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Identification of resistance sources against *Plasmopara viticola* in *Vitis vinifera* cultivars for breeding programs

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Abstract

The Eurasian grapevine (*Vitis vinifera*), an Old World species now cultivated worldwide for high-quality wine production, is extremely susceptible to the agent of downy mildew, *Plasmopara viticola* (Berk. et Curt.) Berl. and de Toni. This Oomycete is one of the most important pathogens of European grapevine. The discovery of resistant cultivars for breeding programs could be a solution to decreasing fungicides application for downy mildew disease worldwide. Extensive evaluation of Georgian cultivated grapevine germplasm has highlighted unique resistance behavior through the reduction of disease severity and pathogen sporulation. Unraveling the genetic architecture of grapevine response to *P. viticola* infection is crucial to develop resistant varieties. The aim of this project was to identify loci related to *P. viticola* resistance traits and to obtain new insights in the mechanism of resistance of Georgian germplasm. To address the first aim a genome-wide association (GWA) approach has been applied to a panel of Georgian-derived accessions phenotyped for *P. viticola* susceptibility and genotyped with Vitis18kSNP chip array. GWA identified three new loci (*Rpv*29, *Rpv*30 and *Rpv*31) associated with a low level of disease incidence. *Rpv*29, *Rpv*30, and *Rpv*31 loci appeared to be associated with genes related to plant defense mechanism against biotic stresses (pathogen recognition and signal transduction). Regarding the second objective, the role of leaf VOCs in the resistance mechanism of two resistant cultivars (Mgaloblishvili, a pure Georgian *V. vinifera* cultivar, and Bianca, an interspecific hybrid) has been investigated. The leaf VOC profiles analyzed through solid-phase microextraction gas chromatography-mass spectrometry analysis, and the expression of six terpene synthases (TPSs), through, real-time RT-PCR, were determined upon pathogen inoculation. In both cultivars, an increment of VOCs (such as farnesene, nerolidol, ocimene and valencene) has been detected after pathogen inoculation, contextually to an increment of the expression pattern of six TPSs. Finally, the transcripts of *P. viticola* in the early interaction with grapevine cultivars have been characterized. In this study, the early *P. viticola* development in susceptible host cells (2008-059-020, *Rpv*–) was compared two resistance 2008-059-121 (carrier of *Rpv3* and *Rpv10*) and 2011-003-013 (homozygous for the locus *Rpv10*) using RNA sequencing data and microscopic observation. In total six novel genes of TAR 1 protein, cellulose synthase, a regulator of G-protein in signaling and Ras-related proteins were identified in *P. viticola* which are differentially expressed during the initial infection. This primary signaling induction by the pathogen in host cell could be used in the future coupled with the first report on resistance loci in *V. vitinifera*, VOC induction and genome regions involved in resistance response, for further genetic study of *V. vinifera* and breeding programs.

Acknowledgment

I would like to express my sincere gratitude and appreciation to my supervisory committee Doctor Gabriella De Lorenzis, Doctor Silvia Laura Toffolatti and Prof. Osvaldo Failla for their continuous support, understanding and guidance. I am very happy and grateful for being given the chance to pursue my PhD in their research group. I also highly appreciate Milan university for their financial support through the University PhD student grant. My profound gratitude is also extended to doctoral coordinator Prof. Daniele Bassi for his kind assistance during my study. I will forever be thankful to Dr. Emanuele Quattrini for all his kind support during my stay in Arcagna. Many thanks to all my dear friends and lab mates especially Dr. Remo Chiozzotto. I would like to extend my sincere thanks to Prof. Eva Zyprian for hosting me in the institute of Julius Kühn Institute and my dear friend Dr. Sarah Fröbel, which helped me carry out my experiments at JKI. Last but not least I would like to offer my heartfelt thanks to my dearest parents and my lovely husband who have always believed in me and made me believe in myself to perform to my maximum ability.

Contents

Acronyms

What this means?	Abbreviation
Asparagine (amino acid)	N
Aspartic acid (amino acid)	D
Base pair	bp
Carboxylesterases	CXEs
Chromosome	Chr
Cluster regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems	CRISPR/Cas9
Cold semi-arid climate	Bsk
CRISPR RNA	crRNA
Cysteine-rich RLKs	CRK1
Downy mildew	DM
Effector triggered immunity	ETI
Fixed and random model Circulating Probability Unification	FarmCPU
fold change	FC
for open reading frame	ORF
Gene of interest	GOI
Gene ontology	GO
Generalized Linear Model	GLM
Genome Wide Association Study	GWAS
GenTrain score	GT
Guide RNA	gRNA
Homologous recombination	HR
Hot-summer humid continental climate	Dfa
Humid subtropical climate	Cfa
Hypersensitive response	HR
Indole-3-acetic acid	IBA
Inositol transporter 1	INT1
Kilo base pair	kb
Large Gap Read Mapping	LGRM
Leucin rich repeats domain	LRRs
Linkage disequilibrium	LD
Linkage group	LG
LOB domain-containing protein	LBD
Magnesium-dependent phosphatase	MDP
Marker-assisted selection	MAS
MDIS1-interacting receptor like kinase 2	MIK2
Measurement ton	mt
Mega base pair	Mb
Million hectars	Mha
Mixed Linear Model	MLM
Multiple Locus Mixed Linear Model	MLMM

New Breeding Technologies	NBTs
Nitric oxide	NO
Nucleotide	nt
Nucleotide-binding site leucine-rich repeat	NB-LRR
Pathogen recognition receptors	PRRs
Pathogenesis-related proteins	PRs
Patterns-Triggered Immunity	PTI
Photosynthetically active radiation	PAR
plant growth regulator PGR	PGR
Plasmopara viticola	*P. viticola*
Polymerase chain rection	PCR
Polyvinylpolypyrrolidone	PVPP
Principal Component Analysis	PCA
Quantile-quantile plots	QQ
Quantitative real time PCR	qRT-PCR
Quantitative Trait Loci	QTL
Reactive oxygen species	ROS
Reads per kilo base per million mapped reads	RPKM
Receptor-like proteins	RLPs
Resistance genes	R-genes
Resistance level	RL
Resistance locus to *Plasmopara viticola*	*Rpv*
Sequence Manipulation Suite	SMS
Settlement of MLM Under Progressively Exclusion Relationship	SUPER
Simple-sequence repeats	SSR
Single-nucleotide polymorphism	SNP
Subarctic climate	Dfc
Subfamily Protein Architecture Labeling Engine	SPARCLE
Subpolar oceanic climate	Cfc
Systemic acquired resistance	SAR
Temperate oceanic climate	Cfb
Transcription activator-like effector nuclease	TALENs
Tukey Honest Significant Difference test	HSD
Tundra climate	ET
Unweighted Pair Group Method with Arithmetic Mean phylogenetic tree	UPGMA
Wall-Associated Kinase	WAK
Warm-summer humid continental climate	Dfb

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Chapter 1: General introduction

1.1 Grapevine: a high socio-economic impact crop strongly threatened by climate change

The genus *Vitis* is present in ten distribution areas, all in the northern hemisphere: five in North of America, where 29 species have been described, four in Asia, with at least 11 species, and only one, the *Vitis vinifera*, in a large range that includes the Mediterranean, sub-Mediterranean and Caucasian floristic regions with a dilatation toward the Pontic, Caspian and Central Asiatic ones, making this species one of the most widely cultivated plant species of agricultural interest.

Among the *Vitis* genus, grapevine (*V. vinifera*) is the only species greatly used in the global wine industry. It approximately covered approximately 7.4 mha in 2018, producing more than 77.8 mt of grapes (wine, table and dried grapes) with a world wine trade of around EUR 32 billion (http://www.oiv.int/). It is usually cultivated in an area roughly located between 35th and 55th northern parallel and between the 25th and 35th southern parallel where the average annual temperatures range between 10 and 20 °C. These environments are characterized by the alternation of a favorable growing season and an unfavorable cold one. However, the cold winters are not too intense (minimum temperatures range from -10 to15 °C) and the favorable season (average temperature higher than 10 °C) is long enough (> 200 days).

Viticulture depends on environmental factors, such as temperature, soil, rain, etc., in terms of yield and quality (van Leeuwen & Darriet, 2016). The recent scenario prospected by climate change, such as the increase of average global temperature, represents an impending threat to agriculture. Consequently, the risks of paying severe price increase dramatically if humans fail to dampen its consequences. Due to the socio-economic impact of the wine sector in Europe and around the world, over the past years, there has been an increase in works aimed to study the impact of climate change on viticulture.

Breeding programs for new varieties could be one of the most promising solutions towards managing future environmental conditions. An appropriate cultivar selection could reduce the required input for plant management by increasing the sustainability of the productions. For these reasons, this review aims to assess the potential of Georgian cultivars (South of Caucasus) as a source of useful traits for new breeding programs, aiming to face the future challenges that await viticulture worldwide. Thus, the peculiar genetic and phenotypic (such as berry traits and resistance to the pathogen) aspects of Georgian germplasm have been reviewed, hoping to provide a better understanding of the diversity and quality of the genetic resources available to viticulturists, coming directly from the cradle of domestication.

1.2 South Caucasus, the first grapevine domestication center

V. vinifera is indigenous of Eurasia and it is suggested to have the first *Vitis* genus ancestral appearig about 65 million years ago (Olmo et al., 1995). Nowadays, *V. vinifera* species includes both cultivated (*V. vinifera* subsp. *sativa* Beck.) and wild (*V. vinifera* subsp. *silvestris* Beck.) subspecies, where the latter is considered as the progenitor of subspecies

sativa. Its domestication process seems to be strongly linked to the alcoholic and gustative superiority of its fermented juice (the wine) in comparison to other pulpy fruits (fruit wines). However, this has been debated amongst researchers and not much certainly is available regarding which process predated the other (Terral et al., 2010). The main changes driving the grapevine domestication were identified in flower morphology (appearance of hermaphrodite flowers), larger berry size, higher berry sugar content, a wider range of berry color and aromatic content. These parameters could provide adequate yield, quality and greater sugar content for better fermentation. The major issue about grapevine domestication is related to the number of events that occurred during the process and the geographical location in which the events took place. The most accredited hypothesis declares that *V. vinifera* was domesticated from its wild form in the South Caucasus, between the Caspian and Black Seas, around 6,000–5,800 BC, and then spread throughout Europe and Mediterranean areas thanks to civilization (McGovern et al., 2017). Moreover, secondary domestication centers in the Mediterranean basin have also been hypothesized (Arroyo-García et al., 2006; Grassi et al., 2003). So far, molecular analysis has provided new insights on grapevine domestication and genetic diversity inside the *V. vinifera* species. The occurrence of an East-to-West grapevine gene flow after the first domestication process has been comprehensively indicated by the literature where genetic relationships between wild and cultivated accessions, especially in the Mediterranean Basin and Central Asia were evident (Myles et al., 2011; Riaz et al., 2018). Moreover, geographic origin and human usage were found to strongly shape the genetic structure of grapevine germplasm (Bacilieri et al., 2013).

1.3 Georgian germplasm as a source of genetic variability

Historical information coupled with archaeological, palaeobotanical and molecular findings pointed out Georgia as a cradle for grapevine domestication (McGovern, 2003; McGovern et al., 2017; Zohary & Hopf, 2000). Genetic diversity of Georgian germplasm was extensively investigated, by both SSR (Simple Sequence Repeat) and SNPs (Single Nucleotide Polymorphism) molecular markers. However, several autochthonous cultivars, collected in local ampelographic collections, remain to be studied. Thanks to GrapeGen06 (2007-2010) (Lacombe et al., 2011), COST Action FA1003 (2011-2014) (Failla, 2015), and European research programs, a strong ongoing network of scientific collaborations have been developed between European and Georgian researchers. The fundamental aim is to genetically characterize and preserve Georgian genetic resources. All of the outcomes related to the genetic characterization of Georgian germplasm reported the uniqueness and originality of this germplasm when compared to the European and Central Asia germplasm (Bacilieri et al., 2013; De Lorenzis et al., 2015, 2019; Imazio et al., 2013; Myles et al., 2011; Riaz et al., 2018). The Georgian cultivars showed the distinctive features of a domestication center, such as a high level of genetic diversity and heterozygosity, alleles absent or poorly represented in other countries, and differentiation from the European varieties, clustering in a well-separated branch (Figure 1.1). Based on the geographical origin of cultivars, a differentiation inside the germplasm was also identified indicating the varieties putatively originated in Kartli and Kakheti (Eastern regions) differed from the ones that originated in the Abkhazeti, Samegrelo and Guria (Western regions). The origin of this subdivision lies in the geographical subdivision of Georgia in two major parts due to the Likhi Mountains stretching from North-to-South direction across Georgia (De Lorenzis et al., 2015; Imazio

et al., 2013). This confirms that despite long-standing cultivation, the Georgian cultivars maintain their originality.

Genetic variation provides the foundation for any breeding programs, and natural genetic diversity historically represented the major source of variability for crop improvement and adaptation to changing environmental conditions. Given the uniqueness of Georgian germplasm, its strong link with origin regions coupled with the fact of this country being the center of domestication makes this germplasm very attractive to be investigated in terms of phenology, grape phenotype and resistance to biotic and abiotic stresses, as a source of new variability for the further breeding programs.

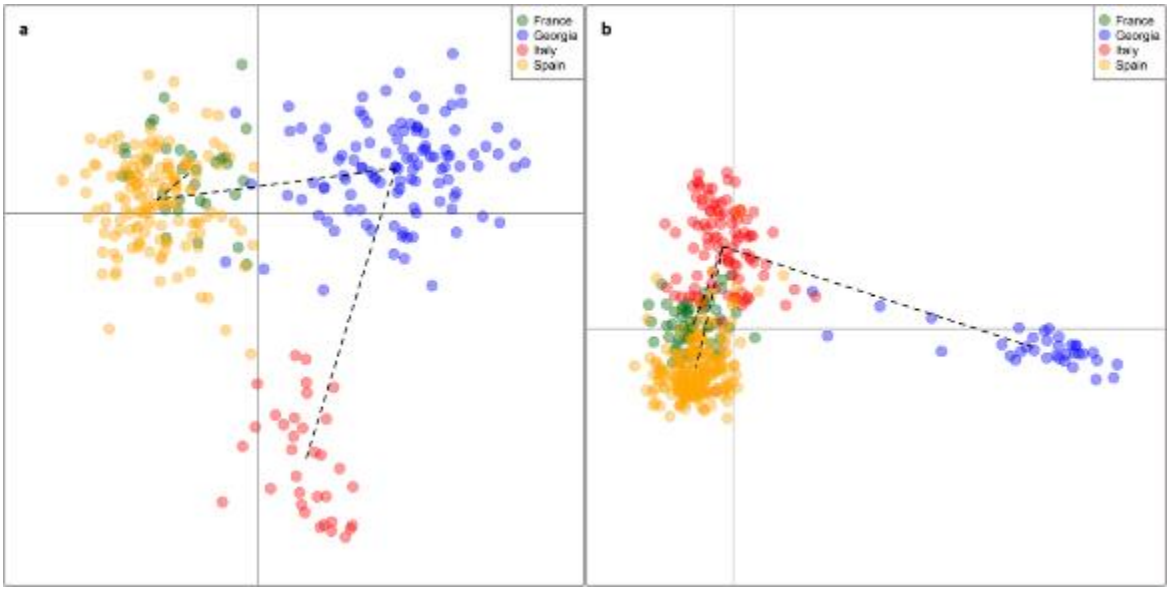


Figure 1.1 Two-dimension DAPC (Discriminant Analysis of Principal Component) scatter plot. Results of DAPC performed on grapevine cultivars coming from France, Georgia, Italy and Spain, genotyped by 20 SSRs (a) and 18k SNPs (b), using data reported in references (De Lorenzis et al., 2015, 2019; Laucou et al., 2018; Riaz et al., 2018). Black dotted lines represent a minimum-spanning tree.

1.4 Georgian climate and its relationship with grapevine

Georgia is a large basin of the mid-latitudes, bordered by the Greater Caucasus at North and the Lesser Caucasus at South and opened towards the Black Sea at West and towards the Caspian depression at East. These geographical features strongly characterize the climate of Georgia that, following the Köppen –Geiger classification (Köppen & Geiger, 1936), can be divided into continental climates (Dfa, Dfb, Dfc), temperate climates (Cfa, Cfb, Cfc), Dry climates (Bsk) and Polar Climates at the highest elevations (ET).

Regarding climate change, in 1994 Georgia faced a sudden rise in temperatures, similar to what happened in Western Europe in the late 1980s (Bonnefoy et al., 2013; P. Reid et al., 2016) being 1987 as the most likely year of change (Mariani et al., 2012). This delay could be explained as the progressive dilution of the Atlantic circulation signal as it moves into the European continent (Cola et al., 2017).

The increase of temperature indicated an advance in grapevine phenology, being more significant at higher altitudes, where more favorable thermal conditions were established.

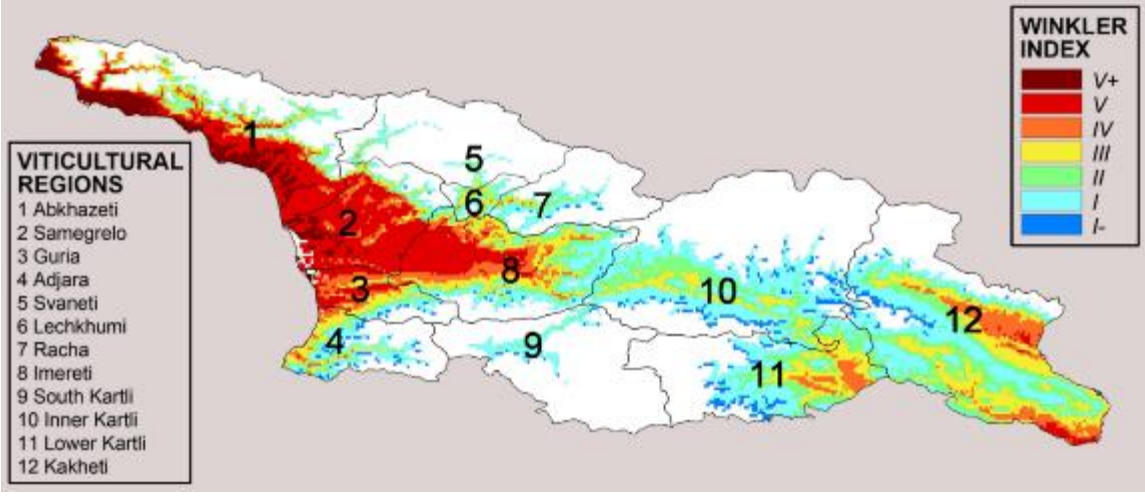
On the other hand, at lower altitudes, the phenological advance was partially depleted by the increase of super-optimal thermal conditions. For instance, in the case of the widely diffuse cultivar Rkatsiteli, the average advance of veraison was less than 6 days for the 250–500 m asl elevation belt and around 18 days for the 750–1000 m one (Cola et al., 2017).

The current thermal context of Georgia is really interesting since Georgian viticultural regions (Figure 1.2) cover all the classes of the Winkler Regional Classification (Table 1.1), meaning that Georgian viticulture, with its local varieties, exploits a wide variety of environmental conditions. In parallel, it is worth noticing the high variability in the plant phenology among Georgian cultivars, both in the spouting date and in the ripening period. A delayed budburst period could represent an avoidance mechanism against spring frosts. Considering Georgian cultivars, bud swelling of 'Partala' vines were recorded at the end of March, while the other cultivars sprouted in April (Maghradze et al., 2014). Global warming generally results in super-optimal temperatures in summertime, during grape ripening. A delay in the maturation process, obtained through the selection of late-ripening cultivars, could ensure more suitable thermal regimes for the berry metabolisms. Maghradze et al.(2012) studied the phenology of Georgian cultivars in North Italy, and they found a relatively late ripening for the reference varieties. Nevertheless, a very wide range of variability was maintained. Similar results were reported by Maghradze et al. (2014) and Rustioni et al. (2014). Some extreme cases are: early ripening cultivars – Karaleva vinogradnikov, Kartuli Saadreo, Khalili, Cheliagis Tsiteli, Meskhuri Mtsvane, Buza and Budeshuri Tsiteli, Ganjuri and Daisi; late-ripening cultivars – Ojaleshi, Akomshtali, Kamuri, Shavi, Tavkara, Khushia Shavi, Satsuravi, Maghlari Tvrina, Mtevandidi, Argvetula, Dziganidzis Shavi, Adanasuri, Mamukas Vazi, Otskhanuri Sapere, Gorula, Saperavi Meskhuri, Ghrubela and Shavtita.

Table 1.1 Description of Winkler classes.

Winkler class	**GDD**	**Viticultural climate**	**Vinicultural aptitude**
I -	< 850	very cool	Very early ripening grapes for fresh and fruity wines or sparkling wine bases
I	850 - 1400	cool	Early ripening grapes for fresh and fruity wines or sparkling wine bases
II	1400 - 1650	temperate cool	Early ripening grapes for wines to be aged. Medium ripening grapes for white or red wines ready to drink.
III	1650 - 1950	temperate	Medium ripening grapes for white or red wines ready to be aged.
IV	1950 - 2200	temperate warm	Late ripening grapes for white or red wines ready to be aged.
V	2200 - 2700	hot	Late ripening grapes for bodied red wines to be aged.
V +	> 2700	very hot	Very late ripening grapes for bodied red wines to be aged.

Figure 1.2 Winkler classification based on yearly average Winkler index calculated for the period 1994-2013 in Georgia (Caucasus). The analysis is limited to the areas below 1250 m above sea level.



similar results were obtained from comparing the phenological timing of Georgian and international varieties, such as Chardonnay and Cabernet sauvignon, through phenological modeling (Cola et al., 2017; Mariani et al., 2013). Figure 1.3 shows, for the Italian site of Perugia, the average phenological timing (1990-2019) of three relevant Georgian varieties such as Saperavi, Rkatsiteli, Mtsvane Kakuri, compared with Chardonnay and Cabernet Sauvignon.

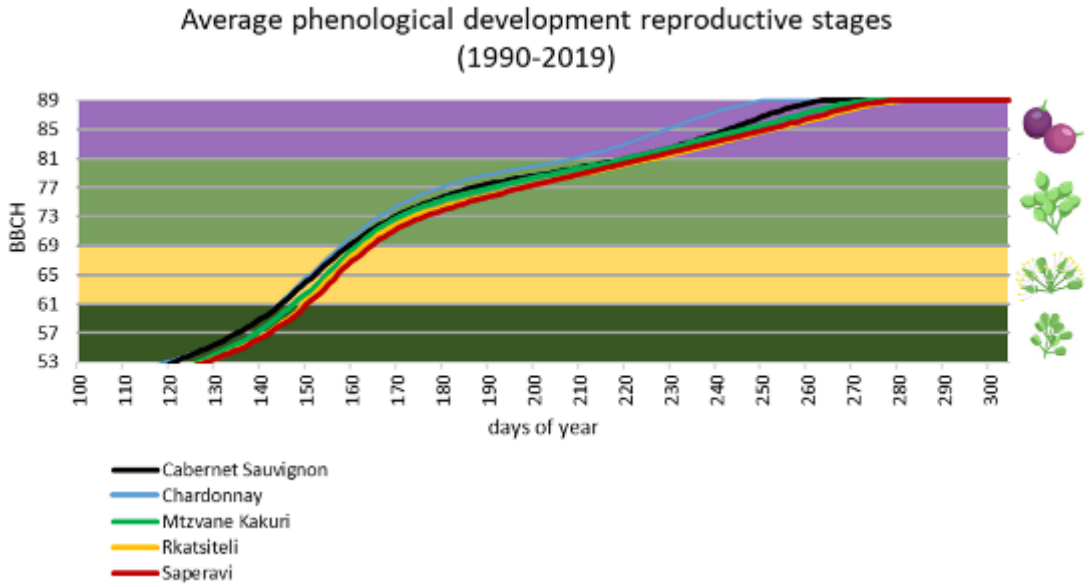


Figure 1.3 Phenological timing simulation for three relevant Georgian cultivars, compared with Chardonnay and Cabernet sauvignon, using meteorological data of Perugia (Italy) (years 1990-2019). Phenology is represented following the reference BBCH scale [42]: i) 53-59 development of flowers; ii) 60-69 flowering; iii) 70-79 development of fruits; iv) 80-89 ripening.

1.5 Georgian grapevine ampelographic traits

Georgia is noticed as an important source of grapevine ampelographic biodiversity (Chkhartishvili & Maghradze, 2012; Tsertsvadze, 2012). Ampelographic comparison has been conducted in a joint Asia and European research; meanwhile, numerous autochthonous cultivars have been described (Abashidze et al., 2015; Aroutiounian et al., 2015; Cornea & Savin, 2015; Goryslavets et al., 2015; Maghradze et al., 2015; Popescu et al., 2015; Ujmajuridze & Mamasakhlisashvili, 2015). Furthermore, the phenotypic variabilities are considered as a result of the genotype, environmental growing condition and their interaction.

Such as seed shape which in Georgia mostly round or slightly elongated, small berries have been selected by winemakers to be cultivated based on the ancient traditions in millennia (Chkhartishvili & Maghradze, 2012; McGovern et al., 2017). Also, Georgian grapevine observations reported a high level of both sugar and acid while nowadays the climate change could affect these levels by influencing anticipated ripening conditions (de Orduña, 2010; Keller, 2010; van Leeuwen & Destrac-Irvine, 2017). Which presence of late-ripening Georgian cultivars (Maghradze et al., 2012) could be interesting even for sugar levels counterbalanced by the acidity. Furthermore, there are adaptations of Georgian grapes due to the proportion of skin thickness, seeds and pulp. From which the berry shin thickness could be a barrier against climate changes (van Leeuwen & Destrac-Irvine, 2017) and other environmental stresses.

Epical waxes on the outer side of the grape skin protective role have been reported against dehydration (Di Matteo et al., 2000; Doymaz I., 2004; Doymaz, 2006; Doymaz & Pala, 2001; Mahmutoğlu et al., 1996; Muganu et al., 2011; Pangavhane et al., 1999) and pathogen infections (Marois et al., 1986; Percival et al., 1993; Rosenquist & Morrison, 1988). Furthermore, a study conducted on Georgian cultivars suggested a possible eco-physiological role of epicuticular waxes in reducing heating stresses by their interaction with infrared radiation (Rustioni et al., 2012). Often, plants face stresses through secondary metabolites, and the crucial role of phenolics against photodamages is well known (Close & McArthur, 2002; Graham et al., 2004; Rustioni, 2017). The Georgian cultivars showed a low amount of total phenolic compound accumulation (Abashidze et al., 2015; Rustioni et al., 2019) which in correlation with climate change impacts on Georgian cultivars, could be considered as a positive trait during future difficult ripening conditions of the future.

1.6 Resistance to grapevine diseases

The grapevine varieties cultivated worldwide belong to the Eurasian grapevine, *V. vinifera*, and are susceptible, at different levels, to several pathogens (fungi, bacteria and viruses). On the other hand, non-*vinifera* species, from North American and Asia, are resistant to fungi and tolerant to viruses and some bacteria (Armijo et al., 2016; Oliver & Fuchs, 2011). Amongst various diseases, which directly affect grapevines, powdery mildew (caused by the ascomycete *Erysiphe necator* Schwein.) and downy mildew (caused by the oomycete *Plasmopara viticola* (Berk. *et* Curtis) Berl. and de Toni) are considered as two of the most important threats. It was in the second half of the nineteenth century that pest management becomes an inevitable task for European viticulture due to the introduction of powdery and downy mildew agents in those regions. (Töpfer et al., 2011). The search for suitable tools for disease management rapidly became a priority for the viticulturists. The discovery of the

efficacy of sulfur and copper in controlling the diseases was a key point. However, great attention was also paid to the development of resistant cultivars. The American *Vitaceae* soon proved to be the best sources of resistance, due to co-evolution between the pest and pathogens. Also, extensive breeding programs, based on interspecific crosses between American *Vitis* species (e.g. *Vitis riparia*, *Vitis rupestris*, *Vitis berlandieri* and *Vitis labrusca*) and *V. vinifera*, were undertaken at the beginning of the XX century. Nevertheless, the interest in searching for resistant plants decreased over time, probably due to the discovery of fungicide efficacy (Russell, 2005), that was largely employed for disease control, and the inheritance of the specific foxy off-flavors from the non-*vinifera* parent species.

Recently, the public concern about sustainability in agriculture and new regulations on plant protection products renewed the interest of growers in the cultivation of resistant varieties. Despite that viticulture in the EU allocates a low percentage of arable land, it uses high amounts of fungicides to fight downy mildew infections (Eurostat 2007, http://ec.europa.eu/eurostat/de). Furthermore, studies on the effect of CO_2 and temperature on downy and powdery mildews showed that the disease incidence of downy mildew increased with the rising of gas and temperature, whereas an increase in CO_2 was not influencing powdery mildew incidence (Pugliese et al., 2011). Also, because of climate change, which will potentially favor the pathogen's development, it is important to search for new resistance genes, focusing on alternative species, such as *V. vinifera*, to the non-*vinifera* ones.

1.7 *V. vinifera* resistant cultivars against *P. viticola*

The identification of *P. viticola* dates to 1838, when Schweinitz, one of the founders of American mycology, collected the first samples from wild *Vitis* species in South Carolina. In Europe, downy mildew was first reported during 1878 in Bordeaux and then it spread all over the old continent, reaching Australia and New Zealand between 1919 and 1926 (Emmett et al., 1992). All traditional European grapevine cultivars showed high susceptibility against the pathogen, leading to a severe pandemic across Europe (Boso & Kassemeyer, 2008; Gessler et al., 2011). Nowadays, the pathogen is detected in warm and humid climates worldwide.

Symptoms of downy mildew (Figure 1.4) are observable on infected organs in various forms such as yellowish oily lesions (sometimes red, in black cultivars) on the upper surface of the leaves (Figure 1.4a and b) followed by sporulation on the underside of the leaf (Figure 1.4c), malformations and necrosis on herbaceous shoots and inflorescences (Figure 1.4d and e), change of color to violet and withering on berries (Figure 1.4f), that detach from the rachis leaving a dry stem scar. The disease negatively impacts grape production at both qualitative and quantitative levels. For instance, the loss of photosynthetic tissues limits the sugar amount in berries, which produces low-quality wines and the shoot and bunches damage leads to a poor yield. Severe infections, in absence of disease control, can result in total loss of leaves and some cases total yield loss (Toffolatti, De Lorenzis, et al., 2018; Töpfer et al., 2011).

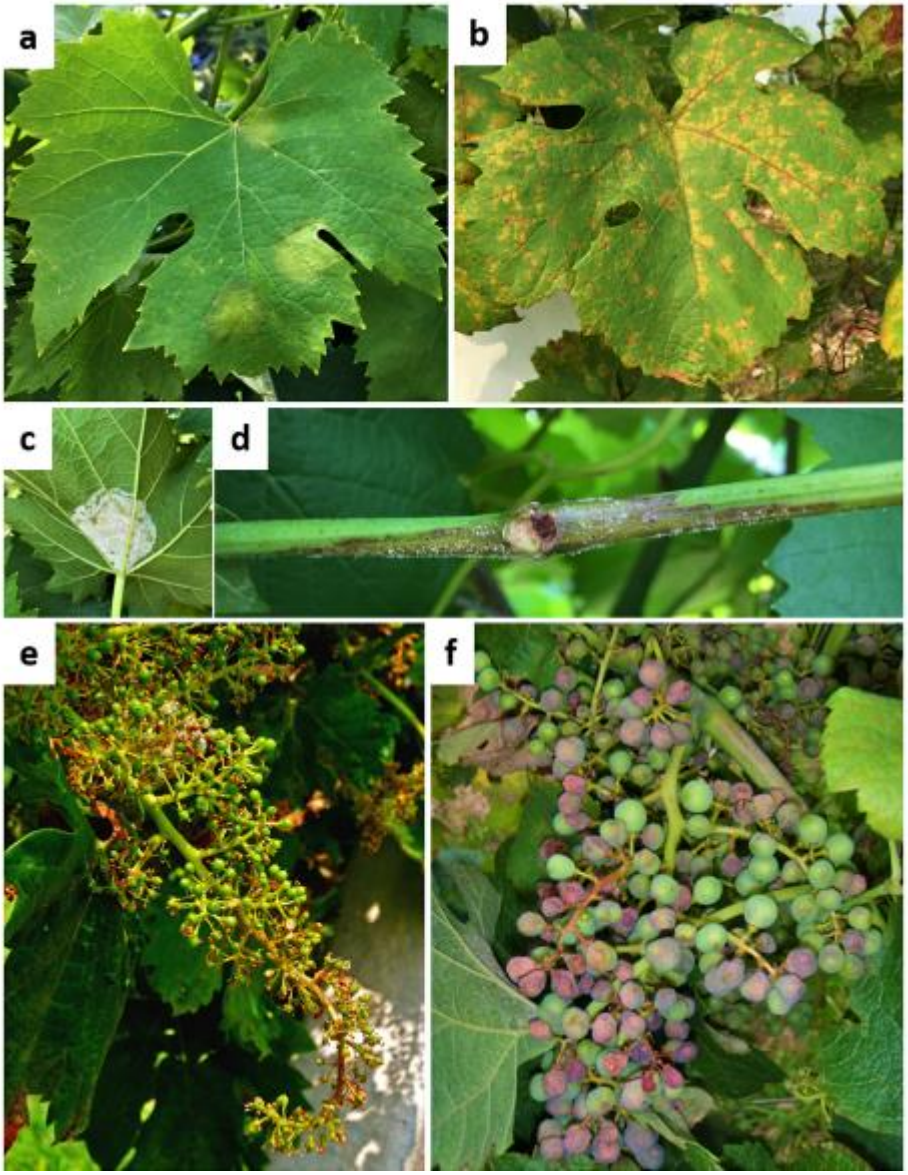


Figure 1.4 Symptoms of grapevine downy mildew on leaves (a-c), shoot (d) and bunches (e,f). a) oil spot (yellow circular spots with an oily appearance) on the upper side of the leaf; b) mosaic symptom (yellow spot restricted by veins to form yellow-to-brown small, angular spots in a mosaic pattern) on the upper side of the leaf; c) sporulation (sporangiophores and sporangia appearing as a bright white, fluffy growth) on the undersides of leaves; d) shoot covered by sporulation turning brown; e) distorted bunch (U-shaped) turning necrotic; f) shrinking berries turning violet.

Most of the *Vitis* taxa related to North America are to some extent resistant to *P. viticola* (Unger et al., 2007). The resistance response to *P. viticola* results in rapid plant cell death after pathogen recognition and local necrosis induction. This mechanism, known as the hypersensitive response (HR), is an active triggered procedure initiated by fungal elicitors or other elicitors (Balint-Kurti, 2019) that leads to bursts of production of reactive oxygen species (ROS) and nitric oxide (NO). Consequently, the host cells collapse and shrink, hampering the fungal infection (Toffolatti et al., 2016). Cell death appears as small necrotic spots on plant tissues.

The Georgian grapevine germplasm is characterized by very high genetic diversity, with cultivars differing from major European ones (Imazio et al., 2013). Considering that this high variability could also be a source of resistance to important pathogens, some studies have been undertaken to assess the resistance levels of Georgian accessions to *P. viticola*. The first one, carried out by Bitsadze et al. (2015), showed that 20 accessions were characterized by medium to high levels of resistance to downy mildew in a collection of 61 native Georgian varieties. The promising results showed the importance and value of screening for additional Georgian germplasm. In a study by Toffolatti et al. (2016), a total of 93 accessions were studied over three years both in the field and laboratory. A small group of varieties, including Kamuri Shavi, Mgaloblishvili and Ubakluri, showed reduced disease severity values. However, only Mgaloblishvili showed strong and constant phenotypical resistance against the pathogen. In Table 1.2, a list of Georgina resistant varieties is reported. Indeed, recent studies on the transcriptome of Mgaloblishvili showed that the cultivar possesses a unique response to *P. viticola* that is based on the overexpression of genes that are not modulated or downregulated in susceptible (Pinot noir, a *V. vinifera* cv) and resistant (Bianca, interspecific hybrid) cultivars (Toffolatti, De Lorenzis, et al., 2018). The resistance mechanism of Mgaloblishvili is based on the overexpression of genes encoding: i) receptors for pathogen recognition (PAMP-Pathogen Associated Microbial Patterns-receptors and for damages at the cell wall (DAMP-Damage Associated Microbial Patterns); ii) a NB-LRR receptor of fungal effectors (named Lr10); iii) ethylene signaling; iv) synthesis of terpenes, such as valencene, and flavonoids; v) strengthening of the cell wall. Besides genes involved in resistance, susceptible genes were also identified. Susceptibility genes are essential for plant-pathogen interaction and their disruption leads to resistance, as with *mlo* gene, whose knockdown is involved in resistance to *E. necator* (Pessina et al., 2016). The candidate gene related to susceptibility to *P. viticola* in *V. vinifera* encodes a LOB domain-containing (LBD) protein (Toffolatti et al., 2020) that has been previously found in the interaction between *Arabidopsis thaliana* and *Fusarium oxysporum* (Thatcher et al., 2012). The new genome-editing tools, providing several protocols to introduce knockout on target sequences, make the understanding of plant pathogen-resistance mechanism mediated by susceptibility genes a very attractive alternative for the development of durable disease-resistant varieties (Zaidi et al., 2018).

Table 1.2 List of Georgian grapevine accessions (wild and cultivated) showing resistance to *P. viticola*, ranging from very high to high degree (Bitsadze et al., 2015; Toffolatti et al., 2016).

Variety	**Berry color**	**Usage**	**Region of origin**	**Distribution**	**Resistance to *P. viticola***
Ikaltos Tsiteli	blue black	wine	Kakheti	Germplasm	high
Krakhuna Clone	blue black	table grapes	Imereti	Minor	
Ktsia	blue black	wine	Kartli	Germplasm	
Mtsvane Kakhuri	green yellow	wine	Kakheti	Major	
Tsitska	green yellow	wine	Imereti	Major	
Tsitska, clone	green yellow	wine	Imereti	Minor	
Rkatsiteli	dark red	wine	Kakheti	Minor	
Vardisperi	violet				
Saperavi	green yellow	wine	Kakheti	Minor	
Skra	-	-	Kartli	Wild	
Tedotsminda 10	-	-	Kartli	Wild	
Tedotsminda 15	-	-	Kartli	Wild	

Tskobila	blue black	wine	Kakheti	Germplasm	
Goruli Mtsvane	green yellow	wine	Kartli	Major	
Mgaloblishvili	blue black	wine	Imereti	Germplasm	very high
Chkhikoura	green yellow	wine	Imereti	Germplasm	
Dondghlabi Shavi	blue black	wine	Imereti	Germplasm	
Dondghlabi Mtsvane	grey	wine	Imereti	Germplasm	
Kakhis Tetri	green yellow	wine and tables grapes	Kakheti	Germplasm	
Kesi	green yellow	wine	Kakheti	Germplasm	
Muradouli	green yellow	wine	Imereti	Germplasm	
Tsirkvalis Tetri	green yellow	wine	Imereti	Germplasm	

1.8 Loci associated with the resistance to *P. viticola*

So far, the investigation of the genetic basis of *P. viticola* resistance has led to the identification of 28 resistance (R) loci in different regions (Figure 1.5). These R loci (designated *Rpv*), identified through QTL (Quantitative Trait Loci) analysis on a range of North American and Asian *Vitis* species, confer different degrees of disease resistance, ranging from partial to total resistance (Dry et al., 2019). The major loci of this list are: i) *Rpv1*, identified in *Muscadinia rotundifolia*, confers partial resistance to *P. viticola* infection and is associated with a gene encoding TIR-NB-LRR protein (MrRPV1) (Feechan et al., 2013; Merdinoglu et al., 2003); ii) *Rpv2*, identified in *M. rotundifolia*, confers total resistance to downy mildew and is associated to a cluster of TIR-NB-LRR genes (Dry et al., 2019); iii) *Rpv3*, identified in *V. labrusca*, *Vitis lincecumii*, *V. riparia and V. rupestris*, confers partial resistance to downy mildew (Bellin et al., 2009; Gaspero et al., 2011; Welter et al., 2017); iv) *Rpv8* and *Rpv12*, identified in *V. amurensis*, confer high resistance to *P. viticola* infection and are associated with the cluster of genes encoding NB-LRR proteins (Blasi et al., 2011; Venuti et al., 2013); v) *Rpv15*, identified in *Vitis piasezkii*, confers strong resistance to *P. viticola* infection (Dry et al., 2019). The other R loci are considered minor loci due to their capability to confer low degrees of resistance and their usefulness is proved only in combination with major R loci. To date, from which 28 loci (*Rpv*1-28), except *Rpv* 15, 16 and 28, the rest have been identified on chromosomal genome location of grapevine (Figure 1.6) under different genetic backgrounds (Bellin et al., 2009; Di Gaspero et al., 2012; Divilov et al., 2018; Fischer et al., 2004; Marguerit et al., 2009; Merdinoglu et al., 2003; Moreira et al., 2011; Ochssner et al., 2016; Sapkota et al., 2019; Schwander et al., 2012; van Heerden et al., 2014; Venuti et al., 2013; Welter et al., 2007; Wiedemann-Merdinoglu et al., 2006; Zyprian et al., 2016). This map could be used to study the majority of linkage maps and marker-assisted selection (MAS).

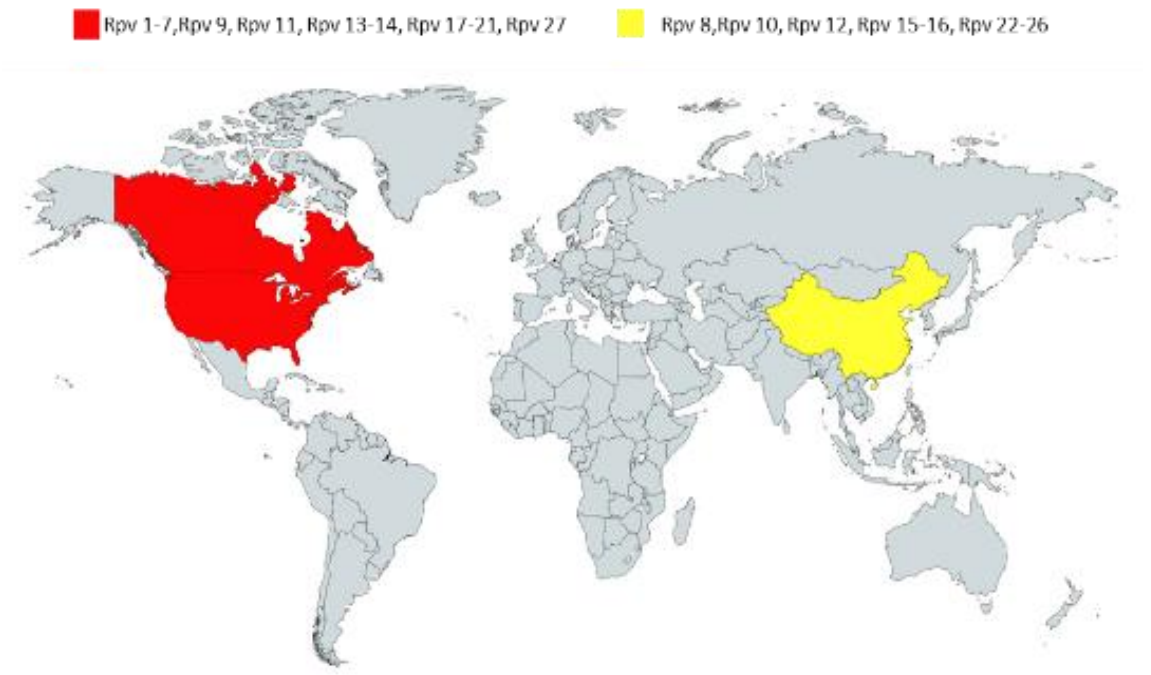


Figure 1.5 Distribution of resistance loci to *P. viticola* (*Rpv*) in grapevine genetic background which have been identified in Northern American and Asian *Vitis* species.

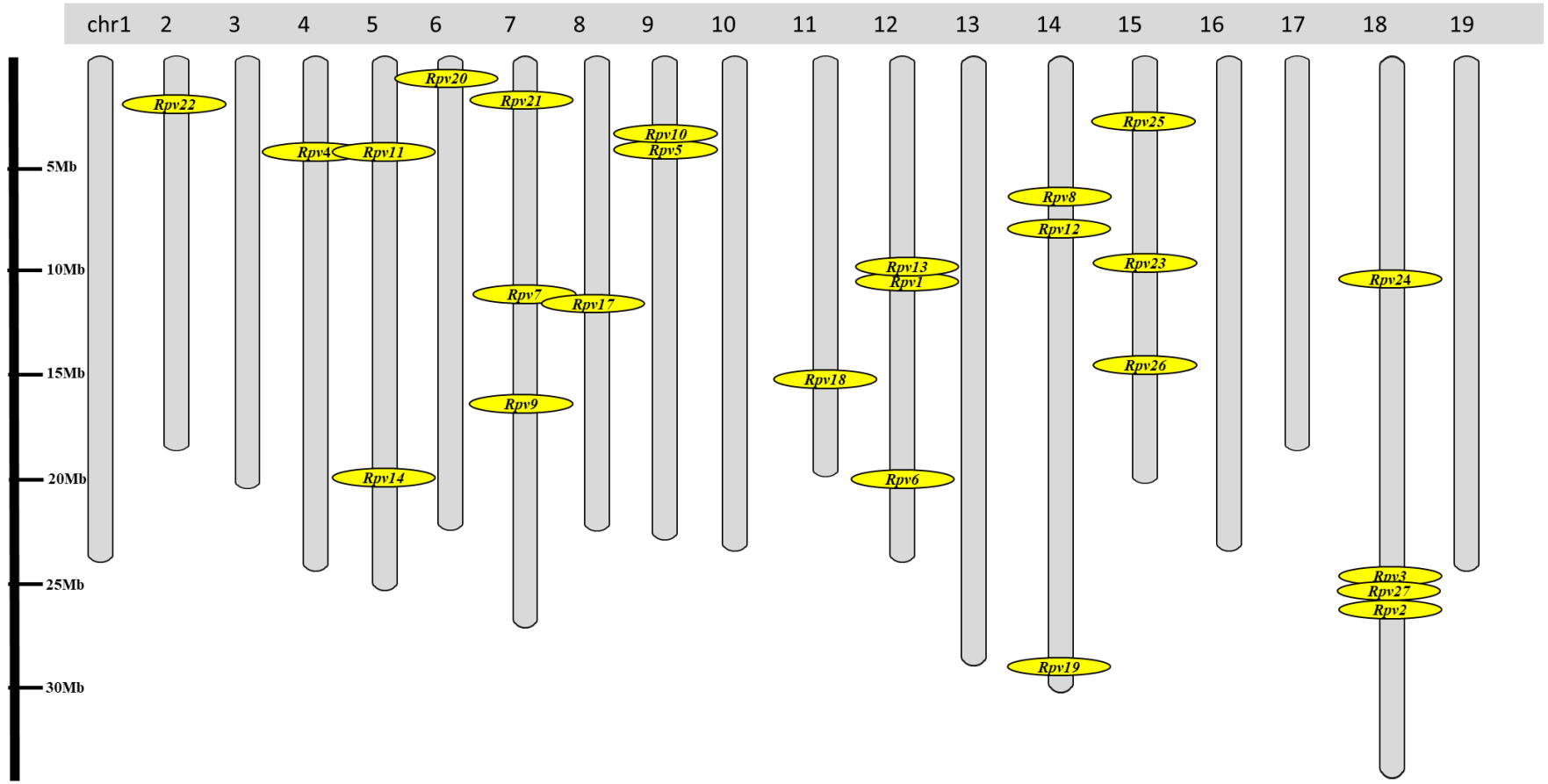


Figure 1.6 Identification of pathogen-resistance loci in *Vitis*. 28 reported *Rpv* on *Vitis* reference genome (12X v2.0) (Canaguier et al., 2017) marked on chromosomal map (chr1-19). Ruler on the left side indicates the Mb distance.

1.9 Conclusion, Problem and aim of project

The Georgian germplasm is considered as cultivars characterized by late ripening, which could potentially reduce issues related to excessive temperatures in summertime, distinctive eno-carpological traits, which affect the grape and wine quality, specific responses to abiotic stresses, such as sunburn; and resistance traits related to biotic stresses, such as oomycete *P. viticola* (Berk. & M.A. Curtis) Berl. & De Toni. The interestingly huge genetic variability in Georgian grapevine made them a worthwhile resource for breeding programs. Amongst which, in primary pathogen development, Mgaloblishvili has been determined with unique resistance behavior of overexpression of genes related to pathogen recognition, signaling and defense response (Toffolatti, De Lorenzis, et al., 2018).

Given the reasons stated in this work, the screening and assessment of Georgian germplasm for resistance response to *P. viticola* pathogens should be promoted. In this way, it will be possible to exploit the valuable traits enclosed by this unique source of genetic variability for new varieties giving them the ability to properly face the challenges awaiting viticulture in the era of resistance to *P. viticola*. In line with this, a modified version of chapter one has been published as a review paper in Frontiers in Plant Science entitled "Georgian Grapevine Cultivars: Ancient Biodiversity for Future Viticulture" authored by Maryam Sargolzaei, Laura Rustioni, Gabriele Cola, Valentina Ricciardi, Piero A. Bianco, David Maghradze, Osvaldo Failla, Fabio Quaglino, Silvia L. Toffolatti and Gabriella De Lorenzis . Aside from traditional breeding programs, these invaluable resources could be exploited in breeding programs based on the use of New Breeding Technologies (NBTs); i.e. through genome editing applied on both resistance and, susceptibility candidate genes (which even have more practical advantages) to abiotic and biotic stresses.

However, during the plant-pathogen interaction, both plant and pathogen evolve for survival. While there are lots of investigations on grapevine response to *P. viticola*, there is less focus on the molecular reaction of the pathogen with its host. The understanding of pathogen virulence mechanism with different resistance grapevines originated from various regions could be a prerequisite to developing pathogen strategies.

The overall aim of the project breaks down into three objectives which are proposed as to identify loci related to resistance to *P. viticola* by GWA study (chapter 2); to thoroughly reveal grapevine cultivar resistance mechanism against *P. viticola* (chapter 3) and to characterize transcripts of *P. viticola* in the early interaction with grapevine cultivars (chapter 4).

To breed grapevines with specific features, marker-assisted selection (MAS) of either qualitative or quantitative trait could be used as a tool. Markers related to disease-resistance genes are currently used in large-scale breeding programs of grapevine. From an economic point of view, the identification of inheritance and the subsequent development of molecular markers linked to resistance genes to *P. viticola* in *V. vinifera* may have a very important impact on the grapevine breeding programs via marker-assisted selection (MAS) due to the reduction of the time needed to obtain resistant varieties characterized by high-quality standards. Therefore, the identification of the loci related to downy mildew resistance in Mgaloblishvili (*V. vinifera*) by GWA approach was set as the first objective for the current study. Chapter 2 is part of the publication titled "*Rpv*29, *Rpv*30 and *Rpv*31: three novel

genomic loci associated with resistance to *Plasmopara viticola* in *Vitis vinifera*" authored by Maryam Sargolzaei, Giuliana Maddalena, Nana Bitsadze, David Maghradze, Piero Attilio Bianco, Osvaldo Failla, Silvia Laura Toffolatti and Gabriella De Lorenzis and published on Frontiers in Plant Sciences (11:562432; 2020).

Resistance mechanism could be traced by RNA-sequencing, which is a high-throughput method to find regions with differentially transcribed genes. In the previous work of Toffolatti et al. (2018), two genes of valencene synthase and a cytochrome P450 (CYP72A219 element) showed a remarkable expression pattern. Valencene synthase is a terpene synthase, involved in the biosynthesis of (+)-valencene, a sesquiterpene, and its isomer (-)-7-epi-α-selinene, by using farnesyl diphosphate as a substrate (Lucker et al., 2004). This project explores the involvement of Volatile Organic Compounds (VOCs) emitted by two resistant varieties, Bianca (an interspecific hybrid obtained by crossing American species with *V. vinifera*) and Mgaloblishvili (*V. vinifera*) in response to *P. viticola* infection. Therefore, the ultimate aim was to propose an eco-sustainable approach regarding to VOCs act against pathogens and herbivores. The results reported in chapter 3 were part of publication titled "From plant resistance response to the discovery of antimicrobial compounds: the role of volatile organic compounds (VOCs) in grapevine downy mildew infection to Plant Physiology and Biochemistry" authored by Valentina Ricciardi, Demetrio Marcian`o, Maryam Sargolzaei, Giuliana Maddalena, David Maghradze, Antonio Tirelli, Paola Casati, Piero Attilio Bianco, Osvaldo Failla, Daniela Fracassetti, Silvia Laura Toffolatti, Gabriella De Lorenzis.

However, the study of plant-pathogen interaction to deduce alternative plant-protective solutions is not confined to the study of plant response to a pathogen, rather it also includes the study of the molecular reactions of the pathogen during the interaction. Thus, the last objective was to apply the RNA sequencing data with next-generation sequencing technology (NGS) to identify transcripts and genes activity in the early *P. viticola* development on susceptible (state of *Rpv*-, 2008-059-020) in comparison to two resistance (heterozygous (*Rpv*3/*Rpv*10) and homozygous (*Rpv*10/*Rpv*10) hosts. This study aimed for understanding the encoding transcripts and genes of pathogen signal, apoplast and effectors proteins combined with its virulence mechanisms, to develop novel strategies of pathogen control. The results reported in chapter 4 were submitted to the European Journal of Plant Pathology.

Chapter 2: Genome Wide Association (GWA) study to identify loci related to resistance to pathogen

2.1 Introduction

Vitis vinifera L. is one of the most widely cultivated fruit tree species of agricultural interest and it is the only species of the *Vitis* genus extensively used in the global wine industry. According to the data collected in 2018, viticulture covers approximately 7.6 million hectares worldwide and produces more than 67 million tons of grapes (http://www.oiv.int/). Unfortunately, *V. vinifera* is also known as the most susceptible *Vitis* species to *Plasmopara viticola* (Berk. *et* Curt.) Berl. and de Toni, the oomycete causing grapevine downy mildew. *P. viticola* was introduced into France from North America during the XIX century together with American wild *Vitis* species and rapidly spread across Europe dividing into two genetically distinct groups (Fontaine et al., 2013; Maddalena et al., 2020). Structure analysis indicated that the European and Italian *P. viticola* populations are formed by two separate genetic clusters, distributed according to a geographical gradient (East-West) and climatic conditions (Fontaine et al., 2013; Maddalena et al., 2020). *P. viticola* is a polycyclic pathogen able to biotrophically grow on tissues (leaves, shoots and clusters) of susceptible *Vitis* species and, particularly, *V. vinifera*. If adequate disease management strategies are not applied, the disease seriously affects yield in terms of grape quality and quantity (Toffolatti, Russo, et al., 2018).

Resistant accessions within the North American *non-vinifera* species, such as *Vitis riparia* Michx., *Vitis cinerea* (Engelm. ex A.Gray) Engelm. ex Millard and *Vitis labrusca* L., and the Northeast Asian species (*Vitis amurensis* Rupr.), exhibit varying levels of resistance, ranging from moderate to high, due to co-evolution with the pathogen (Jürges et al., 2009). Several QTL (Quantitative Trait Loci), conferring downy mildew resistance, at different levels ranging from weak to total, were discovered in *Vitis* species background: *Rpv1* and *Rpv2* in *Muscadinia rotundifolia* Michaux (Merdinoglu et al., 2003; Wiedemann-Merdinoglu et al., 2006); *Rpv3* and *Rpv19* in *Vitis rupestris* Scheele (Bellin et al., 2009; Divilov et al., 2018; Foria et al., 2020; Vezzulli et al., 2019; Welter et al., 2007); *Rpv4*, *Rpv7*, *Rpv11*, *Rpv17*, *Rpv18*, *Rpv20* and *Rpv21*, in unspecified American species (Bellin et al., 2009; Divilov et al., 2018; Fischer et al., 2004; Welter et al., 2007); *Rpv5*, *Rpv6*, *Rpv9* and *Rpv13* in *V. riparia* (Marguerit et al., 2009; Moreira et al., 2011); *Rpv8*, *Rpv10*, *Rpv12*, *Rpv22*, *Rpv23*, *Rpv24*, *Rpv25* and *Rpv26* in *V. amurensis* (Blasi et al., 2011; Lin et al., 2019; Schwander et al., 2012; Song et al., 2018; Venuti et al., 2013); *Rpv14* in *V. cinerea* (Ochssner et al., 2016); *Rpv15* and *Rpv16* in *Vitis piasezkii* Maxim. (Pap et al. unpublished); *Rpv27* in *Vitis aestivalis* Michx. (Sapkota et al., 2015, 2019); and *Rpv28* (Bhattarai et al., in preparation; www.vivc.de).

The management of downy mildew on traditional *V. vinifera* varieties requires regular application of fungicides. It is estimated that in the European Union, viticulture accounts for approximately 70% of all agrochemicals used, most of which are applied to contain the agents of downy and powdery mildews. Nevertheless, the intensive use of chemicals is becoming more and more restricted because of their high costs, their risks to human health and their negative environmental impact due to the chemical residues detected in grapes,

soil and aquifers. Also, disease control could be difficult to attain in the future because some *P. viticola* strains could develop site-specific fungicide resistances, leading to great difficulties in the management of disease, while the discovery of new modes of action is rare (Hollomon, 2015). The EU Directive 2009/128 for sustainable management of diseases caused by plant pathogens in Europe strongly recommends a reduction in the number of treatments in the field. Moreover, the application of Regulation 1107/2009, concerning the placement on the market of plant protection products, is causing a reduction in the active substances available. The exploitation of resistance sources is the best way to decrease the use of chemicals for disease management and to achieve effective protection from *P. viticola* in an environmentally friendly way. Breeders had already started crossing the susceptible *V. vinifera* varieties with American species in the XIX century, first in the US and then in Europe (Eibach & Töpfer, 2015; Merdinoglu et al., 2018; Migicovsky et al., 2016; Yobrégat, 2018). Nowadays, numerous varieties combining resistant traits from American and Asian species and the quality traits of *V. vinifera* are available (A Reynolds, 2015). A comprehensive list of new resistant varieties can be accessed from the *Vitis* International Variety Catalogue website (VIVC; www.vivc.de).

Finding new sources of resistance is of paramount importance in breeding for biotic stress resistance in a perennial crop, which has to be productive for years while maintaining its resistance characteristics at the same time: the main strategy for preventing the selection of pathogen strains able to overcome resistance is pyramiding resistance genes in the crop variety (Delmotte et al., 2016; Eibach et al., 2007; Zini et al., 2019). Recently, unique resistance traits to the downy mildew agent have been reported in *V. vinifera* varieties (Bitsadze et al., 2015; Toffolatti et al., 2016) coming from the first domestication center of the species: Georgia, Southern Caucasus (Imazio et al., 2013). The resistance mechanism for one of these resistant cultivars, named Mgaloblishvili, has been studied in detail (Toffolatti et al., 2020; Toffolatti, De Lorenzis, et al., 2018). After artificial inoculation, *P. viticola* growth and sporulation are significantly affected in Mgaloblishvili: the mycelium degenerates, sporangiophores show altered morphology and lower numbers of sporangia are produced, without any evidences of the hypersensitive response that occurs in American species. From the transcriptomic point of view, its defense mechanism shows overexpression of genes related to pathogen recognition through PAMP (pathogen-associated molecular patterns), DAMP (damage-associated molecular patterns), and effector receptors and ubiquitination, signaling pathway through ethylene, synthesis of antimicrobial compounds (such as monoterpenes and flavonoids) and fungal wall degrading enzymes, and the development of structural barriers (such as cell wall reinforcement). The discovery of resistance to *P. viticola* in *V. vinifera* promises fresh opportunities for grapevine breeding in terms of new resistant loci.

Breeding for disease resistance is a very time-consuming process (up to 25-30 years are required for a breeding program) because it needs the evaluation of resistance levels of the progeny and other important characteristics (yield and quality of vines), which is typically not achieved until the third year after planting. A way to considerably decrease the length of the breeding process (accelerating the process by up to 10 years) is the adoption of the marker-assisted selection (MAS) approach, which allows the targeted selection of progeny harboring the resistance loci (Eibach & Töpfer, 2015).

Identification of genomic loci associated with complex quantitative and qualitative traits was enabled by the development of QTL (quantitative trait locus) and GWA (genome-wide association) mapping approaches, combining genetic and phenotypic data. QTL mapping is

performed using segregating biparental populations, while GWA approach relies on historical recombination events that occurred in natural populations, germplasm collections and breeding materials (Korte & Farlow, 2013). Over the last 10 years, NGS (next-generation sequencing) technologies have made available numerous (from thousands to hundreds of thousands) SNP (single nucleotide polymorphism) markers to be used for GWA study (GWAS) in various plant and animal species (Bhat et al., 2016).

In grapevine, at least three high-density SNP arrays have been set up (Laucou et al., 2018; Marrano et al., 2017; Myles et al., 2010), and the most used SNP set is the Vitis18kSNP chip array, developed by the GrapeReSeq Consortium, re-sequencing the genome of 47 *V. vinifera* genotypes and 18 genotypes belonging to American *Vitis* species and holding 18,071 SNPs. This high-density SNP array has been demonstrated to be a valid method for mapping both quantitative and qualitative traits (Laucou et al., 2018).

In the present work, the Vitis18kSNP chip array was used to genotype a panel of *V. vinifera* Georgian accessions to identify genomic regions and/or putative markers associated with *P. viticola* resistance in *V. vinifera*, through a GWA approach, to be used for MAS in further breeding programs.

2.2 Material and methods

2.2.1 Plant material

The panel of accessions analyzed in this study (Table S.1) accounted for 132 genotypes: 84 are seedlings of the Mgaloblishvili self-pollinated population, and 48 are genotypes belonging to the Georgian germplasm collection which were in order to increase the accuracy of the GWA study and rich the minimum number of individuals (>100). The selection of cultivars was randomized regarding sample availability. The breeding-derived genotypes are maintained in the greenhouse of the Department of Agricultural and Environmental Sciences (DiSAA), located in Arcagna (Lodi, Italy), and the germplasm genotypes are planted in the DiSAA germplasm collection vineyard, located in Torrazza Coste (Pavia, Italy). Mgaloblishvili self-progeny was obtained in the spring of 2012, by enclosing Mgaloblishvili inflorescences in paper bags before flowering. At harvesting, bunches were collected, and the seeds were extracted from berries to be vernalized at 5°C for two months in humid sand. The vernalized seeds were placed in plates of polystyrene cups filled with rockwool and maintained at 20 to 25 °C up to germination in a screenhouse. The seedlings were transplanted into 8-cm pots filled with a sand–peat mixture (7:3 in volume) and after one year were moved in 20-cm pots. The plants were regularly irrigated and maintained without mineral fertilization practice. In Figure 2.1, some stages of Mgaloblishvili self-pollination, seedling germination and plant maintenance in the greenhouse are shown.

Figure 2.1 Some stages of Mgaloblishvili self-pollination (A, B), seedling germination (C) and plant maintenance in greenhouse (D).

2.2.2 Phenotyping

The degree of susceptibility to *P. viticola* was evaluated through experimental inoculation on leaf samples collected at the beginning of 2015, 2016, and 2017 grapevine growing seasons, using the protocol described by Toffolatti et al. (2016). To maximize the genetic variability of the pathogen and allow the detection of accessions that were resistant to a wide range of pathogen strains, field populations of *P. viticola* were used for the experimental inoculations (Toffolatti et al., 2016). Recent studies demonstrated that the European and Italian *P. viticola* population is divided into two genetic clusters, separated over an east-west gradient (Fontaine et al., 2013; Maddalena et al., 2020). In this study, east and west populations of *P. viticola* coming from Italy, at S. Maria della Versa (Pavia; East population) and Casarsa della Delizia (Pordenone; West population), and Georgia (West), were mixed to perform experimental inoculations. Phenotypical evaluations were performed in triplicate. Briefly, three leaf discs (1.5 cm in diameter) were cut from three leaves collected from the 3rd-5th leaf starting from the shoot apex of the plants. The leaf disks were sprayed with 1 mL *P. viticola* sporangia suspension ($5x10^4$ sporangia·mL^{-1}) and incubated in a humid chamber at 22 °C for 10 days. Disease severity was estimated from the area covered by sporulation by calculating the Percentage Index of Infections (I%I) (Townsend &

Heuberger, 1947). The accessions with an average I%I lower than 25% along the three sampled years were considered resistant. The 25% threshold was chosen based on the I%I distribution. Box plot distribution of the three replicate values of the samples showed that only nine samples (ID 124, ID 122, ID LIB 56, ID 138, ID 109, ID L22A, ID M22F, ID M22A, ID M22E) showed I%I<40%, while the others reached higher values (Supplementary Figure S.1). The average I%I of these samples was 20±5% (95% confidence interval). Therefore, 25% was the chosen threshold. The existence of differences between I%I recorded in different years was analyzed by Pearson's correlation coefficient.

Resistance levels (RLs), expressed in percentage, were calculated for each accession by using the following formula:

$RL = 100 - (\frac{I\%I_x}{I\%I_{MAX}} \times 100)$ where $I\%I_x$ is the average disease severity of sample x and $I\%I_{MAX}$ is the maximum value of disease severity recorded (accession ID 157 M, I%I=85.8%).

2.2.3 SNP genotyping

The 132 genotypes were genotyped using the Vitis18kSNP array (Illumina Inc., San Diego, CA, USA), containing 18,071 SNPs. The genotyping of breeding-derived accessions (Mgaloblishvili seedlings) was performed in this work, while for germplasm genotypes the data were obtained by De Lorenzis et al. (2015). Genotyping was carried out on 200 ng of genomic DNA extracted from 100 mg of freeze-fresh young leaf tissue using NucleoSpin® Plant II (MACHEREY-NAGEL, Germany), according to the manufacturer's protocol. DNA concentration and quality were checked by electrophoresis on an agarose gel and by spectroscopy using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Quant-iT dsDNA HS assay kit for Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Genotyping analysis was performed by the laboratory of Fondazione Edmund Much (San Michele all'Adige, Trento, Italy).

2.2.4 Data analysis

SNP data produced in this work (84 Mgaloblishvili seedlings) were filtered for samples showing a call quality value (p50GC) lower than 0.54 and loci with a GenTrain (GT) score value lower than 0.6 and a marker missing rate > 20% (De Lorenzis et al., 2015). The Mgaloblishvili self-pollinated population dataset and the SNP profiles of 48 varieties reported in De Lorenzis et al. (2015) were merged and filtered for minor allele frequency (MAF) > 5%.

MEGA 7.0 software (S. Kumar et al., 2016) was used to design a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) phylogenetic tree, based on the Dice's coefficient (Dice, 1945) distance matrix generated by PEAS 1.0 software (Xu et al., 2010). Principal Component Analysis (PCA) was carried out using *adegenet* package (Jombart, 2008) of R software (R Core Team), and the first two components values were plotted on a 2-D scatterplot. Structure analysis was carried out using LEA package (Frichot & François, 2015) of R software by varying the number of ancestral genetic groups (K) from 1 to 10 in ten repetition runs for each K value. The most likely K value was detected based on LEA cross-validation method.

The LD (linkage disequilibrium) estimation as Pearson's squared correlation coefficient (r^2) between each pair of molecular markers (Zhao et al., 2005) was evaluated using PLINK (Purcell et al., 2007) software. The pair-wise LD as r^2 was calculated using the parameters -

-ld-window-r2 0, --ld-window 99999, --ld-window-kb 10000. The distances between loci were categorized into intervals of a fixed length (100 kb) and, for each interval, average r^2 was calculated. The LD decay was visualized by plotting the average r^2 per interval from 0 up to 10 Mb by R software.

Association analysis was performed in R software using GAPIT package (Lipka et al., 2012). GLM (Generalized Linear Model), MLM (Mixed Linear Model), MLMM (Multiple Locus Mixed Linear Model), FarmCPU (Fixed and random model Circulating Probability Unification) and SUPER (Settlement of MLM Under Progressively Exclusion Relationship) algorithms were tested. For fixed effect, Q-matrix (for K = 3), detected by LEA, was used as the covariate for association analysis accounting for population structure. The GWA algorithm performances were evaluated through quantile-quantile (QQ) plots. A conservative threshold for assessing SNP significance was calculated based on Bonferroni correction for a type I error rate of 0.05. The SNPs fitting a logistic regression, performed in PLINK software, were selected.

2.2.5 Candidate gene mining

Gene associated with SNP loci passing the Bonferroni-adjusted threshold were predicted based on the LD r^2 threshold of 0.2 (X. Li et al., 2014), using the grapevine reference genome PN40024 (12X.v2 version) (Canaguier et al., 2017). The SNP loci mapping to reference genome was conducted using CLC Genomic Workbench software (v. 20.0) in advance sequence finder toolbox including negative strand. Nearby genes in linkage regions of stable SNP-trait associations with putative functions supposedly related to the *P. viticola* resistant trait were selected as candidates.

2.3 Results

2.3.1 Phenotypic and genetic diversity of accession panel

Phenotyping evaluations were performed for three years (2017-2019) and only genotypes scored with an I%I<25% in the three years of the evaluation were classified as resistant. Evaluation trials have shown an overall high susceptibility to *P. viticola* infection, with some accessions showing a large distribution of the data (Supplementary Figure S.1). Nine out of 132 genotypes were resistant: five Mgaloblishvili seedlings (ID 124, 122, LIB 56, 138, 109), Mgaloblishvili and three varieties (Jani Bakhvis, Zerdagi and Kamuri shavi) (Figure 2.2A; Table S.1). The samples showed a significant correlation among years ($r>0.991$; $N=3$; $P<0.043$). RLs of the nine resistant genotypes ranged from 70 to 84% (Table S.1). None of the resistant genotypes showed HR in leaf tissues.

The SNP genotyping data of the Mgaloblishvili self-pollinated population were merged with the ones of 48 Georgian cultivars (De Lorenzis et al., 2015). The final dataset accounted for 132 genotypes and 12,825 SNP loci. Clustering analysis discriminated the genotypes into two well distinct main groups (Figure 2.2B). In each main group, both breeding-derived genotypes and germplasm cultivars were included, though they were mainly clustered in well-separated sub-groups. Resistant genotypes were distributed between the two main groups. The range of identity varied from 95% to 88%. PCA strongly differentiated Mgaloblishvili self-pollinated and germplasm individuals into two distinct groups (Figure 2.2C). The first two principal components (PCs) captured 33% of total explained variance (PC1 = 29% and PC2 = 4%). The two groups were separated along the PC1. As expected, the germplasm individuals showed a variability higher than the breeding-derived accessions.

According to the cross-validation plot, structure analysis identified three ancestral populations (K = 3), one for Mgaloblishvili seedlings (group 1) and two for germplasm individuals (groups 2 and 3) (Figure 2.2D). The three resistant cultivars were assigned one to group 2 (Zerdagi) and two to group 3 (Jani Bakhvis and Kamuri shavi). The percentage of admixed genotypes (with a membership probability < 80%) was 28%. All the admixed genotypes were detected among the cultivars (Table S.2). All the nine resistant genotypes showed a membership probability higher than 80%. LD decay was estimated for the entire dataset (Figure 2.2E). LD decreased with the increase in the physical distance between marker loci. Average LD decay (r^2 = 0.11) was observed after ~2Mb. The LD value dropped to 0.2 after ~100kb.

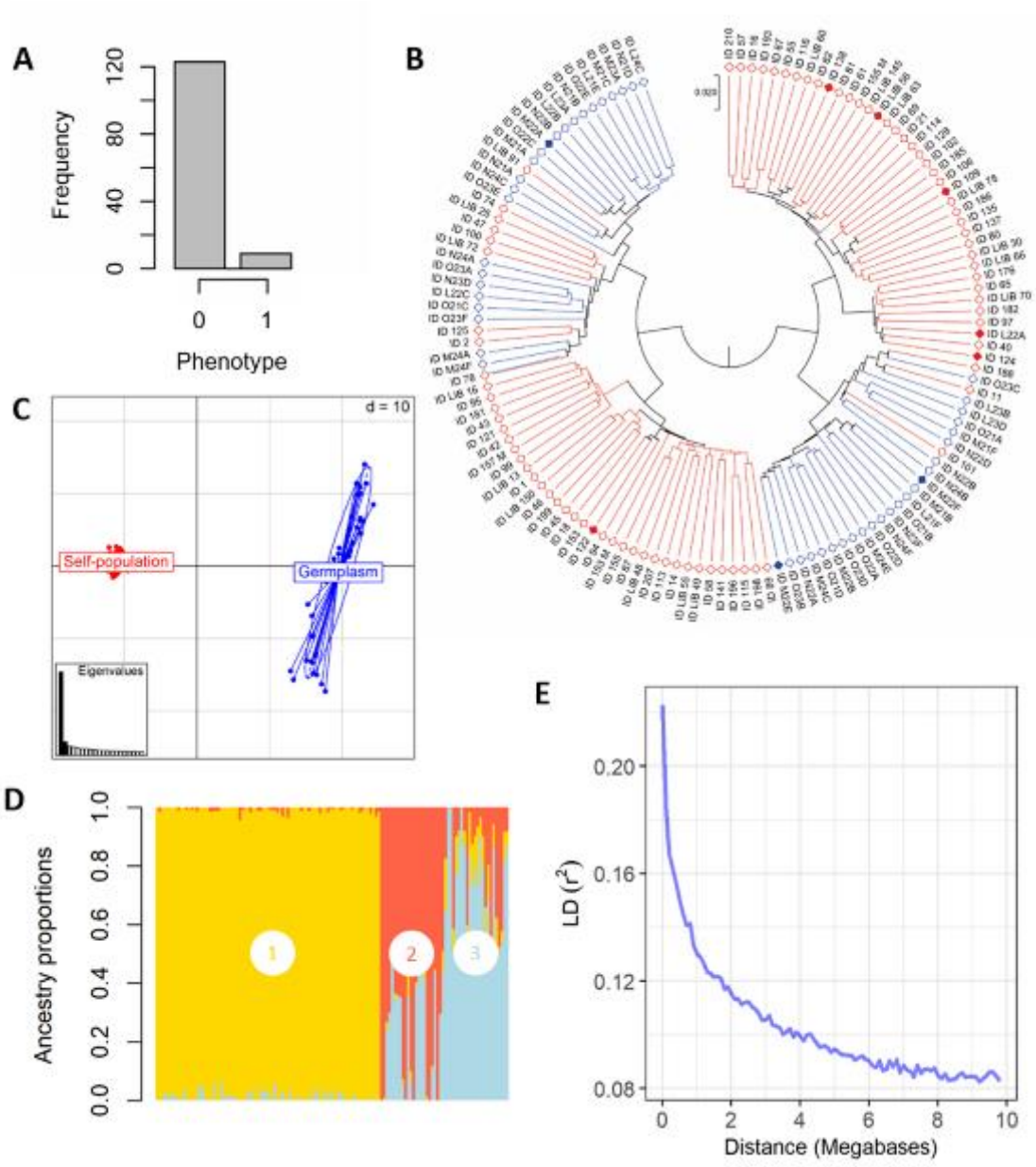


Figure 2.2 Phenotypical and genetic diversity in the panel of 132 grapevine accessions, belonging to the Mgaloblishvili self-population (84) and Georgian germplasm population (48), used for GWA analysis. The individuals were phenotyped for resistant trait to *P. viticola* infection and were genotyped using the Vitis18kSNP array. A. Histogram summarizing the frequency of susceptible (0) vs resistant (1) phenotypes.

B. UPGMA dendrogram showing relationships among individuals of Mgaloblishvili self-population (red) and Georgian germplasm population (blue). Filled rhombus indicate resistant accessions C. Scatterplot relationships among individuals of Mgaloblishvili self-population (red) and Georgian germplasm population (blue), as represented by the first two principal components (PC1 along the horizontal axis, PC2 along the vertical axis) of PCA. D. Admixture proportions as estimated by LEA package at K = 3, displayed in a barplot. Each sample is represented as a vertical bar, reflecting assignment probabilities to each of the three groups. Group 1: Mgaloblishvili self-population individuals. Group 2 and 3: Georgian germplasm population individuals. E. Decay of average linkage disequilibrium (LD r2) over distance (Mb).

2.3.2 GWA analysis

Different statistical models (GLM, MLM, MLMM, FarmCPU and SUPER) were tested for detecting associations for *P. viticola* resistance. Because structure analysis was able to capture the differences among the Georgian germplasm cultivars better than PCA, Q-matrix for K = 3 was used as a covariate in the GWA analysis. The application of GLM, MLM and SUPER models allowed to account for stratification, although a relevant number of false positives was detected (Figure 2.3A, B, E). A significant SNP associated with *P. viticola* infection was identified in the three tested models: the SNP (chr14_21613512_C_T) located in the chromosome 14 at position 21,613,512 with a *p*-value of 4.01e-07, 5.09e-07 and 3.68e-10, respectively for GLM, MLM and SUPER models. MLMM and FarmCPU models reduced false-positive associations (Figure 2.3C, D). MLMM models detected one significant SNP associated with *P. viticola* infection, with a $-\log_{10}$ *p*-value above the Bonferroni-adjusted threshold, and two SNPs below the Bonferroni-adjusted thresholds. The first SNP was the same detected by the GLM, MLM and SUPER models, with a *p*-value of 1.25e-08. The remaining two SNPs were li_T_C_chr16_21398409, located on chromosome 16 at position 21,398,409 and a *p*-value of 7.9e-06 and cn_C_T_chr3_16229046, located on chromosome 3 at position 16,229,046 and a *p*-value of 1.25e-05. FarmCPU model detected the same SNPs detected by MLMM model. chr14_21613512_C_T and cn_C_T_chr3_16229046 were above the Bonferroni-adjusted threshold, with *p*-values of 8.23e-08 and 8.18e-04, respectively, while li_T_C_chr16_21398409 was slightly below the threshold, with a *p*-value of 6.25e-03.

For an approximate estimation of allelic effect, logistic regression was fitted for the three significant SNPs. As observed by the odds ratio, a highly significant association was confirmed for chr14_21613512_C_T locus, followed by li_T_C_chr16_21398409 and cn_C_T_chr3_16229046 (Table 2.1).

Table 2.1 Allelic effect estimation by logistic regression for SNP loci associated to *P. viticola* resistant traits. Odds ratio and *p*-values are reported.

SNP ID	**Chromosome**	**Genome position (bp)**	**Odds ratio**	***p*-value**
chr14_21613512_C_T	14	21,613,512	28.39	0.00021
cn_C_T_chr3_16229046	3	16,229,046	3.74	0.00143
li_T_C_chr16_21398409	16	21,398,409	7.33	0.00179

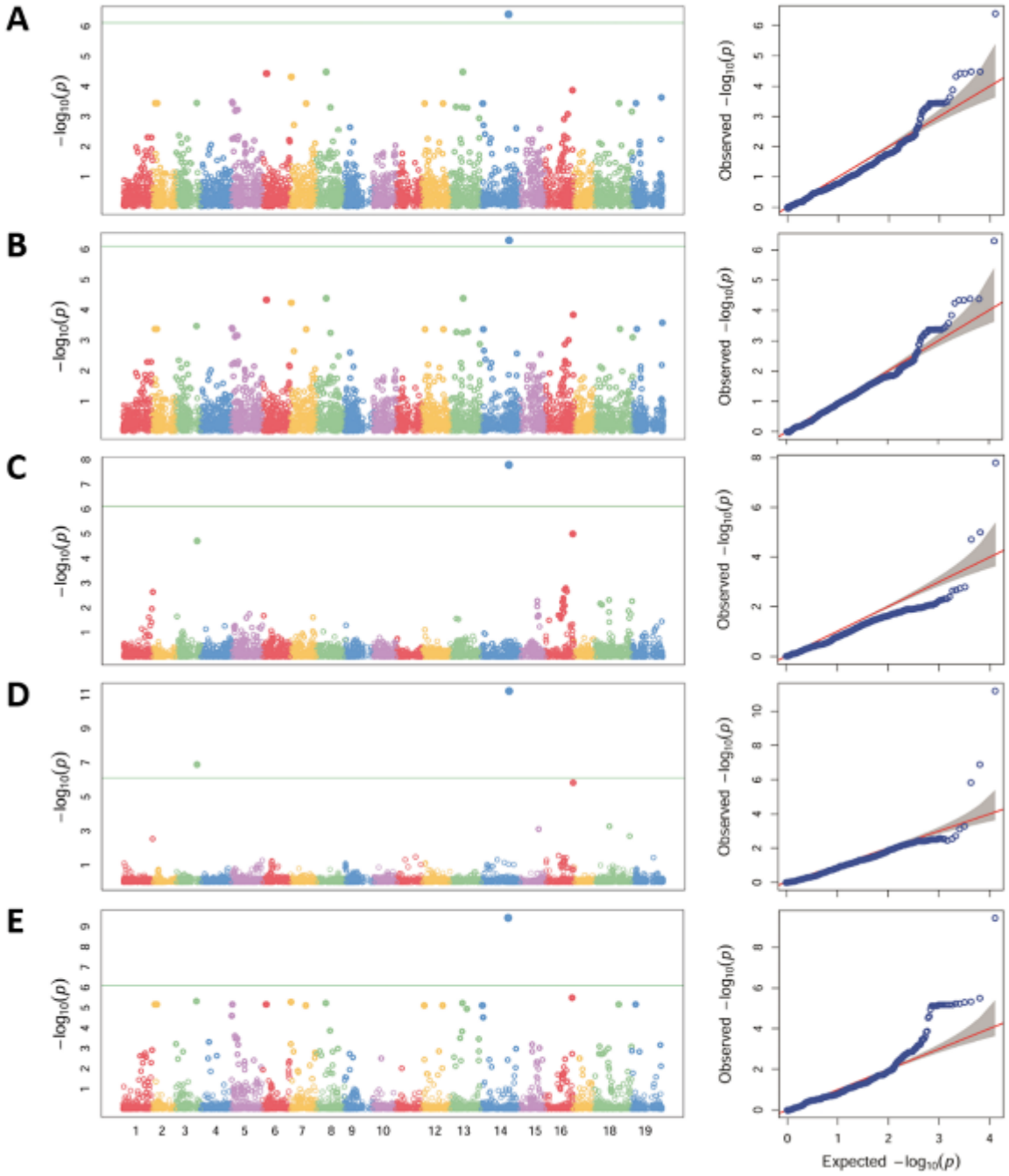


Figure 2.3 Manhattan plot (left) of -log10 p-values estimated for binary (resistant vs. susceptible) coded phenotypic response to *P. viticola* infection in the panel of 132 accessions genotyped by 18k SNPs. Significant SNPs are circles above the Bonferroni-adjusted threshold (green horizontal line). Quantile-quantile plot (right) of expected vs observed -log10 p-values. Association analysis results of GLM (A), MLM (B), MLMM (C), FarmCPU (D) and SUPER (E) algorithms.

2.3.3 Candidate gene prediction

The three SNP loci passing the Bonferroni-adjusted threshold were mapped to *V. vinifera* reference genome (PN40024 12X) to identify putative genes related to the *P. viticola* resistant trait (Figure 2.4). The LD value (r^2) dropped to 0.2 after ~100kb, for this reason, a

window of 100kb upstream and downstream the most significant SNPs were chosen to search for candidate genes. Supplementary Table S.3 reports the list of candidate genes in a window of 100kb upstream and downstream the three SNPs associated to *P. viticola* resistance trait. Supplementary Table S.4 reports the SNP allele information associated with these three regions.

The chr14_21613512_C_T locus mapped in the coding region of HEAT repeat-containing 5B protein (VIT_214s0006g03120) (Figure 2.5). The polymorphism (G → A) was non-synonymous giving rise to a change in the encoded amino acid, from aspartic acid (D) to asparagine (N). Upstream of this locus were annotated five genes: three of them encode for uncharacterized proteins (VIT_214s0006g03076, VIT_214s0006g03080 and VIT_214s0006g03100), and two for a probable cellulose synthase A catalytic subunit 8 [UDP-forming] (VIT_214s0006g03090) and an acyl-CoA-binding domain-containing protein 3-like (VIT_214s0006g03110). Downstream of this locus were annotated two genes, encoding for a probable carboxylesterase 17 and a plant cadmium resistance 4 protein (VIT_214s0006g03180 and VIT_214s0006g03190, respectively).

cn_C_T_chr3_16229046 and li_T_C_chr16_21398409 loci were mapped in intragenic regions (Figure 2.6 and 2.7). The first locus was localized in a region including, upstream, an uncharacterized protein (VIT_203s0017g00420), a magnesium-dependent phosphatase 1 (VIT_203s0017g00410), an ubiquitin carboxyl-terminal hydrolase 21 (VIT_203s0017g00396), a MADS-box protein JOINTLESS-like (VIT_203s0017g00390), and a magnesium-dependent phosphatase 1-like (VIT_203s0017g00380), downstream, an uncharacterized protein (VIT_203s0017g00440), a MADS-box protein JOINTLESS-like (VIT_203s0017g00450) and an inositol transporter 1 (VIT_203s0017g00460). The second locus mapped in the genomic region including, upstream, two rust resistance kinase Lr10-like genes (VIT_216s0148g00020, VIT_216s0148g00010) and two genes encoding for uncharacterized proteins (VIT_216s0050g02810, VIT_216s0050g02800), and downstream, two rust resistance kinase Lr10-like genes (VIT_216s0148g00030 and VIT_216s0148g00040).

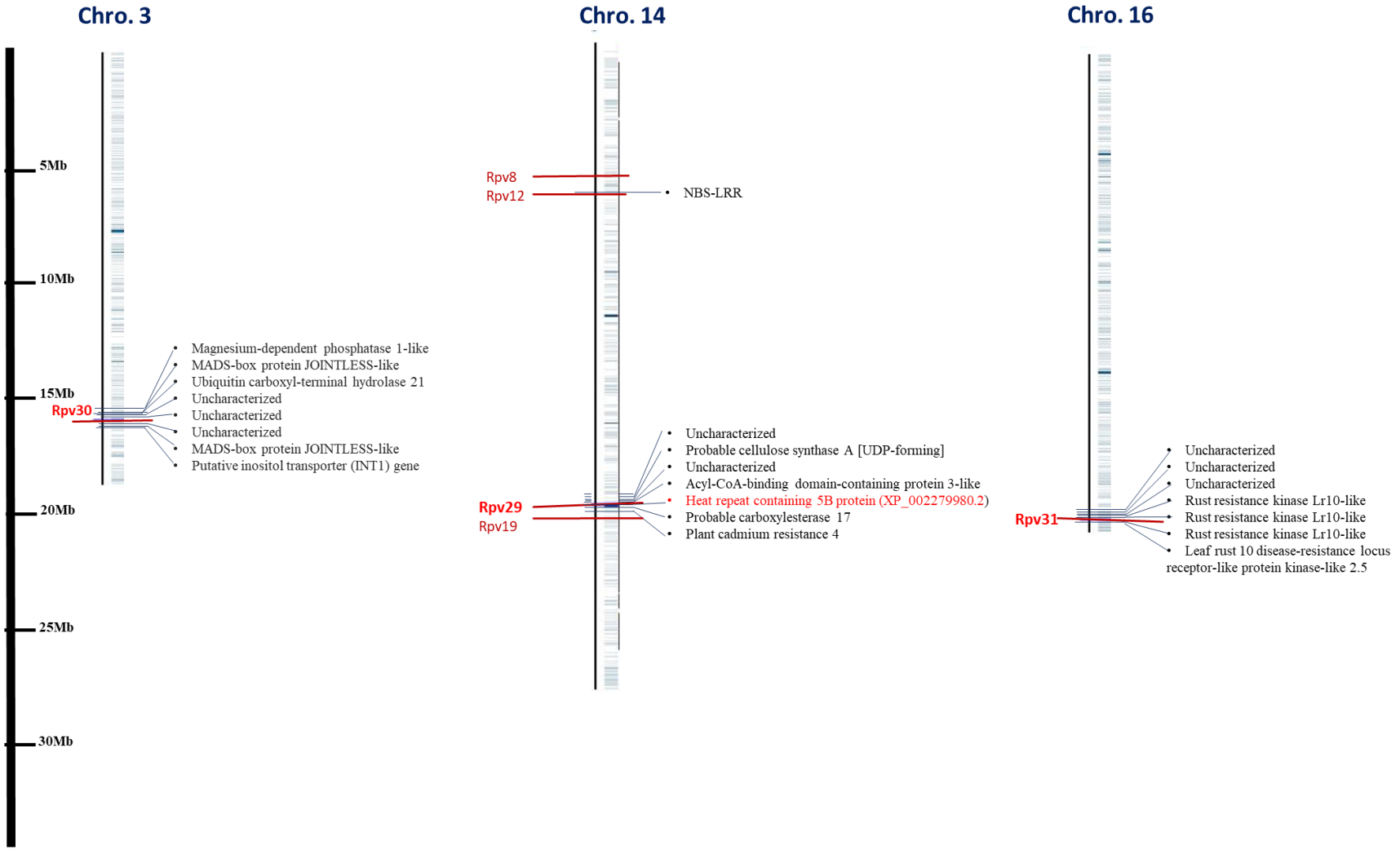


Figure 2.4 Genomic locations of detected *Rpv* (29, 30 and 31) loci for resistance to downy mildew resulted from GWA study (marked in bold red). The genes in genomic position of *Rpv* loci are indicated in 1 Mb around distance. The *Rpv29* on chromosome 14 indicates close distance (approximately 1Mb) to *Rpv* 19 and more distance to *Rpv 12* and *Rpv 8* (approximately 15 Mb).

2.4 Discussion

Downy mildew is one of the most important diseases affecting grapevines worldwide. So far, the sources of resistance were searched for in non-*vinifera* species, such as *V. labrusca*, *V. aestivalis*, *V. riparia*, *V. rotundifolia* and *V. amurensis*. The identification of resistant cultivars in the *V. vinifera* Georgian germplasm gave us the possibility to explore this promising material. In this work, a first insight was provided into quantitative resistance loci affecting downy mildew resistant traits in *V. vinifera* using an association mapping approach.

2.4.1 Grapevine resistant cultivars belong to different Georgian regions

Experimental inoculations on 132 grapevine individuals belonging to the Mgaloblishvili seedling population and Georgian germplasm confirmed the high susceptibility of *V. vinifera* to *P. viticola* infection. Almost all breeding-derived and germplasm accessions were severely affected by the pathogen, developing medium to high I%I. Several accessions showed large variability in the I%I distribution: this variability is frequently occurring in-field assessment and in bioassays (Cadle-davidson, 2008; Calonnec et al., 2013; Toffolatti et al., 2016) and could be related to several factors among which are the physiological state of the plant and the virulence of the pathogen. It is due to this variability that the experimental inoculations have been carried out in different years with mixed inocula: to identify those accessions that consistently showed a resistant behavior. A limited number of accessions (five breeding-derived and four germplasm accessions) clearly showed a reduced disease severity, which ranged from 5 to 25%. None of the accessions showed any necrotic spots, which are associated with HR, confirming that the defense mechanism different from the one observed for North American and Asian *Vitis* species (Dry et al., 2019; Toffolatti, De Lorenzis, et al., 2018). The resistant cultivars showed different genetic origins. They were grouped into two different clusters and ancestral groups, characterized by cultivars having the same geographical provenance (De Lorenzis et al., 2015; Imazio et al., 2013). Zerdagi, a variety that originated from Samegrelo province in Western Georgia, was grouped with cultivars coming from Southern regions and Jani Bakhvis and Kamuri shavi with cultivars coming from the Western and Eastern regions.

2.4.2 Multi-locus GWA models are the best for studying complex traits

The GWA approach was applied by genotyping 132 grapevine individuals with the 18k SNP genotyping array. A recent study has demonstrated the power of this array in detecting both known (such as berry color) and novel (such as acidity) loci related to phenotypic traits *via* GWA (Laucou et al., 2018). GWAS requires a genomic map with a marker density higher than the LD extent (Brachi et al., 2011). In our dataset, the average LD declined with the increase of the physical distance between markers, as already estimated in grapevine (Laucou et al., 2018; Myles et al., 2010) (Figure 2.2E). The high LD levels observed in grapevine and the average inter-SNP spacing (about one SNP every ~47 kbp, (Laucou et al., 2018)), appear to be enough to tag associated loci.

Regarding the accuracy, the effectiveness of the GWA approach is strongly influenced by population stratification. Breeding-derived and germplasm accessions clearly showed genetic differentiation (Figure 2.2B-D). Since structure analysis was better able than PCA to capture the level of stratification, structure results were used as covariates for association analysis. Accounting for the complexity of phenotypic dataset and known population stratification, different algorithms, both single- (GLM, MLM and SUPER) and multi-locus

(MLMM and FarmCPU) for modeling marker-trait associations were tested. It is widely accepted that multi-locus GWAS models are superior to single-locus GWAS methods to identify association (Cui et al., 2018). In our study, multi-locus GWAS models detected the highest number of significant SNPs: FarmCPU = two (plus one just above the Bonferroni-adjusted threshold; MLMM = one (plus two Bonferroni-adjusted thresholds); GLM, MLM and SUPER = one (Figure 2.3). Furthermore, our results confirm the usefulness of the *Vitis* SNP genotyping array in detecting loci associated with phenotypical traits (Laucou et al., 2018).

2.4.3 Three novel SNP-trait associations to *P. viticola* resistance were identified

To date, up to 28 QTL conferring resistance to downy mildew have been identified within wild *Vitis* species (Dry et al., 2019; www.vivc.de), but only two, *Rpv1* and *Rpv3* were characterized (Eisenmann et al., 2019; Feechan et al., 2013), mapping on chromosomes 12 and 18, respectively. *Rpv1* is a NB-LRR (nucleotide-binding site leucine-rich repeat) receptor, while *Rpv3* is associated with the biosynthesis of stilbenes. In our study, clear signals were identified on chromosomes 14, 3 and 16. The signal on chromosome 14, related to chr14_21613512_C_T locus, was recorded in all the five tested models, while the other two, related to cn_C_T_chr3_16229046 and li_T_C_chr16_21398409, were recorded only in MLMM and FarmCPU models, with some differences in the *p*-value. Among the 27 QTL already identified, three (*Rpv8*, 12 and 19) map on chromosome 14, while no QTL were found to map on chromosomes 3 and 16. *Rpv8* and *Rpv12* both mapped on the upper arm of chromosome 14 (Blasi et al., 2011; Venuti et al., 2013), while *Rpv19* mapped on the lower arm, at around position 24 Mb (Divilov et al., 2018). Because the SNPs identified in this work do not physically co-locate to the QTL already identified, it is possible to conclude that the three loci are novel associations. We designated the locus on chromosome 14 (for chr14_21613512_C_T) *Rpv29*, the locus on chromosome 3 (cn_C_T_chr3_16229046) *Rpv30* and the locus on chromosome 16 (li_T_C_chr16_21398409) *Rpv31*.

The logistic regression values (Table 2.1) indicated that the *Rpv29* locus is the one having a major effect on the phenotype. Furthermore, the other two loci, *Rpv30* and *Rpv31*, showed a statistically significant *p*-value as well, although the allelic effect estimation is lower. Nevertheless, since the resistance mechanism of accessions analyzed in this work did not show HR, it suggests that more than one locus is necessary to acquire the resistance.

2.4.4 *Rpv*29, *Rpv*30 and *Rpv*31 are markers associated with genes related to *P. viticola* resistance in *V. vinifera*

NB-LRR genes appeared to be associated with *Rpv12* locus in the upper arm of chromosome 14 (Venuti et al., 2013). The SNP located on chromosome 14 (*Rpv29*) mapped in the coding region of HEAT repeat-containing 5B protein and the polymorphism leads to a non-synonymous amino acid substitution from aspartic acid to asparagine. Further studies are needed to better understand the effect at the protein level. HEAT motifs are tandemly repeated sequences of about 50 amino acid residues identified in a wide variety of eukaryotic proteins (Andrade et al., 2001). It was demonstrated that repeat proteins possess an intrinsic ability to bind peptides, acting as an integral component of protein complexes (M. Sharma & Pandey, 2016). HEAT repeat proteins, such as ILA, are required for plant immunity. In *Arabidopsis thaliana*, ILA is required for both non-host and basal resistance against *Pseudomonas syringae*, for resistance mediated by NB-LRR proteins and for systemic acquired resistance (SAR) (Monaghan & Li, 2010). NB-LRR proteins act as specific receptors of pathogen effectors, activating defense mechanisms leading to effector-triggered

immunity (ETI) (Jones & Dangl, 2006). It is therefore tempting to speculate that the chr14_21613512_C_T locus could be involved in both primary plant-pathogen interactions leading to both ETI and SAR. Nevertheless, further investigations are needed to confirm this result.

In a region spanning ~100kb upstream and downstream the *Rpv29* locus, four genes, encoding for a probable cellulose synthase A catalytic subunit 8 [UDP-forming], an acyl-CoA-binding domain-containing protein 3-like, a probable carboxylesterase 17 and a plant cadmium resistance 4 protein, as well as three genes encoding for uncharacterized proteins, were mapped. All the candidate genes, except plant cadmium resistance 4 protein, appeared to be related to plant defense mechanism, based on the literature.

Cellulose synthases are involved in the secondary cell wall formation (Taylor et al., 2000). Structural modification, such as cell wall thickening, is one of the mechanisms adopted by plants to contrast the pathogen infection (Schulze-Lefert, 2004). Several evidences proving the connection between cell wall structure and stress signaling, leading to enhanced production of hormones (such as jasmonate and ethylene) and to enhanced resistance to a broad range of pathogens were described (Ellis & Turner, 2001). Similar to other organisms, Mgaloblishvili showed an up-regulation of genes, such as cellulose synthase-like protein G3 gene, that are involved in the transition from primary to secondary wall synthesis (Taylor et al., 1999).

Acyl-CoA binding proteins are thought to facilitate the transport of fatty acids/lipids among the cells (Kragelund et al., 1993). They are required for PAMP resistance to fungal pathogens, as described for *A. thaliana* against *Botrytis cinerea* and *Colletotrichum higginsianum* (Xia et al., 2012).

Carboxylesterases (CXEs) are a large family of enzymes, belonging to the α/β hydrolase fold superfamily, that hydrolyze ester, amide, and carbamate bonds (Putterill et al., 2003). They are involved in plant defense responses. *Nicotiana tabacum*, *A. thaliana* and *Capsicum annuum* showed some CXEs involved in the plant-pathogen interaction, some of them related to hypersensitive response (Kim et al., 2001; Pontier et al., 1994; Putterill et al., 2003). In *Vitis flexuosa*, some CXEs were upregulated in response to *Botrytis cinerea*, *Elsinoe ampelina* and *Rhizobium vitis* infection, indicating a putative role in defense mechanism during pathogen infection (Islam & Yun, 2016).

The cn_C_T_chr3_16229046 locus on chromosome 3 (*Rpv30*) was annotated close to predicted genes, such as MADS-box protein JOINTLESS-like, ubiquitin carboxyl-terminal hydrolase 21, magnesium-dependent phosphatase 1 and 1-like (MDP-1 and MDP-1-like) and inositol transporter 1 (INT1), and two genes encoding for uncharacterized proteins. All the candidate genes, except INT1, appeared to be related to plant defense mechanism, based on the literature.

MADS-domain transcription factors are proteins involved in multiple developmental pathways in plants, animals, and fungi (Castelán-Muñoz et al., 2019). JOINTLESS is a MADS-domain transcription factor, that together with MACROCALYX, induces the expression of AP2/ERF (ethylene response factor) 52 transcription factor in tomato during pre-abscission and abscission stages of the pedicel (Nakano et al., 2014). Transcriptional data revealed that Mgaloblishvili defense mechanism is mediated mainly by ethylene (Toffolatti, De Lorenzis, et al., 2018). This MADS-domain transcription factor can be related to *P. viticola* resistance mechanism in *V. vinifera*.

Ubiquitin-protein hydrolases are involved in the processing of ubiquitinated proteins. Ubiquitination in plant cells modulates signaling mediated by PAMP receptors and leads to the accumulation of NB-LRR receptors (Furlan et al., 2012). In Mgaloblishvili, the ubiquitination process appeared to be activated, upregulating genes encoding for RING H2-type E3 ligases (Toffolatti, De Lorenzis, et al., 2018), activated in response to biotic and abiotic stresses and involved in ubiquitination (Mazzucotelli et al., 2006).

Protein phosphorylation, by a combined action of protein kinases and phosphatases, is a rapid post-translational control mechanism in the response to environmental stimuli, such as pathogen elicitors, playing a major role in signal transduction pathways (Friso & van Wijk, 2015). Some DNA-binding proteins, with phosphatase activity, can bind defense-related genes and take part in their transcriptional regulation (*i.e.* DBP1 controlling transcription of the defense-related CEVI1 gene in *A. thaliana* during plant–virus interactions) (Carrasco et al., 2003). MDP-1 and MDP-1-like genes can be involved in the transcriptional regulation of some defense-related genes in the *V. vinifera-P. viticola* interactions.

The locus named li_T_C_chr16_21398409 (*Rpv31*) was annotated in the linkage group including several rust resistance kinase Lr10-like genes. As already described above, Mgaloblishvili could recognize *P. viticola* through specific NB-LRR receptors, such as several Lr10 genes. It was demonstrated the Lr10 confers enhanced resistance to *Puccinia triticina* in *Triticum aestivum* (Feuillet et al., 2003). Frequently, NB-LRR genes occur in clusters. In *Vitis*, the *Rpv12* locus accounts for 13 NB-LRR genes in a region of about 600 kb and it is part of a cluster of 46 NB-LRRs in the upper arm of chromosome 14 (Venuti et al., 2013). In our study, three Lr10-like genes (LOC100251517, LOC100256646, LOC100242248), spanning a region of about 47 kb on chromosome 16, appeared to be associated with the *P. viticola* resistance trait. Also, these three Lr10-like genes are part of a wider region, including a higher number of NB-LRR genes. In Toffolatti et al.(2018), seven Lr10-like genes were differentially expressed (with a log2 fold-change value higher than 2) in Mgaloblishvili after *P. viticola* inoculation. Among them, four are located on chromosome 16, spanning a region of about 6 Mb. GWA results corroborate the involvement of these receptors in triggering the plant response. Indeed, during the infection process *P. viticola* has shown the expression of numerous different cytoplasmic and apoplastic effectors (Toffolatti et al., 2020) and their interaction with the NB-LRR receptors of the plant should be further investigated. Since no hypersensitive response (HR) was observed in the Georgian resistant accessions, due to the absence of co-evolution with the pathogen, the involvement of the effector receptor Lr10 could be associated with an effector-triggered immunity not associated with HR. Indeed, HR is not always occurring in ETI (Jones & Dangl, 2006).

2.5 Conclusion

In this study, for the first time in *V. vinifera*, GWAS was used to identify loci associated with the resistance to *P. viticola* attack. The analysis provided evidence of three novel resistant loci (*Rpv29*, *Rpv30* and *Rpv31*) in a panel of Georgian accessions, that they could be utilized for further genetic and breeding studies to select genotypes showing resistance to *P. viticola* infection. The three loci were found to co-locate within genomic regions enriched genes associated with plant defense mechanism against biotic stress, suggesting both PAMP-triggered immunity and ETI-HR free response. Nevertheless, this suggestion must be validated, by functional characterization of the candidate genes. Functional

genomics approaches, such as CRISPR-based (Clustered Regularly Interspaced Short Palindromic Repeats) or RNA interference technologies, can help to functionally validate the candidate genes and, thus, to investigate which gene(s) is essential for resistance to *P. viticola* infection.

The great advantage provided by sources of resistance in *V. vinifera* germplasm compared to the *non-vinifera* one lies in the possibility to obtain crosses with cultivated varieties showing a good resistance level against a specific pathogen and, at the same time, able to provide a product free from the unpleasant characteristics usually imparted by the American vines, first of all, the foxy flavor of the grapes. The discovery of resistant sources in the *V. vinifera* background is crucial to exploit favorable alleles already present in germplasm, coupling at the same time good resistance to the pathogen and good agronomic traits. Indeed, Caucasian accessions show very attractive characteristics for high-quality production also in the perspective of climate change, such as late-ripening, medium-size berries, avoidance of excessive sugar accumulation, smooth tannin and ability to maintain a good level of acidity.

Chapter 3: Identification of grapevine's volatile organic compounds (VOCs) role in downy mildew resistance

3.1 Introduction

Plants are exposed to different environmental and biological stresses and they can thrive against threats through various pathways, including the production of secondary metabolites. Secondary metabolites are synthesized by different plant species not only as a defense mechanism against biotic and abiotic stresses but also for reproducibility and dissemination of their offspring (Alarcon et al., 2015). These bioactive metabolites can be alkaloids, flavonoids, saponins, tannins, terpenes and others.

In particular, terpenes are the largest and most investigated class of secondary metabolites that plants produce. They derive from the condensation of two or more isoprenic units, the precursor isopentenyl pyrophosphate (C5) and its allylic isomer dimethylallyl pyrophosphate, to form mono- (C10), sesqui- (C15) and diterpene (C20) precursors, through two alternative pathways: the mevalonate pathway and the methylerythritol phosphate pathway (MEP). The MEP pathway, localized in the plastids, leads to the biosynthesis of hemiterpenes, monoterpenes and diterpenes, while the cytosol-localized mevalonate pathway leads to sesquiterpene biosynthesis. The last step of the pathway catalyzes the conversion of each precursor to the primary representatives of each class by a large family of an enzyme known as terpene synthases. Finally, some terpenes are formed by oxidation, dehydrogenation, acylation, and other reaction types (Dudareva et al., 2004).

Terpenoids thus synthesized, together with alkanes, alkenes, alcohols, esters and acids, belong to the class of volatile organic compounds (VOCs). In the plant defense systems, secondary metabolites having antifungal properties are synthesized immediately after pathogen infection (F. Brilli et al., 2019). Among these secondary metabolites, VOCs can act against pathogens and herbivores either by a direct, as defense metabolites, or indirect mechanisms, mediating the signals between different parts of the same plant, from plant to plant and other organisms (Pierik et al., 2014).

The effectiveness of VOC-mediated induced resistance has been demonstrated in several plant pathosystems, such as: tobacco and *Ralstonia solanacearum* (Dorokhov et al., 2012); *Arabidopsis thaliana* and *Trichoderma* spp. (Estrada-Rivera et al., 2019). Furthermore, numerous studies reported the ability of leaf VOCs to inhibit spore germination and mycelial growth of fungal pathogens. For instance, citral, carvacrol, and *trans*-2-hexenal showed inhibitory activity against *Monilinia laxa* (Neri et al., 2007).

The involvement of VOCs in response to pathogens, such as the oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, has been demonstrated in grapevine, as well. *P. viticola* is the causal agent of downy mildew, one of the most destroying diseases affecting the Eurasian grapevine cultivars (*Vitis vinifera*). It originated in North America, where autochthonous species, such as *V. labrusca, V. aestivalis, V. riparia*, have been developed resistance traits due to the co-evolution with the pathogen. At the end of the 19th century, *P. viticola* reached Europe, leading to substantial quantitative and qualitative damages due to the high susceptibility of the *V. vinifera* species. It has been demonstrated

that *P. viticola* infection is inhibited in leaf tissues by some VOCs (2-ethylfuran, 2-phenylethanol, β-cyclocitral or *trans*-2-pentenal) (Lazazzara et al., 2018). On the other hand, non-*vinifera* resistant genotypes (Kober 5BB, SO4) showed to emit specific VOC profiles in response to *P. viticola* infection (Alarcon et al., 2015; Lazazzara et al., 2018).

Mgaloblishvili is a *V. vinifera* cultivar native to Georgia (Caucasus, the first grapevine domestication center), showing unique resistance traits against *P. viticola* (Toffolatti et al., 2016; Toffolatti, De Lorenzis, et al., 2018). This cultivar shows a limitation of *P. viticola* growth and sporulation (up to 80 % in comparison to the susceptible *V. vinifera* cultivar Pinot noir) and overexpression of genes related to the synthesis of antimicrobial enzymes and compounds such as terpenes (Toffolatti et al., 2020; Toffolatti, De Lorenzis, et al., 2018). In particular, two genes showed a remarkable expression pattern: valencene synthase and a cytochrome P450 (CYP72A219 element). Valencene synthase is a terpene synthase, involved in the biosynthesis of (+)-valencene, a sesquiterpene, and its isomer (-)-7-epi-α-selinene, by using farnesyl diphosphate as a substrate (Lucker et al., 2004).

In this study, the role of VOCs in the resistance mechanism of grapevine to *P. viticola* has been investigated. To this purpose, the VOC profile and biosynthetic pathway of two resistant varieties, Bianca (an interspecific hybrid obtained by crossing American species with *V. vinifera*) and Mgaloblishvili (*V. vinifera*), experimentally inoculated with *P. viticola* has been investigated, as well as the inhibitory effect of some VOCs against *P. viticola* infection.

3.2 Material and Methods

3.2.1 Plant material and experimental inoculation with *P. viticola*

The study of VOC biosynthesis in response to *P. viticola* inoculation was carried out on leaves of Mgaloblishvili (the Georgian *V. vinifera* cultivar showing unique resistance behavior against *P. viticola* (Toffolatti, De Lorenzis, et al., 2018) and Bianca (a *Vitis* interspecific hybrid variety), artificially inoculated with *P. viticola*. Mgaloblishvili and Bianca plants were four-years-old, maintained in the greenhouse (24 °C, 16-h photoperiod) at the Department of Agricultural and Environmental Sciences (University of Milan, Italy) in 5 L pots filled with sand-peat mixture (7:3 v/v), regularly drip watered. The plants were regularly treated against powdery mildew with azole fungicides and did not show any other disease symptoms.

Two strains belonging to the two different *P. viticola* genetic populations (one from the Western and the other from the Eastern population) were identified in Italy (Maddalena et al., 2020) which separated over an East-West gradient. Both identified *P. viticola* populations were mixed and used for the experimental inoculations. Mixing of both populations was to achieve a plant response of representative genetic variability in the Italian *P. viticola* population. *P. viticola* strains were isolated from single sporangia (obtained from serial dilutions of a single sporangiophore) of two populations sampled in Northern Italy, namely Lombardy (S. Maria della Versa, western location) and Friuli (Casarsa della Delizia, eastern location), and routinely propagated on the underside of detached leaves of grapevine (cv Pinot noir). The inoculated leaves were placed in Petri dishes (9 cm diameter) containing moistened filter paper and incubated in a growth chamber at 22 °C with a 12/12 photoperiod (Toffolatti et al., 2012). After 7 days of incubation, sporangia were collected with sterile distilled water and counted in Kova chambers to estimate the number of sporangia contained in one mL of water.

Three plants per variety and one shoot per plant were used in the experimental procedure. Four leaves of each shoot were sampled, three leaves were inoculated with *P. viticola* and as a control, one leaf was not inoculated. Experimental inoculation was carried out using an airbrush spraying of 2.5 x 10^4 *P. viticola* sporangia suspension per leaf underside of three leaves located between the second and the fifth leaf from the shoot apex. A single leaf per shoot was not inoculated with *P. viticola* but sprayed with sterile distilled water. Inoculated shoots were covered with transparent plastic bags to keep a high percentage of humidity. Three leaf disks (15 mm in diameter) were excised with a cork borer from a single inoculated leaf per shoot at 1, 2 and 3 days post-inoculation (dpi) and incubated, in Petri dishes (as described above) to assess the disease occurrence through the estimation of the area covered by sporulation at 7 dpi. The remaining leaf material was stored at -80 °C for further VOCs and gene expression analysis. The percentage of the sporulating area (PSA) was estimated by visually assigning a class of 0 (absence of sporulation) to 7 (75-100% of the leaf disc covered by sporulation) for each leaf disc and using the following formula $PSA = \frac{\sum(n \times v)}{7 \times N} \times 100$, where n = number of leaf discs in each class, v = numerical value of each class and N = total number of leaf discs in the sample (Toffolatti et al., 2012). Experimental inoculation was performed on Pinot noir (a *V. vinifera* variety susceptible to downy mildew) as well, to evaluate the level of resistance of the two Mgaloblishvili and Bianca cultivars.

3.2.2 Volatile compound determination

Free VOCs from inoculated and non-inoculated leaf tissues, collected at 0, 1, 2 and 3 dpi, were assessed by gas chromatography coupled with mass spectrometry using solid-phase microextraction technique (SPME-GC/MS) following the procedure reported by Griesser et al. (2015) with some modifications. Inoculated and non-inoculated leaves were homogenized with liquid nitrogen and 100 mg of tissue were placed in a glass vial that was immediately hermetically closed. Leaf samples were added with 5 µl of 1-heptanol (12.5 µg 20 ml-1 in 10 % ethanol; Sigma-Aldrich, Germany), as an internal standard. The fiber was a carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30 µm x 1 cm) (Supelco, Bellefonte, PA, USA). The SPME was carried out with an autosampler (HTA autosampler, Brescia, Italy) set at the following conditions: incubation for 30 min at 90 °C without agitation; extraction for 60 min; desorption for 20 min. The GC/MS equipment was a Perkin Elmer Autosystem XL Gas Chromatograph coupled with a Turbomass Mass Spectrometer (Perkin Elmer, Italy). The separation was achieved by a Stabilwax-MS column (30 m x 0.250 mm x 0.25 µm) (Restek, Bellefonte, PA, USA) and using helium as carrier gas at 1 mL min-1 flow rate. The oven temperature was initially set at 40 °C and held for 5 min, ramped at 5 °C min-1 up to 220 °C and held for 5 min. The transfer line temperature was set at 230°C and the source temperature at 250 °C. The MS detector registered the m/z in the range from 33 up to 350 Da. The ions used for the identification of target metabolites were chosen according to the National Institute of Standards and Technology (NIST) MS Search 2.0 library. Only ions showing a fixed fitting value (R) of 90 % to the library spectra were recorded except for valencene. The latter compound was confirmed by the analysis of pure standards (Pub Chem SID 24901709, Sigma-Aldrich, St. Louis, MO, USA). Semi-quantitative data (referred to µg ml^{-1} of internal standard) were revealed considering the ratio between the peak area of each identified compound and the peak area of internal standard and referred to the internal standard. For the latter, a 5-point calibration curve was obtained in the range 0-125 µg 20 ml^{-1} using a leaf sample to exclude any possible matrix effect. Data were expressed as µg 100 mg^{-1} of the leaf.

3.2.3 RNA extraction and real-time RT-PCR

Total RNA was extracted from 100 mg of three leaf samples non-inoculated (0 dpi) and inoculated with *P. viticola* (1, 2 and 3 dpi). The samples were ground with liquid nitrogen into a fine powder using mortar and pestle and RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), according to the manufacturer's instructions.

Quantity and quality of RNA were verified by NanoDrop Spectrophotometer (Thermo Scientific, MA) and agarose gel electrophoresis. For samples showing a 260/230 ratio lower than 1.8, a lithium-chloride purification was performed (Toffolatti, De Lorenzis, et al., 2018).

Candidate genes belonging to monoterpene and sesquiterpene biosynthetic pathways were selected according to their expression profile in mature grape leaves, as reported in the literature (Matarese et al., 2013; Toffolatti, De Lorenzis, et al., 2018). Six candidates, *VvGwECar2* ((E)-β-caryophyllene synthase), *VvGwaBer* ((E)-α-bergamotene synthase), *VvCSaFar* ((E, E)-α-farnesene synthase), *VvCSbOciM* ((E)-β-ocimene synthase), *VvTer* ((-)-α-terpineol synthase) and *VvVal* (valencene synthase), were consequently chosen and their expression investigated through the technique of semi-quantitative real-time reverse transcriptase (RT)-PCR. Primer setsfor the first five candidate genes were obtained from Matarese et al. (Matarese et al., 2013), while for *VvVal* gene was designed using related nucleotide sequence in Mgaloblishvili genome, using the Primer3Plus webtool (https://primer3plus.com/cgi-bin/dev/primer3plusPackage.cgi). Ubiquitin (Fujita et al., 2007) and actin (K. Reid et al., 2006) genes were used as references for data normalization. Table 3.1 reports forward and reverse primer sequences.

Total RNA (500 ng) was reverse-transcribed with SuperScript®IV Reverse Transcriptase (Thermo Fischer) following the manufacturer's instructions. Real-time PCR was carried out on QuantStudio® 3 Real-Time PCR Systems (Thermo Fischer). Each reaction was carried out in a volume of 20 µL, using 10 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 4 µL of cDNA diluted 1:10, 500 nM of each primer and water up to the final volume of reaction. Each reaction was performed in triplicate. Thermal cycling conditions were obtained from Matarese et al. (2013). Ubiquitin was selected to normalize the Ct (cycle threshold) values due to PCR efficiency value (97 %) in the range of target genes (ranging from 92 to 95 %), The expression of each gene in different varieties and treatments was calculated by comparing their $2^{-\Delta\Delta Ct}$ values (Livak & Schmittgen, 2001).

Table 3.1 Forward and reverse primers sequences of two reference genes (actin and ubiquitin) and six encoding terpene synthas genes ((E)-β-caryophyllene synthase, (E)-α-bergamotene synthase, (E,E)-α-farnesene synthase, (E)-β-ocimene synthase, (-)-α-terpineol synthase and valencene synthase) involved in biosynthesis of terpenes in grapevine leaves.

Gene	Sequence 5'-3'	Reference
Actin	F: CTTGCATCCCTCAGCACCTT R: R: TCCTGTGGACAATGGATGGA	(K. Reid et al., 2006)
Ubiquitin	F: TCTGAGGCTTCGTGGTGGTA R: AGGCGTGCATAACATTTGCG	(Fujita et al., 2007)
(E)-β-caryophyllene synthase	F: TGCCTCAGCTGTTGAATGCT R: TGAGGACGGTCATCGGAACA	(Matarese et al., 2013)
(E)-α-bergamotene synthase	F: CCTAGCATTTGGGGCAATAC R: CCGTTGAACTGCATCGATAA	(Matarese et al., 2013)
(E,E)-α-farnesene synthase	F: GGGTGCACGTTGCTTCTAGT R: TGGCATCAGCACTGGTGTAG	(Matarese et al., 2013)
(E)-β-ocimene synthase	F: GGAACATCACTGGATGAGTTGA	(Matarese et al., 2013)

	R: ATCTCCATGCTGATACATGCAC	
(-)-α-terpineol synthase	F: AGAGTCTCCATTCCCTGAAACA	(Matarese et al., 2013)
	R: GGGCTCAACGAGTAATGACAA	
Valencene synthase	F: AGTTGTGGATGCATGGAAGG	The present work
	R: TTTGGTCATGCGATAGGGTG	

3.2.4 Efficacy test of pure terpene solutions against *P. viticola* under laboratory conditions

The efficacy of pure terpene solutions against *P. viticola* infection was evaluated on Pinot noir leaf disks experimentally inoculated with a sporangia suspension. Grapevine leaves (3^{rd}-5^{th} leaf from the shoot apex) were detached from five-year-old three healthy plants of Pinot noir, grown in the greenhouse, as above mentioned. Leaf disks were soaked in terpene solution for 2 min and then were placed upward facing in 9 cm diameter Petri dishes containing moistened filter papers. The farnesene (a mixture of isomers; Pub Chem SID: 24901903, Sigma-Aldrich), nerolidol (a mixture of *cis* and *trans*; Pub Chem SID: 24895721, Sigma-Aldrich), ocimene (a mixture of isomers; Pub Chem SID: 329830629, Sigma-Aldrich) and valencene (Pub Chem SID: 57652542, Sigma-Aldrich) were tested at four concentrations (0.01, 0.1, 1 and 5 g l^{-1}). Each terpene was diluted to reach a concentration of 50 g l^{-1} with 2 % DMSO (Sigma-Aldrich) and serially diluted with sterile distilled water to obtain the final experimental concentration per treatment. A negative control (only distilled water) and a DMSO control (distilled water with 0.2 % g l^{-1} of DMSO) were included in each assay. Each treatment was repeated by three technical repeats. The experimental inoculations were carried out airbrush spraying of 0.2 ml of a suspension of *P. viticola* sporangia ($5x10^4$ sporangia ml^{-1}) per disk abaxial surface. The samples were incubated at 22 °C in light with a 12-h photoperiod. The percentage of the sporulating area (PSA) was estimated at 7 dpi, as previously described by Toffolatti et al. (2012).

3.2.5 Data analysis

3.2.5.1 Statistical analysis to evaluate disease severity

In order to evaluate the significant differences of disease severity among accessions (Mgaloblishvili, Bianca and Pinot noir), one way ANOVA and posthoc test (REGWF) were carried out on transformed PSA values ($arcsin\sqrt{PSA}/100$). Statistical analysis was carried out with SPSS v. 26 (IBM Statistics Italia, Milano).

3.2.5.2 Statistical analysis to determine volatile compounds

VOCs profiles were subjected to Levene's test to assess homogeneity of variance and tested for statistical significance through a GLS (generalized least squares) model, accounting for inhomogeneity of variance, with *nlme* R package (Pinheiro et al., 2020). *p*-values were obtained through a post-hoc test carried out with *multcomp* R package (Hothorn et al., 2008). Graphs were generated using IBM SPSS Statistic v.21 software. Principal component analysis (PCA) and clustered heatmap were produced by *ggbiplot* (https://github.com/vqv/ggbiplot) and *gplots* (Warnes et al., 2014) R packages, respectively.

3.2.5.3 Statistical analysis to determine gene expression levels of six terpene synthases

Gene expression values were subjected to Levene's test to assess the homogeneity of variance and were statistically tested for significance through a GLS model, using *nlme* R

package. *p*-values were obtained through a post-hoc test carried out with *multcomp* R package. Graphs were generated using IBM SPSS Statistic v.21 software.

3.2.5.4 Statistical analysis to determine efficacy test of pure terpene solutions against *P. viticola*

Statistical analysis (ANOVA with multiple comparison REGW post-hoc test) was performed on transformed PSA percentages ($\text{asin}(\sqrt{\%/100})$) to understand if the treatment with terpenes or DMSO caused a significant reduction in disease severity compared to the untreated control sample.

3.3 Results

3.3.1 Disease severity evaluation

Leaf disks of Mgaloblishvili and Bianca were inoculated with a suspension of *P. viticola* sporangia and the disease severity (PSA) was evaluated at 7 dpi. Pinot noir leaf disks were inoculated as a positive control. No disease symptoms were observed on Bianca, where numerous necrotic areas were present because of the hypersensitive response (Figure 3.1A). A few areas with sporulation, covering 22 % of the leaf disks on average, were observed on Mgaloblishvili samples (Figure 3.1B). While, a uniform presence of sporulation, covering 84 % of the leaf disks on average, was observed in Pinot noir (Figure 3.1C). Statistical analysis showed a significant four-times reduction of PSA in Mgaloblishvili compared to Pinot noir ($F=148.9$; $df=2,6$; $P<0.001$) (Figure 3.2).

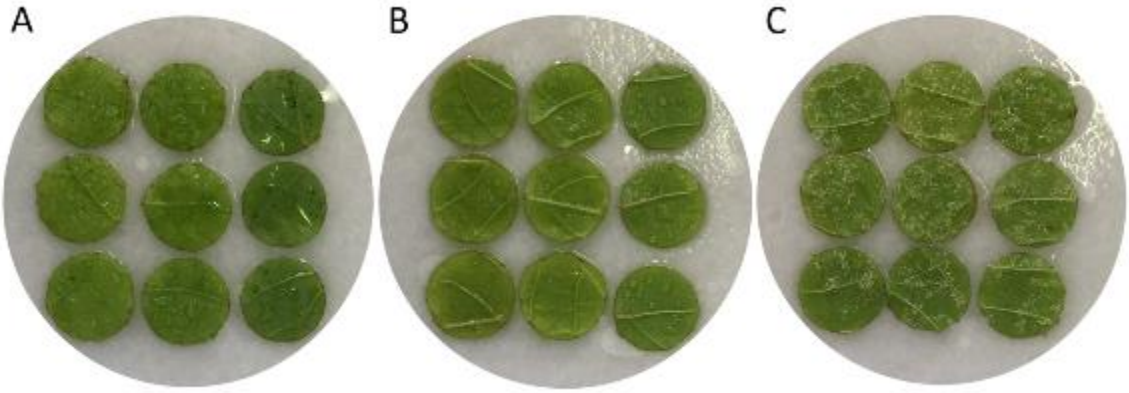


Figure 3.1 *P. viticola* sporulation (in white) on the inoculated leaf disks of Bianca (A), Mgaloblishvili (B), Pinot noir (C) at seven days after inoculation. Brown spots in Bianca correspond to necrotic areas, where hypersensitive response (HR) occurred.

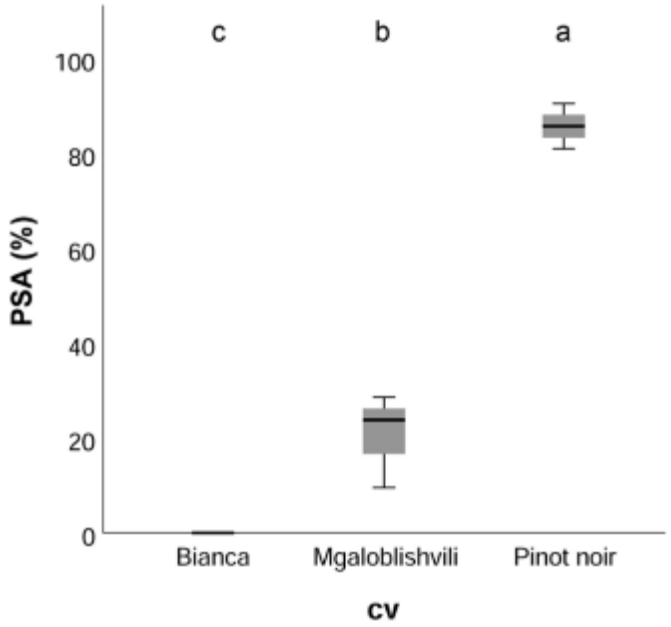


Figure 3.2 Box plot distribution of the percentages of sporulating area (PSA) estimated 7 days post inoculation with *P. viticola* on Bianca, Mgaloblishvili and Pinot noir leaf disks and results of statistical analysis (different letters correspond to a significant difference among mean PSA values for P<0.001).

3.3.2 VOCs detection in leaves inoculated with *P. viticola*

In total, 54 VOCs were identified during SPME-GC/MS analysis of 24 Mgaloblishvili and Bianca leaf samples collected at 0, 1, 2 and 3 dpi with *P. viticola*. This dataset was filtered for those compounds identified in all three biological replicates. The final dataset accounted for 33 VOCs. Based on the biochemical features, the VOCs were categorized into three main groups: alcohols (6 compounds), aldehydes (11 compounds), terpenes (10 compounds). The fourth group of 6 compounds included alkenes and esters. Table 3.2 reports the amount of each VOC (µg/100 mg of leaf sample) per cultivar and treatment. Most of the 33 VOCs were detected in both cultivars and overall the treatments, except for 1-hexanol, 2-ethyl- and phenylethyl alcohol among alcohols, benzeneacetaldehyde and dodecanal among aldehydes, farnesene and *p*-menth-1-en-8-ol among terpenes and 1-octadecene, 1-(4-bromobutyl)-2-piperidinone and *trans*-2-(2-pentenyl)furan among other VOCs. The highest detection of total VOCs was detected at 2 dpi and 3 dpi for Mgaloblishvili and Bianca, respectively. In both cultivars, the amount of some VOCs increased (such as benzyl alcohol) and some other decreased (such as farnesene) in correlation with an increment of inoculation (Table 3.2).

Bianca showed statistically significant values at 1 dpi for the amount of other VOCs and the total VOCs, at 2 dpi for the amount of aldehydes, terpenes, other VOCs and total VOCs, at 3 dpi for the amount of aldehydes, terpenes and total VOCs. At 1 dpi, a statistically significant increase for hexanal, 2-n-octylfuran, *trans*-2-(2-pentenyl)furan and methylhydrazine was detected. At 2 dpi, Bianca showed a statistically significant increase for 2-hexenal, hexanal and farnesene. While at 3 dpi, 3-hexen-1-ol, 2-hexenal, hexanal, farnesene, 3-buten-2-one-4-(2,6,6-trimethyl-1-cyclohexen -1-yl), 4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one, 2-n-octylfuran and methylhydrazine showed a statistically significant increase (Table 3.2).

In comparison to the 0 dpi samples, Mgaloblishvili showed statistically significant abundances at 1 dpi for the amount of other VOCs and at 2 dpi for the amount of terpenes, other VOCs and total VOCs. At 1 dpi, Mgaloblishvili showed a statistically significant increase for 2-undecanone, 6,10-dimethyl, 1-(4-bromobutyl)-2-piperidinone and methylhydrazine. At 2 dpi, statistically significant increase was detected for 4-(2,6,6-trimethylcyclohexa-1,3-dienyl) but-3-en-2-one, 1-(4-bromobutyl)-2-piperidinone and methylhydrazine. Farnesene was detected at 2 (the highest amount) and 3 dpi. None of the VOC amounts significantly increased at 3 dpi (Table 3.2).

PCA was performed to detect significantly influenced VOC categories after *P. viticola* inoculation. The first two principal components (PC) explained about 85 % of the total variance (Figure 3.3). Bianca and Mgaloblishvili samples differentiated mainly along the PC2. Bianca samples collected at 0 dpi were differentiated from 1 dpi samples and 2 and 3 dpi samples. 1 dpi Bianca samples appeared differentiated based on other VOCs variable, while 2 and 3 dpi samples for alcohol and aldehyde variables. Mgaloblishvili samples appeared more homogeneous, with a slight differentiation of 1 and 2 dpi samples from 0 and 3 dpi ones (Figure 3.4). Mgaloblishvili samples were differentiated based on other VOCs (mainly) and terpene (less) variables.

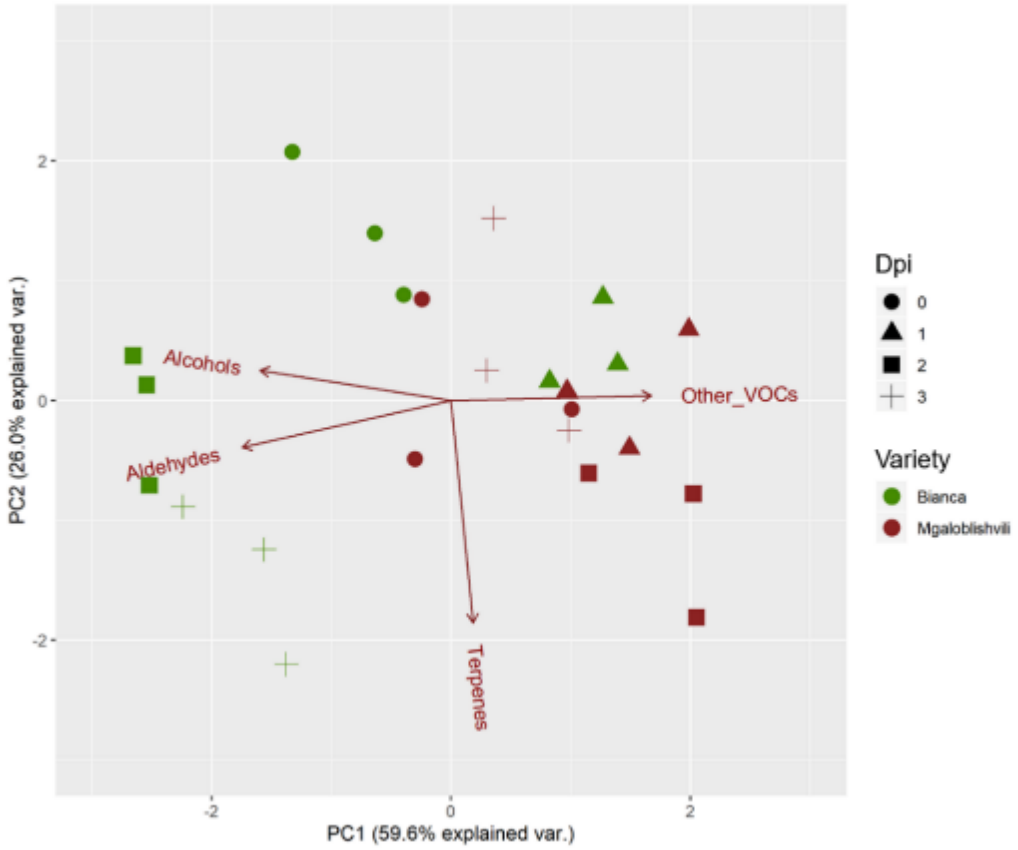


Figure 3.3 Principal Component Analysis (PCA) along the first two components (PC) obtained using the amount of volatile metabolites (alcohols, aldehydes, terpenes and other VOCs) detected in Mgaloblishvili and Bianca leaves collected at 0, 1, 2 and 3 days post inoculation (dpi) with *P. viticola*.

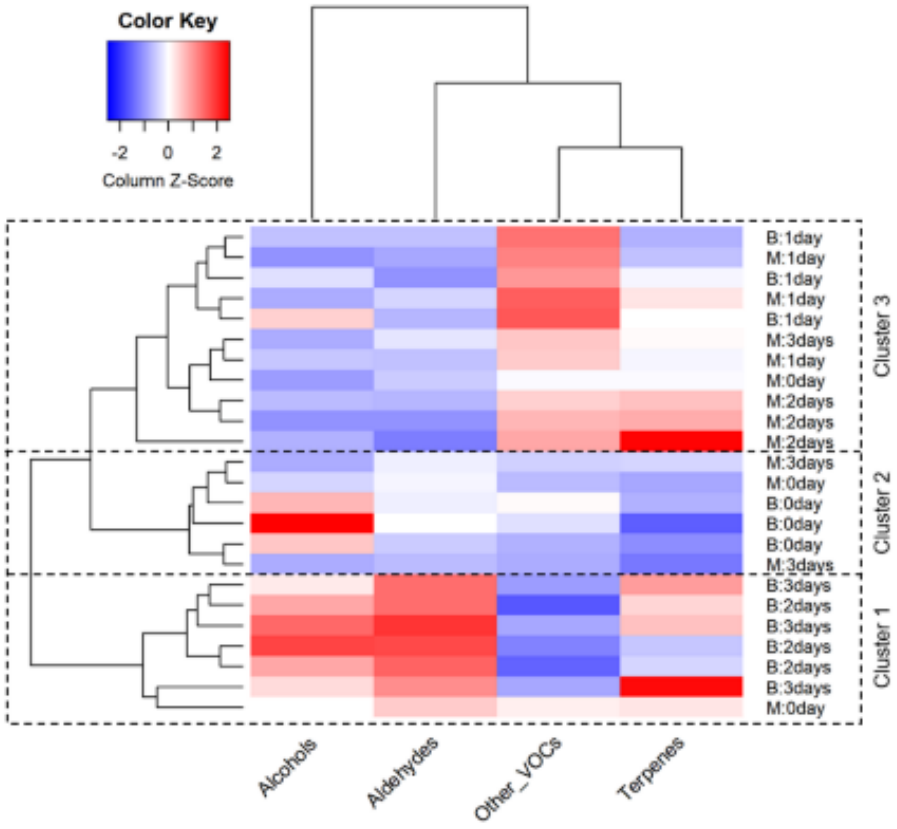


Figure 3.4 Hierarchical clustering and heatmap visualization for volatile metabolites (alcohols, aldehydes, terpenes and other VOCs) detected in Mgaloblishvili (M) and Bianca (B) leaves collected at 0 (0day), 1 (1day), 2 (2days) and 3 (3days) days post-inoculation with *P. viticola* represent the accumulation pattern of volatile compounds during *P. viticola* infection clustered by hierarchical cluster analysis. Tree well distinct clusters (Cluster 1, Cluster 2 and Cluster 3) have been highlighted. Cluster 1 grouped Bianca samples collected at 2 and 3 dpi, Cluster 2 grouped Bianca samples collected at 0 dpi and Mgaloblishvili samples collected at 3 dpi, while Cluster 3 grouped 1 dpi Bianca samples and 1 and 2 dpi Mgaloblishvili samples. Cluster 1 showed a positive correlation with the amount of alcohols, aldehydes and terpenes and a negative correlation with the amount of other VOCs. Cluster 2 showed mainly a negative correlation with all the four VOC categories. Cluster 3 showed a positive correlation with the amount of other VOCs and a negative correlation with the amount of alcohols and aldehyde.

Table 3.2 VOCs accumulation (μg 100 mg^{-1} leaf sample) in Bianca and Mgaloblishvili leaves at 0, 1, 2, and 3 days post inoculation (dpi). Statistical analysis was performed on subtotal and total amounts per each cultivar. Values followed by '*' significantly differ from the values recorded at 0 dpi, according to gls method (*** *P*=0.000; ** *P*=0.001; * *P*=0.01). n.d. = not detected.

VOC ID	VOC	Bianca				Mgaloblishvili			
		0 dpi	**1 dpi**	**2 dpi**	**3 dpi**	**0 dpi**	**1 dpi**	**2 dpi**	**3 dpi**
	Alcohols								
VOC1	1-Hexanol, 2-ethyl-	16.19±3.10	6.63±1.46 *	12.59±4.18	8.24±2.11 *	n.d.	n.d.	n.d.	n.d.
VOC2	1-Nonol	5.33±1.51	3.30±1.73	3.51±1.69	2.95±1.62	8.35±2.28	4.69±0.36 *	6.67±3.89	5.31±2.34 *
VOC3	4,8-Dimethyl-1,7-nonadien-4-ol	2.12±0.47	1.95±0.46	2.39±0.53	2.73±0.66	5.61±1.60	4.27±0.65	4.38±0.91	2.27±0.77 *
VOC4	3-Hexen-1-ol	3.98±0.88	6.68±1.44	8.07±2.39	9.98±2.19 *	1.94±0.84	1.51±1.02	2.19±1.72	3.89±1.54
VOC5	Benzyl alcohol	18.73±4.33	9.96±1.31 *	14.74±3.76	10.56±3.59	11.13±2.46	9.55±4.47	6.75±2.57 **	10.69±1.03
VOC6	Phenylethyl alcohol	16.10±6.67	5.47±3.70 *	19.50±1.98	16.21±5.12	n.d.	n.d.	n.d.	n.d.
	Subtotal	**62.45±21.23**	**33.98±11.23**	**60.80±10.09**	**50.68±13.76**	**27.03±9.45**	**20.02±4.87**	**19.99±4.09**	**22.16±1.89**
	Aldehydes								
VOC7	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	6.05±0.34	6.11±0.63	6.97±1.14	7.97±1.47	11.64±1.93	11.54±0.49	12.05±1.91	6.34±1.17 *
VOC8	2-4 Heptadienal, (E,E)-	n.d.	n.d.	n.d.	n.d.	3.12±0.15	4.87±1.32	5.12±1.89	1.91±0.40 *
VOC9	2-Hexenal	199.96±19.52	112.11±17.56 *	340.56±26.38 ***	353.10±44.73 ***	169.93±25.10	142.45±22.53	119.33±14.88 *	201.40±23.42
VOC10	2,4-Hexadienal	18.86±2.01	19.57±7.37	30.54±1.46	21.84±14.94	27.43±5.77	23.84±5.84	16.07±6.95	14.73±11.73
VOC11	Benzeneacetaldehyde	n.d.	20.12±2.44	24.29±7.78	22.55±4.95	n.d.	n.d.	n.d.	n.d.
VOC12	Benzaldehyde	14.80±1.58	6.47±1.63 *	9.57±1.72	12.31±1.08	10.48±0.51	11.08±2.13	4.90±0.29 *	7.53±2.23
VOC13	Decanal	4.99±0.31	4.18±0.64	2.30±1.97	3.59±1.73	17.42±5.85	13.41±0.17	11.85±2.06	7.87±1.91 **
VOC14	Dodecanal	n.d.	n.d.	n.d.	n.d.	18.88±3.81	9.61±3.47 *	13.87±2.94	9.94±5.37
VOC15	Furfural	10.79±3.64	12.66±1.73	11.72±3.03	7.32±1.04	14.16±4.25	7.23±1.90	7.25±1.39	5.57±3.34
VOC16	Hexanal	12.62±6.23	26.48±5.74 *	39.46±4.38 **	27.45±7.40 *	24.95±7.32	17.67±4.57	11.10±2.00 **	16.27±2.84
VOC17	2-Furancarboxaldehyde,5-(hydroxymethyl)	18.36±8.6	20.79±6.36	9.97±2.09 *	11.43±7.13	8.50±6.45	4.14±1.25	4.13±1.12	1.67±1.73 **
	Subtotal	**286.42±24.68**	**228.49±35.90**	**475.38±19.47 *** **	**467.55±46.02 *** **	**306.52±55.36**	**245.85±24.89**	**205.66±27.68**	**273.24±27.31**
	Terpenes								
VOC18	Farnesene	8.26±4.78	22.40±6.83	86.88±11.04 **	115.91±6.81 **	n.d.	n.d.	108.67±18.03	34.11±13.32

VOC19	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	75.23±9.58	64.60±31.54	20.29±1.54 *	25.34±2.41 *	81.72±8.61	61.87±7.76	77.53±14.38	84.20±14.53
VOC20	*p*-Menth-1-en-8-ol	2.71±0.53	4.22±2.33	4.93±1.91	4.42±1.03	n.d.	n.d.	n.d.	n.d.
VOC21	3-Buten-2-one-4-(2,6,6-trimethyl-1-cyclohexen -1-yl)	48.64±15.36	75.08±29.20	78.53±25.37	112.40±23.60 **	100.82±12.62	68.87±5.92	79.71±24.55	68.25±26.52
VOC22	1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate	1.14±0.28	1.53±0.27	2.44±1.03	2.00±0.08	3.21±0.38	3.36±0.69	3.57±1.55	2.18±0.45
VOC23	5,9-Undecadien-2-one, 6,10-dimethyl-(E)-	24.92±4.28	37.40±7.47	23.07±2.92	35.77±12.35	13.08±5.35	34.83±11.66	18.65±4.33	8.52±3.10
VOC24	3,7,11-trimethyl-1,6,10-dodecatrien-3-ol	4.11±0.27	5.75±0.15	5.50±1.79	4.83±0.95	7.95±1.15	7.78±2.51	3.11±1.74	3.60±1.86
VOC25	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	3.54±0.73	9.12±4.90	8.31±3.52	13.86±4.53 *	7.27±1.72	6.17±0.37	14.18±3.56 *	5.68±1.71
VOC26	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	13.75±5.48	12.16±2.01	11.97±2.96	14.52±3.77	18.49±6.27	16.22±1.92	16.44±1.85	8.16±4.41
VOC27	2-Undecanone, 6,10-dimethyl	7.57±2.78	6.69±2.28	4.16±2.85	5.70±1.87	11.33±4.27	49.15±11.91 **	10.19±2.67	9.04±2.35
	Subtotal	**189.85±23.24**	**238.95±24.75**	**246.10±37.58 ***	**334.75±54.27 *****	**243.88±34.99**	**248.25±25.82**	**332.05±57.07 ****	**223.74±41.91**
	Other VOCs								
VOC28	1-Octadecene	n.d.	21.63±6.51	16.19±5.31	13.89±4.25	n.d.	15.70±6.05	11.04±3.20	8.16±3.71
VOC29	1-(4-Bromobutyl)-2-piperidinone	n.d.	4.13±0.94	4.96±0.63	7.72±0.62	3.21±0.42	34.87±7.69 ***	36.14±7.02 ***	n.d.
VOC30	Decanoic acid, ethyl ester	5.32±1.01	4.62±1.63	6.46±3.93	3.67±2.33	4.89±1.03	9.75±8.63	5.93±2.94	6.88±2.44
VOC31	2-n-Octylfuran	7.32±3.44	19.46±5.54 *	14.38±2.82	17.75±2.53 *	37.35±5.81	18.44±5.95 *	17.13±6.59 *	39.25±10.98
VOC32	*trans*-2-(2-Pentenyl)furan	0.84±0.11	8.46±3-87 **	0.71±0.17	11.04±3.77 **	n.d.	n.d.	n.d.	n.d.
VOC33	Methylhydrazine	197.57±31.39	292.10±31.63 ***	76.27±7.02 ***	110.49±10.02	158.46±33.78	253.79±34.95 **	226.91±15.34 *	165.54±51.04
	Subtotal	211.05±34.51	350.40±23.02 ***	118.98±16.72 ***	164.56±4.04	203.90±36.31	332.55±44.69 **	297.14±18.11 *	219.82±60.84
	Total	749.78±66-45	851.83±56.26 **	901.26±31.29 ***	1017.54±38.32 ***	781.34±41.3	846.68±75.74	854.85±20.79 *	738.96±39.87

3.3.3 Relative expression of terpene synthases in leaves inoculated with *P. viticola*

The expression pattern of six genes involved in the biosynthesis of monoterpenes and sesquiterpenes (*VvGwaBer*, *VvGwECar2*, *VvCSaFar*, *VvCSbOciM*, *VvTer* and *VvVal*) were investigated in leaf samples of Bianca and Mgaloblishvili collected at 0, 1, 2 and 3 days after inoculation with *P. viticola*. Both varieties showed a similar pattern of expression, characterized by an increase in the expression level in response to the pathogen inoculation (Figure 3.5). For every gene and variety, apart from *VvTer* in Mgaloblishvili, the highest expression level was obtained at 1 dpi, followed by a drop at 2 dpi and another minor increase at 3 dpi. Overall, Mgaloblishvili appeared to show a greater increment in the gene expression of candidate genes compared to Bianca, with double or triple values. Compared to the other genes, *VvVal* in Mgaloblishvili exhibited a remarkably high increase in its expression level at 1 dpi, with a value equal to 120 times the non-inoculated sample value (0 dpi). A similar difference in the gene expression is shared, at the same time point and in the same variety, by *VvGwaBer* (80 times the 0dpi sample value). The only exception to this behavior is *VvGwECar2*, in which the Mgaloblishivili gene expression resulted lower than Bianca and it showed a decrease throughout the time points.

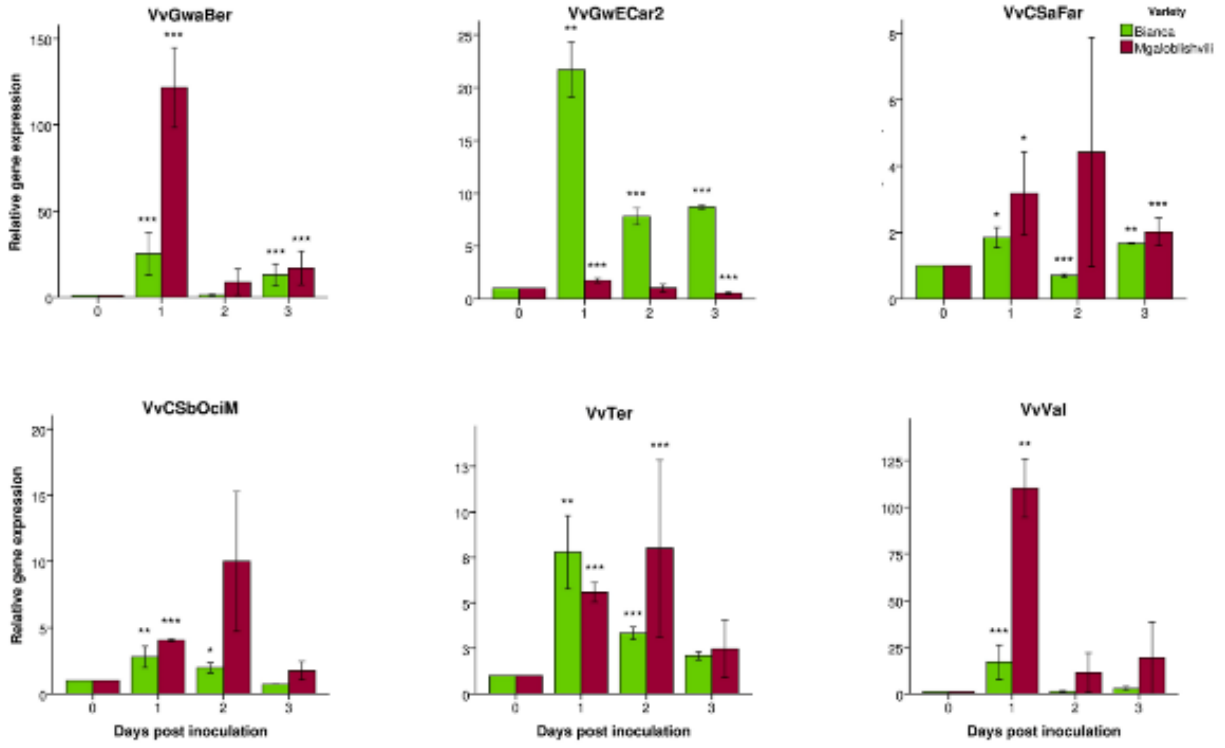


Figure 3.5 Expression level of genes involved in the biosynthesis of terpenes in Mgaloblishvili (violet bars) and Bianca (green bars) leaves at 0, 1, 2 and 3 days post-inoculation with *P. viticola*. The expression of each gene has been normalized using the gene expression values of actin at each time point. The relative gene expression has been determined based on the $2^{-\Delta\Delta Ct}$ method. Standard error bars are visualized. Bars followed by asterisks indicate significant differences from the values recorded at 0 day after inoculation, according to gls test (* *P*= 0.01; ** *P*=0.001; *** *P*= 0.000). *VvGwECar2*: (E)- β-caryophyllene synthase; *VvGwaBer*: (E)-α-bergamotene synthase; *VvCSaFar*: (E,E)-α-farnesene synthase; *VvCSbOciM*: (E)-β-ocimene synthase; *VvTer*: (-)-α-terpineol synthase; *VvVal*: valencene synthase.

3.3.4 Efficacy of pure terpene solutions in containing *P. viticola* infections

Based on the VOC profiles and gene expression data, the efficacy of pure solutions of farnesene, nerolidol, ocimene and valencene in reducing *P. viticola* infection on Pinot noir leaves were evaluated (Supplementary Figure S.2). The average I%I of the untreated control was 62 % (Table 3.3). No significant differences were found between the I%I recorded on the untreated and DMSO controls (F=1.6; df=1-4; P=0.28). Significant reduction in *P. viticola* sporulation (I%I) was observed following treatment with each terpene starting from 0.01 g l^{-1} (F>4.9; df=4-10; P<0.018). Indeed, the I%I showed a significant 3-, 4-folds reduction until 17-26 % between 0.01 and 1g l^{-1} and a further significant decrease to 0 % at 5 g l^{-1} of nerolidol and ocimene (Table 3.3). No further reductions in I%I values occurred between 0.01 and 5 g l^{-1} of farnesene and valencene (Table 3.3). It must be pointed out that some signs of phytotoxicity, visible as brown spots, were visible at 5 g l^{-1} of ocimene (Supplementary Figure S.2).

Table 3.3 Average disease severity (I%I) recorded on Pinot noir leaves infected with *P. viticola* and untreated (0) and treated with DMSO (0.2 %) and farnesene, nerolidol, ocimene and valencene at four different concentrations. Untreated and treated with DMSO leaves were considered as controls. Letters indicate statistically different PSA values (P<0.05) following ANOVA and multiple comparison REGW post-hoc test.

			Concentration (g l^{-1})			
Treatment	**0**	**DMSO**	**0.01**	**0.1**	**1**	**5**
Farnesene	62±10 a	57±12 a	19.0±11 b	16.7±15 b	14.3±19 b	11.7±11 b
Nerolidol			16.3±4 b	26.3±4 b	14±7 b	0±0 c
Ocimene			14±12 b	11.7±8 b	9.3±8 b	0±0 c
Valencene			16.7±11 b	21.3±7 b	16.7±8 b	12±9 b

3.4 Discussion

3.4.1 The VOCs biosynthesis in response to *P. viticola* is cultivar-specific

VOCs play a crucial role in the plant-pathogen interaction mechanism (F. Brilli et al., 2019). In grapevine, their biosynthesis was associated with resistance against *P. viticola* infection (Alarcon et al., 2015; Lazazzara et al., 2018). Their role in grapevine defense mechanism against downy mildew was confirmed by the detection of a high amount of VOCs in resistant genotypes, harboring the American species background, in comparison to the susceptible ones following the pathogen infection (Lazazzara et al., 2018). Transcriptomic data on the *V. vinifera* cultivar Mgaloblishvili leaves inoculated with *P. viticola* revealed the overexpression of genes involved in the biosynthesis of terpenoids, such as several cytochrome P450s and valencene synthase (Toffolatti et al., 2020; Toffolatti, De Lorenzis, et al., 2018).

In response to the pathogen infection, the two cultivars analyzed in this work showed a different behavior (Figure 3.3 and 3.4). In both cultivars, the increased accumulation of VOCs was found in the inoculated samples, but with different timing: Mgaloblishvili showed the highest amount at 2 dpi and Bianca at 3 dpi (Table 3.2). The detection of the highest amount of VOCs in response to *P. viticola* inoculation suggested that their biosynthesis can be related to the plant-pathogen interaction mechanism as proposed for other resistance cultivars (Lazazzara et al., 2018). Indeed, the plant response in Mgaloblishvili occurs at 1 dpi, as demonstrated by the high transcriptomic changes, but the damages to *P. viticola* structures are visible starting from 3 dpi (Toffolatti, De

Lorenzis, et al., 2018). At 1 and 2 dpi, regular hyphae and haustoria were observed in Mgaloblishvili. The transcriptomic data indicated that the genes encoding VOCs were overexpressed at 1 dpi. However, the antifungal molecules, which lead to the alterations of the vegetative structures observed at 3 dpi (Toffolatti, De Lorenzis, et al., 2018), were synthesized at 2 dpi. On the contrary, Bianca transcriptome showed greater changes in its transcriptome between 1 (when hypersensitive response, HR, is observed) and 3 dpi (Toffolatti, De Lorenzis, et al., 2018). However, the VOCs accumulation at 3 dpi could be an indication of a late response of the plant to pathogen inoculation.

In Mgaloblishvili, the class of terpenes was mostly affected VOC class with the inoculation, at 2 dpi was the (Table 3.2, Figure 3.3 and 3.4). Terpenes are recruited to several ecological roles in plants. Many of these substances have antimicrobial and anti-herbivore properties, suggesting their role in defending the most important parts of the plant (Z Li et al., 2020). The increase of terpenes was mainly due to farnesene discovery, which was not detected in the not inoculated samples (0 dpi) and the 1 dpi samples (Table 3.2). Farnesene is a sesquiterpenes, being one of the principal compounds of some essential oils, extracted from seeds, fruits, flowers, leaves or roots, showing a good antimicrobial activity: for example, *Vitex agnus-castus* essential oil is active against *Streptococcus mutans* (Gonçalves et al., 2017). Among the other VOCs showing a statistically significant increase after the *P. viticola* inoculation, 1-(4-bromobutyl)-2-piperidinone and 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one (β-ionone) are noteworthy for their proved antimicrobial activities. Indeed, the synthesis of 1-(4-bromobutyl)-2-piperidinone in *Trichoderma asperellum* has been correlated to the biocontrol of *Fusarium oxysporum* (Wu et al., 2017). Meanwhile, the antimicrobial activity of β-ionone has been ascertained against some organisms, such as *Microcystis aeruginosa* (Shao et al., 2011).

Alcohols and aldehydes, on the contrary, were the two classes mostly discriminating the Bianca response to *P. viticola* infection (Table 3.2, Figure 3.3 and 3.4). Alcohols and aldehydes arise from fatty acid metabolism and are commonly referred to as "green leaf volatiles", synthesized in plant green organs in response to wounding (Dudareva et al., 2004, 2006). Among them, 3-hexen-1-ol and hexenal are two known compounds t involved in the plant-pathogen interactions with key roles in insect repelling and deterring (Wei & Kang, 2011), and remarkable antimicrobial properties against *Aspergillus flavus* (Gardini et al., 2001), respectively.

3.4.2 The expression of terpene synthases correlates with the pathogen colonization

The biosynthesis of VOCs occurs in every grapevine organ, though each organ shows a different VOC profile, and basically terpene synthase (TPSs) genes are expressed in all organs, while only some showed an organ-specific expression pattern (Matarese et al., 2013). *VvGwaBer*, *VvGwECar2*, *VvCSaFar*, *VvCSbOciM*, *VvTer* and *VvVal* genes were selected because they showed a gene expression in grapevine leaves at juvenile and mature stage (Matarese et al., 2013; Toffolatti, Russo, et al., 2018). Our real-time RT-PCR data revealed that all the analyzed TPSs had detectable transcripts in both not inoculated and inoculated samples (Figure 3.5), confirming their involvement in response to *P. viticola* infection, by producing metabolites that act as antifungal compounds (Dudareva et al., 2004, 2006). In both cultivars, the TPSs showed a peak of expression at 1 dpi, except for *VvTer*. This peak of expression, already described in previous transcriptomic studies, can be correlated to the timing of infection. At 1 dpi, *P. viticola* produces the first haustorium and activates the plant response (Perazzolli et al., 2012; Polesani et al., 2010; Toffolatti et al., 2012, 2020; Toffolatti, Russo, et al., 2018).

The gene expression patterns were consistent with the highest quantity of VOCs being detected at 2 and 3 dpi (Table 3.2). Unfortunately, it was not able to correlate analyzed TPSs gene expression with metabolites extracted from leaves, apart from farnesene, the main product of *VvCSaFar*, and nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol), one of the *VvGwaBer* products. The inability to detect the products of some TPSs is not unusual (Falara et al., 2011; Matarese et al., 2013), and it can be due to the extremely sensitive methods required to detect the compounds present at very low concentration, or a low level of compounds, or further conversion to other metabolites.

The genes showing the highest expression level were *VvGwaBer* and *VvVal* (Figure 3.5). *VvGwaBer* was identified as the functional gene responsible for the biosynthesis of α-bergamotene as a major product, and nerolidol as a minor product (Martin et al., 2010). The antimicrobial activity of this compound was widely demonstrated (Chan et al., 2016). In grapevine, nerolidol was synthetized following inoculation with *Phaeoacremonium parasiticum*, as well as the increase of VvPNLinNer1 transcripts, gene responsible of (E)-nerolidol biosynthesis (Escoriaza et al., 2019). A biosynthesis of nerolidol was also found in grape leaves (*Vitis labrusca*) attacked by *Popillia japonica* (Loughrin et al., 1997). Nerolidol was detected in our experimental conditions, in both not inoculated and inoculated samples, although the detected concentrations were not statistically changed after inoculation (Table 3.2).

VvVal catalyzes the conversion of farnesyl diphosphate to valencene, a sesquiterpene with antimicrobial activity (Manter et al., 2006). In *V. vinifera*, this gene was only expressed in flower buds and no transcripts were detected in the vegetative tissues of young leaves (Lucker et al., 2004; Matarese et al., 2013). Our results demonstrated that some *VvVal* transcripts were detected in Mgaloblishvili and Bianca leaves not inoculated, and they increased after the inoculation with *P. viticola* (Figure 3.5). Nevertheless, neither valencene nor its isomer (-)-7-epi-α-selinene was detected in inoculated samples.

3.4.3 New natural bioactive molecules against *P. viticola* infection

The identification of natural bioactive molecules is a key point in developing sustainable crop production. Due to their antimicrobial activity, natural VOCs can be a valid eco-friendly strategy to implement green agricultural practices and limiting the use of synthetic molecules, representing to date the most common disease management strategy (F. Brilli et al., 2019). Indeed, the efficacy of Oregano essential oil and other molecules, such as 2-ethylfuran, 2-phenylethanol, β-cyclocitral, *trans*-2-pentenal, in reducing the development of grapevine downy mildew symptoms has been already demonstrated (Lazazzara et al., 2018; Rienth et al., 2019). In this work, the efficacy of four terpenes (farnesene, nerolidol, ocimene and valencene) that are specifically synthesized by Mgaloblishvili upon pathogen inoculation, in counteracting *P. viticola* was proved in *ad hoc* experimental inoculations where the pathogen sporulation was significantly hampered compared to the untreated control, confirming their role as bioactive compounds in the resistance mechanism.

However, the direct involvement of these terpenes in the resistance mechanism needs to be further established through more deep investigations, *e.g.* by coupling microscopic observations, sporangia production and *P. viticola* quantification at different time points, as well as their effectiveness to enhance plant defenses in the field. Furthermore, the possibility of using mixtures of VOCs other protocols for the terpene application could be evaluated. It was demonstrated that VOCs work in a blend rather than alone in inhibiting the pathogen infection, by acting in an additive or synergistic way with different plant secondary metabolites, such as phenolics and terpenoids, in resistance establishment (Henriquez et al., 2012). Thus, the discovery of new

antimicrobial molecules and the availability of a wide range of bioactive molecules are crucial to set up new blends able to effectively contain the disease in the field in an eco-friendly and sustainable way.

3.5 Conclusions

In this study, the investigation of the resistance mechanism of two grapevine cultivars characterized by different genetic backgrounds (American and Eurasian) demonstrated that VOCs biosynthesis increased in leaves following the infection with *P. viticola*, although we cannot exclude that a fraction of detected VOCs was emitted by the pathogen. Moreover, the results obtained on the antifungal activity of four selected VOCs confirmed that farnesene, ocimene, nerolidol and valencene are indeed able to reduce disease severity in *in vitro* conditions. Further investigation is needed to establish the mode of action of these molecules and their toxicity profile. The identification of compounds biologically active against *P. viticola*, such as those reported here, opens new perspectives for sustainable viticulture. Cultural practices are scarcely effective in reducing downy mildew incidence, whereas fungicide treatments more efficiently protect grapevine against the disease. In the next few years, the use of some synthetic substances active against *P. viticola* will be banned or strictly regulated in Europe due to the application of the regulation concerning the placement on the market and the use of pesticides (Regulation (EC) No 1107/2009; Directive 2009/128/EC). To assure acceptable protection against the pathogen, the discovery of new bioactive molecules is, therefore, strictly needed. In this view, the exploitation of molecules that are naturally produced by the plant in response to the pathogen could be one of the possible to accomplish with this need.

Chapter 4: Characterization of *Plasmopara viticola* transcripts in the early interaction with resistant and susceptible grapevine cultivars

4.1 Introduction

Oomycetes are a large group of eukaryotes phylogenetically related to brown algae and diatoms which can be found in the natural and managed ecosystem (Kamoun et al., 2015). More than 60% of oomycetes are plant pathogens and among the 33 species reported, the top ten of relevance in agriculture include *Plasmopara viticola* (Kamoun et al., 2015; Strullu, 2011).

The oomycete *P. viticola* [(Berk. & Curt.) Berl. & de Toni] causes downy mildew disease in grapevine. The infection spreads by sporangia that are distributed by water splashes. When landed on grapevine tissues, sporangia liberate biflagellate zoospores that swim to stomata, encyst at the stomatal rim and germinate hyphae that enter the substomatal cavity. Mycelium emerges from a substomatal infection vesicle and proliferates intercellularly, e.g. in the spongy mesophyll of leaves. As an obligate biotrophic pathogen, *P. viticola* hyphae absorb nutrients by the formation of haustoria that invaginate living plant cells. These intimate cellular contact triggers responses of the host and the pathogen. After successful propagation, the hyphae grow out of stomata with branched sporangiophores that constrict asexually produced new sporangia at their termini for dispersal. Several cycles of this vegetative propagation can happen during a growing season and give rise to the epidemic (Gessler et al., 2011). Late in summer, *P. viticola* undergoes sexual recombination to generate oospores that can survive winter conditions and start the primary infections in the following year (Kortekamp & Zyprian, 1999; Welter et al., 2017).

The downy mildew disease is usually defeated with several fungicide sprays during the growing season; however, these have a negative impact on the environment (Zubrod et al., 2019) and need to be reduced. Effective pest-management requires detailed knowledge of the physiology of the interaction between plant and pathogen. Field isolates of *P. viticola* exhibit phenotypic (Gómez-Zeledón et al., 2013) and genetic diversity as recently studied with nuclear SSR (Simple sequence repeats)- and SNP (Single nucleotide polymorphisms) markers. High genetic variability in *P. viticola* strains is observed, possibly due to primary infections by sexually recombined oospores (Boso et al., 2014; Gessler et al., 2011; Gobbin et al., 2005; Matasci et al., 2010). Pathogens like *P. viticola* release "effectors" to overcome the first level of plant immunity (PTI or MTI, pathogen-triggered immunity or microbe-triggered immunity) in host plants. In the "first-level" resistance, plants respond to microbe-associated molecular patterns (MAMPs) or damage-induced molecular patterns (DAMPs) from attacked plant cells by a set of defense reactions. Pathogen effectors developed to interfere with these defense responses and raise virulence (leading to ETS–effector triggered susceptibility). However, in resistant host plants, products of specific resistance genes (R-genes), typically NBS (nucleotide-binding site)-LRR (Leucine rich repeat) proteins, act as receptors and recognize the effector proteins. This effector-receptor interaction initiates a signaling cascade that leads to more potent defense responses in host cells than the first level reactions and results in effector-triggered immunity (ETI) (Jones & Dangl, 2006; Tabima & Grünwald, 2019). The interaction between pathogen and host is strain-specific since each pathogen strain releases a

specific set of effectors. Resistant host plants may have one or several receptors encoded in resistance loci, to intercept a "fitting" effector and transmit this signal to the plant pathways of induced defense.

Genetic mapping and quantitative trait locus (QTL) analysis of downy mildew resistant grapevines (Emanuelli et al., 2013; Maul et al., 2015; Schwander et al., 2012; Welter et al., 2007) revealed resistance determining genomic regions which they typically encode NBS-LRR proteins. However, the functions of genes found in resistance-associated genomic regions of the host plant are yet to be completely understood (Divilov et al., 2018; Fanizza et al., 2016). QTLs effective on downy mildew were named "Resistance to *P. viticola* (*Rpv*)" (Fischer et al., 2004) and so far 27 *Rpv* loci are identified on several *Vitis* chromosomes (www.vivc.de/ data on breeding and genetics) in different *Vitis* species. The *Rpv3* locus from American *Vitis* relatives was found on chromosome 18 (Fischer et al. 2004; Welter et al. 2007; Foria et al., 2019), while *Rpv10* is located on chromosome 9 (Schwander et al., 2012). The locus *Rpv10* is from Asian *Vitis amurensis* origin and mediates resistance by inducing a large number of genes including those encoding defensive phytoalexins (Fröbel et al., 2019). In resistant grapevines, a restriction of the infestation was observed by microscopic observation of early host-pathogen interaction (Kortekamp & Zyprian, 2003). However, individual resistance loci present in new grapevine cultivars may overcome because of pathogen strains adaptation (Kast, 2001; Peressotti et al., 2010). Therefore, for grapevine resistance breeding, a combination of several diverse resistance genes is desirable to obtain durable resistance. The analysis of molecular markers linked to resistance loci allows to "pyramid" more than one resistance locus in breeding programs (Eibach et al., 2009). However, additional strategies to raise resistance through the interference of the host-pathogen interaction could be highly supplemental.

The understanding of pathogen evolution combined with its virulence mechanisms is prerequisite to develop novel strategies of pathogen control (R. Sharma et al., 2015). High numbers of effector proteins are predicted from genomic sequences in oomycetes (Dussert et al., 2019; Kamoun, 2006; Monteiro et al., 2013; X. Yin et al., 2017). In the case of *Plasmopara viticola*, genomic sequences encoding several apoplast proteins and effectors have been determined (F. Brilli et al., 2019; Dussert et al., 2019; X. Yin et al., 2017). However, there is a lack of functional knowledge on *Plasmopara viticola* effectors. RNA-Seq with next-generation sequencing technology (NGS) provides a technique for comprehensive transcriptome analysis. Both pathogen resistance and -susceptibility of a host plant are reflected by specific transcriptional responses. In this study, genes of *P. viticola* with differential expression patterns in the interaction with resistant host was compared to susceptible host plants and identified by transcript mapping to the *P. viticola* genome (Dussert et al., 2019; X. Yin et al., 2017) and *de novo* transcriptome assembly of RNA-sequences. The RNA sequences were obtained from inoculated leaf samples of resistant *Rpv10*- and *Rpv3*-carriers and susceptible grapevine plants at 6 hours post-inoculation (6 hpi). Selected differentially expressed genes were re-analyzed to confirm their expression pattern by quantitative Real-time PCR from isolated pathogen sporangia and at 6 and 24 h of host/pathogen interaction.

4.2 Material and Methods:

4.2.1 Plant material, growth conditions and inoculation

The RNA sequencing (RNA-Seq) study was conducted during the early interaction of the pathogen with its host plants. Three different grapevine genotypes, 2008-059-020 (susceptible, *Rpv–*), 2008-059-121 (resistant, carrier of *Rpv3* and *Rpv10*, each in heterozygous state) and 2011-003-013 (resistant, homozygous for the locus *Rpv10*) were analyzed at six hours after experimental inoculation (Fröbel et al., 2019). Three biological replicates were done for each interaction. The genotypes 2008-059-020 and 2008-059-121 are full sibling descendants from the *Rpv10* and *Rpv3* segregating cross of GF.GA-52-42 x 'Solaris'. The plant 2011-003-013 resulted from the self-pollination of 'Solaris'. All plants were raised in the greenhouse and adapted in a climate chamber before experimental inoculation.

The *P. viticola* inoculum was obtained from the field of JKI (Julius Kühn Institute for Grapevine Breeding Geilweilerhof) from the leaves of several susceptible grapevine cultivars. Naturally infected leaves with typical "oil spots" disease symptoms were collected and incubated overnight at high moisture to induce sporulation. Sporangia were collected by carefully brushing the surface of the freshly developed sporangial lawns with water. The resulting sporangial suspensions were microscopically checked in a counting chamber and adjusted for their density.

4.2.2 RNA sequencing

RNA-Seq analysis was performed in service and data were processed at JKI with CLC Genomics Workbench 6.5.1 (CLC bio, Aarhus, Denmark) as reported previously by Fröbel et al. (2019). The reads mapping to the *V. vinifera* genome were removed from the data set. The remaining reads were aligned to *P. viticola* genomic DNA sequences (using the most advanced assembly in 2165 contigs covering 101 MB, described by Yin et al. (2017). To facilitate transcript mapping, the *P. viticola* genome was analyzed using the AUGUSTUS tool (http://bioinf.uni-greifswald.de/augustus/) to predict genes based on the organism *Galdieria sulphuraria* (red algae), the closest model organism to *Plasmopara viticola* available in this database.

The CLC mapping parameters were Minimum length fraction: 0.9; Minimum similarity fraction: 0.95; Maximum number of hits for a read: 10; Minimum distance 80; Maximum distance: 150. The selected organism was ''Eukaryote'' and ''Exon discovery'' was checked (Settings: Required relative expression level: 0.2; Minimum number of reads: 10; Minimum length: 50). For the comparative characterization of transcriptional activity in the different host/pathogen interactions, the analysis packages (Function ''Set up Experiment'', Settings: ''Two-group comparison'' and ''Paired'') were employed. The expression level of the RNA-Seq reads was characterized by the value RPKM (Reads per kilo base per million mapped reads) (Mortazavi et al., 2008).

In addition, the "Large Gap Read Mapping" (LGRM) algorithm in the "Transcriptomic Analysis" tool was applied to predict transcripts (and potential splice variants) *de novo* and to match them to regions of the *P. viticola* genome. The parameters were set to Minimum length fraction 0.9, Minimum similarity fraction 0.95 and a Maximum number of hits for a read of 10.

4.2.3 Prediction of proteins and their characterization

The putative *P. viticola* genes predicted from the genomic sequence and those resulting from the mapping of RNA-Seq reads were processed by modules of the CLC Genomic Workbench to identify coding domains. The resulting predicted proteins were characterized by the three tools

SignalP (Almagro Armenteros et al., 2019), ApoplastP (Sperschneider et al., 2017), and EffectorP (Sperschneider et al., 2015).

The sequences of predicted transcripts from "Large gap read mapping" were searched for an open reading frame (ORF) using the Sequence Manipulation Suite (SMS, Version 2; Stothard 2000) (https://www.bioinformatics.org/sms2/orf_find.html). These open reading frame (ORF) sequences were translated *in silico* to identify effector-typical repeats and motifs in the encoded proteins. Nucleotide and protein sequences were analyzed by BlastX and BlastP searches at https://blast.ncbi.nlm.nih.gov/Blast.cgi to identify similar proteins with annotated functions in databases (RefSeq, NR). The ''Venny 2.1'' software (http://bioinfogp.cnb.csic.es/tools/venny/; Oliveros 2007-2015) was used to represent the number of predicted transcripts and encoded proteins overlapping between the pathogen responses on the three different grapevine genotypes.

4.2.4 Experimental inoculation and RNA extraction

For quantitative Real-Time PCR (RT-qPCR) assays, healthy, unsprayed leaf samples from greenhouse-propagated plants were used. Samples were collected from the *Rpv*-carrying genotypes 'Rondo' (*Rpv10*), 'Solaris' (*Rpv10/Rpv3*) and the susceptible 'Chasselas blanc' (*Rpv*-) before and after inoculation with a field isolate of *P. viticola* (collected from 'Pinot Blanc' in the vineyards of the Institute). These genotypes correspond to the genotypes used for RNA-sequencing concerning the *Rpv* loci and their phenotypes.

From each genotype, the third and fourth leaf basipetal from the tip of the shoot of four plants were collected and prepared for the leaf disc assay. Leaf discs (15 mm diameter) were cut by a cork borer and placed upside down on 1% Agar in a culture plate (23 cm x 23 cm). Three biological replicates from each genotype were inoculated with 50 µl of *P. viticola* sporangia (28,500 sporangia/ml). Pathogenesis could proceed for 6 hpi, 24 hpi and 7 dpi. During this time, the leaf discs were kept in a climate chamber (16 h day, 65 kLux, 80% humidity, 24 °C,) and 8 h night (100% humidity, 22°C). After 6 hpi, the droplet of the sporangial suspension was removed. Total RNA was extracted at 6 and 24 hpi and converted to cDNA. The infestation stage achieved at 7 dpi was analyzed by microscopy (see below) to confirm successful host-pathogen interaction. For a comparison to the pathogen not yet interacting with its host (called "time point 0 hpi"), total RNA was extracted from freshly collected *P. viticola* sporangia.

RNA extraction was conducted using sporangia samples or leaf discs which were shock-frozen in liquid nitrogen, ground into fine powder in the presence of a spade point of PVPP (Polyvinylpolypyrrolidone, Carl Roth GmbH +Co. KG, Karlsruhe, Germany), and homogenized in 1.5 ml centrifuge tubes using the homogenization buffer of the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, Missouri, USA). The kit was used according to the specifications of the supplier and manuscript. To eliminate any residual DNA, an "on column" DNaseI treatment was performed. For this purpose, 2.5 µl DNaseI (6.8 Kunitz units) (QIAGEN GmbH, Hilden, Germany), 10 µl RDD buffer and 87.5 µl nuclease-free water were mixed and transferred to each sample. After 15 minutes, the samples were centrifuged at 14000 g for one minute. Elution was performed twice in 35 µl elution buffer. The RNA concentration was determined photometrically (λ = 260 nm).

4.2.5 Quantitative real-time PCR

The kit of ''High Capacity cDNA Reverse Transcription Kit™'' (Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesize from 5 ng of total RNA samples extracted from

sporangia ("0 hpi") and at 6 hpi and 24 hpi of host/pathogen interaction. Primer design for the genes of interest employed "Primer3" (ver. 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). The resulting oligonucleotides were mapped *in silico* to the *P. viticola* and (for control) to the *Vitis* genome. There were no hits on the *Vitis* genome. For each gene, three primer pairs (as indicated by Table S.5) were designed and tested for efficient amplification and specificity by reverse transcription-PCR on cDNA of *P. viticola* sporangia (Figure 4.1). The resulting amplification products were sequenced (in-service). The primer pairs yielding the most abundant reverse transcript were employed for quantitative real-time PCR (Table 4.1).

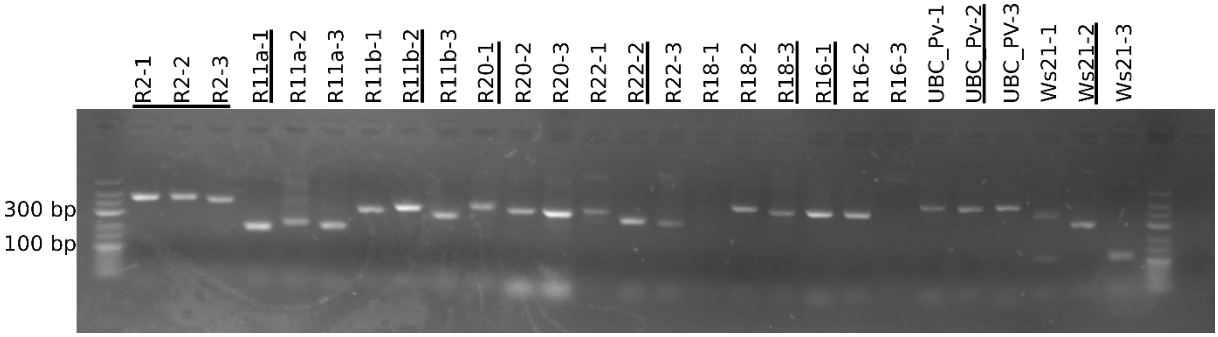


Figure 4.1 Test of primer pairs for amplification of differentially expressed genes. Reverse transcription – PCR products were resolved by 3 % agarose gel electrophoresis. The primer pairs producing the most abundant and most clear products were used for qRT-PCR: These are underlined. R2 did not show the targeted gene region after sequencing and was discarded. The right side of the gel shows amplified fragments of the reference genes *UBC_PV* and *Ws21* (Evangelisti et al. 2013). The size marker was GeneRuler™ Low Range DNA Ladder (Thermo Scientific™)

Table 4.1 Gene-specific primer pairs (including primers for *UBC_Pv_2* and *WS21_Pv_2* as reference genes for RT-PCR, Evangelisti et al. 2013)

	Gene	**Primer sequence**	**Amplicon size**
	R11a-1	F: CAGCAAGAACGTTGTGGAAA	220 bp
		R: CAACCCAATACCGTGAATCC	
	R11b-2	F: CAAGAAGCAGTCTCCGAACC	372 bp
		R: ACGCATCCGTAAGTCGTACC	
	R20-1	F: TACTGCGCTTTGTCACGAAC	389 bp
		R: TCAAAGATTGCTGGCAACAC	
	R22-2	F: ACGAGTTCCTTGACGCAATC	312 bp
		R: CTTCGGCGTAGTGGAGAATC	
	R18-3	F: GCTTCCACAATCCACCACTT	422 bp
		R: ATCGCATACACGGTCGTACA	
	R16-1	F: CGGTACGCAAGACCCATACT	419 bp
		R: GATCGTGTCGTCTGCTTCAA	
Accession No.	**Reference genes**		
CK859493	*UBC_Pv_2*	F: GCCGAAGCCTATAGAGCAGA	470 bp
		R: GCGTACTTGGCAGTCCATTC	
CF891675	*WS21_Pv_2*	F: ACGGCTCAGATTCGTGCTAT	297 bp
		R: GAGCCAGCCATCGACTCTAC	

Quantitative real-time PCR (qRT-PCR) was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with ‘‘Power SYBR Green PCR Master Mix’’ (Thermo Fisher Scientific, Waltham, MA, USA). Reaction mixes (total volume 20 µl) contained 2 µl cDNA, 10 µM of each forward and reverse primers, 7 µl nuclease-free water and 10 µl of 2x Power SYBR Green Master mix. *UBC_Pv* (encoding Ubiquitin-conjugating enzyme E2 protein) and *WS21_PV-2* (encoding 40S ribosomal protein 3a) reference genes (Evangelisti et al., 2013) for the vegetative propagation phase of oomycetes were used to normalize the qRT-PCR results. Expression levels of the six target genes were quantified by amplification over 40 PCR cycles (95 °C for 15 s and 60°C for 60 s). After running the qRT-PCR program, a melting curve analysis of the amplified fragments ensured specific amplification. The 7500 Fast Software v2.3 determined the Ct-value of the examined genes. The Ct- values were used to calculate the relative expression using the ΔΔCt-method (Livak & Schmittgen, 2001). The relative gene expression is given as "Fold change" (FC = $2^{-\Delta\Delta Ct}$), standard error and standard deviation were calculated for the mean fold change (FC) in each genotype with three biological replicates and two technical repetitions.

4.2.6 Microscopic follow-up of leaf disc infestation

The *P. viticola* inoculated leaf discs not used for RNA extraction remained in the climate chamber for seven days for examination by microscopy. The samples were bleached in 30 ml of KOH (1N) overnight (65 °C), washed in distilled water for three times, placed on microscope slides and stained for 10 minutes with Aniline blue 0.05% (w/v) in 1 N KOH (Hood & Shew, 1996). An epifluorescence microscope Leica DM4000B-M (excitation at 395–440 nm, emission filter 470 nm) was used to follow the progress of pathogenesis in each genotype (Figure 4.2).

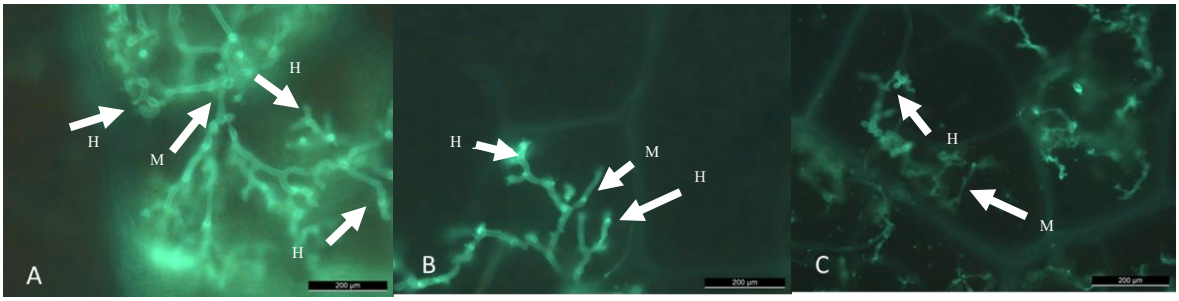


Figure 4.2 Degree of infestation of inoculated leaf samples. A: ‘Chasselas Blanc’ (*Rpv*–,) B: ‘Rondo’ (*Rpv10)*, C: ‘Solaris’ (*Rpv10/Rpv3*) at 7 dpi. *P. viticola* development was followed using epifluorescence at 7 dpi after bleaching with 1 N KOH and Aniline blue staining. (A) *P. viticola* mycelium expanded widely and many haustoria emerged in the susceptible genotype (*Rpv*–). (B) Mycelial growth was strongly decreased in the genotype with the *Rpv10*-locus (*Rpv10*). (C) Mycelial development was also strongly inhibited in the resistant genotype carrying both resistance loci *(Rpv10/Rpv3)*. The space bars correspond to 200 µm. M, mycelium; H, haustoria.

4.3 Results

4.3.1 *P. viticola* genome annotation and read mapping

The assembly of the *P. viticola* genome sequence described by Yin et al. (2017) covers 2165 scaffolds with a total of 101 Mb and was used for transcript mapping. To facilitate the mapping, the genome was first annotated by “Augustus” which predicted 15442 protein-encoding genes.

The *in silico* characterization with the tools EffectorP, SignalP and ApoplastP yielded 2022 putative effectors, 1149 proteins predicted to carry a secretion signal and 802 apoplastic proteins.

After adapter and quality trimming, 99.8% (up to 50 million “reads” per triplicated assay) of the 6 hpi "reads" from each genotype-pathogen interaction were available for analysis. The "reads" mapping to *V. vinifera* (87 %, up to 43 million “reads”) were subtracted from the total set. For the different genotypes the remained 12% in the *Rpv10* homozygote (2011-003-013), 13 % in the *Rpv3/Rpv10* heterozygote (2008-059-121) and 14 % of the “reads” in the susceptible genotype (2008-059-020) unmapped and potentially containing reads from the pathogen. This corresponded to approximately 3 million “single” and “paired-end” unmapped reads. From these reads, 10 to 12 % were successfully mapped to the *P. viticola* genome (Table 4.2).

Table 4.2 Number of reads mapped to the *P. viticola* genome. Three biological repeats per genotype were subjected into RNA-sequencing reads analysis.

Interacting genotype	**No. of reads mapped**	**Percentage reads mapped**
Sus-1	404 151	10.9%
Sus-2	340 553	11.6%
Sus-3	354 931	11.3%
Rpv3/*Rpv10*-1	391 191	12.5%
Rpv3/*Rpv10*-2	304 259	10.4%
Rpv3/*Rpv10*-3	253 095	10.1%
Rpv10/Rpv10-1	361 379	10.5%
Rpv10/Rpv10-2	348 950	11.3%
Rpv10/Rpv10-3	325 831	10.6%

4.3.2 Transcriptional activity in the host/pathogen interactions

The reads from the *P. viticola* genome sequence that matched to annotated transcripts were analyzed for their overlapping presence in the three different host/pathogen interactions. In total, 2877 transcripts from the pathogen sequence were found in the interaction with the susceptible genotype. In the interactions with the two different resistance carriers, more genes from the pathogen were transcribed, 4142 in *Rpv3/Rpv10* and 3171 in *Rpv10/Rpv10*. The number of shared transcripts between the three different pathogen/host interactions is presented in Figure 4.3. A set of 1720 transcripts was active in common during all three interactions. A larger number of shared transcripts appeared in the interactions with both resistant genotypes. While fewer RNA sequences overlapped between the pathogen interacting with the susceptible and the *Rpv10*-homozygote grapevine genotypes, the interaction with the genotype containing the two resistance factors *Rpv3* and *Rpv10* overlapped with 558 transcripts to the susceptible genotype and produced the highest number of “private” transcripts (1143, Figure 4.3).

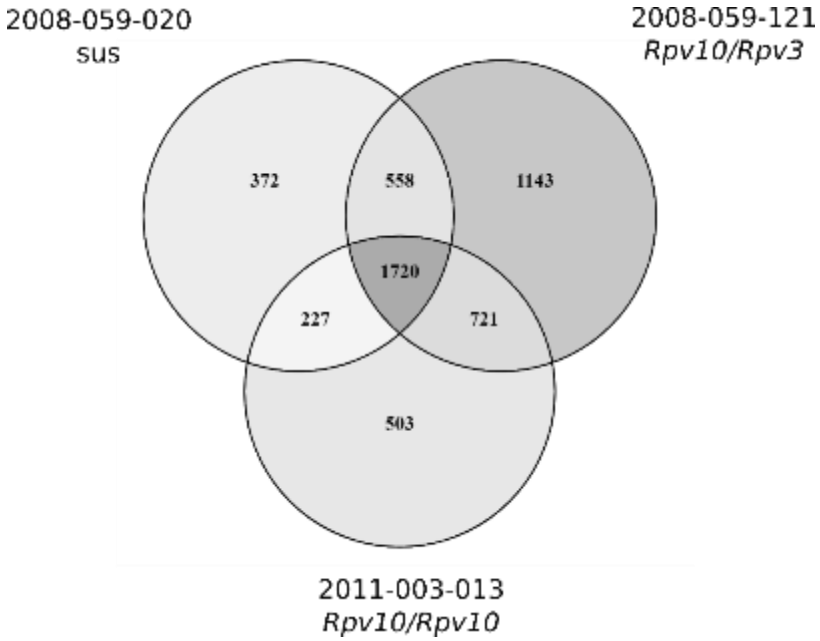


Figure 4.3 Venn diagram showing the amounts of pathogen transcripts after 6 hpi of interaction with the three grapevine genotypes and their overlaps.

4.3.3 Potential effectors

From the pathogenic sequences in the interaction with the susceptible genotype, 729 of the 2877 transcripts were assigned to code for 156 apoplastic proteins, 224 secreted proteins and 349 effector proteins. In the interaction with the *Rpv3/Rpv10* carrier, 912 *in silico* translated transcripts of the pathogen were classified as 197 apoplastic proteins, 279 secreted proteins and 436 putative effectors. From the interaction with the *Rpv10/Rpv10* carrying genotype 809 predicted proteins were classified in the different protein groups (201 apoplastic proteins, 246 proteins with signal sequence, 362 putative effector proteins). Within the classes of the different encoded proteins, some proteins were assigned to more than one class (Table 4.3).

Table 4.3 Assignment of predicted proteins from the *P. viticola* interaction with the three different grapevine genotypes according to their characterization with Effector P, Signal P and Apoplast P. Some of the predicted proteins show more than one assignment.

Interaction of *P. viticola* with	**Effector**	**Signal**	**Apoplastic**	**Effector and apoplastic**	**Effector and Signal**	**Apoplastic and Signal**
Susceptible	349	224	156	35	30	60
Rpv10/Rpv10	362	246	201	46	39	66
Rpv3/Rpv10	436	279	197	39	26	63

The predicted proteins were checked for the presence of typical effector motifs. Searching these motifs, in total 19 Crinkler (CRN) effectors and 123 RxLR type effectors were identified as cytoplasmic effectors. (Table S.6). The approach to predict candidate RxLR and CRN motifs in effectors looked for selected amino acid sequences translated from the effector's open reading

frames (ORFs) in genome for motif of interest. This was able to find 38 conserved motifs of RxLR effectors and 8 for crinkler (CRN) effectors. This method was used to predict 5, 6 and 14 effectors in *P. viticola* interacted with each susceptible (*Rpv-/Rpv-*), resistant homozygote (*Rpv10/Rpv10*) and resistant (*Rpv3/Rpv10*) genotypes, respectively (Table 4.4). In this comparison, 8 RxLR and 1 HVLxxP-CRN motif-containing effectors were observed in common between all genotype's interactions. Most CRN effectors were found in the interaction of *P. viticola* with the *Rpv3/Rpv10* carrier. Furthermore, one HVLxxP-CRN and 3 RxLR effectors were common between both resistance genotypes.

Table 4.4 The table represented the effector proteins and effector-typic RxLR and CRN motifs per *P. viticola*-grapevine host interaction genotype. Values show selected differentially expressed genes from the RNA-Seq study in the three host/pathogen interactions and their annotation according to Blast analysis

Susceptible exclusively	***Rpv10*/*Rpv10* exclusively**	***Rpv3*/*Rpv10* exclusively**	**Susceptible and *Rpv10*/*Rpv10***	**Susceptible and *Rpv3*/*Rpv10***	***Rpv3*/*Rpv10* and *Rpv10*/*Rpv10***	**All three interactions**
g3846 (RxLR)	g12735 (RxLR)	g10502 (RxLR)	g10873 (RxLR)	g10745 (RxLR)	g11308 (CRN-HVLxxP)	g11674 (RxLR)
g7813 (RxLR)	g14474 (RxLR)	g11356 (RxLR)	g13193 (RxLR)	g10789 (RxLR)	g12034 (RxLR)	g11830 (RxLR)
g9894 (RxLR)	g4019 (RxLR)	g14223 (RxLR)		g11523 (RxLR)	g6127 (RxLR)	g11900 (RxLR)
g13720 (RxLR)	g4820 (RxLR)	g1892 (RxLR)		g2535 (RxLR)	g697 (RxLR)	g12648 (RxLR)
g586 (HVLxxP)	g5463 (RxLR)	g4821 (RxLR)				g1861 (RxLR)
	g5589 (RxLR)	g5891 (RxLR)		g8131 (RxLR)		g3974 (RxLR)
		g6249 (RxLR)				g4111 (RxLR)
		g7396 (RxLR)				g9832 (RxLR)
		g7570 (RxLR-CRN)				g8655 (HVLxxP-CRN)
		g7901 (RxLR)				
		g8495 (RxLR)				
		g8158 (CRN)				
		g3619 (CRN)				
		g14055 (CRN)				

4.3.4 Identification of candidate genes

The regions mapping to the annotated transcripts with an expression level of RPKM >2 overall three host/pathogen interactions were selected and transferred to a library in CLC. The sequences

from these regions were checked via BlastN algorithm for annotated functions at NCBI (nr nucleotide collection).

In this way, 128 transcripts were predicted and were annotated on the sequence of the pathogens. Gene functions including acidic chitinase, nucleoside triphosphate hydrolase, serine protease, peptidase, trehalose-phosphate synthase (PHALS_09303), INRA-310 trehalose-phosphatase, glucosidase, serine protease, peptidase, growth factor like domain, putative PITG, transmembrane superfamily, sporangia induced phosphatidyl inositol kinase (PIK-E1), CRN-like protein, P-loop containing nucleoside triphosphate hydrolase (PHALS_14339), CRN-like protein (PHALS_08610), amino acid/auxin permease-like protein, ribosome biogenesis regulatory protein (PHALS_11950), T30-4 ribosome biogenesis regulatory protein (PITG_04384) were annotated in the three host-pathogen interactions (Table S.7). Candidate genes were searched especially in these highly transcriptionally active regions re-analyzed by LGRMs. From the 128 total annotated transcripts, six candidate genes were selected based on the predicted function of homologous genes, that seem to play a role during pathogen-plant interaction. The genes of interest (GOI) were selected from the highly differentially expressing and annotated genomic regions of *P. viticola* for confirmatory evaluation by RT-qPCR. These genes include some coding for avirulence proteins, kinases, regulatory proteins, signal proteins and cellulose synthase enzymes. Grapevines corresponding to the *Rpv* carriers of the original RNA-Seq study were selected. To represent the non-interacting state ("0 hpi"), freshly obtained sporangial suspensions of *P. viticola* were included in the analysis. RNA extracted from sporangia and at 6 and 24 hpi was reverse transcribed and applied for gene expression analysis.

4.3.5 Differential gene expression

The selected genes (strongly differentially induced genes) were examined by qRT-PCR. Further expression investigation was validated by qRT-PCR after the design and test of specific primer pairs (Table 4.1, Figure 4.1). The RPKM value for each gene corresponds to the level of gene expression by the number of mapped reads in this region. The region shows the localization of the gene region on the contigs and the contig number in genome assembly of reference genome of red algae, MTPI01.1 (g13823, g13827, g12648, g 14640, g8655 and g3932) and P.v_INRA-PV221 (PVIT_0024729, PVIT_0024734 , PVIT_0002314 , PVIT_0010433, PVIT_0012441 and PVIT_0017700) were identified (X. Yin et al., 2017) (Figure 4.5).

Table 4.5 The gene contigs of the pathogen and their activity (RPKM level) within the RNA-Seq analysis between the three different grapevine genotypes in interacting with *P. viticola*.

Gene	Region	Contig no. in genome assembly			RPKM 2011-003-013 (*Rpv10/Rpv10*)	RPKM 2008-059-121 (*Rpv10/Rpv3*)	RPKM 2008-059-020 (*Rpv-/Rpv-*)	Motif	Function according to Blast Analysis	Accession of homologue
		MTPI01.1_augustus-v331	Contig No.	P.v_INRA-PV221						
R11a	8590..9088	MTPI01000899	g13823	PVIT_0024729	3968254	9259259	0	non	TAR 1 protein *Aphanomyces invadans*	XP_008881610.1
R11b	38268..39504	MTPI01000899	g13827	PVIT_0024734	3071087	4519275	220750,55	non	TAR 1 protein *Aphanomyces invadans*	XP_008881610.1
R16	5120..6863	MTPI01000643	g12648	PVIT_0002314	6684257	6708866	0	RxLR	TAR 1 protein *Aphanomyces invadans*	XP_008881610.1
R18	6887..9940	MTPI01001194	g14640	PVIT_0010433	7743128	7847694	0	non	Cellulose Synthase 1 *P. viticola*	ADD84670.1
R20	7433..8510	MTPI01000317	g8627	PVIT_0012441	18947668	15290573	32951101	CRN	Ras-related protein Rab5 *Phytophthora nicatianae*	KUF69269.1
R22	8932..9768	MTPI01000925	g3932	PVIT_0017700	5790643	4954216	19391119	non	Regulator of G-protein signaling *Phytophthora infestans* T30-4	XP_002898207.1

4.3.6 Validation of the RNA-Seq analysis – qRT-PCR

For the validation of the RNA-Seq results, a gene expression analysis of selected genes was performed. For this study inoculated leaf discs from 'Chasselas blanc' (susceptible), 'Rondo' (*Rpv10*) and 'Solaris' (*Rpv10/Rpv3.3*) were used on different time points (before inoculation, 6- and 24-hour post-inoculation (hpi)). For the time point before the inoculation, new-collected *P. viticola* spores were used. Thus, the gene expression could be evaluated at the time 0 hpi (from the pathogen spores), at the time 6 hpi as in the RNA-Seq analysis and after 24 hpi. The primers used for qRT-PCR were tested and sequenced. The basic presence of the six genes was shown by reverse transcriptase PCR (Figure 4.1) and then the genes were further submitted to quantitative Real-Time (RT-qPCR) assays (Table 4.6) including a time point of 24 hpi. The C_T value output was used to calculate the expression level using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) in calibration to reference genes (to WS21-Pv (Table 4.6) and to UBC-Pv (data not shown here)).

The expression results for most of the genes (Figure 4.4) shown expressed after 6 hpi than after 24 hp expected in the susceptible genotype and the genes 11b and R22a. In the resistance genotypes, the genes are up-regulated at 6 hpi and after 24 hpi most are down-regulated, while, only two genes of R16 and R18 are insignificant. The R22 encoding gene shown the highest activity which in both resistance genotypes is early induced after 6 hpi. Meanwhile, in the susceptible genotype low expression of gene R22 was observed at 24 hpi. Also, R20 was shown higher activity in the susceptible genotype as a 2-fold more increasing than in the resistance ones.

Table 4.6 Activity of the six identified differentially expressed genes in interaction with the three grapevine genotypes at 6 and 24 hpi of on-going interaction. The fold change (FC) of gene expression was determined using the $2^{-\Delta\Delta CT}$ calculation by quantitative Real Time PCR assays normalizing to WS21 (homologous to *Phytophthora* sp. genes encoding 40S ribosomal protein 3a) as reference gene. Expression changes of less than two-fold (FC<1) were considered as insignificant.

Interacting genotype	**Gene ID**	**6 hpi**		**24 hpi**	
		FC			
Chasselas blanc, *Rpv-*	R11a	9,04	up	3,28	down
	R11b	1,08	up	12,44	up
	R16	0,92	insignificant	0,51	insignificant
	R18	0,65	insignificant	0,01	insignificant
	R20	14,51	up	7,37	down
	R22	5,84	up	207,51	up
‚Rondo' *Rpv10*	R11a	7,19	up	1,94	down
	R11b	2,91	up	0,43	down
	R16	0,10	insignificant	0,25	insignificant
	R18	0,14	insignificant	0,06	insignificant
	R20	2,86	up	1,84	down
	R22	61,75	up	35,78	down
‚Solaris' *Rpv10/Rpv3*	R11a	4,61	up	1,40	down
	R11b	2,34	up	1,11	down
	R16	0,55	insignificant	0,11	insignificant
	R18	0,14	insignificant	0,02	insignificant

	R20	2,25	up	0,60	down
	R22	111,12	up	10,12	down

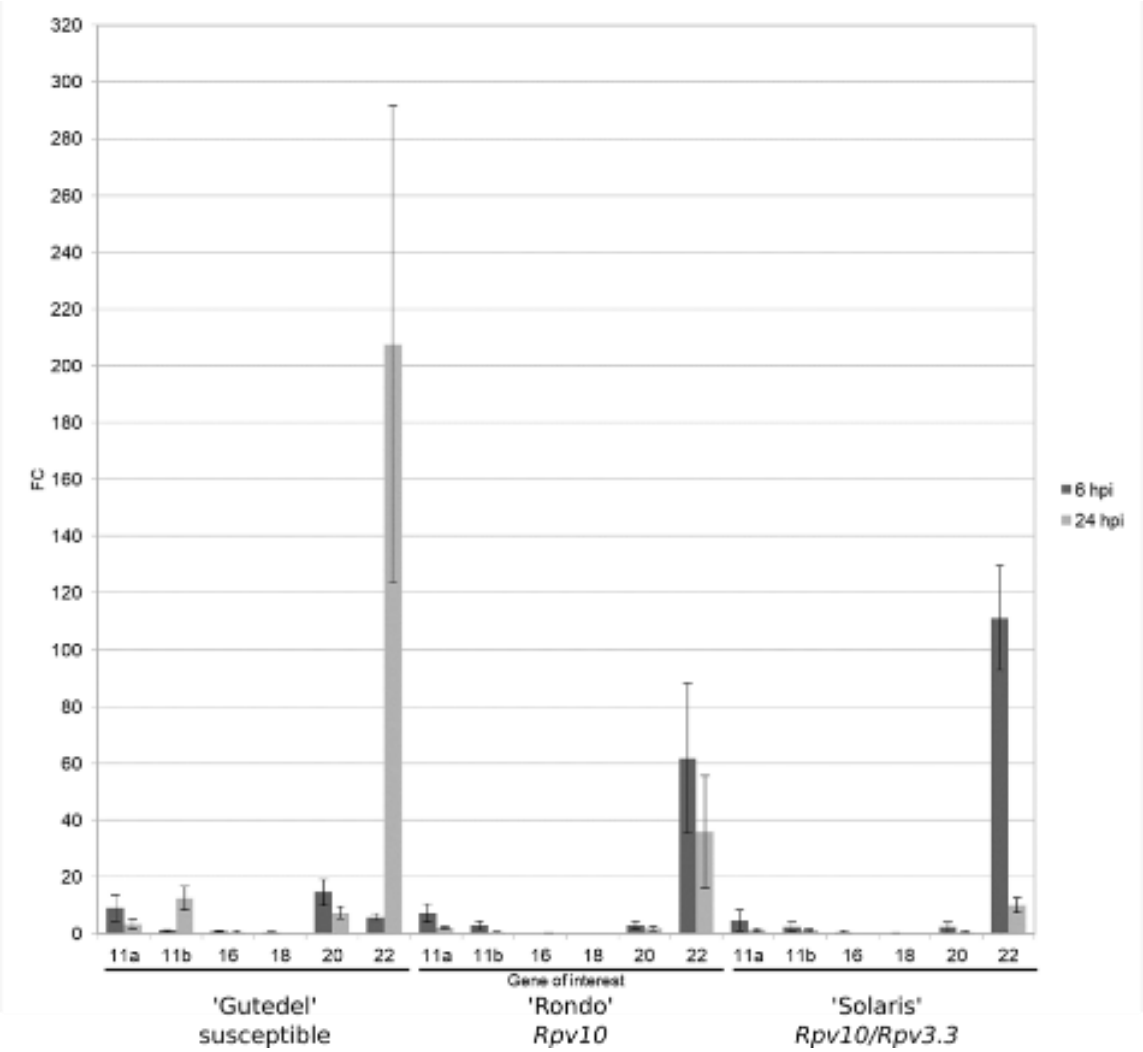


Figure 4.4 Gene expression of the genes of interest in three different genotypes. Illustrated are the gene expression of the GOI on the two different time points 6 hpi and 24 hpi in one susceptible referent genotype and two resistance genotypes one with the *Rpv10* locus and the other with the *Rpv3.3* locus in addition to the *Rpv10*. The data is calculated with the reference gene WS21_Pv

4.4 Discussion

Despite its worldwide economic impact, little knowledge is available about the pathogenicity of *P. viticola* on a molecular basis. In this study, the primary goal was to identify genes involved in early pathogen development in resistant and susceptible host tissues. RNA sequencing is a sequence-based technology with the ability of *de novo* transcript assembly, transcript fusion detection, and specific expression study (Dillies et al., 2013). The RNA-Seq data used in the

current research was obtained from Fröbel (2019) on grapevine genotypes inoculated with *P. viticola.* The genotypes used for RNA-Seq carried the Asian resistance locus *Rpv10* in homozygous state and a combination of heterozygous *Rpv*10 with the American locus *Rpv3* (heterozygous) in comparison to a susceptible genotype from the same progeny. Moreover, grapevine genes belonging to the GO category "Biological process" were identified as "Signaling", "Defensive" and "Receptor" classes, which included PR-, ERF5-, WRKY75-, PAL- and STS genes (Fröbel et al., 2019). Furthermore, from 870 million reads obtained in the original experiment, 7 percent did not match with *Vitis vinifera* genome, and were considered as transcripts of the pathogen expressed during the interaction with host cells. Therefore, the work represented here has re-used the RNA sequencing data to acquire a better understanding of the molecular responses of the pathogen level in relation with the genotypes of different *Rpv*-carriers. The pathogenic attack of the host is facing and counteracting host cell defense responses. Induced defense responses of host cells include the closure of stomata (Allègre et al., 2009), enzymatic activities, callus deposition (Hamiduzzaman et al., 2007), accumulation of phytohormones (Gessler et al., 2011), and the expression of defense genes, which have been described as plant defense mechanisms during pathogen attack. Little information is available regarding pathogen related secretum, whereas oomycetes cytoplasmic and apoplastic effectors are reported as plant immune system triggers (M. Brilli et al., 2018; Du et al., 2015; L. Yin et al., 2015).

In the current study, a total of 10190 transcripts were observed in the pathogen genome during early pathogen-host interaction. The deduced coding frames were grouped into three protein classes of "Apoplast", "Signaling", and "Effector". Generally, *P. viticola* has expressed a high number of effector and apoplast genes during interaction with genotypes containing *Rpv loci*. Not much information is available regarding these protein classes. However, recently the "Effector" activity was screened through gene cloning and suppression of host immune responsive genes during infection, and their contribution to pathogen virulence were characterized. In addition, the effectors impact on hormones signaling were indicated in plant defense (Xiang et al., 2016). The study of "effectors" could help produce stable transgenic plants and can also be used for selecting appropriate fungicides in various breeding programs. Based on previous studies, the percentage of effectors in pathogens putative proteins has varied for different varieties (Fabro et al., 2011; Zhengpeng Li et al., 2015; Wang et al., 2011). The *P. viticola* encodes effector proteins during *V. vinifera* infection and suppresses the immunity. However, an immune system can recognize the effectors probably through effectors specific resistance gene (Xiang et al., 2016). In the current work, the highest numbers of effector and signaling proteins were identified in 2008-059-121-*Rpv10_Rpv3,* while the lowest number of apoplast proteins was reported for 2008-059-02-*Rpv-_Rpv-* genotype through genomic comparative approach using annotated genes in *P. viticola* genome sequence. Total reads with high RPKM levels were common between genotypes, while during infection 2008-059-121-*Rpv10_Rpv3* induced the highest amount of secretum. The increase of effector proteins in *P. viticola* could be related to *Rpv* existence in host cells. However, further investigation is required to fully understand this relationship. Several studies have attempted to identify immune pathways in host cells, and recently the metabolic targeting pathway of pathogen effectors in host cells has also been taken into account. The current study determined subcellular effectors related proteins that were co-expressed with signal and apoplast proteins in primary disease. CRN motif at C-terminal of cytoplasmic effectors exhibits virulence activities as host cell death, whereas the N- terminal are required for delivery inside plant cells (Haas et al., 2009).

Reverse transcriptase PCR was used at different *P. viticola* infection cycles, which represented the expression of identified genes in RNA-Seq data (Figure 4.1). Similar genotypes genetically and

phenotypically were selected to identify the GOI resulted from RNA-Seq during *P. viticola* interaction with host cells. Importantly, among all expressed genes identified by RNA-Seq data, six genes corresponding to TAR 1 protein, Ras-related protein, G-protein signaling, cellulose and avirulent proteins were found (Table 4.5). Here six novel genes are reported, which have high query cover with *Plasmopara* putative proteins. The data from the qRT-PCR showed that these genes were expressed upon infection. A better understanding of the genes could be obtained by its molecular characterization.

High quality genome development can improve the detection of involved pathogenicity genes and can shed light on understanding the resistance mechanism in *Vitiaceae*. Most of the identified apoplastic effectors in the oomycete *P. viticola* genome, with high similarity to glucanase inhibitors, are grouped as hydrolysis involved proteins. The RNA sequencing analysis of the current work showed high expression levels for the R22 signal protein with high RPKM levels. Furthermore, the real-time results demonstrated a very high expression level at 6 hpi in comparison to time point 0 hpi. The expression level increased even after 24 hpi in susceptible variety, whereas it decreased 10 folds for genotype contains *Rpv10/-*. However, *Rpv10/Rpv3* contained genotype had the maximum expression level amongst all genotypes and dropped almost 50 times at 24 hpi. The gene activity was confirmed by query cover in BLAST search analysis and R20 is a probable signal effector with lower expression compared to R22, and the expression level decreased smoothly after 24 hpi. The increase of R22 expression level in susceptible genotype could be due to the lack of *Rpv* loci and induced immune responses in host cells. In addition, after an increase in expression levels at time point 6 hpi, a decrease was observed at 24 hpi for all the genotypes. Therefore, it could be suggested that R22 is not dependent on the existence of *Rpv* loci. In the current study, genes R16 with high query to effectors in oomycetes was expressed almost steady, which is in contrast with work reported in (M. Brilli et al., 2018). We have identified gene R18 encoding putative cellulose synthases which by BLASTN search tool and comparing the R18 gene, we have found the high similarity with *PvCesA* gene causes resistance to fungicide in *Plasmopara* (Blum et al., 2010). The 11a and 11b avirulence novel genes with query to signal proteins represented increased expression at 6 hpi and while increased continuously in susceptible genotype, dropped down in other partly resistant genotypes. The correlation between disease development based on leaf disc assay and interested gene expressions in *P. viticola* can be an important feature for defining the pathosystem during the pathogenicity. As it was reported in correlation between host cell gene DEG and disease symptom development in the past and even in other oomycetes. In similar studies, the RxLR proteins and apoplastic effectors expression are known to be upregulated upon infection (Haas et al., 2009). Furthermore, the expression of *P. viticola* effector genes were observed in early interaction stages and also zoospores germination (Dong et al., 2011; Haas et al., 2009). Our work is in line with the findings reported in different qRT-PCR and expression studies for effector genes. In general, the laboratory results agreed with RNA sequencing data analysis results, although the exact genotype was not inoculated with *P. viticola*, but the *P. viticola* genes identified and expressed in RNA seq data and qRT-PCR results have the same effect on the plants. The disease development mechanism is in line with the RNA seq data. The early *Plasmopara* development on leaf discs of corresponding plant genotypes was studied by microscopy and compared between the plant genotypes (Blum et al., 2010). On the susceptible host cells *P. viticola* propagated and produced sporangia within 7 dpi. In grapevine genotypes carrying the *Rpv* loci understudy, this process seemed to be slowed down with less or weak development of haustoria in host cells. These findings are in line with genetic studies that

suggested the *Rpv* loci as responsible genetic factors for the resistance of host cells. However, so far little attention was paid to the pathogen response to the resistance factors RNA-sequencing.

4.5 Conclusion

Undoubtedly, a broad spectrum of genome sequencing of oomycete species can make a way to the robust genetic population of oomycetes wide groups-genome (Thines & Kamoun, 2010). We assembled the first step for the genomic comparative study of *P. viticola* to expand the knowledge about pathogenicity mechanisms. However, the conclusion from these results is difficult because host plant DEG is not included here. Most studies on transcriptomic gene expression between grapevine genotypes and *Plasmopara* involved *Rpv*-mediated genes as reported in previous work as background (Fröbel et al., 2019). These pathogens' identified genes might be specific markers for successful interaction with the host or can be marker genes for plants' defense signaling. Indeed, it was the first report that underlay more pathogenic gene-mediated studies to unravel the resistance mechanism against downy mildew disease.

Chapter 5: Concluding Remarks and Future Perspectives

The literature on Georgian germplasms have shown various interesting traits such as resistance to downy mildew disease, which is one of the most important grapevine diseases in the world. In this study, a Georgian *Vitis vinifera* cultivar (Mgaloblishvili) was selected because of its unique resistance traits against *P. viticola* and was subjected to further studies of in-genomic and molecular pathway response to the pathogen. While the reported resistance sources in the literature were from non-vinifera species, the use of *V. vinifera* as a resistance source is reported here for the first time.

Breeding for disease resistance is a very time-consuming process and could take up to 25 to 30 years. The evaluation of progeny's resistance levels and other important characteristics such as yield and quality of vines is necessary, which is typically not achieved until the third year after planting. A way to considerably decrease the length of the breeding process and accelerate the process by up to 10 years is the adaption of the marker-assisted selection (MAS) approach. This method allows the targeted selection of progeny harboring the resistance loci (Eibach & Töpfer, 2015). To breed grapevines with specific features, MAS of either qualitative or quantitative trait could be used. Markers related to disease-resistance genes are currently used in large-scale breeding programs of grapevine. From an economic point of view, the identification of inheritance and the subsequent development of molecular markers linked to resistance genes to *P. viticola* in *V. vinifera* may have a very important impact on the grapevine breeding programs via marker-assisted selection (MAS). This could be due to the reduction of the time needed to obtain resistant varieties characterized by high-quality standards. In the present work, the Vitis18kSNP chip array was used to genotype a panel of *V. vinifera* Georgian accessions (GWAS approach) to identify genomic regions and/or putative markers associated with *P. viticola* resistance in *V. vinifera*.

The first GWA study used the *Vitis* 18kSNP chip array on *Vitis vinifera* self-pollinated Mgaloblishvili and Georgian accessions applying structure analysis for association analysis. Marker-trait modeling was conducted using both single- (GLM, MLM and SUPER) and multi-locus (MLMM and FarmCPU) algorithms, which resulted in the identification of three novel loci associated with downy mildew resistance. For this purpose, a total of 132 chosen grapevine individuals (84 self-pollinated Mgaloblishvili cultivars and 48 Georgian resistant accessions) were selected originating from two different genetic ancestry clusters, which accounted for 12,825 SNP loci. Recent studies have identified up to 28 QTL conferring resistance to downy mildew mapped on grapevine chromosomal genomes. However, only *Rpv1* and *Rpv3* were characterized as nucleotide-binding site leucine-rich repeat and associated stilbenes biosynthesis locus. *Rpv* loci were looked up in the model genotype PN40024, which resulted in the identification of three novel loci of *Rpv29*, *Rpv30* and *Rpv31*. From these three loci, *Rpv29* represented the most phenotypic effect based on *P*-value and allelic effect estimation. The designated *Rpv* loci were co-located with in-genomic regions enriched genes associated with a plant defense mechanism against biotic stress, suggesting both PAMP-triggered immunity and ETI-HR free response. The genome positions of the *Rpv*s surrounding encoded genes were identified, from which the rust resistance kinase Lr10-like (NB-LRR protein) and ethylene-responsive transcription factor 1B were associated with *Rpv*29, *Rpv*30 and *Rpv*31 loci. Furthermore, *Rpv29* located on chromosome 14 was mapped in the

coding region of HEAT repeat-containing 5B protein, which is a required plant immunity protein (such as ILA). The other genes including probable cellulose synthase A catalytic subunit 8 [UDP-forming], acyl-CoA-binding domain-containing protein 3-like, probable carboxylesterase 17 and a plant cadmium resistance 4 protein, and some uncharacterized genes were mapped, which are genes involved in stress signaling and PAM resistance to the fungal pathogen. The *Rpv*30 locus chromosomal region was in close distance to MADS-box protein JOINTLESS-like, ubiquitin carboxyl-terminal hydrolase 21, magnesium-dependent phosphatase 1 and 1-like (MDP-1 and MDP-1-like) with plant defensive role and also inositol transporter1 (INT1) and two uncharacterized genes. The *Rpv31* locus was annotated in the linkage group of several rust resistance kinase Lr10-like genes on chromosome 16, associated with the *P. viticola* resistance trait. Moreover, the gene contained LRR domain encodes ubiquitination and ERT involves in stress signaling in the host cell. Furthermore, these genes were proposed as good candidate genes regarding their expression level in Mgaloblishvili inoculated with *P. viticola*. In the future, the target genes could be functionally analyzed in the grapevine genome using CRISPR/Cas9 technology, which is a powerful, fast and cheap genetic approach. Moreover, the candidate genes could be edited to approve the involvement of the genes in plant-pathogen interaction.

The identification of resistance sources at genetic and biomolecular levels is a must for breeding programs aiming for effective and environmentally friendly protection against *P. viticola*. The study of host cells' response mechanism to the pathogen, was coupled with the discovery of VOCs from leaf tissue inoculated with *P. viticola*. This finding could be a new perspective for sustainable viticulture in the future against downy mildew disease by using the biologically active VOCs during pathogen attack. However, the VOCs accumulation at 3 dpi was an insight for a late response of host plants to the pathogen. Between the six TPs subjected to expression study, four encoding genes related to farnesene, ocimene, nerolidol and valencene represented up-regulation in primary pathogen development. Furthermore, disease severity reduction was observed in the presence of these four VOCs in *in vitro* conditions. This investigation was a preliminary study for VOCs and specially TPs in the resistance response of grapevine (*V. vinifera*) to *P. viticola*. However, further study is needed for functional analysis of interacted biologically active compounds and genes in resistance mechanism to downy mildew with different approaches.

The host mechanism of grapevine against *P. viticola* has been widely studied, whereas less attention has been paid to the pathogenicity mechanism. The pathogen genome assembly and classification are important methods in resistance breeding programs. In a parallel work, the *P. viticola* isolations from different origins and hosts were collected and subjected to RNA-sequencing studies. Also, in this survey, the isolates obtained from JKI (Julius Kühn Institute for Grapevine Breeding Geilweilerhof) were subjected to the identification of genes involved in early pathogen development. This work has re-used the RNA sequencing data of *Rpv*-10 and *Rpv*-3 loci contained genotypes to identify *Rpv*-mediated gene of interest from *P. viticola*. Six novel genes (genes encoding: TAR 1 protein, Cellulose Synthase, Ras-related protein 1, Regulator of G-protein**)** were identified that could be involved in primary signaling regarding expression level and blast function analysis. While the immunity system of the host can recognize the effectors probably through effector specific resistance genes, the expression level of pathogen genes was changed during pathogenicity. Therefore, the *P. viticola* pathogenicity mechanism between different genome assemblies contigs was compared in gene expression level. The results represented the effectiveness of *Rpv* loci on the expression of genes encoding TAR 1 protein Aphanomyces, Ras-related protein Rab5 Phytophthora nicatianae, and Regulator of G-protein signaling Phytophthora infestans T30-4 in primary pathogenicity.

The *P. viticola* involved *Rpv*-mediated (*Rpv*3 and *Rpv*10) genes, could be used as marker for further investigation of the pathogen-host interaction. The reported transcribed genes and encoding genes involved in *P. viticola* pathogenicity can be used in the future as references to elucidate the *P. viticola* pathogenicity mechanism and to choose best fungicides against downy mildew. Furthermore, in sustainable agriculture, the understanding of pathogen effectors' target in host cells could be an effective strategy to improve the resistance of grapevine to this pathogen. The detection of *P. viticola* novel genes could be used in the development of high-quality genome assembly of this pathogen to trace the pathogenicity molecular mechanism more clearly.

In summary, plant protection against pathogen could be achieved through the identification of resistant factors and mechanisms in resistance sources and their comparison with susceptible plants. Three loci related to resistance against *P. viticola* were identified in *V. vinifera* Mgaloblishvili cultivar by the GWA study. Moreover, emitted biomolecules during the *P. viticola* attack were identified and studied in resistance Mgaloblishvili and Bianca cultivars, which were compared with the susceptible Pinot noir cultivar, in both genetic and biomolecular levels. Consequently, this will raise hope in introducing the constantly resistant source of *V. vinifera* for further studies such as marker selection (MAS), resistant genes characterization, genome editing, RNA interfering, and then recombinant plant production, crossbreeding and other related breeding programs. Furthermore, biomolecules related to this resistance mechanism such as VOCs could make the possibility to introduce new sources of effective biomolecules for breeding programs. A couple of these biomolecules and related genes with pathogen effectors would be a step forward for a better understanding of *P. viticola* pathogenicity. This understanding of pathogen effectors could provide a suitable and effective approach to control the pathogen. In conclusion, both host and pathogen were subjected to genetic and biomolecular levels studies during plant-pathogen interaction. Hence, it could shed light on finding effective methods to control one of the most distractive grapevine diseases, downy mildew.

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Supplementary material

Table S.1 List of grapevine accessions phenotyped for their resistance/susceptibility to *P. viticola* infection and genotyped by the Vitis18kSNP genotyping array. Phenotype column reports the resistance (1) or susceptibility (0) to *P. viticola* infection and resistance levels. The accessions showing a percentage of infection lower than 25%, in the three years of sampling (2015, 2016 and 2017), were considered resistant. Phenotypical evaluations were performed in triplicate.

Accession ID	Population	Cultivar name	Phenotype	Resistance level
ID 109	Mgaloblishvili self-pollinated	-	1	70
ID 122	Mgaloblishvili self-pollinated	-	1	71
ID 124	Mgaloblishvili self-pollinated	-	1	82
ID 138	Mgaloblishvili self-pollinated	-	1	73
ID L22A	Mgaloblishvili self-pollinated/Germplasm	Mgaloblishvili	1	81
ID LIB 56	Mgaloblishvili self-pollinated	-	1	76
ID M22A	Germplasm	Zerdagi	1	83
ID M22E	Germplasm	Kamuri shavi	1	84
ID M22F	Germplasm	Jani bakhvis	1	72
ID 1	Mgaloblishvili self-pollinated	-	0	53
ID 100	Mgaloblishvili self-pollinated	-	0	30
ID 101	Mgaloblishvili self-pollinated	-	0	47
ID 102	Mgaloblishvili self-pollinated	-	0	28
ID 106	Mgaloblishvili self-pollinated	-	0	53
ID 11	Mgaloblishvili self-pollinated	-	0	44
ID 113	Mgaloblishvili self-pollinated	-	0	12
ID 114	Mgaloblishvili self-pollinated	-	0	52
ID 115	Mgaloblishvili self-pollinated	-	0	16
ID 116	Mgaloblishvili self-pollinated	-	0	44
ID 121	Mgaloblishvili self-pollinated	-	0	40
ID 125	Mgaloblishvili self-pollinated	-	0	40
ID 128	Mgaloblishvili self-pollinated	-	0	46
ID 135	Mgaloblishvili self-pollinated	-	0	28
ID 137	Mgaloblishvili self-pollinated	-	0	28
ID 14	Mgaloblishvili self-pollinated	-	0	8
ID 141	Mgaloblishvili self-pollinated	-	0	43
ID 153	Mgaloblishvili self-pollinated	-	0	65
ID 153 M	Mgaloblishvili self-pollinated	-	0	20
ID 155	Mgaloblishvili self-pollinated	-	0	36
ID 155 M	Mgaloblishvili self-pollinated	-	0	57
ID 157 M	Mgaloblishvili self-pollinated	-	0	0
ID 16	Mgaloblishvili self-pollinated	-	0	14
ID 176	Mgaloblishvili self-pollinated	-	0	62

ID 18	Mgaloblishvili self-pollinated	-	0	17
ID 181	Mgaloblishvili self-pollinated	-	0	25
ID 182	Mgaloblishvili self-pollinated	-	0	32
ID 185	Mgaloblishvili self-pollinated	-	0	14
ID 186	Mgaloblishvili self-pollinated	-	0	61
ID 188	Mgaloblishvili self-pollinated	-	0	2
ID 193	Mgaloblishvili self-pollinated	-	0	17
ID 196	Mgaloblishvili self-pollinated	-	0	25
ID 198	Mgaloblishvili self-pollinated	-	0	16
ID 199	Mgaloblishvili self-pollinated	-	0	27
ID 2	Mgaloblishvili self-pollinated	-	0	21
ID 207	Mgaloblishvili self-pollinated	-	0	0
ID 21	Mgaloblishvili self-pollinated	-	0	16
ID 210	Mgaloblishvili self-pollinated	-	0	51
ID 40	Mgaloblishvili self-pollinated	-	0	46
ID 42	Mgaloblishvili self-pollinated	-	0	1
ID 43	Mgaloblishvili self-pollinated	-	0	21
ID 45	Mgaloblishvili self-pollinated	-	0	36
ID 46	Mgaloblishvili self-pollinated	-	0	21
ID 47	Mgaloblishvili self-pollinated	-	0	34
ID 55	Mgaloblishvili self-pollinated	-	0	11
ID 57	Mgaloblishvili self-pollinated	-	0	24
ID 58	Mgaloblishvili self-pollinated	-	0	12
ID 61	Mgaloblishvili self-pollinated	-	0	40
ID 65	Mgaloblishvili self-pollinated	-	0	32
ID 67	Mgaloblishvili self-pollinated	-	0	18
ID 69	Mgaloblishvili self-pollinated	-	0	43
ID 74	Mgaloblishvili self-pollinated	-	0	31
ID 78	Mgaloblishvili self-pollinated	-	0	36
ID 80	Mgaloblishvili self-pollinated	-	0	24
ID 81	Mgaloblishvili self-pollinated	-	0	30
ID 82	Mgaloblishvili self-pollinated	-	0	27
ID 87	Mgaloblishvili self-pollinated	-	0	44
ID 89	Mgaloblishvili self-pollinated	-	0	39
ID 94	Mgaloblishvili self-pollinated	-	0	4
ID 95	Mgaloblishvili self-pollinated	-	0	31
ID 97	Mgaloblishvili self-pollinated	-	0	46
ID 99	Mgaloblishvili self-pollinated	-	0	41
ID L21E	Germplasm	Saperavi grdzelmtevana	0	29
ID L21F	Germplasm	Zakatalis tsiteli	0	24
ID L22B	Germplasm	Marguli sapere	0	49
ID L22C	Germplasm	Gabekhouri tsiteli	0	58

ID L23A	Germplasm	Khushia shavi	0	57
ID L23B	Germplasm	Orona	0	26
ID L23D	Germplasm	Okhtoura	0	57
ID L24C	Germplasm	Zakatalis tetri	0	49
ID LIB 13	Mgaloblishvili self-pollinated	-	0	13
ID LIB 145	Mgaloblishvili self-pollinated	-	0	8
ID LIB 150	Mgaloblishvili self-pollinated	-	0	41
ID LIB 16	Mgaloblishvili self-pollinated	-	0	25
ID LIB 25	Mgaloblishvili self-pollinated	-	0	2
ID LIB 30	Mgaloblishvili self-pollinated	-	0	37
ID LIB 48	Mgaloblishvili self-pollinated	-	0	20
ID LIB 49	Mgaloblishvili self-pollinated	-	0	56
ID LIB 55	Mgaloblishvili self-pollinated	-	0	42
ID LIB 60	Mgaloblishvili self-pollinated	-	0	60
ID LIB 63	Mgaloblishvili self-pollinated	-	0	54
ID LIB 66	Mgaloblishvili self-pollinated	-	0	44
ID LIB 70	Mgaloblishvili self-pollinated	-	0	22
ID LIB 72	Mgaloblishvili self-pollinated	-	0	25
ID LIB 78	Mgaloblishvili self-pollinated	-	0	34
ID LIB 91	Mgaloblishvili self-pollinated	-	0	28
ID M21A	Germplasm	Ghvinis tsiteli	0	56
ID M21B	Germplasm	Kharistvala shavi	0	48
ID M21C	Germplasm	Tqupkvirta	0	49
ID M21F	Germplasm	Goruli mtsvane	0	63
ID M22B	Germplasm	Paneshi	0	61
ID M23A	Germplasm	Tkbili kurdzeni	0	54
ID M24A	Germplasm	Asuretuli shavi	0	52
ID M24C	Germplasm	Argvetula	0	50
ID M24E	Germplasm	Ananura	0	31
ID M24F	Germplasm	Tchvitiluri	0	70
ID N21A	Germplasm	Gorula	0	43
ID N21B	Germplasm	Tita kartlis	0	21
ID N21D	Germplasm	Shavkapito	0	51
ID N22A	Germplasm	Otskhanuri sapere	0	38
ID N22B	Germplasm	Orbeluri ojaleshi	0	41
ID N22D	Germplasm	Rkatsiteli	0	44
ID N23B	Germplasm	Bazaleturi	0	62
ID N23D	Germplasm	Vertkvitchalis tetri	0	63
ID N23F	Germplasm	Adanasuri	0	50
ID N24A	Germplasm	Ojaleshi	0	68
ID N24B	Germplasm	Aladasturi	0	57

ID N24C	Germplasm	Tchumuta	0	58
ID N24F	Germplasm	Adjaruli tetri	0	76
ID O21A	Germplasm	Tamaris vazi	0	24
ID O21B	Germplasm	Saperavi atenis	0	63
ID O21C	Germplasm	Tkvlapa shavi	0	48
ID O21D	Germplasm	Tavkveri	0	60
ID O22A	Germplasm	Sapena	0	53
ID O22C	Germplasm	Rkatsiteli vardisperi	0	38
ID O22D	Germplasm	Tskobila	0	31
ID O22E	Germplasm	Danakharuli	0	42
ID O23A	Germplasm	Maghlari tvrina	0	47
ID O23B	Germplasm	Rko shavi	0	52
ID O23C	Germplasm	Dziganidzis Shavi	0	60
ID O23D	Germplasm	Didshavi	0	58
ID O23E	Germplasm	Kvelouri	0	40
ID O23F	Germplasm	Samarkhi	0	50

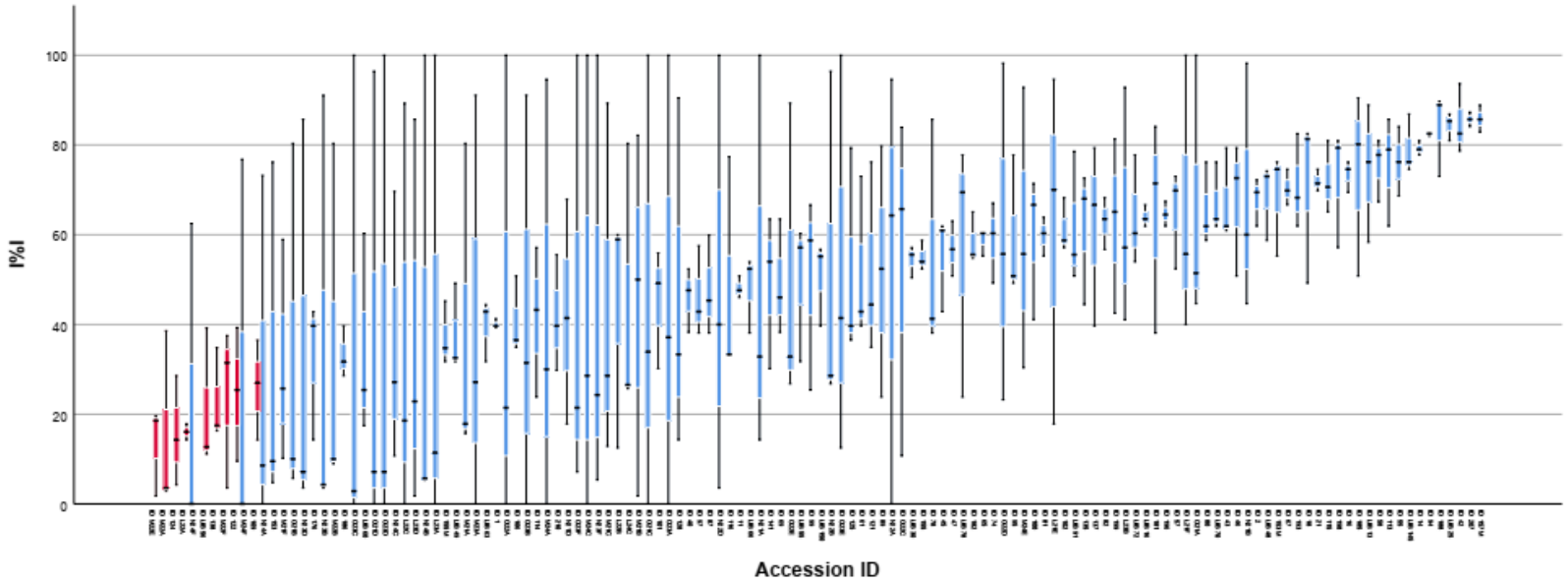

Figure S.1 Box-plot distribution of the I%I recorded by each grapevine accessions, belonging to a Mgaloblishvili self-pollinated (84) and Georgian germplasm population (48), following *P. viticola* inoculation. Resistant accessions are highlighted in red.

Table S.2 Ancestry values at K = 3 detected for SNP profiles of 132 grapevine accessions, belonging to a Mgaloblishvili self-pollinated (84) and Georgian germplasm population (48), genotyped at 18k loci.

Accession ID	G1	G2	G3
ID 40	0.997	0.000	0.003
ID 181	0.986	0.014	0.000
ID LIB 66	0.999	0.000	0.001
ID 99	0.975	0.000	0.025
ID 65	1.000	0.000	0.000
ID 157 M	0.989	0.000	0.011
ID 124	0.982	0.000	0.018
ID 95	0.988	0.000	0.012
ID LIB 70	0.972	0.014	0.014
ID 42	1.000	0.000	0.000
ID 182	0.968	0.000	0.032
ID 121	0.986	0.010	0.004
ID L22A	1.000	0.000	0.000
ID LIB 16	0.995	0.005	0.000
ID 176	0.986	0.001	0.014
ID 43	0.974	0.022	0.004
ID 97	0.960	0.000	0.040
ID LIB 13	0.953	0.000	0.047
ID 69	1.000	0.000	0.000
ID 122	0.997	0.000	0.003
ID 21	0.986	0.014	0.000
ID 153	0.932	0.008	0.060
ID LIB 56	0.990	0.010	0.000
ID 94	0.974	0.025	0.002
ID 61	0.930	0.000	0.070
ID 207	0.994	0.000	0.006
ID 116	0.992	0.000	0.008
ID 87	0.985	0.000	0.015
ID LIB 60	0.987	0.000	0.013
ID 155	0.986	0.002	0.012
ID 114	0.990	0.000	0.010
ID 153 M	0.944	0.056	0.000
ID 210	0.992	0.007	0.001
ID 89	1.000	0.000	0.000
ID LIB 63	0.967	0.002	0.030
ID 199	0.975	0.015	0.010
ID 67	0.971	0.000	0.029
ID 115	1.000	0.000	0.000
ID 155 M	0.988	0.012	0.000

ID 14	0.941	0.015	0.044
ID 138	0.970	0.000	0.030
ID LIB 48	0.969	0.000	0.031
ID 55	0.996	0.004	0.000
ID 196	0.976	0.006	0.018
ID LIB 145	0.941	0.008	0.051
ID 113	0.983	0.017	0.000
ID 81	0.995	0.004	0.000
ID LIB 49	0.948	0.031	0.020
ID 16	1.000	0.000	0.000
ID 141	0.964	0.036	0.000
ID 106	0.968	0.000	0.032
ID LIB 150	1.000	0.000	0.000
ID 57	0.989	0.000	0.011
ID 198	1.000	0.000	0.000
ID 137	0.977	0.017	0.006
ID 18	1.000	0.000	0.000
ID 82	1.000	0.000	0.000
ID LIB 55	0.974	0.017	0.010
ID 193	1.000	0.000	0.000
ID 58	0.981	0.000	0.019
ID 109	0.954	0.000	0.046
ID 1	0.979	0.021	0.000
ID 128	0.997	0.003	0.000
ID LIB 25	0.973	0.000	0.027
ID 80	0.970	0.026	0.004
ID 45	0.956	0.004	0.040
ID 186	0.990	0.010	0.000
ID LIB 72	1.000	0.000	0.000
ID 102	0.974	0.000	0.026
ID 74	0.960	0.010	0.030
ID LIB 30	0.996	0.003	0.000
ID 2	1.000	0.000	0.000
ID 135	0.996	0.000	0.004
ID 100	0.986	0.009	0.005
ID LIB 78	0.982	0.000	0.018
ID 46	1.000	0.000	0.000
ID 188	0.979	0.021	0.000
ID 125	0.990	0.009	0.000
ID 11	1.000	0.000	0.000
ID 78	0.985	0.000	0.014
ID 185	0.975	0.025	0.000
ID 47	1.000	0.000	0.000

ID 101	0.963	0.035	0.003
ID LIB 91	0.989	0.011	0.000
ID N22B	0.000	1.000	0.000
ID O21C	0.013	0.987	0.000
ID M21F	0.030	0.703	0.268
ID N23D	0.171	0.186	0.643
ID N24B	0.000	0.000	1.000
ID N24A	0.000	0.000	1.000
ID O21A	0.068	0.405	0.527
ID L22C	0.139	0.106	0.755
ID N22D	0.000	0.679	0.321
ID O23A	0.040	0.072	0.889
ID L23D	0.025	0.517	0.457
ID O23F	0.015	0.002	0.983
ID O23C	0.040	0.071	0.889
ID M24A	0.037	0.389	0.574
ID L23B	0.147	0.022	0.831
ID M24F	0.134	0.130	0.737
ID L21F	0.013	0.611	0.376
ID L23A	0.103	0.100	0.796
ID O22A	0.000	0.621	0.379
ID N24C	0.203	0.070	0.727
ID N24F	0.068	0.029	0.903
ID M21C	0.000	0.628	0.372
ID M24C	0.105	0.091	0.804
ID N21D	0.014	0.932	0.054
ID O23B	0.089	0.342	0.569
ID N23B	0.117	0.205	0.678
ID O21B	0.000	1.000	0.000
ID L21E	0.069	0.433	0.498
ID O21D	0.065	0.557	0.378
ID M21A	0.032	0.892	0.076
ID O22D	0.000	1.000	0.000
ID N21A	0.013	0.582	0.405
ID M22F	0.078	0.054	0.868
ID N21B	0.089	0.360	0.551
ID M24E	0.007	0.471	0.521
ID L22B	0.032	0.422	0.547
ID M21B	0.001	0.501	0.498
ID O22E	0.000	0.510	0.490
ID O23D	0.024	0.562	0.414
ID M22A	0.026	0.974	0.000
ID N22A	0.000	1.000	0.000

ID M23A	0.007	0.886	0.108
ID M22E	0.104	0.093	0.803
ID L24C	0.009	0.537	0.455
ID M22B	0.000	1.000	0.000
ID O22C	0.000	0.679	0.321
ID N23F	0.037	0.078	0.885
ID O23E	0.084	0.482	0.433

Table S.3 List of candidate genes in a window of 100kb upstream and downstream the three SNPs associated to resistance trait to *P. viticola* infection.

chr.	Position from .. To (bp)	Locus Tag	Gene description	a gene symbol
16	21289025..21291597	VIT_216s0050g02750	rust resistance kinase Lr10-like	LOC100854184
	21291598..21292606	VIT_216s0050g02760	rust resistance kinase Lr10-like	LOC100243072
	21296417..21297482	VIT_216s0050g02770	leaf rust 10 disease-resistance locus receptor-like protein kinase like 2.3	LOC100854673
	21297543..21298094	VIT_216s0050g02780	uncharacterized	
	21309145..21309516	VIT_216s0050g02800	uncharacterized	
	21312366..21319643	VIT_216s0050g02810	uncharacterized	
	21379089..21381377	VIT_216s0148g00010	rust resistance kinase Lr10-like	LOC100251517
	21381945..21385100	VIT_216s0148g00020	leaf rust 10 disease-resistance locus receptor-like protein kinase like 2.5	LOC100256646
	21421886..21425581	VIT_216s0148g00030	rust resistance kinase Lr10-like	LOC100242248
	21498263..21501414	VIT_216s0148g00040	rust resistance kinase Lr10-like	LOC100251011
14	21533965..21534998	VIT_214s0006g03076	uncharacterized	
	21553355..21554075	VIT_214s0006g03080	uncharacterized	
	21558910..21560892	VIT_214s0006g03090	probable cellulose synthase A catalytic subunit 8 [UDP-forming]	LOC117918711
	21607164..21608222	VIT_214s0006g03100	uncharacterized	
	21611382..21612188	VIT_214s0006g03110	acyl-CoA-binding domain-containing protein 3-like	LOC117907480
	21612866..21742251	VIT_214s0006g03120	uncharacterized	
	21742614..21743660	VIT_214s0006g03180	probable carboxylesterase 17	LOC117931162
	21746749..21749225	VIT_214s0006g03190	protein plant cadmium resistance 4	LOC100246421
3	16125132..16127240	VIT_203s0017g00380	magnesium-dependent phosphatase 1-like	LOC117910398
	16137049..16137935	VIT_203s0017g00390	MADS-box protein JOINTLESS-like	LOC117911100

16166870..16167220	VIT_203s0017g00396	ubiquitin carboxyl-terminal hydrolase 21	LOC117926659
16190988..16192049	VIT_203s0017g00410	magnesium-dependent phosphatase 1	LOC100264015
16192103..16206554	VIT_203s0017g00420	uncharacterized	
16235590..16246518	VIT_203s0017g00440	uncharacterized	
16246761..16247367	VIT_203s0017g00450	MADS-box protein JOINTLESS-like	LOC117911336
16314218..16334275	VIT_203s0017g00460	inositol transporter 1	LOC100268023

Table S.4 Allele information on SNP loci detected in a region spanning 100kb upstream and downstream the three loci associated (highlighted in grey) to the *P. viticola* resistance. In red: resistant genotypes.

Accession ID	Resistant level	Chromosome 3			Chromosome 14							Chromosome 16										
		lb_G_T_chr3_16133045	ae_T_C_chr3_16164211	cn_C_T_chr3_16229046	chr14_21534631_C_T	chr14_21540043_C_T	chr14_21613512_C_T	chr14_21632108_C_T	chr14_21633763_C_T	chr14_21666228_G_T	ae_C_T_chr14_21693204	chr16_21305250_A_G	chr16_21324783_A_G	chr16_21348749_G_T	chr16_21374358_A_G	chr16_21396785_A_G	li_T_C_chr16_21398409	chr16_21405690_A_C	chr16_21452310_A_G	chr16_21473117_A_G	chr16_21496611_A_G	chr16_21499064_A_G
ID 40	46	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 181	25	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 66	44	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 99	41	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB

ID 65	32	BB	AA	BB	BB	BB	BB	NC	AB	NC	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 157 M	0	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 124	82	BB	AA	BB	BB	BB	AB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AB	AA	AA	BB	AA	BB
ID 95	31	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID LIB 70	22	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 42	1	BB	AA	AB	BB	BB	BB	BB	BB	AA	BB	NC	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 182	32	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 121	40	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID L22A	81	BB	AA	AB	BB	BB	AB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AB	AA	AA	BB	AA	AB
ID LIB 16	25	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 176	62	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 43	21	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 97	46	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 13	13	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 69	43	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 122	71	BB	AA	AB	BB	BB	AB	AA	AA	BB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 21	16	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 153	65	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID LIB 56	76	BB	AA	BB	BB	BB	AB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AB	AA	AA	BB	AA	AA
ID 94	4	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 61	40	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 207	0	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AB	AA	AA	BB	AA	AB
ID 116	44	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 87	44	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 60	60	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 155	36	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 114	52	BB	AA	AB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 153 M	20	BB	AA	BB	BB	BB	BB	NC	NC	AB	BB	AB	BB	BB	AA	BB	AA	AA	NC	BB	AA	AB
ID 210	51	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 89	39	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AB	AA	AA	BB	AA	AB
ID LIB 63	54	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB

ID 199	27	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 67	18	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 115	16	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 155 M	57	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 14	8	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 138	73	BB	AA	AB	BB	BB	AB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 48	20	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 55	11	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 196	25	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AB	AA	AA	BB	AA	AB
ID LIB 145	8	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 113	12	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 81	30	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID LIB 49	56	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 16	14	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 141	43	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 106	53	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AB	AA	AA	BB	AA	BB
ID LIB 150	41	BB	AA	AB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 57	24	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 198	16	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 137	28	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 18	17	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 82	27	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 55	42	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 193	17	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 58	12	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 109	70	BB	AA	AB	BB	BB	AB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AB	AA	AA	BB	AA	AA
ID 1	53	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 128	46	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 25	2	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 80	24	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 45	36	BB	AA	BB	BB	BB	AB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AB	AA	AA	BB	AA	AA

ID 186	61	BB	AA	AB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID LIB 72	25	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 102	28	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AA
ID 74	31	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID LIB 30	37	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 2	21	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 135	28	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	BB	BB	BB	AA	BB	AB	AA	AA	BB	AA	BB
ID 100	30	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 78	34	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 46	21	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 188	2	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 125	40	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 11	44	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	BB	BB	BB	AA	BB	AA	AA	AA	BB	AA	BB
ID 78	36	BB	AA	BB	BB	BB	AB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 185	14	BB	AA	AB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 47	34	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID 101	47	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID LIB 91	28	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID N22B	41	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID O21C	48	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID M21F	63	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AB	BB	AA	AA	AA	AB	AA	BB
ID N23D	63	BB	AA	BB	AB	AB	BB	BB	BB	AA	BB	AB	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB
ID N24B	57	BB	AA	BB	BB	BB	AB	AB	AB	AB	BB	AA	BB	AB	AA	BB	AA	AA	AA	BB	AA	AB
ID N24A	68	BB	AA	AB	BB	BB	BB	AB	AB	AB	BB	AA	BB	AB	AA	BB	AB	AA	AA	BB	AA	AB
ID O21A	24	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	AB	AA	BB	AA	AB	AB	AB	AA	AB
ID L22C	58	BB	AA	BB	BB	NC	BB	BB	BB	AA	BB	AA	AB	AB	AB	BB	AA	AA	AA	BB	AA	BB
ID N22D	44	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	BB	BB	AA	AA	AA	BB	AA	BB
ID O23A	47	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AB	BB	AA	AA	AA	BB	AB	AA
ID L23D	57	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AA	BB	AA	AB	AB	AB	AA	AB
ID O23F	50	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AB	BB	AB	AB	BB	AA	AA	AB	BB	AA	BB
ID O23C	60	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AB	BB	AA	AA	AA	BB	AB	AA

ID M24A	52	BB	AA	BB	BB	NC	BB	AB	AB	AB	BB	AA	BB	AA	AB	BB	AA	AB	AB	AB	AB	AA
ID L23B	26	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	AA	AA	AB	AB	AA	AB	AB	BB	AA	AB
ID M24F	70	BB	AA	AB	AB	AB	BB	BB	BB	AA	BB	AA	AB	AA	AA	AB	AA	BB	BB	AB	AA	AA
ID L21F	24	BB	AA	BB	BB	NC	AB	NC	AB	AB	BB	AA	BB	AB	BB	BB	AA	AA	AA	BB	AB	AA
ID L23A	57	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AB	BB	AA	AA	AA	BB	AA	AB
ID O22A	53	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AB	BB	AB	AA	AA	BB	AA	BB
ID N24C	58	BB	AA	BB	BB	NC	BB	BB	BB	AA	BB	AA	AB	AA	AA	AB	AA	AB	AA	BB	AA	BB
ID N24F	76	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AA	BB	AA	AB	AB	AB	AA	AA
ID M21C	49	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	AB	BB	BB	AA	AA	AA	BB	AB	AA
ID M24C	50	BB	AA	BB	BB	NC	BB	BB	BB	AA	BB	AA	AB	AB	AB	BB	AA	AA	AA	BB	AA	AB
ID N21D	51	BB	AA	BB	BB	BB	AB	AB	BB	AA	BB	AB	BB	AB	AB	BB	AA	AA	AA	BB	AA	BB
ID O23B	52	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	AA	AB	AA	AB	BB	AA	AB	AB	AB	AA	AB
ID N23B	62	BB	AA	BB	AB	AB	BB	BB	BB	AA	BB	AA	BB	AB	AA	BB	AA	AB	AB	AB	AA	AA
ID O21B	63	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID L21E	29	BB	AA	BB	BB	BB	BB	AA	AB	AB	BB	AB	BB	AA	AB	BB	AA	AB	AB	AB	AA	AB
ID O21D	60	BB	AA	BB	BB	NC	BB	AB	AB	AB	BB	NC	BB	AA	AB	BB	AA	AB	AB	AB	AB	AA
ID M21A	56	BB	AA	BB	BB	NC	BB	BB	BB	AA	BB	AB	BB	NC	AB	BB	AA	AA	AA	BB	AA	BB
ID O22D	31	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID N21A	43	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AB	BB	AA	AA	AA	BB	AA	AB
ID M22F	72	BB	AA	BB	BB	BB	AB	AB	BB	AA	BB	AA	AB	AA	AA	NC	AB	AB	AB	BB	AA	AB
ID N21B	21	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AA	BB	AA	AA	BB	AA	BB	BB	AA	AA	AA
ID M24E	31	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	AB	AB	BB	AA	AB	AB	AB	AA	AB
ID L22B	49	BB	AA	BB	BB	BB	BB	AA	AA	BB	BB	AA	BB	AA	BB	BB	AA	AA	AA	BB	AB	AB
ID M21B	48	BB	AA	BB	BB	BB	AB	AB	AB	AB	BB	AA	BB	AB	AB	BB	AA	AB	AB	AB	AA	AB
ID O22E	42	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	AB	AB	BB	AA	AB	AB	AB	AA	AB
ID O23D	58	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID M22A	83	BB	AA	AB	BB	BB	AB	BB	BB	AA	BB	BB	BB	BB	BB	BB	AB	AA	AA	BB	AA	BB
ID N22A	38	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID M23A	54	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	AB	BB	AA	AA	AA	BB	AA	BB
ID M22E	84	BB	AA	AB	BB	BB	BB	AB	BB	AA	BB	AA	BB	AA	AA	BB	AB	AB	AB	AB	AA	AB
ID L24C	49	BB	AA	BB	BB	BB	BB	AB	AB	AB	BB	AA	BB	BB	AB	BB	AA	AA	AA	BB	AA	BB

ID M22B	61	BB	AA	BB	BB	BB	BB	AB	BB	AA	BB	BB	BB	AB	BB	BB	AA	AA	AA	BB	AA	BB
ID O22C	38	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	BB	BB	BB	AA	AA	AA	BB	AA	BB
ID N23F	50	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	AB	AB	BB	AA	AA	AA	BB	AB	AA
ID O23E	40	BB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	BB	BB	AA	BB	AA	AA	AA	BB	AA	AB

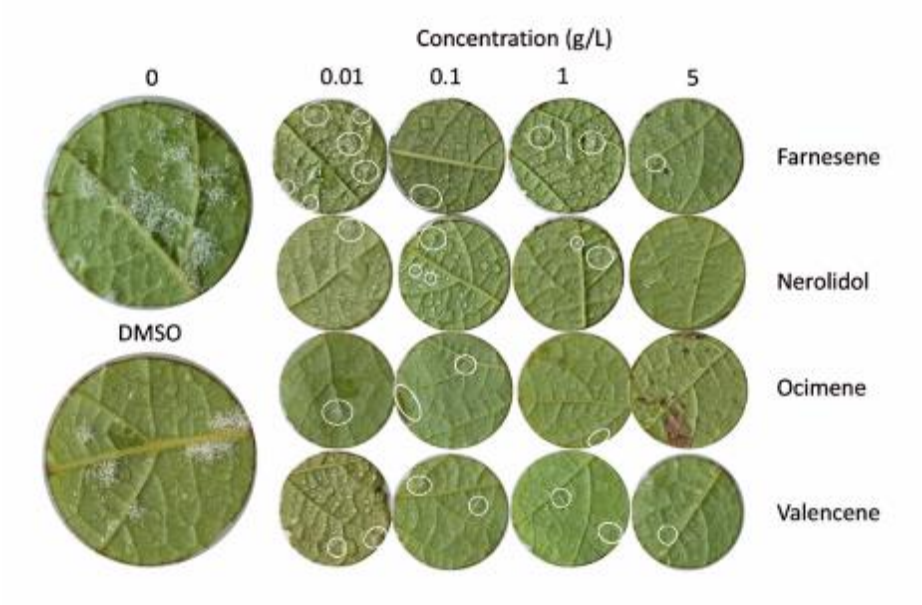

Figure S.2 Pictures of the leaf disks inoculated with *P. viticola* and covered by white sporulation at 7 days post inoculation. White circles indicate the presence of sporulation on the leaf disks treated with increasing concentrations of farnesene, nerolidol, ocimene and valencene. Leaf disks untreated (0) and treated with DMSO (0.2 %) were considered as controls.

Table S. 5 All three sets of primers for each gen are represented here.

Primer name	Expected band size	GC%	Tm	Sequence	Quantity
R2.1	442	60.04	45	F: TTGGCTGCCCAGATATTTTC	22,69
		60.05	50	R: CACGGCAAAGTGAGTAAGCA	
R2.2	452	60.04	45	F: TTGGCTGCCCAGATATTTTC	35,54
		60.07	45	R: TTTCCCTATTCACGGCAAAG	
R2.3	428	60.06	45	F: AACGCTTCGAAACTGCAACT	12,26
		59.72	50	R: TCCGTAATGTCGTTGTGAGC	
R11A.1	220	59.88	45	F: CAGCAAGAACGTTGTGGAAA	12,72
		60.05	50	R: CAACCCAATACCGTGAATCC	
R11A.2	232	59.88	45	F: CAGCAAGAACGTTGTGGAAA	1,32
		59.90	50	R: CGTATAACTTGCACGCCTGA	
R11A.3	212	60.19	50	F: CGTAAGTGACGCAGCAAGAA	21,45
		60.05	50	R: CAACCCAATACCGTGAATCC	
R11B.1	343	60.01	45	F: CCAACGAACGTGACCTTTTT	16,13
		59.99	55	R: GTTCGGAGACTGCTTCTTGG	
R11B.2	372	59.99	55	F: CAAGAAGCAGTCTCCGAACC	30,49
		60.02	55	R: ACGCATCCGTAAGTCGTACC	
R11B.3	303	60.07	55	F: CTGTCCGTCAGGGATTAGGA	16,66
		59.99	55	R: GTTCGGAGACTGCTTCTTGG	
R20.1	389	60.06	50	F: TACTGCGCTTTGTCACGAAC	12,04
		59.85	45	R: TCAAAGATTGCTGGCAACAC	
R20.2	369	59.85	55	F: GTGTTGCCAGCAATCTTTGA	20,8
		59.90	50	R: TACGCCACGTTCTTGTCTTG	
R20.3	360	59.85	45	F: GCAATCTTTGACGACGTTGA	4,48

		59.90	50	R: TACGCCACGTTCTTGTCTTG	
R22.1	385	60.08	50	F: AGTTGCCATCCGATCTGTTC	7,16
		60.23	40	R: TTTTTCAATGTCGTGGAGCA	
R22.2	312	60.26	50	F: ACGAGTTCCTTGACGCAATC	8,51
		59.84	55	R: CTTCGGCGTAGTGGAGAATC	
R22.3	301	60.13	50	F: TCGCTCTTGAACCGACTTCT	4,72
		59.58	50	R: ACTGGGGATCTTGTGCACTT	
R18.1	467	59.97	45	F: CACCTTTTTCAGGCAACCAT	11,73
		59.99	45	R: CGCTTCGTCATGACTTCAAA	
R18.2	465	59.97	45	F: CCTTTTTCAGGCAACCATGT	8,46
		59.99	45	R: CGCTTCGTCATGACTTCAAA	
R18.3	422	59.97	50	F: GCTTCCACAATCCACCACTT	20,96
		60.02	50	R: ATCGCATACACGGTCGTACA	
R16.1	419	60.02	55	F: CGGTACGCAAGACCCATACT	9,63
		59.99	50	R: GATCGTGTCGTCTGCTTCAA	
R16.2	410	59.95	50	F: GCACTGCTCGTGTGTTTTGT	7,2
		59.99	50	R: TGTACCACGGCTGTAATGGA	
R16.3	412	59.95	50	F: TCTTCATCGCGACATCAGTC	
		59.93	50	R: GCCCCAGACAAATAGGTTGA	
UBC_Pv_1	475	59.71	55	F: GCCGAAGCCTATAGAGCAGA	5,33
		60.32	55	R: AAGTGGCGTACTTGGCAGTC	
UBC_Pv_2	470	59.71	55	F: GCCGAAGCCTATAGAGCAGA	16,26
		60.67	55	R: GCGTACTTGGCAGTCCATTC	
UBC_Pv_3	485	59.71	55	F: GCCGAAGCCTATAGAGCAGA	7,4
		61.12	55	R: AGCGTGCTCTAAGTGGCGTA	
WS21_Pv_1	107	59.91	50	F: AAGTTCGACCTTGTGCGTCT	17,46
		59.98	60	R: GAGCCAGCCATCGACTCTAC	
WS21_Pv_2	297	59.87	50	F: ACGGCTCAGATTCGTGCTAT	21,25
		59.98	60	R: GAGCCAGCCATCGACTCTAC	
WS21_Pv_3	113	59.87	50	F: ACGGCTCAGATTCGTGCTAT	15,53
		59.93	45	R: CCTTGCCAATGATTTCTGGT	

Table S. 6 The table represented the effector proteins full sequences and effector-typic RxLR and CRN motifs per *P. viticola*/ grapevine host interaction genotype. Each of a) Susceptible (*Rpv*-/*Rpv*-), b) Resistance (*Rpv*3/*Rpv*10) and c) Resistance (*Rpv*10/*Rpv*10) represents effectors with identified motifs.

a)

Name	Description	Size	Motif	Sequence
g10745	Effector probability: 0.697	199	RXLR	MTRKACTAWEPSLKFPRTTMIMGQLRTFSSK YDVTISLSARKNNSLSFRVRGSLTKRSLRLVS GCNGLQKFXSREFWFVIHARWMKRWSLFVG KEGQEPGPITNCELLMPGFIAGVNPNQAVTV RSDIEVMKDFRFVTPMVWSLLAALHGPGDA PPIARFSLDINSDAPEDVNEVLSEAKGQAKGL ATSLREKCKVVVR
g10789	Effector probability: 0.755	143	RXLR	MVTSGPQSDRLAFGEYIQKNMKLYELRNGV TLNGPATANYVRNELARFLRRAPYQVNLLIG AVDATPEQEAEPSLHWIDYLGAMVKVNFGA HGYGAHFCLSIFDREWKPNMTLDEAKDVFK KCRAELDMRFLVRNGQWAYTVR

g10873	Effector probability: 0.568	182	RXLR	MQPPKTAAALTPLTLNEQVAAGLKRITEELE ESSDHRKRVLLDHLAATPTEQILDPAIICGRG RPAGALNRRNRNNRNNRNAYSTQRDLPAFE HVKNAFGENPTGGRRCGRCREQGHNSRSSST VIADIADDAAAATAANASRVARIQRMARAA WLGVDVQIPQRSHKLDSKVASRSLRAFE
g11523	Effector probability: 0.604	211	RXLR	MNGSSSLRMTIETVMKNTSAAVKTMRLESD GRRLSRYMLLDLQGRDVGDTVTDRYAHAL DFTVEGNFLRAQHGNSTLCFWDLRQRAGTR VTSSNLLRMLRWQTHSVSIGHDVSGLALTTN DFAVANSTQGLTLVLTGNGDLNLVPFPCSPP PHAASWLTRRPSVCVWDFALRGDIVVTGSQ FDPALCQWHVDKELADVRASTRLPIRSAPS
g11674	Effector probability: 0.702	217	RXLR	MVRKGRNKENVAGERYDSNAIQMPQSTSSR TRPYPSQFNPADLMGMMGGGGGGMPDMSQ MMEMMQSMGGMPSMGGMPGMPGMPDMS AEGTPTSKFMTFYPNYIDKEKTLHQGRRIPKE KACETPIADEMSEYPRDWMVPGRIRVRLLRD DGTPENREFPSRKTLMIKMAELIPQLQSRKVR LENEAEEARKRLAAAAEAAAPASGGGKKKS KKKGRR
g11830	Effector probability: 0.575	157	RXLR	MCATLTKKFMDAMKDYQKAQQKYKSDMK TKVKRQVQIVKPDASEAEIDAVMRSGDPGSI YKSAILQGGAADSITDVFFHCQDKYQDVLKA AGRTAGSDRQSSAYSRQLRRTRQSGGAEGH QVSEVLSKENVLPAWHRRLYPCGDRSHCAG AQEGVVK
g11900	Effector probability: 0.911	204	RXLR	MSCCKTPHFPFIRRVLRSRFFHSTTKVAMTD NQVDNFCSASQNVATFAAGCFWGVQLAFDR LPGVLETSVGYTQGDVDSPTYRQVCTGRTN HAEAIRIVYDESKTSYESLLRKFWAIHDPTTL NRQKNDKGTQYRSGIYYNNEGQRKMALAS KEEHQKKLSKPIVTEILEAKQFWDAEDYHQK YLEKGGQCADKGCDTPIRCYG
g12648	Effector probability: 0.74	181	RXLR	MDTEPSQATHLRGLLDKIVHTEPARRTPVRV LAASWEDGDVGVARKLRPIAEKIVDGETVV MFTDRQAAGEMESAVVNFDVRSSPNCVVSV CGEMSKWYLGAKEKCFDLRIAPKSADQKEN GLIRGVSAAYSGLRPVATNLYLKFDETNFID GSKCEYEIISGSKTVRKEEITSDSQAVTA
g13193	Effector probability: 0.622	169	RXLR	MVKPVVNANMNKTFREVVGRYVGVPPQLS HKQKVQRLYKKSLKTLESWVIDRRLWNDEA TKIRAEFDANGKLDPQSGYVPYRYLYQYRLL REAQDKVDHSTHPDRYIFNYMPGGSLFMRN APIPLDVCFPDGVIPDDVEVSPLEGINIDMTPL PAKETVFVDFSKKGYD
g13720	Effector probability: 0.852	230	RXLR	MGKKGRQGVAKGKPASVGGSKKAARMRGI QQVVPVQKVGKGTINSKAKTLSPAGRLAEM RRKIDGGKFRMLNEQLYTTTGSDAFSTFQQD PKLFDVYHQGFREMANKWPTNPLDTFIDYIE SHPKAVVADFGCGDARLAESVSNKVHSFDL VSRKPAVTACNIADVPLEENSIDIAVYCLAL MGTNVREYVREGYRVLRPGGVLKVAEVKSR FETEALGGIGGTNATRCL
g1861	Effector probability: 0.644	196	RXLR	MHTGHPSLGQFLFLSVNVYLVMYVHLPPLV GDRTGLLVSTAVHVDDPTCVKSYDLIPSNPS LFCVKTAGWLLELAYQAYFDPPGCPSLSGYG ELSLEQHGFELITHLRNNRTDTHVVVAWSQA DHRRLVISFRGTTSKENWKSNLRADQTVLW MKSRGLRWRKSCLEKAKDVAAKIPLLNMAL PRVHRGDCKIAIF
g2535	Effector probability: 0.857	178	RXLR	MSDACFLPMGALSRINSDHSRINGHVSLSAH KNHGMTTLTDPGIIHSLGQLNETEIEDESATIE MEGGQLLHSKEMLASRKRSLRRRYCDICGIT QDSSTDHCEDCGVCVAGYDHHCPWMGKCI GRDNMHAFKMFNMAWIMYVCFVLVIAITH MDWGQTAVNTLKHSASGNWGTSRIP

g3846	Effector probability: 0.746	193	RXLR	MFCLTIMVDAFVLFEFIKFRRSGTSKETAPAL AETPRSTYAAFNGERPHMQSLLQETLEIHKM DDNSAKLLPAVGVIVSGPSALKMATNEAVY ALGIVTKHPFFVREYKWDSKVATSRQNFDAL GQLHCKYIWTLVYKTKVPGGTDLILGVDGPP LCRTNLLSLPSLVTLHVTLRSLRRSDGPVLNR SSFLLV
g3974	Effector probability: 0.872	142	RXLR	MERVDVWNPANKRKETAVVKLVVENGAAD RVGVTIGSTVVAINRKSVDSEPYLAILNLIKA APRPLRMRFERGSQNMDTTQGNILTRISIRID YFADGTFSVGNLTSGNATWTTKYFAFGGSK MDVLQLFVSRAAYHEVGRI
g4111	Effector probability: 0.596	279	RXLR	MSARRLREAINNPPRGTLHKAAENEHDYLR QCVDPILMPLIESLLLYQPESVYHFIHDFVDD NKNTRFVHHSRSVGYAKKLTTRRKMADFMS TSVIPVMDELAKQXXXXXXXXCQIFYSRRG VCDRLSATLFGVNDRVWCRYKGRPRFYPAV ITNIDDNGNFTVLYDDGKLESNVHSMCLKQY VNDTVNRNDVDNQKRPKATCSKDTVYLIIG VDGAGKTTLLSTLQGDLDKEHVPSAGFTSVT FQTDKGSATFFDLGGGPAFRNVWKEYYADV KTLAF
g586	Effector probability: 0.638	198	HVLxx P	MEYGRRSTGVSATNLHVLDRPLSRGKSDVSL SAFSFLFSEMVQYFQGRVQNISDLENRLDDA GFGVGVRIVELLCHREKFGRRETRLLAMLQF IVSTCWKALFGKAADALERSTENEDEYMIHE LEPLTNKFVSVPSDLGQLDCAAYIAGIVRGIL CSSGFVAAVTAHTVEVPGGLRDKTVFLVKF DESVIRRERVQT
g7813	Effector probability: 0.634	240	RXLR	MTLRVSNAXKRFINDKTAGIFVEPIQGEGGV YPADPAFMRDLRALCDKHDALLITDEVQCG LGRTGKLFAHELYDVTPDVMTLAKPLAGGL PIGAILLSNKVASAIAPDGPHENHGRRLFGQG AHTRRVLGHRVEKVAAQVSKANCRRSGQQ GPLLCARVPPTRGTTDQVCTRETGAHHLGRE QVRVAWLLGVDDATGLILFSLLFVCSTIRLCP PLVVDEKDIDQLLHVFDQAFREEIL
g8131	Effector probability: 0.597	187	RXLR	MRMRIIMDDRNCPMCKQPLERVVVSTSPRPY ESFELWGDAAGPESVMDEPSEMIFVDCRAH YYELRSLREYKCRMKRCREVKHSLGQLKEH LQHDHGVEFCELCLHHQSFFIQEQEVFTKGA LKGHSIGRSRGGSVGQKHANMGKDFHPMCQ FCRKRYYGDKELYEHLERDHFKCHICKVENE YFRN
g8655	Effector probability: 0.603	251	HVLxx P-CRN	MKTIATAILGSLAIGSVSAILGGKVVPPHESN YTSFLSLSKSGDPICGAALINPRHVLTSASCTL QKPTYVVVGAQSLSNTSNADVLKIKKTQIHP KYDAQYLAYDLAVLTLEKKSSFTPANLPVTE DSSQTIKAKDLATALGWGSTNWELYYELSE KLYSVELPVIENGACRNLTGLSFINRQYLCAG GLKDKGLNRVDLGGPLIAKNKPSKGKDMVV GVASYAQSGHDGEISIFARVSAELDWIRSQL Q
g9832	Effector probability: 0.838	196	RXLR	MCVGLPLNQACEISVYAPSSAAKGVLSTVTF EIQQVLTRAQGNELRVVDCILLKDVFEAEYT HQVFCCGTAHGSKMRRSSAAVATRMLRVGT YLHGAIINFTKGKDAAIRLANQSSGMTRTVF KPDFDFTKLGIGGLDKEFNDIFRRAFASRLFP TDVIQKLGIQHVRGMPAFGPPGCGKTLIARKI SQALTAQGA
g9894	Effector probability: 0.697	232	RXLR	MNALKLDEVASYFDKDDEYVGDGEGLRIAL RFLALDDGRLVIIDLPTSVHESTAVEFTKKFF RAAGNDDEVAGRGSMTARRAVNPNKEADA TFGPKGSTPLRTPPPAPRTIADWVTLAVEVGR SQSWASLPDAARWWFGYAGIQYVLLLKINA PGTQIRYALYDSTTRPTPSIRPTASGTFRHNA AGAAVNVTFDMRRILSIPASQALPTGVNAIA VVDLRTVMNQVTRSLR

b)

Name	Description	Size	Motif	Sequence
g10502	Effector probability: 0.882	177	RxLR	MGVCCRPCQIHGGAITSGNDRDAAYSAGVGLWPR TASDYNKEVLELTTCPNVNENVHDLYSKAEFYAG AWSSMSQYMKQEESQTEDQLHFDLILTAETLYTED VAVELYQTIKRHLRRSRDARALVAAKKYYFGTNG SVQHFMGVVEADNVLRADIVWEERNCRSNIRAIV QLTYIY
g10745	Effector probability: 0.697	199	RxLR	MTRKACTAWEPSLKFPRTTMIMGQLRTFSSKYDV TISLSARKNNSLSFRVRGSLTKRSLRLVSGCNGLQK FXSREFWFVIHARWMKRWSLFVGKEGQEPGPITN CELLMPGFIAGVNPNQAVTVRSDIEVMKDFRFVTP MVWSLLAALHGPGDAPPIARFSLDINSDAPEDVNE VLSEAKGQAKGLATSLREKCKVVVR
g10789	Effector probability: 0.755	143	RxLR	MVTSGPQSDRLAFGEYIQKNMKLYELRNGVTLNG PATANYVRNELARFLRRAPYQVNLLIGAVDATPEQ EAEPSLHWIDYLGAMVKVNFGAHGYGAHFCLSIF DREWKPNMTLDEAKDVFKKCRAELDMRFLVRNG QWAYTVR
g10871	Effector probability: 0.698	231	CRN	MSAHLGARRHQRSSYGGSLRRMSIDAINTLSTRSL GDLNQLSQATREFGEVYSGSPDGSRRSPRLLQRLF DPLENRSVEKCLFRVRISGIQCRNLQGRLFSGKSDP YVEFFWDDPEETAPYVTPVIKADLNPNYKGVLIAF EYEAPLNELQKRKLLVKVXSXXXXXXXXLIXKFN AKYLIGEAKVDLWSIATGPVHHDHHLVGCDNGRV VFNCYMEQCNEWNIVVSDVGTA
g11308	Effector probability: 0.616	277	HVLxxP	MRFPAKAKVLSHSALATAGIAALASYFSSSLLVQC EESEGSKVALSPKEYRSFTVSKVETVNHNTKRITFA LPSPEYEMGLPTPSCLMARARVNGKMVAKPYTPT NVNAEKGFLELVVKGYPMGRMSKHIVNLEEGDSL EMKGPFTKFKYYPNKYKSIGMIAGGSGITPMLQLI KTICRNPEDCTKITLIYCSVSEEDIILREEVEAMMYL YPQVSVIHVLSNPSAKWEGLSGFISKEMIENFMPEP SDDNLVCVCGPPPMMYHVSGDKDKDKTQGF
g11356	Effector probability: 0.575	249	RxLR	MAEPLAAIIASMAAATGNASPFEKTAPKTDDLADV NTACREKKRDREPKFTPPSGIWNPQAKMPLSASAL SKLEGAARTCTVNPAMEVVRQKMFLKMCNKIRRA SEDLGIGKLSNSVYEIWQFTSQFMVKEQDPSIFHAE CDNTRFFERLFCKAGATRSEAIKKCKELTRESERM LRKFGQQNFVVGKKKVQLRVINDVSAEHFAKLHE LYARKHGLNGDGSSMALKDQRQFDFRYRAVTDF QLNNGGF
g11523	Effector probability: 0.604	211	RxLR	MNGSSSLRMTIETVMKNTSAAVKTMRLESDGRRL SRYMLLDLQGRDVGDTVTDRYAHALDFTVEGNFL RAQHGNSTLCFWDLRQRAGTRVTSSNLLRMLRW QTHSVSIGHDVSGLALTTNDFAVANSTQGLTLVLT GNGDLNLVPFPCSPPPHAASWLTRRPSVCVWDFAL RGDIVVTGSQFDPALCQWHVDKELADVRASTRLPI RSAPS
g11674	Effector probability: 0.702	217	RxLR	MVRKGRNKENVAGERYDSNAIQMPQSTSSRTRPY PSQFNPADLMGMMGGGGGGMPDMSQMMEMMQ SMGGMPSMGGMPGMPGMPDMSAEGTPTSKFMTF YPNYIDKEKTLHQGRRIPKEKACETPIADEMSEYPR DWMVPGRIRVRLLRDDGTPENREFPSRKTLMIKM AELIPQLQSRKVRLENEAEEARKRLAAAAEAAAPA SGGGKKKSKKKGRR
g11830	Effector probability: 0.575	157	RxLR	MCATLTKKFMDAMKDYQKAQQKYKSDMKTKVK RQVQIVKPDASEAEIDAVMRSGDPGSIYKSAILQGG AADSITDVFFHCQDKYQDVLKAAGRTAGSDRQSS AYSRQLRRTRQSGGAEGHQVSEVLSKENVLPAWH RRLYPCGDRSHCAGAQEGVVK
g11900	Effector probability: 0.911	204	RxLR	MSCCKTPHFPFIRRVLRSRFFHSTTKVAMTDNQVD NFCSASQNVATFAAGCFWGVQLAFDRLPGVLETS VGYTQGDVDSPTYRQVCTGRTNHAEAIRIVYDESK TSYESLLRKFWAIHDPTTLNRQKNDKGTQYRSGIY YNNEGQRKMALASKEEHQKKLSKPIVTEILEAKQF WDAEDYHQKYLEKGGQCADKGCDTPIRCYG

g12034	Effector probability: 0.748	134	RxLR	MSLMLLDGTIGEHAEFALQSDRRLKDQHQRLRSC DDSFDALLASYNAAARQDTENPALKSPLTPYTEYL DCIGNALCPKALVEWKTCMASVMTGEKYMQECA LTKRLLERCLRSKSEELLQGSQPQVFRPNATP
g12648	Effector probability: 0.74	181	RxLR	MDTEPSQATHLRGLLDKIVHTEPARRTPVRVLAAS WEDGDVGVARKLRPIAEKIVDGETVVMFTDRQAA GEMESAVVNFDVRSSPNCVVSVCGEMSKWYLGA KEKCFDLRIAPKSADQKENGLIRGVSAAYSGLRPV ATNLYLKFDETNFIDGSKCEYEIISGSKTVRKEEITS DSQAVTA
g14055	Effector probability: 0.58	249	CRN	MKRLPREHADLNTRRNSKQDNDDKAEILIAERIPL KTWNKFAQLEKLPCGLRQLVWDDDKVLVAECPLS RTICRCFPSEVCITGAADITGSGPDGQLITCQPDMSC SPCRNAPSIQAPQGIDFDDWRTVIVEVLNTRTWPSV RRKVHAYRQRIGVQYILCVKMAEQLKSWSYEFYD FGDAPTPPFPHMANHHAFRLGTVGAQDHRVVFVS RRVLGLPAHAPLPAGWPHNLTIDTVDLARHVTPYC LNAN
g14223	Effector probability: 0.574	292	RxLR	MPQVEELGTEHSTDVEYDVEWSEGPLGCELKQRN GLPAVKSVTGTGVTPSVAQIAAGDILVSINGLRVEE IGFKSTVTLMMRATKPVYLRFLRGGARQSISGSSL NDLPLHKGPQNINRRPYHTEGGTGASAALLEAKQ YTVLWRDGPLGIQIRTSSKGRVVVARLTGAGAPNV NDAVKAGDIFLRQKDGMAQRKYAWYCKLKEAY MTAATTFTKHYQDKVGDDESVIGAKHKCEIAGSA CPVRTEERVQRLYSGLGFTTEAEYVTEVIVKSDPE GAGEKALSKPKATHENT
g1861	Effector probability: 0.644	196	RxLR	MHTGHPSLGQFLFLSVNVYLVMYVHLPPLVGDRT GLLVSTAVHVDDPTCVKSYDLIPSNPSLFCVKTAG WLLELAYQAYFDPPGCPSLSGYGELSLEQHGFELIT HLRNNRTDTHVVVAWSQADHRRLVISFRGTTSKE NWKSNLRADQTVLWMKSRGLRWRKSCLEKAKD VAAKIPLLNMALPRVHRGDCKIAIF
g1892	Effector probability: 0.669	181	RxLR	MGRARRLRGQAWVTFDDILSASNALRSANGSILFE KPAVIHFAKEKADVIARREGTFVPREKRKREPKPT APQQQATKKKANENGSANAKGSFGATQPRMAQN VPNKILFLEELPESCNRDMLGVLFKQYQGFKEVRM VPGKKGLAFVEFGDEAQAAIALQGLYGFKLTPTDV LRVSFAKK
g2535	Effector probability: 0.857	178	RxLR	MSDACFLPMGALSRINSDHSRINGHVSLSAHKNHG MTTLTDPGIIHSLGQLNETEIEDESATIEMEGGQLL HSKEMLASRKRSLRRRYCDICGITQDSSTDHCEDC GVCVAGYDHHCPWMGKCIGRDNMHAFKMFNMA WIMYVCFVLVIAITHMDWGQTAVNTLKHSASGN WGTSRIP
g3619	Effector probability: 0.788	191	CRN	MDNHPMWGLDRVWSFTARNYFFWGCRNLARIEE VLTASDLKSKDKLLKKDLTVSNNTLIGERDSDAST DKMCGTSQDQSPPTRLNDRTLCKSVGLESLFIAEN GFLDLSGTDFGAAIKLSNSVALLDAPQITAAIFATSI KKRNDANSLIQKEIRLGADMNHTLENAALRELDPP SRVLLVAKVFRAKQKV
g3974	Effector probability: 0.872	142	RxLR	MERVDVWNPANKRKETAVVKLVVENGAADRVG VTIGSTVVAINRKSVDSEPYLAILNLIKAAPRPLRM RFERGSQNMDTTQGNILTRISIRIDYFADGTFSVGN LTSGNATWTTKYFAFGGSKMDVLQLFVSRAAYHE VGRI
g4111	Effector probability: 0.596	279	RxLR	MSARRLREAINNPPRGTLHKAAENEHDYLRQCVD PILMPLIESLLLYQPESVYHFIHDFVDDNKNTRFVH HSRSVGYAKKLTTRRKMADFMSTSVIPVMDELAK QXXXXXXXXCQIFYSRRGVCDRLSATLFGVNDRV WCRYKGRPRFYPAVITNIDDNGNFTVLYDDGKLES NVHSMCLKQYVNDTVNRNDVDNQKRPKATCSKD TVYLIIGVDGAGKTTLLSTLQGDLDKEHVPSAGFTS VTFQTDKGSATFFDLGGGPAFRNVWKEYYADVKT LAF

g4821	Effector probability: 0.949	174	RxLR	MLVVYVAVAMSSMAVIGRFKSLDAETDEDHRVFG IVGYPKEFERINEVDAEGFQLPIRAAKAASRSQVTS TWYNAGSVVVHYRRNQLNDDNDSDTIAFDRLSVH CEDGSQFSALESFMQKQTVVTHEKAESLSTKMFIP VIGGVLIGGVASFFAIRILRNRPFLRAQVGVGPHQ
g5891	Effector probability: 0.843	142	RxLR	MDRLLRALEYADLDSRRNSKLDGGNGAEMLIAER IPLQTWNKFAQLEKLPRSLRLRQLVWDDDKVWIV EYPLSSCHEGAVGYICRSFPKEVYGTGAADITGPGP NGQPITCQPDMSCCPCRNVPGVQAPQARYVHTNG RMRI
g6127	Effector probability: 0.578	150	RxLR	MLASETPGVSSSAPSNESQQPSFPSQIVADVHTVLS VLMDALEQRHEGIKKDLTMLFDAGDLNHDRVLTL YEFSAIIRNGKPHFSDRRILRMFREALMGGADQSFA LSMEAFVNVCNDHGLVSLLPDDRMVDPFALPQAA AKKLLSKPVG
g6249	Effector probability: 0.582	215	CRN	MIPRMTSTCRNGISRSITMTWRRLPGAEFDNXXXX XXXXDKEPKNFVRSAVAGACFSRVEPTPIASPELVI ASPNSLLLVGIEVKESLNQSDEQQIEGKDEDFQPIET LVPVLAGNKLLPGSESAAQCYCGHQFGYFSGQLG DGAALYLGEVVLEDERWEVQLKGSGLTPYSRTAD GRKVLRSTLREFFVYENMHALGVPTTRAGSVVMS RETQV
g697	Effector probability: 0.642	290	RxLR	MXXXXTAMRLLHVVVATVSLTGAITSLIAAQHYN VSSDVIGGIEKEDFGVGCNSRTKYPTAADTSKLQP RFAYLITNSLADFIAVHFIKFNLPDNDFVQIRAADPS AVDNRVLRYRGNESNGVFFADALSTKSVIVELFTN ASSSAQKTNSSKCVGFAVDSYQYLGEGSTLNGSKE EVCGADNSREASCYSGYTNAFRASNAVHPAGWG XXXXXXXXNGFGTVTSLTMGXXXXXXXXYYLDT QGGSSGSPVLXAVVALHHCGGCPNTAINSYKLVN DMKWRGILPANACT
g7396	Effector probability: 0.708	234	RxLR	MRPKSDVSAYYSSCLDADEAFTEALRVNLQRLIVY ARSADTALQREVAEKLANEAVKPDRQVQIVELDG LQLLLPLTKSRDTEVQRLAAHALANLSVNSENQSK MATEGGIDMLIDLLSSTNEHVQRQAAKALANLGV NGAVLSCLERIAKAGGIKPLIDLASSRQIGVAVEAI AALANLAVNGDIWKLFVDANEVEIARKGGLKPIID GAHSESIELQSQVARALRNLSVNRT
g7570	Effector probability: 0.625	180	RxLR-CRN	MREACIFGALMVANDNLDGNTCSGNCYNGGRRL RVWSHYFSTRGGVLSLPDDQTAVLIDPLSDEGKTL DKVSGRIQIENVSFAYPSRPEIQVCRNYSLTIEPGET VALVGPSGSGKSTMVSLLERFYDPLSGSVSIDGVD ATAALHGRGDSVYTNENESLNVMFNPVDPPRGDG WYRKPA
g7901	Effector probability: 0.712	208	RxLR	MTSSPITTHPPDATSVHSTQVTRVTSFFGREVEVNI ELWNSSMCQQWALVLSSTARSRASQVGPECLDAF ACTDRVFESRFTFLMQHDGLLLGFFMTSPTATLMT KFKDGKAMPLELLLRFHCNSRVAMATVAPVFLYA ESPKLVGKKTAAQKQAIYSLLLQLNRAHGHTADV SLRALRAKNFIDAGVGQVTKKKPRVAGAFSGLDG Y
g8131	Effector probability: 0.597	187	RxLR	MRMRIIMDDRNCPMCKQPLERVVVSTSPRPYESFE LWGDAAGPESVMDEPSEMIFVDCRAHYYELRSLR EYKCRMKRCREVKHSLGQLKEHLQHDHGVEFCEL CLHHQSFFIQEQEVFTKGALKGHSIGRSRGGSVGQ KHANMGKDFHPMCQFCRKRYYGDKELYEHLERD HFKCHICKVENEYFRN
g8158	Effector probability: 0.836	163	CRN	MQRAQTLLQTPKFNQEIAGAYDAMLHINKYGLRE HYRGLTAILCRNGPGNMLFFGLRGPIRAMLPNGDS ATAMMTNDFICGAMLGAVISTFTFPINGSGTDTNA KRLWPTISRAVGGTSVDVQGAWMQREVLVSRRA NEFFPIVNVMGHNQLYVREAEVDYMR

g8495	Effector probability: 0.597	190	RxLR	MNSLGLAPSVIVAVSPTQSANKCALAKSVSSPNDA QSFRCVDAKASIPVKPDVTTDLAASTNDEDDKMLL SRAGVPPAVTPNDASEAQQRVVRFLRLQQDRGKD FQTTLQDKKEVRNPYILEKVVEYFGIDQLHSNFSPD VFDPHGLPLHEFADALALEQKKRADARAQRQLQQ QRDGDFRKLQFLSSNP
g8655	Effector probability: 0.603	251	HVLxx P-CRN	MKTIATAILGSLAIGSVSAILGGKVVPPHESNYTSFL SLSKSGDPICGAALINPRHVLTSASCTLQKPTYVVV GAQSLSNTSNADVLKIKKTQIHPKYDAQYLAYDLA VLTLEKKSSFTPANLPVTEDSSQTIKAKDLATALG WGSTNWELYYELSEKLYSVELPVIENGACRNLTGL SFINRQYLCAGGLKDKGLNRVDLGGPLIAKNKPSK GKDMVVGVASYAQSGHDGEISIFARVSAELDWIRS QLQ
g9832	Effector probability: 0.838	196	RxLR	MCVGLPLNQACEISVYAPSSAAKGVLSTVTFEIQQ VLTRAQGNELRVVDCILLKDVFEAEYTHQVFCCGT AHGSKMRRSSAAVATRMLRVGTYLHGAIINFTKG KDAAIRLANQSSGMTRTVFKPDFDFTKLGIGGLDK EFNDIFRRAFASRLFPTDVIQKLGIQHVRGMPAFGP PGCGKTLIARKISQALTAQGA

c)

Name	Description	Size	Motif	Sequence
g10871	Effector probability: 0.698	231	CRN	MSAHLGARRHQRSSYGGSLRRMSIDAINTLST RSLGDLNQLSQATREFGEVYSGSPDGSRRSPR LLQRLFDPLENRSVEKCLFRVRISGIQCRNLQ GRLFSGKSDPYVEFFWDDPEETAPYVTPVIKA DLNPNYKGVLIAFEYEAPLNELQKRKLLVKV XSXXXXXXXXLIXKFNAKYLIGEAKVDLWSI ATGPVHHDHHLVGCDNGRVVFNCYMEQCNE WNIVVSDVGTA
g10873	Effector probability: 0.568	182	RxLR	MQPPKTAAALTPLTLNEQVAAGLKRITEELEE SSDHRKRVLLDHLAATPTEQILDPAIICGRGRP AGALNRRNRNNRNNRNAYSTQRDLPAFEHV KNAFGENPTGGRRCGRCREQGHNSRSSSTVI ADIADDAAAATAANASRVARIQRMARAAWL GVDVQIPQRSHKLDSKVASRSLRAFE
g11308	Effector probability: 0.616	277	CRN-HVLxx P	MRFPAKAKVLSHSALATAGIAALASYFSSSLL VQCEESEGSKVALSPKEYRSFTVSKVETVNH NTKRITFALPSPEYEMGLPTPSCLMARARVNG KMVAKPYTPTNVNAEKGFLELVVKGYPMGR MSKHIVNLEEGDSLEMKGPFTKFKYYPNKYK SIGMIAGGSGITPMLQLIKTICRNPEDCTKITLI YCSVSEEDIILREEVEAMMYLYPQVSVIHVLS NPSAKWEGLSGFISKEMIENFMPEPSDDNLVC VCGPPPMMYHVSGDKDKDKTQGF
g11674	Effector probability: 0.702	217	RxLR	MVRKGRNKENVAGERYDSNAIQMPQSTSSRT RPYPSQFNPADLMGMMGGGGGGMPDMSQM MEMMQSMGGMPSMGGMPGMPGMPDMSAE GTPTSKFMTFYPNYIDKEKTLHQGRRIPKEKA CETPIADEMSEYPRDWMVPGRIRVRLLRDDG TPENREFPSRKTLMIKMAELIPQLQSRKVRLE NEAEEARKRLAAAAEAAAPASGGGKKKSKK KGRR
g11830	Effector probability: 0.575	157	RxLR	MCATLTKKFMDAMKDYQKAQQKYKSDMKT KVKRQVQIVKPDASEAEIDAVMRSGDPGSIY KSAILQGGAADSITDVFFHCQDKYQDVLKAA GRTAGSDRQSSAYSRQLRRTRQSGGAEGHQV SEVLSKENVLPAWHRRLYPCGDRSHCAGAQE GVVK
g11900	Effector probability: 0.911	204	RxLR	MSCCKTPHFPFIRRVLRSRFFHSTTKVAMTDN QVDNFCSASQNVATFAAGCFWGVQLAFDRL PGVLETSVGYTQGDVDSPTYRQVCTGRTNHA EAIRIVYDESKTSYESLLRKFWAIHDPTTLNR QKNDKGTQYRSGIYYNNEGQRKMALASKEE HQKKLSKPIVTEILEAKQFWDAEDYHQKYLE KGGQCADKGCDTPIRCYG

g12034	Effector probability: 0.748	134	RxLR	MSLMLLDGTIGEHAEFALQSDRRLKDQHQRL RSCDDSFDALLASYNAAARQDTENPALKSPL TPYTEYLDCIGNALCPKALVEWKTCMASVM TGEKYMQECALTKRLLERCLRSKSEELLQGS QPQVFRPNATP
g12648	Effector probability: 0.74	181	RxLR	MDTEPSQATHLRGLLDKIVHTEPARRTPVRV LAASWEDGDVGVARKLRPIAEKIVDGETVV MFTDRQAAGEMESAVVNFDVRSSPNCVVSV CGEMSKWYLGAKEKCFDLRIAPKSADQKEN GLIRGVSAAYSGLRPVATNLYLKFDETNFIDG SKCEYEIISGSKTVRKEEITSDSQAVTA
g12735	Effector probability: 0.586	173	RxLR	MQQLAYFQARRPSGIILGDPQFPHAANTLSTA VAVQQIKVPVVFISIDAFQTIRRKLRDLKADA TISSKRGSGMHIHVEFSGENALEHQWKELASL AVESNWPATEAARDRLFHRMLKDQAALDDH DLQLSLVKNERYEALVASYWRAQRYYSARA DEIAQGSQQEGNEAQDN
g13193	Effector probability: 0.622	169	RxLR	MVKPVVNANMNKTFREVVGRYVGVPPQLSH KQKVQRLYKKSLKTLESWVIDRRLWNDEAT KIRAEFDANGKLDPQSGYVPYRYLYQYRLLR EAQDKVDHSTHPDRYIFNYMPGGSLFMRNAP IPLDVCFPDGVIPDDVEVSPLEGINIDMTPLPA KETVFVDFSKKGYD
g14474	Effector probability: 0.797	184	RxLR	MFADGVTPPKMYNTNVSCAILQSFLKNTCAT DVKNMCKQKCIQLGIELDALNKALQTAPSNT STAPLAAGKSAALAHSVSVGPRPLRNVSSPLP ITPKKNVDSRKLADDDVAEIVAQKTTIEYQLE VIAGASKLANEVLAGTAVVDLADDLGKRLN LDMNGDERANLLLKSRAQYTVVAVSEFS
g1861	Effector probability: 0.644	196	RxLR	MHTGHPSLGQFLFLSVNVYLVMYVHLPPLVG DRTGLLVSTAVHVDDPTCVKSYDLIPSNPSLF CVKTAGWLLELAYQAYFDPPGCPSLSGYGEL SLEQHGFELITHLRNNRTDTHVVVAWSQADH RRLVISFRGTTSKENWKSNLRADQTVLWMKS RGLRWRKSCLEKAKDVAAKIPLLNMALPRV HRGDCKIAIF
g3974	Effector probability: 0.872	142	RxLR	MERVDVWNPANKRKETAVVKLVVENGAAD RVGVTIGSTVVAINRKSVDSEPYLAILNLIKAA PRPLRMRFERGSQNMDTTQGNILTRISIRIDYF ADGTFSVGNLTSGNATWTTKYFAFGGSKMD VLQLFVSRAAYHEVGRI
g4019	Effector probability: 0.638	287	RxLR	MKLAVLTCTLVAQSWAARMFDPIQFRRNAQ SDVSEKSEAVTGNDVVTPIICASPAATAFLGF ECAQHSSSVIKPRQRYLTPQRTPCTHLLLVTG GVDLHLGSCCNVYRYTGTGNLEYYKDQQAQ TNIAHLLALSAKKLQPKVILGHGDNFYWNGL GSDDVEYRFLNSFETMYSDPALLNIKWLNVA GNHDLGGSMFICGKRDNQFVECSGKTDLLKQ XWMRSLRGRASXXXXXXXXWXXXXXXXVE TLEDPDSXXXXXXXXIDTNAAAVHGAHQTC CQCXXXXXXXX
g4111	Effector probability: 0.596	279	RxLR	MSARRLREAINNPPRGTLHKAAENEHDYLRQ CVDPILMPLIESLLLYQPESVYHFIHDFVDDNK NTRFVHHSRSVGYAKKLTTRRKMADFMSTS VIPVMDELAKQXXXXXXXXCQIFYSRRGVCD RLSATLFGVNDRVWCRYKGRPRFYPAVITNI DDNGNFTVLYDDGKLESNVHSMCLKQYVND TVNRNDVDNQKRPKATCSKDTVYLIIGVDGA GKTTLLSTLQGDLDKEHVPSAGFTSVTFQTD KGSATFFDLGGGPAFRNVWKEYYADVKTLA F
g4820	Effector probability: 0.729	180	RxLR	MRQHLGLTSSIGALHAVKQRVLRARHLQATF ATLEQDLMHLLTSVSDYGPLFIVSDKGHKYH DQCPCPWPAYAISVSSRKDRMATRVTAKSLP GSTFSWYTQELIESQAGKFGLMVVSPHQTDIY CVSQHVQSGTVRRRKECARSKRTAFDARRLC VAPTNLAEYVPQAVPCDTSFAIQL

g5463	Effector probability: 0.8	229	RxLR	MLGDETALPKRTVSDYLNVRELREEAEGMH KRAKGAGSPAKGAPLYSTGGGTVPRSIATLS HSVKATAIGKSLTSSASYQDAMNPPHWETIFF NKLIVVTGGDNKAIAFIATQLWLLGANVLLTF SNVAALDEFNTKHLGRFPDPCEREETPRGVM LPVLGSFRTQESIVEWCNSIATKYQRVDYFIN YAGSEMIEAPSSACGAKEGLKNCITSSVYESI GSLRDGSVN
g5589	Effector probability: 0.679	196	RxLR	MAMRLVPQSDTHVLCQLRNDWVVVVDYLC GSIVKLHTFIRGSVRKENVKTGGSLSVLSTAL TSENQSANSLRSLRSSWLSCHRCAATLLFDDT IMCTGIHDANHVNMIDLHQLRRHHGSALPAK DNADEKKATKLGKDEVQAVERLDRFRIATDS IITAVTAHPDQHAVICGGANMKLQILGVMGP THSQERTMYN
g6127	Effector probability: 0.578	150	RxLR	MLASETPGVSSSAPSNESQQPSFPSQIVADVH TVLSVLMDALEQRHEGIKKDLTMLFDAGDLN HDRVLTLYEFSAIIRNGKPHFSDRRILRMFREA LMGGADQSFALSMEAFVNVCNDHGLVSLLP DDRMVDPFALPQAAAKKLLSKPVG
g697	Effector probability: 0.642	290	RxLR	MXXXXTAMRLLHVVVATVSLTGAITSLIAAQ HYNVSSDVIGGIEKEDFGVGCNSRTKYPTAA DTSKLQPRFAYLITNSLADFIAVHFIKFNLPDN DFVQIRAADPSAVDNRVLRYRGNESNGVFFA DALSTKSVIVELFTNASSSAQKTNSSKCVGFA VDSYQYLGEGSTLNGSKEEVCGADNSREASC YSGYTNAFRASNAVHPAGWGXXXXXXXXN GFGTVTSLTMGXXXXXXXXYYLDTQGGSSG SPVLXAVVALHHCGGCPNTAINSYKLVNDM KWRGILPANACT
g8655	Effector probability: 0.603	251	HVLxx P-CRN	MKTIATAILGSLAIGSVSAILGGKVVPPHESNY TSFLSLSKSGDPICGAALINPRHVLTSASCTLQ KPTYVVVGAQSLSNTSNADVLKIKKTQIHPK YDAQYLAYDLAVLTLEKKSSFTPANLPVTED SSQTIKAKDLATALGWGSTNWELYYELSEKL YSVELPVIENGACRNLTGLSFINRQYLCAGGL KDKGLNRVDLGGPLIAKNKPSKGKDMVVGV ASYAQSGHDGEISIFARVSAELDWIRSQLQ
g9832	Effector probability: 0.838	196	RxLR	MCVGLPLNQACEISVYAPSSAAKGVLSTVTFE IQQVLTRAQGNELRVVDCILLKDVFEAEYTH QVFCCGTAHGSKMRRSSAAVATRMLRVGTY LHGAIINFTKGKDAAIRLANQSSGMTRTVFKP DFDFTKLGIGGLDKEFNDIFRRAFASRLFPTD VIQKLGIQHVRGMPAFGPPGCGKTLIARKISQ ALTAQGA

*CRN proteins contained the ‘’ LxLFLAK-DWL’’ sequence at the N-terminal and followed by ‘’ HVLXXP’’.

Table S.7 The contig genes on MTPI01.1 genome assembly reported by Yin et al. (2015) and P-tools identified proteins on reference genome of red algae. The proteins related to apoplast, signal and effectors identified at 6 hpi in each a)susceptible, b) caring *Rpv*10/*Rpv*3 c) carring *Rpv*10/*Rpv*10 genotypes and d)For time point 0hpi, there is only some areas matched while once with coding region.

a)

Contig	gene-ID	CD	Protein Charaterization	Protein
MTPI01001774	g15180, g15181	no	-	Apoplast, other
MTPI01001404	region without annotation			
MTPI01001296	region without annotation			

MTPI01001132	region without annotation			
MTPI01001075	region without annotation			
MTPI01001060	region without annotation			
MTPI01000922	region without annotation			
MTPI01000899	g13826	no	plant cell wall degrading enzymes that directly facilitate colonization of plant tissue.	Other
MTPI01000666	g12802	no	hydrolytic enzymes and effectors	Apoplast
MTPI01000639	region without annotation			
MTPI01000606	region without annotation			
MTPI01000462	5 regions without annotation			
MTPI01000399	g10289	no	cell wall or hydrolitic	Apoplast/signal
MTPI01000340	g9157	no	avirulence genes	Apoplast
MTPI01000289	g8291, g8293	Trypsin-like peptidase domain. Trypsine-like superfamily	Protease	Apoplast, signal, apoplast, signal, signal
MTPI01000288	g8286	no	membran (Peroxisomal) proteins	Apoplast
MTPI01000009	g697, g698	Trypsin-like peptidase domain. Trypsine-like superfamily	peptidase	apoplast, signal, other

b)

Contig	gene-ID	CD	Pro. Charaterization	Protein
MTPI01001774	g15180, g15181	no	-	Apoplast, other
MTPI01000606	region without annotation		subunit ribosomal RNA sequences	
MTPI01001075	region without annotation			
MTPI01000462	region without annotation		plant infection proteins like hydrolises	
MTPI01000639	region without annotation		Ribosomal subunit RNA genes	

MTPI01001404	region without annotation		avirulence genes	
MTPI01001296	region without annotation		-	
MTPI01000922	region without annotation		-	
MTPI01000009	g697, g698	Trypsin-like peptidase domain. Trypsine-like superfamily	peptidase	
MTPI01001772	region without annotation		Tetranychus urticae titin-like	
MTPI01000399	g10289	no	cell wall or hydrolitic	other
MTPI01000340	g9157	no	avirulence genes	
MTPI01000666	g12802	no	hydrolytic enzymes and effectors	
MTPI01002041	region without annotation			
MTPI01000899	region without annotation		-	
MTPI01000943	g14005	P-loop NTPase domain	NTPase,nucleotide/nucleoside kinase	other
MTPI01000288	g8286	no	membran (Peroxisomal) proteins	Apoplast
MTPI01001060	region without annotation		-	
MTPI01000289	g8291, g8293	no…Trypsin-like peptidase domain. Trypsine-like superfamily	Protease	
MTPI01001132	region without annotation	88% similar to non Oomycete fungi like Sporisorium scitamineum		
MTPI01001027	region without annotation			
MTPI01000418	g10540	PLN02432 super family	putative pectinesterase	Apopplast/Signal/effector
MTPI01000761	g15396	no	-	other
MTPI01000615	g12547	PRK02106 super family	choline dehydrogenase	other
MTPI01000317	g8627	no	-	other

MTPI01001912	g15258			other
MTPI01001464	region without annotation		hydrolytic enzymes and effectors	
MTPI01000801	region without annotation		chitinase like	
MTPI01001121	region without annotation		ribosomal protein	
MTPI01001129	region without annotation		avirulence and suppression of cell death	
MTPI01002103	g15396	no	-	signal
MTPI01000643	g12631	no	ATPase	other
MTPI01000044	g1794	no		Effector
MTPI01001927	region without annotation			
MTPI01001525	region without annotation		-	
MTPI01001522	g14901	TGase_elicitor super family	Transglutaminase elicitor	other
MTPI01001792	region without annotation			

c)

Contig	gene-ID	CD	Pro. Charaterization	Protein
MTPI01001774	g15180, g15181	no	-	
MTPI01001075	region without annotation			
MTPI01000606	region without annotation		subunit ribosomal RNA sequences	
MTPI01000462	region without annotation		plant infection proteins like hydrolises	
MTPI01000639	region without annotation		Ribosomal subunit RNA genes	
MTPI01001404	region without annotation		avirulence genes	
MTPI01001132	region without annotation		virulence role	

MTPI01001296	region without annotation		
MTPI01000922	region without annotation		
MTPI01000761	g15396	no	-
MTPI01000666	g12802	no	-
MTPI01000399	g10289	no	cell wall or hydrolitic
MTPI01000009	g697	Trypsin-like peptidase domain. Trypsine-like superfamily	peptidase
MTPI01000340	g9157	no	avirulence genes
MTPI01000899	g13836	no	HAM34-like putative membrane protein
MTPI01000289	g8291, g8293	Trypsin-like peptidase domain. Trypsine-like superfamily	Protease
MTPI01000288	g8286	no	membran (Peroxisomal) proteins

d)

Contig	gene-ID	CD	Protein Charaterization	Protein
MTPI01000606	region without annotation		subunit ribosomal RNA sequences	
MTPI01001075	region without annotation			
MTPI01001132	region without annotation		virulence role	
MTPI01001774	g15180, g15181	no		-