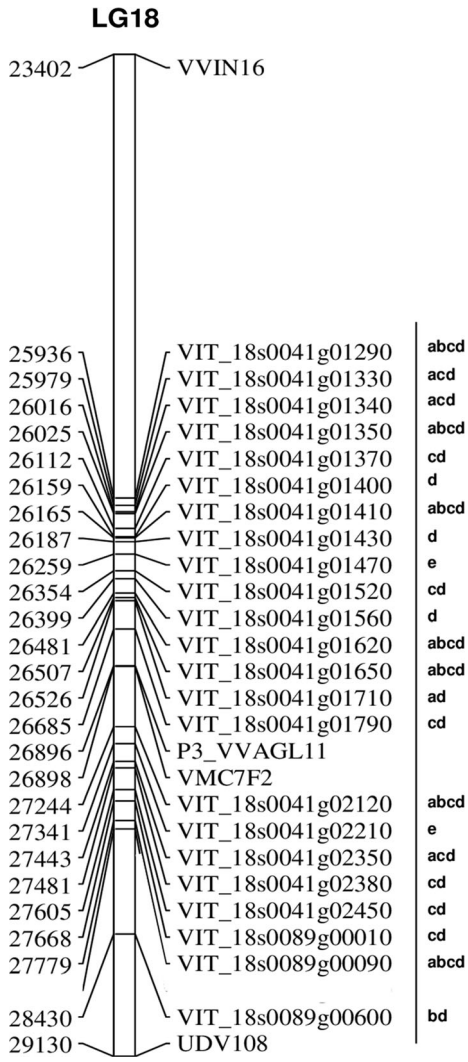

Fig. 1 The position of the candidate genes at *Rpv3* locus on the distal portion of chromosome 18. Letters represent the presence of amplification products for each cultivar, Villard Blanc (VB) and Cabernet Sauvignon (CS), by PCR and by RT-qPCR (a = RNA VB; b = RNA CS; c = DNA VB, d = DNA CS and e = not detected/amplified). Numbers on the left are megabases (MB) and chromosome coordinates are from reference genome PN40024

Rpv3 candidate genes expression in response to P. viticola infection

Transcriptional profiles of candidate genes were obtained by RT-qPCR after spraying *P. viticola* sporangia on leaves from the susceptible cultivar Cabernet Sauvignon and the resistant one Villard Blanc. Out of the twenty-one pairs of specific primers used to evaluate the expression of candidate genes, nine did not show RT-qPCR amplification. For twelve genes it was observed amplification products of expected sizes. The genetic specificity of the twelve PCR amplicons were confirmed by sequencing.

The twelve genes that presented expression were divided into three groups, according to their expression pattern: 1) the ones that presented expression in both cultivars samples (seven genes – Fig. 2a-g); 2) the ones that only had expression in the 'Villard Blanc' cultivar samples (four genes – Fig. 2h-k) and 3) one gene that was only expressed in 'Cabernet Sauvignon' samples (Fig. 2l).

From the first group, the transcriptional profile of the genes VIT_18s0041g01350, VIT_18s0041g01620 and VIT_18s0089g00090 (Fig. 2b, d and g) demonstrated an increase in relative gene expression after challenge with *P. viticola* in both cultivars. The gene VIT_18s0041g01350 presented a transcriptional profile with high levels for 'Villard Blanc' samples with statistical differences after 6 HAI (Fig. 2b). Furthermore, for the Cabernet Sauvignon cultivar, the gene VIT_18s0089g000090 was approximately 2X more expressed after 6 HAI (Fig. 2g). VIT_18s0041g01620 presented a significant difference in 48 and 72 HAI when compared to 0 HAI showing an approximately 2.5X increase in expression in 'Cabernet Sauvignon' (Fig. 2d). VIT_18s0041g02120 presented a higher level of transcripts for 'Cabernet Sauvignon' samples in most time-points while for Villard Blanc cultivar its expression decreased after *P. viticola* inoculation (Fig. 2f). This tendency of differential expression with a decreasing curve happened for other genes, such as VIT_18s0041g01290, VIT_18s0041g01410 and VIT_18s0041g01650 (Fig. 2a, c and e, respectively) for both cultivars.

Interestingly, four genes presented expression only for the resistant cultivar Villard Blanc after *P. viticola* challenge, representing the second group (Fig. 2h-k). Genes VIT_18s0041g01330 and VIT_18s0041g01340 showed a gradual decrease in relative gene expression in the period between 0 and 48 HAI with *P. viticola* (Fig. 2h-i). Moreover, gene VIT_18s0041g01710 presented a statistical difference in relation to 0 HAI with a significant decrease at all time-points for resistant cultivar Villard Blanc (Fig. 2j), while gene VIT_18s0041g02350 presented a variable expression during the experiment time points (Fig. 2k).

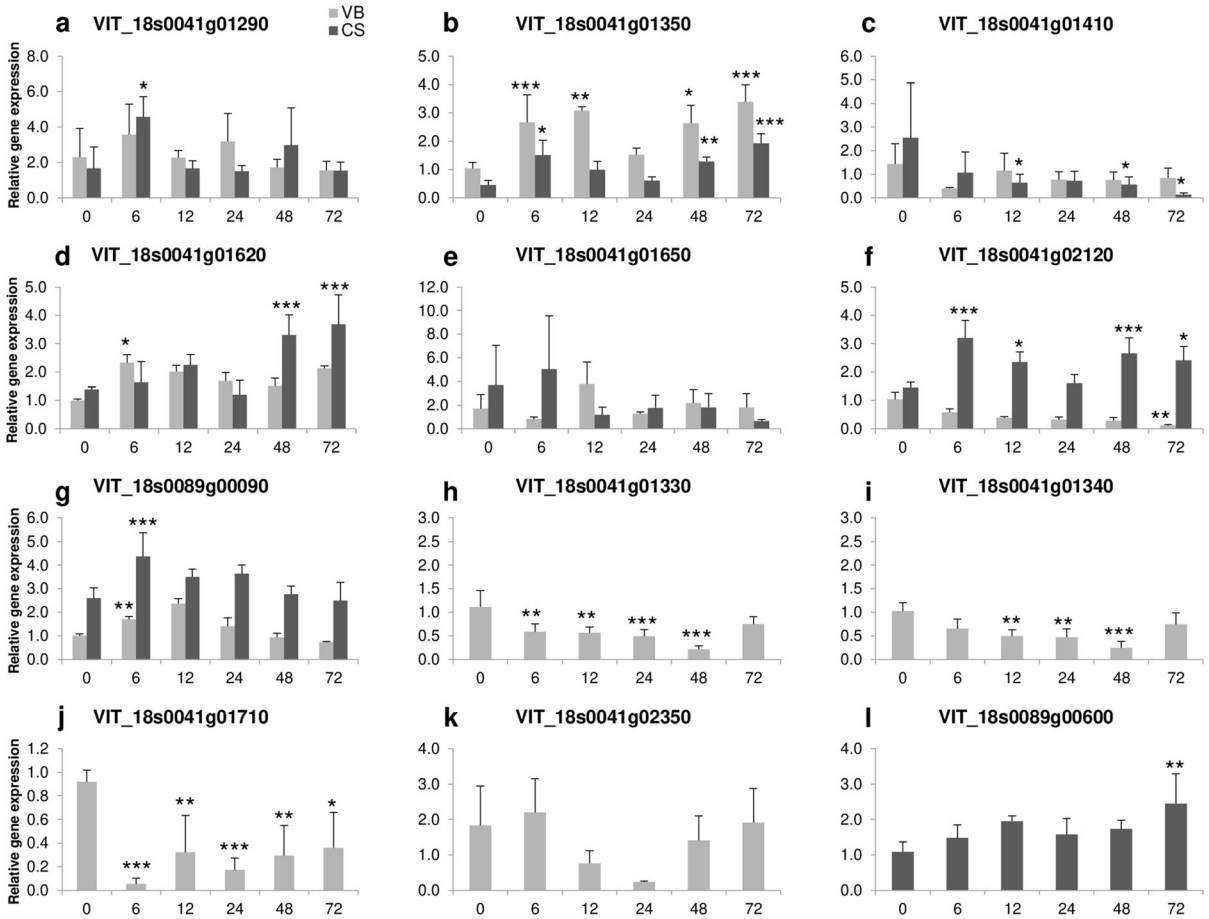

Fig. 2 Transcriptional profile of candidate genes at the *Rpv3* locus after *P. viticola* inoculation. Actin gene expression was used as a reference. Axis *x* shows hours after *P. viticola* inoculation (HAI). VB = Villard Blanc; CS = Cabernet Sauvignon. Standard errors are shown for each time point. One-way ANOVA followed by Dunett's test, in which the 0 h time-point of each cultivar was used as a control for comparison to the following time-points, * $p < 0{,}05$; ** $p < 0{,}001$; *** $p < 0{,}0001$

The VIT_18s0089g00600 gene, that composes the third group, was the only one expressed uniquely in the susceptible cultivar. This gene showed a slight increase in expression after challenge with *P. viticola*, demonstrating a 2.5X higher expression at 72 HAI when compared to 0 HAI (Fig. 2l).

Evaluation of SSR markers for downy mildew resistance

The co-localization of the *Rpv3* locus and the *SdI* locus (Seed development Inhibitor) gave us the opportunity to test molecular markers that were already established for seedlessness assisted selection. These markers (UDV108, VMC7F2, VVIN16, P2_VVAGL11 and P3_VVAGL11) are known for their efficiency (> 80% accuracy) as tools for assisted selection of apirenic grapevines (Doligez et al. 2002; Cabezas et al. 2006; Mejía et al. 2011; Ocarez et al. 2020). In addition, VMC7F2 and VVIN16 were previously described for *P. viticola* assisted selection (Bellin et al. 2009; van Heerden et al. 2014; Vezzulli et al. 2019). For this analysis, we performed the phenotypic characterization of resistant genotypes on 'Villard Blanc' X 'Villard Blanc' population after a *P. viticola* challenge assay (see Materials and Methods—Fig. 3). On our bioassays, we were able to identify reddish-brown tissue of necrotic spots, corresponding to the oil stain, in the upper region of the infected leaves in all the performed assays, a classical symptom of *P. viticola*

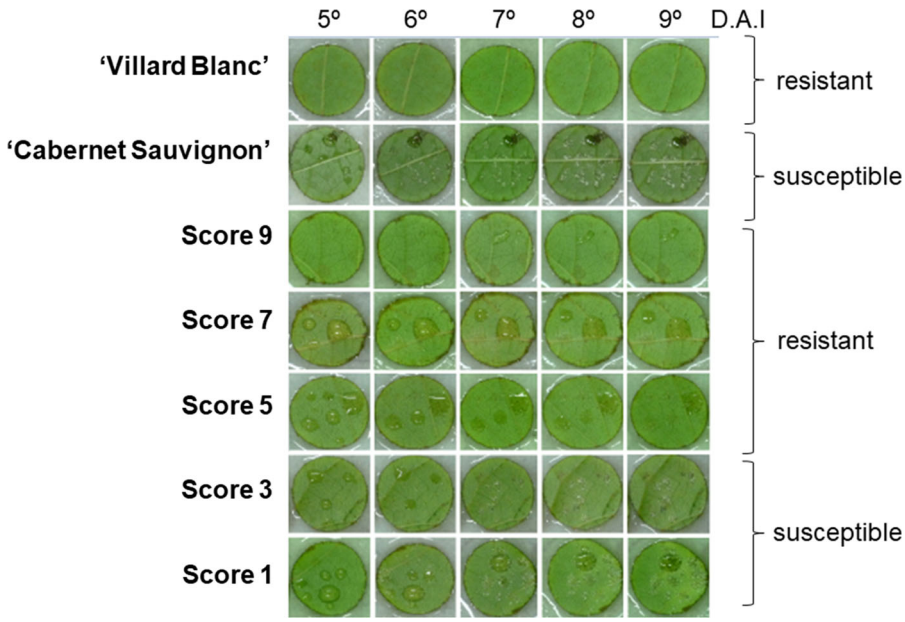

Fig. 3 Downy mildew infection progression on leaf discs. Leaf discs were monitored for 9 days after *P. viticola* inoculation. OIV—452 score was used as reference, in which scores 1 to 3 are defined as susceptible to *P. viticola* and scores ranging from 5 to 9 are defined as resistant to *P. viticola*. Leaves from 5 to 9 DAI are represented for selecting genotypes of each score. Leaf discs from the parent 'Villard Blanc' were used as the resistance control and 'Cabernet Sauvignon' was used as positive control for the infection

infection (Jones et al. 2017). Depending on the severity of lesions, plants were scored for downy mildew resistance or susceptibility, with the resistant plants presenting a score between 5 and 9, while susceptible plants present a score of 1 to 3 (OIV—452) (Fig. 3). After performing the $\chi 2$ adjustment tests, UDV108 and P2_VVAGL11 did not present the expected segregation and were excluded from the analysis. For the loci VMC7F2, VVIN16 and P3_VVAGL11 two segregating alleles per loci were observed in the genotyped population. Analysis of the phenotypic distribution of the evaluated characters versus the allele frequency of the genotyped SSR markers shows the association between the alleles P3_VVAGL11—185 bp ($\chi 2$ calc = 28.9), VVIN16—154 bp ($\chi 2$ calc = 26.81) and VMC7F2—210 bp ($\chi 2$ calc = 36.93) with downy mildew resistance (Fig. 4). The observed recombination rate between these markers within this population was 0.0319 between VMC7F2 X P3_VVAGL11, 0.0455 between VMC7F2 X VVIN16 and 0.0213 between VVIN16 X P3_VVAGL11. Among the recombinants, resistant/tolerant phenotypes were observed in all that kept the alleles significantly linked to medium–high scores of resistance to downy mildew (scores 5–9 OIV452; See Supplementary Table 4). Interestingly, one of the recombinants between P3_VVAGL11 and VMC7F2, that replaced the allele 210 bp by the allele 194 bp presented a sensitive phenotype to downy mildew in the challenge assay nearly as much as the 'Cabernet Sauvignon'. By the evaluation of these marker genotypes, a combination of alleles were identified for resistance to downy mildew. The use of the combination of these three SSR markers, VMC7F2, VVIN16 and P3_VVAGL11, showed a high capacity in the selection of resistant individuals in the samples of plants tested, also contributing to avoid potential false positives.

Development and evaluation of SNP markers for assisted selection of resistance to P. viticola

In order to develop new efficient molecular markers for assisted selection of downy mildew resistance, we chose SNPs that are located within the *Rpv3* locus. These selected mutations were based on a high throughput genotyping of different grapevine backgrounds (Supplementary Table 3). We developed

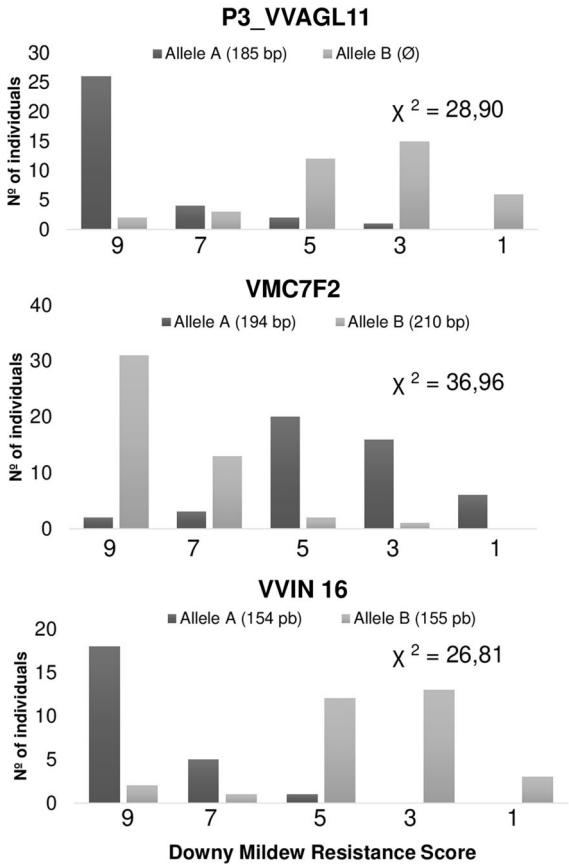

Fig. 4 SSR markers evaluated for downy mildew resistance. Number of F1 individuals from population 'Villard Blanc' X 'Crimson Seedless', and their genotypes versus the score for *P. viticola* resistance (OIV—452)

forty-one markers to be tested by competitive allele PCR (KASP ™). Of those, Rpv3_15 and Rpv3_33 loci presented segregating polymorphisms in the genotyped population (VBXVB). The other markers tested were homozygous in this population. Analysis of the phenotypic distribution versus allele frequency clearly showed ($p < 0.0001$) the association between the G allele of the Rpv3_15 (χ^2 calc = 66, 67, position chr18_26844557) and of the Rpv3_33 (χ^2 calc = 59,56, position chr18_27469511) with downy mildew resistance (Fig. 5). The recombination rate observed between Rpv3_33 and Rpv3_15 was 0.0103. The two observed recombinants presented low scores of resistance, replacing the allele G (guanine) by the allele A (adenine), which demonstrates that the allele G, for both markers, is in coupling with the resistant haplotype in this segregating population (See Supplementary Table 5). All the candidate markers tested are represented at chromosome 18 in Fig. 6.

Discussion

In the current moment of agricultural modernity it is urgent to understand the molecular and physiological mechanisms of plant defence against a large range of abiotic stresses. And more imperative is then to use this knowledge for the development of sustainable and earth-preserving field practices. In this work following two grape cultivar's response to *P. viticola* infection we aim to identify genes associated with resistance to downy mildew exploring expression analysis of putative homologs of defence genes. In addition, we aimed to identify SNPs or INDELs located within the *Rpv3* locus that could be associated with the resistance phenotype. During our bioassays that challenged plant leaf discs with *P. viticola* we followed the presence or absence of reddish-brown stains on the upper part of the leaves as a resistance or susceptibility indicator, respectively. These stains are due to the pathogen recognition system which triggers localized resistance reactions such as the HR (Durrant and Dong 2004). When in nature, over time, these stains increase in size and unite occupying much of the leaf blade with the tissue in the spot's area becoming dry, causing the affected leaves to fall prematurely and depriving the plant to generate photoassimilates (Ribeiro 2001; Garrido, L. da R. Sônego 2007).

Our candidate gene expression analysis results demonstrate the intricacy of the downy mildew resistance mechanism. Studies have shown that the time and intensity with which the infected organism develops the defence process presents a major genotypic component, thus the process of resistance induction is functionally, spatially and temporally complex (Bellin et al. 2009; Pinto et al. 2012; Foria et al. 2020). We identified candidate genes located at the *Rpv3* locus by their predicted function as related to pathogen resistance, which is determinant of plant immune response specificity. Not all the performed PCRs showed amplification for both cultivars analyzed (SFig.1). The absence of amplification products for the 'Villard Blanc' may be related to the fact that this cultivar is a complex hybrid that harbours the Rpv3-1 haplotype and distantly related to 'Cabernet Sauvignon', as a consequence has differences when

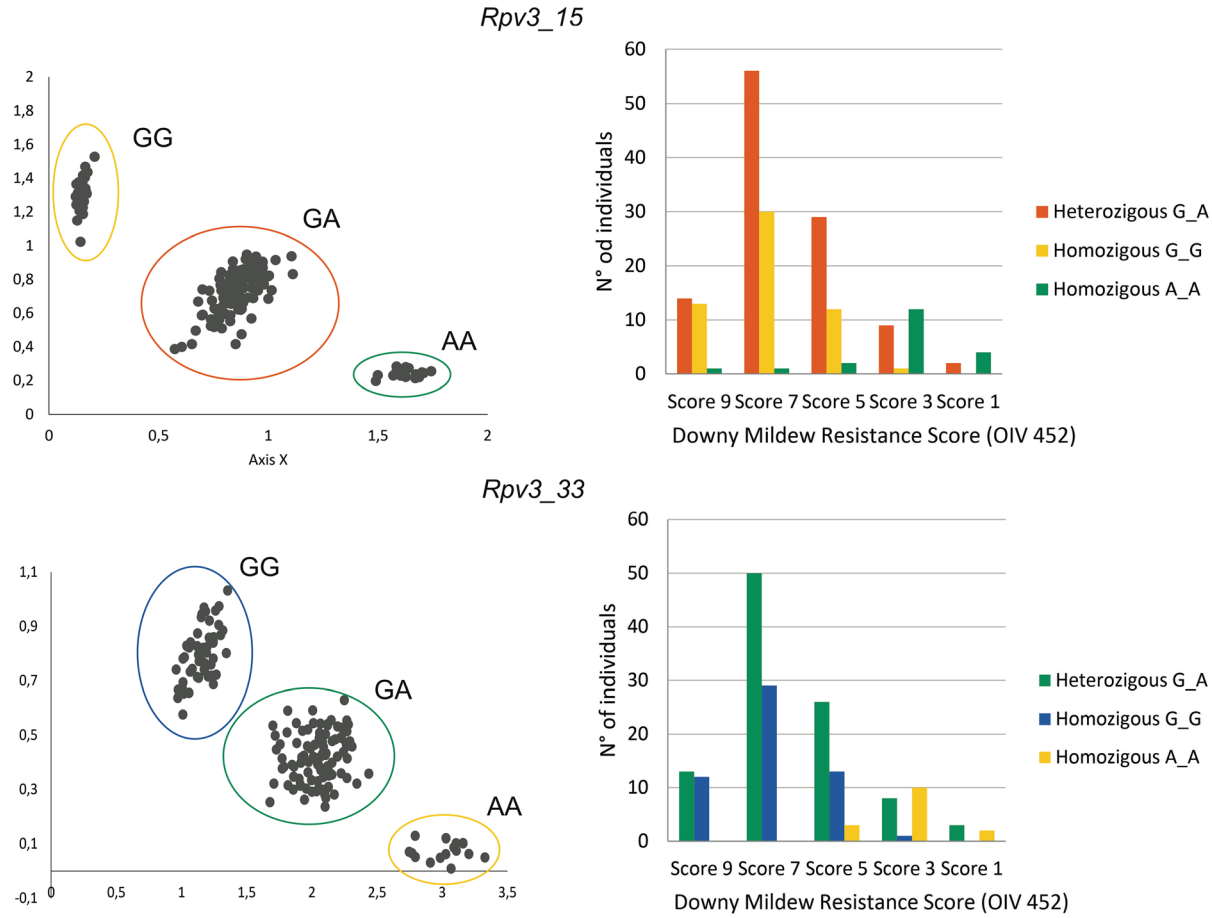

Fig. 5 Evaluation of SNP-type markers in downy mildew resistant segregating population. KASP markers depicted on the left panel show the fluorescence results for FAM and HEX in the axis *x* and *y*, respectively. Right panel demonstrates the relation between the number of individuals and their genotype versus the score for downy mildew resistance (OIV—452)

compared with the inbreeding line PN40024, from which the full genome was sequenced, and from which 'Cabernet Sauvignon' is closely related. Indeed, Foria et al. 2020, unveiled the genetic nature of the Rpv3-1 locus, demonstrating that the movement of transposable elements had a major impact on the generation of haplotype diversity, altering the DNA context and the GC-content in 5'-intergenic regions. These authors mapped the causal genetic factors for resistance to downy mildew in an interval containing a TIR-NB-LRR (*TNL2a* and *TNL2b*) gene pair that originated from an ancestral tandem segmental duplication in wild American grapes. This pair of *TNL* genes corresponds to the VIT_18s0041g01340 locus in the Rpv3-PN40024, which in our challenge assays, showed expression only in 'Villard Blanc' samples. Similarly, the transcriptional profile of VIT_18s0041g01330, that corresponds to the *TNL1* gene in the Rpv3-1 haplotype, was transcriptionally detected only in 'Villar Blanc'. These results are in agreement with what was observed for the Rpv3-1 'Regent' genetic background (Foria et al. 2020). Both genes present the three domains, TIR, NBS and LRR, in their predicted proteins, and both were annotated with GO terms related to processes of apoptosis, binding proteins, innate immune response and stress-causing factors, among other processes. Their transcriptional profiles suggest that these genes may be related to the process of immune response triggered by the effector that results in downy mildew HR.

Among the results comprising genes that were expressed in both cultivars only VIT_18s0041g01350 showed a profile that was predominantly induced upon challenge in 'Villard Blanc' when compared to

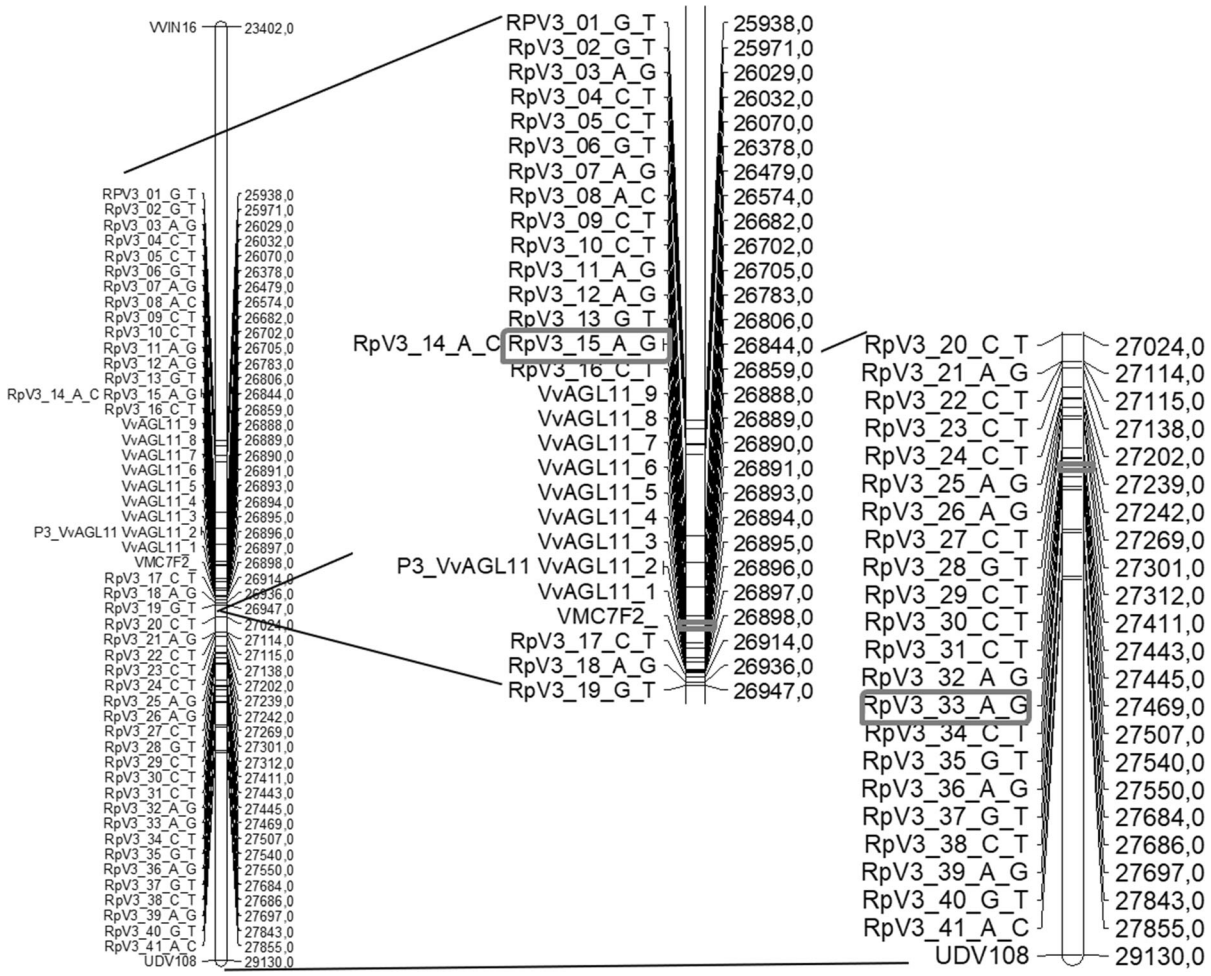

Fig. 6 SNP-type candidate marker's position at the *Rpv3* locus. The markers were developed for competitive allele-specific PCR (KASP) for the evaluation of downy mildew resistant segregation. The SSR markers on the distal portion of chromosome 18 (reference genome PN40024) are the ones tested in this work (VVIN16, P3_VVAGL11, VMC7F2 and UDV108). Markers surrounded by a grey box presented segregating polymorphisms related to downy mildew resistance in the genotyped population

'Cabernet Sauvignon'. Interestingly, leaf discs that were agroinfiltrated with a p35S::VIT_18s0041g01350 construct, showed significantly lower sporulation at 5 days after inoculation than controls (Foria et al., 2020). According to Blast2GO functional terms mapping, the VIT_18s0041g01350 is related to processes of apoptosis, ATP binding, innate immune response, transmembrane activity receptor and signal transduction. The combination of our results confirmed the data reported by Foria et al. (2020) demonstrating that these three genes play pivotal roles in the effector-triggered immune response process that results in the assembly of the HR in resistant individuals harbouring the Rpv3-1 haplotype.

Concerning the time-lapse response observed in our challenge assay, our results deviate from the work of Polesani and collaborators (2010) in which they observed that the resistant genotype, in their case cultivar Gloire (*V. riparia*), starts assembling the defence response mechanism against downy mildew in the first 24 h after contact with the pathogen. This is the period in which the plant innate immune system is activated by a large number of surveillance-type receptors that play a role in detecting the presence of pathogens and transmitting the invasion signal, which

is followed by the establishment of the compatible plant-pathogen interaction (Polesani et al. 2010; Andolfo and Ercolano 2015). In this scenario, the structural defence mechanisms of the host has influence at the early interaction between the pathogen and its host, as observed in a multi-year study for the evaluation of resistance of 'Georgian' grapevine germplasm and *P. viticola* (Toffolatti et al. 2016).

In our evaluation of candidate genes' transcriptional profiles, it was possible to observe that 'Villard Blanc' presented always, though variable, a certain level of transcription of the genes tested. This suggests that the resistant cultivar could be, in a way, always producing the necessary defence response proteins against the pathogen. In the case of *V. vinifera* cultivars, it is known that they are susceptible to the attack of *P. viticola*, although they present defence responses against other pathogens, thus indicating that the defence components specifically against *P. viticola* are not activated in a necessarily short period after infection (Kortekamp 2006). From these data we can hypothesize that the presence of the selected candidate genes in the genome of both cultivars, still demonstrates the absence of one or more components in *V. vinifera* that would be necessary to trigger *P. viticola* defence response, like the four genes expressed only in 'Villard Blanc'. This probably happens because the resistance mechanism is dependent on the activation of R genes, which encode cellular receptors that detect the presence of a particular pathogen, leading to the activation of the signal transduction pathways (Wang et al. 2013). In addition, the susceptibility of this and other species may occur due to failure to assemble the effective defence response, which leads to defective or no recognition of the pathogen (Velasco et al. 2007). Fung et al. (2008) evaluated the interaction between *V. vinifera* and *V. aestivalis* and the *E. necator* pathogen, the agent that causes powdery mildew. These authors observed that for *V. aestivalis* only three genes were regulated as a consequence of the infection, whereas for *V. vinifera*, reprogramming occurred in a high number of genes after pathogen exposure, which is in agreement with the increased expression levels of some genes presented by the 'Cabernet Sauvignon' (genes VIT_18s0041g01290, VIT_18s0041g01350, VIT_18s0041g01620, VIT_18s0041g02120 and VIT_18s0041g00090). Another point is that some varieties might present partial resistance to *P. viticola*, which is much more difficult to characterize. A recent work exampled six important components of partial resistance in a diverse set of grapevine varieties showing that partial resistant varieties presented reduced proportion of inoculation, longer latent period, smaller lesion size, fewer production and number of sporangia, shorter infectious period and lower infectivity of sporangia than the susceptible variety 'Merlot' (Bove and Rossi 2020). Therefore, evaluating if cultivars and individuals are partially resistant to downy mildew should be an interesting approach for future works.

The precocious detection of resistance to pathogens in perennial plants is of great importance for breeding programs and for agricultural development. For this objective many studies focus on discovering new and accurate molecular markers to help plant selection. Recently, a study showed the use of haplotype-tagging insertion/deletions (InDels) for downy mildew resistance of grapevine by observing differences in amplicon size between grapes that carry or do not carry Rpv3-1, which can be analyzed by via standard agarose gel electrophoresis or classical melting curve with fluorescent dyes (Foria et al. 2018). In our study, we initially used SSR markers which confirmed that the P3_VVAGL11, VVIN16 and VMC7F2 markers appear to sufficiently co-segregate with the *Rpv3* locus to allow their use as a molecular marker-assisted selection tool for a downy mildew resistance breeding strategy. These three molecular markers now have a dual purpose: they can be used in the previously known diagnosis of the seedlessness character and also in the evaluation of downy mildew resistance in grapevines. A new study also aimed for selecting seedlessness and downy mildew resistance in wild Chinese grapes, by the use of DNA-probe, SSR and SCAR markers (Li et al. 2020). Even though we achieved good results with the SSR, nowadays there are more efficient genotyping techniques than resolving SSR by electrophoretic apparatus, such as genetic analyzers, even with the availability of automation platforms. One of the alternatives is the KASP ™ genotyping assay. This assay uses a unique form of PCR combined with a homogeneous fluorescence-based information system for the identification and measurement of genetic variation occurring at the nucleotide level to detect single nucleotide polymorphisms (SNPs) or insertions and deletions (INDELs) (He et al. 2014). This methodology may even allow us to transform SSR markers into KASP markers, for fast

one step genotyping. With the evaluation of our forty-one SNP-based molecular markers, we identified two that are heterozygous and segregate in the 'Villard Blanc' self-fertilized population. Markers Rpv3_15 and Rpv3_33 allow for the selection of resistant genotypes by a technique that has several advantages such as a lower genotyping error in positive control DNA samples compared to other techniques and lower per-assay price (7.9–46.1% cheaper) (Semagn et al. 2014). Thus, these markers can generate data on a larger scale, in an automated and cost-efficient fashion (Guimarães et al. 2009). Moreover, the Rpv3_15 and Rpv3_33 markers hereby presented could be combined with other markers, such as VMC7F2, in marker-assisted breeding programs. Taken together, the results presented in this work contribute with key functional data about the *Rpv3/Rpv3-1* loci and its associated resistant genes, demonstrating the biotechnological applicability of molecular markers in downy mildew resistance assisted selection strategy for early plant assortment, therefore long term field sustainability.

Acknowledgements We are grateful for the financial support from the Embrapa Funding System (SEG code 02.08.07.004.00.05.04, 02.13.03.006.00.02.006 and 02.12.12.003.00.00).

Author's contribution statement L.F.R, J.M., A.W. and D.D.P. conceived original screenings, research plans, designed experiments and analyzed resulting data; L.F.R supervised experiments and writing; J.M., A.W and V.B. performed most experiments; L.F.R, R.T. and D.D.P provided technical assistance and performed the statistical analysis. J.M. and A.W wrote the article with contributions from all authors.

Funding We are grateful for the financial support from the Embrapa Funding System (SEG code 02.08.07.004.00.05.04, 02.13.03.006.00.02.006 and 02.12.12.003.00.00).

Declarations

Conflict of interest All authors declare to have no competing interests, both financial and non-financial.

References

Andolfo G, Ercolano MR (2015) Plant innate immunity multicomponent model. Front Plant Sci 6:1–6. https://doi.org/10.3389/fpls.2015.00987

Anonymous (1983) Descriptor list for grapevine varieties and *Vitis* species 452. Organization Internacional de la Vigne et du Vin (OIV), Paris

Belkhadir Y, Subramaniam R, Dangl JL (2004) Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr Opin Plant Biol 7:391–399. https://doi.org/10.1016/j.pbi.2004.05.009

Bellin D, Peressotti E, Merdinoglu D et al (2009) Resistance to *Plasmopara viticola* in grapevine "Bianca" is controlled by a major dominant gene causing localised necrosis at the infection site. Theor Appl Genet 120:163–176. https://doi.org/10.1007/s00122-009-1167-2

Bigeard J, Colcombet J, Hirt H (2015) Signaling mechanisms in pattern-triggered immunity (PTI). Mol Plant 8:521–539. https://doi.org/10.1016/j.molp.2014.12.022

Blasi P, Blanc S, Wiedemann-Merdinoglu S et al (2011) Construction of a reference linkage map of *Vitis amurensis* and genetic mapping of *Rpv8*, a locus conferring resistance to grapevine downy mildew. Theor Appl Genet 123:43–53. https://doi.org/10.1007/s00122-011-1565-0

Blum M, Waldner M, Gisi U (2010) A single point mutation in the novel *PvCesA3* gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*. Fungal Genet Biol 47:499–510. https://doi.org/10.1016/j.fgb.2010.02.009

Bove F, Rossi V (2020) Components of partial resistance to *Plasmopara viticola* enable complete phenotypic characterization of grapevine varieties. Sci Rep 10:1–12. https://doi.org/10.1038/s41598-020-57482-0

Brilli M, Asquini E, Moser M et al (2018) A multi-omics study of the grapevine-downy mildew (*Plasmopara viticola*) pathosystem unveils a complex protein coding- and non-coding-based arms race during infection. Sci Rep 8:757. https://doi.org/10.1038/s41598-018-19158-8

Brun LA, Le Corff J, Maillet J (2003) Effects of elevated soil copper on phenology, growth and reproduction of five ruderal plant species. Environ Pollut 122:361–368. https://doi.org/10.1016/S0269-7491(02)00312-3

Cabezas JA, Cervera MT, Ruiz-García L et al (2006) A genetic analysis of seed and berry weight in grapevine. Genome 49:1572–1585. https://doi.org/10.1139/G06-122

Canaguiera A, Grimplet J, Di Gaspero G et al (2017) A new version of the grapevine reference genome assembly (12X.v2) and of its annotation (VCost.v3). Genom Data 14:56–62. https://doi.org/10.1016/j.gdata.2017.09.002

Costantini L, Battilana J, Lamaj F et al (2008) Berry and phenology-related traits in grapevine (*Vitis vinifera* L.): from quantitative Trait Loci to underlying genes. BMC Plant Biol 8:1–17. https://doi.org/10.1186/1471-2229-8-38

Creste S, Tulmann Neto A, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol Biol Report 19:299–306. https://doi.org/10.1007/BF02772828

Cus F, Basa Cesnik H, Velikonja Bolta S, Gregorcic A (2010) Pesticide residues and microbiological quality of bottled wines. Food Control 21:150–154. https://doi.org/10.1016/j.foodcont.2009.04.010

da Falavigna V, S, Porto DD, Buffon V, et al (2014) Differential transcriptional profiles of dormancy-related genes in ppple

buds. Plant Mol Biol Report 32:796–813. https://doi.org/10.1007/s11105-013-0690-0
Di Gaspero G, Cipriani G (2003) Nucleotide binding site/leucine-rich repeats, Pto-like and receptor-like kinases related to disease resistance in grapevine. Mol Genet Genom 269:612–623. https://doi.org/10.1007/s00438-003-0884-5
Di Gaspero G, Cipriani G, Adam-Blondon A-F, Testolin R (2007) Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for R-gene candidates. Theor Appl Genet 114:1249–1263
Di Gaspero G, Copetti D, Coleman C et al (2012) Selective sweep at the Rpv3 locus during grapevine breeding or downy mildew resistance. Theor Appl Genet 124:277–286. https://doi.org/10.1007/s00122-011-1703-8
Díez-Navajas AM, Wiedemann-Merdinoglu S, Greif C, Merdinoglu D (2008) Nonhost versus host resistance to the grapevine downy mildew, *Plasmopara viticola*, studied at the tissue level. Phytopathology 98:776–780
Doligez A, Bouquet A, Danglot Y et al (2002) Genetic mapping of grapevine (*Vitis vinifera* L.) applied to the detection of QTLs for seedlessness and berry weight. Theor Appl Genet 105:780–795. https://doi.org/10.1007/s00122-002-0951-z
Dry IB, Feechan A, Anderson C et al (2010) Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew. Aust J Grape Wine Res 16:94–105. https://doi.org/10.1111/j.1755-0238.2009.00076.x
Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185–209. https://doi.org/10.1146/annurev.phyto.42.040803.140421
Eisenmann B, Czemmel S, Ziegler T et al (2019) *Rpv3*-1 mediated resistance to grapevine downy mildew is associated with specific host transcriptional responses and the accumulation of stilbenes. BMC Plant Biol 19:1–17. https://doi.org/10.1186/s12870-019-1935-3
Erwin DC, Ribeiro OK (1996) Phytophthora diseases worldwide. American Phytopathological Society (APS Press), St, Paul, Minnesota
Figueira D (2013) Cabernet Sauvignon a casta sagrada. In: Prazeres do vinho. http://www.dfwines.com.br/public/pdf/midia/002.pdf.
Fischer BM, Salakhutdinov I, Akkurt M et al (2004) Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. Theor Appl Genet 108:501–515. https://doi.org/10.1007/s00122-003-1445-3
Flor HH (1971) Current status of the gene-fob-gene concept. Annu Rev Phytopathol 3531:275–296
Foria S, Magris G, Copetti D et al (2018) InDel markers for monitoring the introgression of downy mildew resistance from wild relatives into grape varieties. Mol Breed. https://doi.org/10.1007/s11032-018-0880-4
Foria S, Copetti D, Eisenmann B et al (2020) Gene duplication and transposition of mobile elements drive evolution of the *Rpv3* resistance locus in grapevine. Plant J 101:529–542. https://doi.org/10.1111/tpj.14551
Fröbel S, Zyprian E (2019) Colonization of different grapevine tissues by *Pasmopara viticola* — a histological study. Front Plant Sci. https://doi.org/10.3389/fpls.2019.00951
Fröbel S, Dudenhöffer J, Töpfer R et al (2019) Transcriptome analysis of early downy mildew (*Plasmopara viticola*) defense in grapevines carrying the Asian resistance locus *Rpv10*. Euphytica 215:28. https://doi.org/10.1007/s10681-019-2355-z
Fung RWM, Gonzalo M, Fekete C et al (2008) Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. Plant Physiol 146:236–249. https://doi.org/10.1104/pp.107.108712
Garrido L da R Sônego OR (2007) Manejo de doenças da videira. In: Manejo integrado de doenças de fruteiras. Sociedade Brasileira de Fitopatologia, Brasília, pp 65–86
Gisi U, Waldner M, Kraus N, Dubuis PH, Sierotzk H (2007) Inheritance of resistance to carboxylic acid amide (CAA) fungicides in *Plasmopara viticola*. Plant Pathol 56:199–208. https://doi.org/10.1111/j.1365-3059.2006.01512.x
Götz S, García-Gómez JM, Terol J et al (2008) High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 36:3420–3435. https://doi.org/10.1093/nar/gkn176
Greenberg JT, Vinatzer BA (2003) Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. Curr Opin Microbiol 6:20–28. https://doi.org/10.1016/S1369-5274(02)00004-8
Grenville-Briggs LJ, Van West P (2005) The biotrophic stages of oomycete-plant interactions. Adv Appl Microbiol 57:217–243. https://doi.org/10.1016/S0065-2164(05)57007-2
Guimarães CT, de Magalhães JV, Lanza MA, Schyster I (2009) Marcadores moleculares e suas aplicações no melhoramento genético. Inf Agropecuário 30:24–33
He C, Holme J, Anthony J (2014) SNP genotyping: the KASP assay. Methods Mol Biol 1145:75–86
IBRAVIN (2012) A Vitivinicultura Brasileira. http://www2.agricultura.rs.gov.br.2012.
INRA, IFV, SupAgro M (2013) Montpellier SupAgro. Fiche Variétale Villard Blanc B. Plant Grape. http://www.montpellier.inra.fr/
Jaillon O, Aury JM, Noel B et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–468. https://doi.org/10.1038/nature06148
Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–239. https://doi.org/10.1038/nature05286
Jones DS, McManus PS (2017) Distinctive symptoms and signs of downy mildew on cold-climate wine grape cultivars. Plant Health Prog 18:192–195. https://doi.org/10.1094/PHP-01-17-0009-DG
Jones DA, Takemoto D (2004) Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. Curr Opin Immunol 16:48–62. https://doi.org/10.1016/j.coi.2003.11.016
Kassemeyer HH, Gadoury DH, Hill G, Wilcox WF (2015) Downy mildew In: compendium of grape diseases, eds W. Wilcox, W. Gubler, and J. Uyemoto (St Paul, MN: APS Press), pp 46–52
Kortekamp A (2006) Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol Biochem 44:58–67. https://doi.org/10.1016/j.plaphy.2006.01.008
Kortekamp A, Zyprian E (2003) Characterization of plasmopara-resistance in grapevine using in vitro plants. J Plant

Physiol 160:1393–1400. https://doi.org/10.1078/0176-1617-01021
Lefort F, Douglas GC (1999) An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species Acer, Fraxinus, Prunus and Quercus. Ann for Sci 56:259–263
Li S, Liu K, Yu S et al (2020) The process of embryo abortion of stenospermocarpic grape and it develops into plantlet in vitro using embryo rescue. Plant Cell Tissue Organ Cult 143:389–409.https://doi.org/10.1007/s11240-020-01926-y
Liang Z, Duan S, Sheng J (2019) Whole-genome resequencing of 472 *Vitis* accessions for grapevine diversity and demographic history analyses. Nat Commun 10:1190. https://doi.org/10.1038/s41467-019-09135-8
Marguerit E, Boury C, Manicki A et al (2009) Genetic dissection of sex determinism, inflorescence morphology and downy mildew resistance in grapevine. Theor Appl Genet 118:1261–1278. https://doi.org/10.1007/s00122-009-0979-4
Mejía N, Soto B, Guerrero M et al (2011) Molecular, genetic and transcriptional evidence for a role of *VvAGL11* in stenospermocarpic seedlessness in grapevine. BMC Plant Biol 11:1–18. https://doi.org/10.1186/1471-2229-11-57
Merdinoglu D, Wiedeman-Merdinoglu S, Coste P et al (2003) Genetic analysis of downy mildew resistance derived from *Muscadinia rotundifolia*. Acta Hortic 603:451–456
Moreira FM, Madini A, Marino R et al (2011) Genetic linkage maps of two interspecific grape crosses (*Vitis* spp.) used to localize quantitative trait loci for downy mildew resistance. Tree Genet Genom 7:153–167. https://doi.org/10.1007/s11295-010-0322-x
Ocarez N, Jiménez N, Núñez R et al (2020) Unraveling the deep genetic architecture for seedlessness in grapevine and the development and validation of a new set of markers for *VviAGL11*-based gene-assisted selection. Genes (basel) 11:1–32
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45
Pinto KMS, do Nascimento LC, de Souza Gomes EC, et al (2012) Efficiency of resistance elicitors in the management of grapevine downy mildew *Plasmopara viticola*: Epidemiological, biochemical and economic aspects. Eur J Plant Pathol 134: 745–754. https://doi.org/10.1007/s10658-012-0050-1
Polesani M, Bortesi L, Ferrarini A et al (2010) General and species-specific transcriptional responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. Riparia*) grapevine species. BMC Genom 11:1–16. https://doi.org/10.1186/1471-2164-11-117
Possamai T, Migliaro D, Gardiman M et al (2020) *Rpv* mediated defense responses in grapevine offspring resistant to Plasmopara viticola. Plants 9:1–10. https://doi.org/10.3390/plants9060781
Reid KE, Olsson N, Schlosser J et al (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6:1–11. https://doi.org/10.1186/1471-2229-6-27
Revers LF, Welter LJ, Irala PB, et al (2010) Co-localization of QTLs for seedlessness and downy mildew resistance in grapevine. In: Reisch BI, Londo J (eds) Xth Intl Conf on Grapevine Breeding and Genetics. pp 449–456
Ribeiro IJA (2001) Doenças causadas por fungos e bactérias na cultura da videira. In: BOLIANI AC, CORRÊA L de S (eds) Cultura de uvas de mesa: do plantio à comercialização. ALGRAF, Ilha Solteira, pp 237–239
Ruijter JM, Ramakers C, Hoogaars WMH et al (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:1–12. https://doi.org/10.1093/nar/gkp045
Saifert L, Sánchez-Mora FD, Assumpção WT et al (2018) Marker-assisted pyramiding of resistance loci to grape downy mildew. Pesqui Agropecu Bras 53:602–610. https://doi.org/10.1590/S0100-204X2018000500009
Salmaso M, Malacarne G, Troggio M et al (2008) A grapevine (Vitis vinifera L.) genetic map integrating the position of 139 expressed genes. Theor Appl Genet. https://doi.org/10.1007/s00122-008-0741-3
Santos RF, Ciampi-Guillardi M, Fraaije BA et al (2020) The climate-driven genetic diversity has a higher impact on the population structure of *Plasmopara viticola* than the production system or QoI fungicide sensitivity in subtropical brazil. Front Microbiol 11:575045. https://doi.org/10.3389/fmicb.2020.575045
Sargolzaei M, Maddalena G, Bitsadze N et al (2020) *Rpv29*, *Rpv30* and *Rpv31*: Three novel genomic loci associated with resistance to *Plasmopara viticola* in *Vitis vinifera*. Front Plant Sci 11:1–16. https://doi.org/10.3389/fpls.2020.562432
Schwander F, Eibach R, Fechter I et al (2012) *Rpv10*: a new locus from the Asian Vitis gene pool for pyramiding downy mildew resistance loci in grapevine. Theor Appl Genet 124:163–176. https://doi.org/10.1007/s00122-011-1695-4
Semagn K, Babu R, Hearne S, Olsen M (2014) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol Breed 33:1–14. https://doi.org/10.1007/s11032-013-9917-x
Soanes DM, Talbot NJ (2008) Moving targets: rapid evolution of oomycete effectors. Trends Microbiol 16:507–510. https://doi.org/10.1016/j.tim.2008.08.002
Takken FL, Albrecht M, Tameling WIL (2006) Resistance proteins: molecular switches of plant defence. Curr Opin Plant Biol 9:383–390. https://doi.org/10.1016/j.pbi.2006.05.009
Tessmann DJ, Vida JB (2005) Principais doenças fúngicas da videira. Rev Atualidades Agrícolas 14–15
Toffolatti SL, Maddalena G, Maghradze D, Bianco PA (2016) Evidence of resistance to the downy mildew agent Plasmopara Viticola in the Georgian Vitis Vinifera Germplasm. Vitis. https://doi.org/10.5073/vitis.2016.55.121-128
Unger S, Büche C, Boso S, Kassemeyer HH (2007) The course of colonization of two different *Vitis* genotypes by *Plasmopara viticola* indicates compatible and incompatible host-pathogen interactions. Phytopathology 97:780–786. https://doi.org/10.1094/PHYTO-97-7-0780
van Heerden CJ, Burger P, Vermeulen A et al (2014) Detection of downy and powdery mildew resistance QTL in a 'Regent' × 'RedGlobe' population. Euphytica 200:281–295. https://doi.org/10.1007/s10681-014-1167-4

Velasco R, Zharkikh A, Troggio M et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS ONE. https://doi.org/10.1371/journal.pone.0001326

Venuti S, Copetti D, Foria S et al (2013) Historical introgression of the downy mildew resistance gene *Rpv12* from the Asian species *Vitis amurensis* into grapevine varieties. PLoS ONE 8:e61228. https://doi.org/10.1371/journal.pone.0061228

Vezzulli S, Salacarne GM, Masuero D et al (2019) The Rpv3-3 haplotype and stilbenoid induction mediate downy mildew resistance in a grapevine interspecific population. Front Plant Sci 10:234. https://doi.org/10.3389/fpls.2019.00234

Wang P, Liu C, Wang D et al (2013) Isolation of resistance gene analogs from grapevine resistant to downy mildew. Sci Hortic (amsterdam) 150:326–333. https://doi.org/10.1016/j.scienta.2012.11.035

Welter LJ, Göktürk-Baydar N, Akkurt M et al (2007) Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis vinifera* L). Mol Breed 20:359–374. https://doi.org/10.1007/s11032-007-9097-7

Xiang J, Li X, Wu J et al (2016) Studying the mechanism of *Plasmopara viticola* RxLR effectors on suppressing plant immunity. Front Microbiol 7:709. https://doi.org/10.3389/fmicb.2016.00709

Yin L, An Y, Qu J et al (2017) Genome sequence of *Plasmopara viticola* and insight into the pathogenic mechanism. Sci Rep 7:46553. https://doi.org/10.1038/srep46553

Zeng Y, Yang T (2002) RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant Mol Biol Report 20:417a–417e. https://doi.org/10.1007/BF02772130

Zyprian E, Ochßner I, Schwander F et al (2016) Quantitative trait loci affecting pathogen resistance and ripening of grapevines. Mol Genet Genom 291:1573–1594. https://doi.org/10.1007/s00438-016-1200-5