GENETICS AND MECHANISMS OF POWDERY MILDEW RESISTANCE IN *VITIS RUPESTRIS* B38 AND *VITIS VINIFERA* ‘CHARDONNAY'

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[Paola Leonor Barba Burgos]

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ABSTRACT

The powdery mildew fungus, *Erysiphe necator,* is a major pathogen of cultivated grapevines, causing significant losses worldwide. Natural sources of resistance can be found among North American grapevines that have co-evolved with the pathogen. Introgression of resistance from wild species into cultivated backgrounds is hampered by long generation times and genetic linkage with undesirable traits. Molecular markers are excellent tools that help to overcome these problems by facilitating the introgression of one or several resistant loci, reducing linkage drag, while maintaining the cultivated backgrounds that are associated with good quality.

This work sought to understand the genetics and mechanisms of powdery mildew resistance from the wild *Vitis rupestris* B38 and cultivated *V. vinifera* 'Chardonnay', while developing a dense set of molecular markers located in the physical grape reference map and analyzing their association with powdery mildew resistance. Phenotype segregation among progeny of the cross *V. rupestris* B38 x 'Chardonnay' was studied following natural infection during three growing seasons, as well as in a screenhouse, and by a single-isolate, detached leaf inoculation. Powdery mildew resistance in the progeny of *V. rupestris* B38 x 'Chardonnay' was not controlled by a single resistance gene, but showed quantitative segregation. Evidence for the action of several foliar resistance mechanisms, including penetration resistance, differential response to pathogen genotypes, and ontogenic resistance was found.

Molecular markers were derived from Next-Generation Sequences (NGS) of DNA fragments from parents and progeny obtained by Genotyping-by-Sequencing (GBS), and were used to develop a dense map of 16,834 Single Nucleotide Polymorphisms (SNPs). This map covered both the assembled and unassembled portions of the grape reference genome sequence, with an average density of 36 SNP/Mbp. This is the most dense map published to-date and represents an improvement of about 15-fold over the current standards for grapevine genetics.

A Genome-Wide Association Study (GWAS) was performed to localize and quantify the effect of markers linked with resistance to powdery mildew. GWAS led to the identification of 16 SNPs on 'Chardonnay' associated with susceptibility and 9 resistance-associated SNPs from *V. rupestris* B38. Field observations indicated that resistance loci from *V. rupestris* do not provide an adequate level of protection in the F_1, but single isolate microscopy results suggested that *V. rupestris* resistance could provide protection to specific races of powdery mildew. Moreover, identification of susceptibility loci in a *V. vinifera* background can be used to improve powdery mildew resistance by selecting against their presence among breeding selections. Together, results presented in this work help to understand the nature of resistance in *V. rupestris* B38 and 'Chardonnay', while developing relevant tools for grapevine genomics and breeding.

BIOGRAPHICAL SKETCH

Paola Barba was born to Davioleta del Carmen Burgos and Vicente Barba in Santiago de Chile. Although, Paola lived most of her entire life in a city, since her childhood they spent vacations traveling south into natural areas. The beauty, peace and incredible diversity of life awoke a sense of passion for nature that still remains alive and continues to inspire her.

Paola started her studies in engineering at the Faculty of Physical Science and Mathematics (FCFM - Facultad de Ciencias Físicas y Matemáticas) of the University of Chile in 1997. After three years of the common engineer plan, she moved towards a specialization in biotechnology. There, her interest focused upon molecular biology guided by Professor Oriana Salazar, who gave her the opportunity to accomplish her first research project in a lab, and later accepted her as thesis advisor. During the 6 years of the Engineering program, Paola was twice recognized for ranking in the top 5% of the engineering school. She also enjoyed being an assistant teacher of physics for first year students and for several courses and laboratories in biotechnology. In 2003 she obtained her degree of Civil Engineer in biotechnology with the highest score.

Plant science came into her life in 2007 when she moved to work at the Institute of Agricultural Investigations (INIA) under the leadership of Ph.D. Humberto Prieto. There, she made substantial contributions to projects related to stone fruit transformation for plum pox virus resistance, and grape genomics in response to *Botrytis cinerea*. During this period she realized the need to complement her career with knowledge in plant sciences.

In 2009, Paola started graduate studies in Plant Breeding at Cornell University, a place where she has not only found friends and knowledge, but also great people to work with.

DEDICATION

Para mis padres, cuya vida ha sido siempre un ejemplo de trabajo y dedicación.

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CHAPTER 1

LITERATURE REVIEW

Introduction

The wide variety of uses for the fruit of *Vitis vinifera* – such as table grapes, raisins or processed for juice and wine production – have made the grape one of the top 10 horticultural commodities in the world (FAO, 2008) with global production estimated at 65 million tons in 2007, covering a surface of 7.9 million hectares *(www.oiv.int).* The high value of the crop, combined with the historical importance of its products has been driving selection based on both quality traits and adaptation (Owens, 2008; Riaz, 2007).

Grapevines are hosts to several pathogens and pests. Among them, powdery mildew is a prevalent and widespread disease caused by the biotrophic fungus *Erysiphe necator* and is responsible for economic losses due to the cost of pesticide application, as well as reduction of yield and quality. Pest management has been obtained by the use of chemical pesticides, but chemical applications raise concerns about environmental and human health, making host (genetic) resistance a desirable trait in modern grapevine cultivars due to some consumer's preference for natural over synthetic (Anderson et al., 2011). Some grapevine species that have co-evolved with pathogens have developed host resistance. North American species are an excellent source of powdery mildew resistance that have been used in breeding programs, but the introgression of resistance into a cultivated background must overcome several challenges related with the genetics and biology of the interspecific hybridization.

Nowadays, next-generation sequencing technologies have expanded the set of tools used for analysis of genetic variation. Moreover, the international grape community

has achieved major goals that have given light to understanding grapevine genetics and plant - pathogen interactions. These advances, combined with the genetic diversity found in the genus *Vitis*, allow us to expect that natural resistance to grapevine pathogens will be an attainable achievement in the future. In this chapter, I will review the interaction of powdery mildew with *Vitis rupestris, Vitis vinifera* and other *Vitis* species. The focus will be on the grapevine and pathogen biology, plant-pathogen interaction mechanisms, sources of genetic resistance used in breeding programs, and genetic studies in grapevines using (i) molecular markers, (ii) linkage maps, and (iii) the application of marker assisted selection in grapevine breeding.

Grapevine biology and genetics

Grapevines belong to the genus *Vitis* (family *Vitaceae)* that comprises about 60 species (Mullins et al., 1992). Most *Vitis* species, including *V. vinifera* and *V. rupestris*, are in the *Euvitis* subgenus, have a diploid genome with 38 chromosomes (2n = 38) and can be intercrossed. Species which belong to the subgenus *Muscadinia* have 40 chromosomes (2n = 40). The hybrids between those subgenera are infrequent, but some successful crosses have allowed the introgression of muscadine genes into a *V. vinifera* background (Bouquet, 1986; Riaz et al., 2010). Grapevines are an out-crossing, highly heterozygous perennial species, with strong inbreeding depression that makes the creation of self-pollinated populations difficult. Grapevine varieties are conserved by vegetative propagation, since sexual reproduction does not assure the preservation of parental characteristics in the progeny.

In terms of geographical distribution, *V. vinifera* is often referred to as the European grapevine. The natural range of *V. vinifera* extends from the Mediterranean region to the Transcaucasus region. It was domesticated in the region between the Black and

Caspian Seas, and spread east and west from there (Owens, 2008; Riaz, 2007). The number of *V. vinifera* cultivars is estimated at 5,000 (This et al., 2006), with a diversity of attributes that make them suitable for the production of wine, raisins, juice or fresh fruit. Being the major cultivated grapevine species, *V. vinifera* has been the focus of several genetic studies including the sequencing of the genome of a nearly homozygous genotype (Jaillon et al., 2007) and of the heterozygous variety 'Pinot noir' (Velasco et al., 2007). A study of over 1,000 accessions of domesticated *V. vinifera* grapevines showed that, despite the high genetic diversity within the species, most cultivated varieties are derived from a few elite cultivars that lack genetic sources of pathogen resistance (Myles et al., 2011).

North America has about 30 native *Vitis* species, including *V. aestivalis, V. cinerea, V. riparia, V. berlandieri, V. labrusca, V. rotundifolia,* and *V. rupestris.* Among the most important characteristics of this group is the resistance to a wide range of pathogens responsible for diseases such as powdery mildew, downy mildew, Pierce's disease; phylloxera, nematodes, and abiotic stresses such as cold, high soil pH, and drought (Owens, 2008). Another center of diversity of grapevines can be found in Asia, with about 30 species described (Owens, 2008). Some species have been described to be resistant to powdery mildew (Wan, 2007; Wang et al., 1995). Though less well known in the western world, some wild accessions from Asia have also been integrated into grapevine breeding programs (Ramming et al., 2011a; Riaz et al., 2010).

Powdery mildew

Grapevine powdery mildew (PM) is caused by the biotrophic fungus, *Erysiphe necator* (syn. *Uncinula necator*). The origin of *E. necator* is presumed to be the

eastern United States, from which it spread to Europe and then to other grape-producing regions (Brewer and Milgroom, 2010), including Australia, California, South America and Africa. It is able to infect green tissues (shoots, stems, leaves, buds and berries) in a wide range of host species of the genus *Vitis*, and some isolates can infect multiple genera of Vitaceae (Pearson and Gadoury, 1987). On leaves, powdery mildew mycelia and conidia form a characteristic dusty appearance covering patches or the entire leaf blade. Powdery mildew infection of immature berries may result in cracking as berries mature, making them more susceptible to other diseases and saprophytes, further reducing fruit quality (Pearson, 1988).

Overall, a consequence of the fungal interaction with the plant is a reduction of the rate of photosynthesis, debilitating the vines and resulting in economic losses due to lower yield and quality in wine and table grapes (Calonnec et al., 2004; Owens, 2008; Stummer et al., 2005). Severity of powdery mildew infections will vary between locations and seasons, depending on environmental conditions (Chellemi and Marois, 1991; Delp, 1954; Moyer et al., 2010), fungal genotype (Frenkel et al., 2010; Gadoury and Pearson, 1991) and vine genotype (Cadle-Davidson et al., 2011a; Doster and Schnathorst, 1985; Feechan et al., 2010).

Infection requires conidial germination, appressorium formation and penetration of the plant cell cuticle and cell wall by a penetration peg. Upon reaching an epidermal cell, powdery mildew develops a haustorium whose main function is to obtain nutrients and to suppress host defenses through secretion of effector proteins into the host epidermal cell. In a compatible reaction, plant tissue is colonized by extension and branching of hyphae, which require successful formation of additional appressoria and haustoria. The asexual cycle continues with the formation of conidiophores, which in turn

produce conidia that disperse and establish additional infection sites, initiating a secondary cycle that repeats the above process. Secondary cycles are typically repeated multiple times during the growing season, every 7-14 days, and can lead to severe disease pressure if no action is taken.

Grapevine powdery mildew has two mechanisms for survival during the dormant season. In warm regions, mycelia can overwinter in dormant buds generating primary infection foci called 'flag shoots' early in the season (Ypema and Gubler, 2000). In cool climates, sexual reproduction is required for the formation of overwintering ascocarps. Grapevine powdery mildew has a heterothallic mating system, requiring colonies of two mating types for formation of cleistothecia (syn. chasmothecia) after 24 hr of hyphal contact (Gadoury and Pearson, 1991). This ascocarp structure is able to overwinter on the bark of grapevines, aided by uncinate appendages on the outside of the cleistothecium. After fully maturing, cleistothecia imbibe water and dehisce to release ascospores at the beginning of the next growing season. Therefore, ascospores result from recombination between genotypes and provide the sole source of primary inoculum in cold regions where winter stops the asexual cycle (Pearson, 1988).

The main method to control powdery mildew is through the use of synthetic fungicides and sulfur (Deliere et al., 2010; Erickson and Wilcox, 1997; Gadoury et al., 1994) accounting for around 70% of fungicides applied in Europe (GmbH, 2003). Costs associated with chemical fungicide applications are estimated at 75 million Euros per year in France (Dry et al., 2009). Moreover, pesticide application does not seem to be a sustainable strategy over time—fungicides lose their effectiveness when fungal strains evolve resistance (Baudoin et al., 2008; Erickson and Wilcox, 1997) and some of the current protection practices will be forbidden in Europe due to new

restrictions on the use of certain pesticides (Adam-Blondon et al., 2011). Together, those economic, biological and technical factors indicate that the introduction of resistant cultivars will bring significant benefits (Adam-Blondon et al., 2011; Dry et al., 2009).

Powdery mildew resistance in grapevines

Plant – pathogen interaction

Powdery mildew is an obligate biotrophic fungus that establishes a close plant-pathogen relationship, modulated by specific, fungal effector molecules which have evolved to enable and enhance infection of specific hosts (Spanu et al., 2010). Overall, plants have developed layers of mechanisms to prevent biotrophic invasion (Dodds and Rathjen, 2010; Jones and Dangl, 2006). The first layer of defense is composed of preformed physical and chemical barriers such as leaf surface wax and antimicrobial metabolites (Thordal-Christensen, 2003). Besides these barriers, plants are able to recognize essential pathogen molecules, such as chitin or other components of the pathogen cell wall, known as Pathogen Associated Molecular Patterns (PAMPs). PAMP recognition triggers a cascade of reactions that stimulate an immune response called PAMP-Triggered Immunity (PTI). PTI together with pre-formed and chemical barriers are the main components of non-host resistance. In response to powdery mildews, PAMP recognition induces defense responses that include the expression of pathogenesis-related (PR) genes, such as chitinases and β-glucanases (Giannakis et al., 1998), cytoskeleton rearrangements, and an increase in vesicle trafficking (Dry et al., 2010) in host and non-host plants. Recognition also triggers the induction of antimicrobial compounds and the reinforcement of the cell wall by papillae, callose, phenolics compounds and hydrogen peroxide (Nicholson and

Hammerschmidt, 1992; Schmelzer, 2002; ThordalChristensen et al., 1997). In the vast majority of cases this immune reaction prevents plant colonization.

To overcome PTI, pathogens must develop effector molecules that allow a compatible interaction (Jones and Dangl, 2006). In response to those effectors, plants have evolved a second mechanism based on the expression of Resistance (R) genes, known as Effector-Triggered Immunity (ETI) (Jones and Dangl, 2006). Widely conserved among plants, R-genes contain several conserved motifs: Leucine-rich Repeats (LRRs) thought to be involved in effector recognition, a nucleotide binging site (NBS) involved in the interaction with plant's genome, and variable domains. Most commonly, the inheritance pattern of R-genes and effector genes has indicated that one dominant NBS-LRR is necessary for the recognition of one dominant pathogen effector protein, supporting Flor's gene-for-gene hypothesis (Flor, 1971).

Once the pathogen is detected, the NBS-LRR modulates a new cascade of reactions. Thus, attacked cells undergo programmed cell death (PCD), limiting pathogen growth and survival by blocking nutrient uptake (Jones and Dangl, 2006). Successful pathogens can evade this surveillance system by modifying or eliminating their effectors. This creates a tradeoff between effectors and R-genes that acquire and lose their efficacy during co-evolution, mediating compatible and incompatible interactions between the host and pathogen. From a broader perspective, the expression of a successful R-gene imposes a selective pressure on pathogen populations, which become enriched with individuals that evolve the ability to avoid recognition (Jones and Dangl, 2006). In natural plant populations, when an R-gene fails to provide protection, selective pressure will promote evolution of new R-genes that will respond accordingly. Varieties that have been propagated vegetatively or that have not co-evolved with the pathogen, such as *V. vinifera* varieties, have been left out of this

'arms race', and therefore are more susceptible to a successful plant-pathogen interaction (Brewer and Milgroom, 2010). The relatively simple description of host resistance interactions given above is enriched with mechanisms that have not been as well described at the cellular and molecular level, including quantitative resistance, developmentally-regulated resistance, and environmentally-regulated resistance.

Characterization and use of North American resistant grapevine genotypes

Several grapevine pests from North America, such as phylloxera (*Daktulosphaira vitifoliae* Fitch), powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*), were introduced into Europe during the mid-19th century, hampering the viticulture industry and triggering the adoption of resistant North American germplasm for control. The use of North American species as rootstocks was a successful strategy to obtain control of the phylloxera epidemic and is the best known example of the use of resistant varieties for genetic control of a grapevine pest. *Vitis rupestris, V. riparia, V. berlandieri* (*V. cinerea* var. *helleri*) and *V. aestivalis* have provided excellent sources of phylloxera resistance and have been used in grapevine rootstock breeding (Owens, 2008). North American species were also incorporated in European breeding programs to confer resistance to fungal diseases in grapevines. Many of these interspecific hybrid varieties, commonly called French-American hybrid varieties or just hybrids, were planted in Europe at first, but the quality of the wines did not meet the expectations of European consumers. Therefore, planting of hybrid varieties was discouraged by government officials and grapevine growers, leading to a decrease in the acreage of hybrids in Europe (Owens, 2008). However, those early hybrids have proven useful as parents in breeding programs (Fischer et al., 2004).

Powdery mildew resistance differs among and within *Vitis* species. *Vitis rupestris, V. riparia, V. aestivalis, V. cinerea* and *V. rotundifolia* are North American species generally considered to be resistant (Alleweldt et al., 1991; Pearson, 1988).

Early studies of the inheritance of powdery mildew resistance in *V. rupestris* suggested that the trait was controlled by a polygenic system (Boubals, 1961). Additionally, two hybrids with *V. rupestris* and *V. vinifera* genetic background ('Rubired' and 'Royalty') were resistant, defined as absence of conidiophores on detached leaf assays at 9 days post-inoculation (dpi) and low percent coverage of powdery mildew on clusters and leaves observed in vineyards (Doster and Schnathorst, 1985). Variability within *V. rupestris* has also been reported. Several accessions of *V. rupestris* were screened by observing natural infections in two locations, and by controlled infection of detached leaves using a single isolate of powdery mildew. In the field, the accessions showed variable values of coverage, ranging from 0 to 9, on a scale from 0 to 10, with a mean coverage of 2.2 in Geneva, NY and 3.6 in Fredonia, NY. Some accessions that were rated susceptible in the vineyard were resistant when a single isolate was used to infect detached leaves, suggesting race specificity within the species (Cadle-Davidson et al., 2011a).

Microscopic characterization and comparison of the mechanisms of powdery mildew resistance at early stages of infection in several *Vitis* species showed one genotype of *V. rupestris* as partially resistant, characterized by moderate penetration resistance (only 56% of germinated conidia penetrated host epidermal cells, compared with 81% observed in *V. vinifera*), moderate PCD (10% compared with 45% in *V. rotundifolia*), and little to no sporulation observed at 7 dpi (Feechan et al., 2010). Combined, these

results indicate that some *V. rupestris* accessions are potential sources of quantitative powdery mildew resistance in need of further characterization.

Powdery mildew resistance genes and quantitative trait loci in grapevines

Studies on powdery mildew resistance in *Vitis* species and interspecific hybrids have been conducted and findings can be grouped in 2 main categories: single locus resistance and quantitative resistance. Single locus resistance has been identified, localized and characterized from different sources, and the results suggest the action of one or more clustered R-genes. On the other hand, the molecular mechanisms of quantitative disease resistance are not as well understood due to the complexity added by the interaction of multiple loci (Poland et al., 2009). In grapevines some major quantitative trait loci (QTL) for powdery mildew resistance have been localized, but the mechanisms of resistance remain unknown.

A classic example of single locus resistance, *Run1* was introgressed from *V. rotundifolia* into *V. vinifera*, with the original cross being made in 1917 (Detjen, 1919). After five pseudo-backcrosses (pBC_5) *Run1* was observed to be a dominant resistance to powdery mildew with no macroscopic symptoms (Bouquet, 1986). *Run1* resistance is characterized by a post-haustorial mechanism that rapidly restricts hyphal development through PCD (Dry et al., 2009). Genetic and physical mapping helped to determine the position of *Run1* on chromosome 12 and subsequent studies revealed the presence of 7 full-length, expressed NBS-LRR R-genes (Barker et al., 2005; Donald et al., 2002; Pauquet et al., 2001). Transgenic expression of each NBS-LRR gene is underway in *V. vinifera* in order to study each NBS-LRR separately (Dry et al., 2009). Recently, *Run1* resistance was overcome by naturally occurring isolates in New York State (Cadle-Davidson et al., 2011b).

A second source of single locus, dominant resistance is conferred by the *Ren1* gene that was surprisingly identified in the *V. vinifera* cultivars 'Kishmish vatkana' and 'Dzhandzhal kara'. The origin of the resistance has not been precisely established as the pedigrees of the vines remain unknown (Coleman et al., 2009). Characterization of the resistance through microscopy revealed a post-haustorial resistance mechanism that slowed hyphal growth and reduced conidiophore density (Hoffmann et al., 2008). Co-segregation of this gene with an NBS-LRR-gene cluster and cinnamyl alcohol dehydrogenases (CAD) on chromosome 13 was also demonstrated.

Another single locus, *Ren4*, has been introgressed from *V. romanetii* into a *V. vinifera* background. Vines carrying this locus do not show macroscopic symptoms of powdery mildew, and resistance has been effective against all *E. necator* isolates and populations tested to date. Moreover, microscopic characterization of this source of resistance showed that *Ren4* is able to prevent powdery mildew hyphal development, with a very low rate of successful penetration (Ramming et al., 2011a). *Ren4* has been mapped to chromosome 18 (Mahanil et al., 2011; Riaz et al., 2010).

Ren2 is a major QTL from *V. cinerea* B9 whose mechanism of resistance has not been characterized. It was identified by linkage mapping and QTL analysis in an interspecific hybrid population derived from the cross of 'Horizon' ('Seyval' x 'Schuyler', descending from *V. vinifera, V. labrusca, V. rupestris* and *V. aestivalis*) x Illinois 547-1 (*V. rupestris* B38 x *V. cinerea* B9), and is located on chromosome 14 (Dalbó, 1998; Dalbó et al., 2001).

Ren3 is a major QTL for powdery mildew resistance found in the hybrid cultivar 'Regent', whose pedigree includes the species *V. aestivalis, V. berlandieri, V. cinerea, V. labrusca, V. lincecumii, V. riparia* and *V. rupestris* (Fischer et al., 2004). The mechanism of resistance has not been identified, even though some Resistant Gene Analog (RGA) markers associated with putative R-genes have been co-located with *Ren3* on chromosome 15 (Welter et al., 2007).

Finally, novel QTLs conferring powdery mildew resistance from *V. rotundifolia* 'Magnolia' and 'Trayshed' have been described and named *Run2.1* and *Run2.2* correspondingly (Riaz et al., 2010). These sources of resistance are not related to *Run1*, since they were located on chromosome 18. These two loci partially overlap, but are thought to come from different selections since 32% of the alleles tested were not shared between resistant parents.

Breeding grapevines for powdery mildew resistance.

Characteristics of the breeding process in grapevine.

The high genetic diversity within the genus *Vitis* combined with the viability of progeny from interspecific crosses expands the possible breeding approaches for genetic improvement. Most grapevine breeding strategies focus on wide-crosses between diverse germplasm to introgress traits through several generations, each with selection of progeny combining alleles that account for desired traits while discarding alleles that add negative characteristics. This process may be hampered by the action of several genes with additive effects either positive or negative, interactions between genes and the effect of the environment which may vary from year to year and from location to location.

Cultivated *V. vinifera* and powdery mildew resistant wild species differ significantly in the characteristics of their berries. In interspecific hybrids, negative linkage drag results in the dissimilar quality of the fruit, juice, and wines produced with hybrid vines, relative to pure cultivated background. Wild flavors and aromas are desirable in some markets, such as *V. labrusca* qualities in Chinese, Korean, and Japanese fresh grapes and in Brazilian and U.S. juices. However, in Europe and especially in traditional wine markets, the acceptance of hybrid varieties is low, with exceptions in some areas of Germany, Hungary and the United States where hybrid wine grapevine varieties have been released (Alleweldt and Possingham, 1988). To obtain wine grapes combining traditional quality with adaptive traits from wild species, it is necessary to introgress the traits from wild species into the *V. vinifera* background through several cycles of breeding (Owens, 2008). Because of inbreeding depression, a modified backcross strategy is used for trait introgression, backcrossing to a different *V. vinifera* parent each generation.

Grapevines are perennial species with long juvenile periods. In standard conditions, grapevines reach sexual maturity in two to five years, extending the length of the breeding cycle when compared with annual species. Additionally, grapevines are bigger plants that need more field space and year-round operations in order to maintain the vineyard. This relative increased need for resources limits the number of progeny that can be evaluated under field conditions (Dalbó et al., 2001). Grapevine biology and genetics add some other constrains to the breeding process. While vegetative propagation allows preservation of desired genotypes, heterozygosity and inbreeding depression limit the use on inbred lines in grapevine breeding, which makes it almost impossible to recover the exact same parent's phenotype. Moreover, key traits related to disease resistance, plant physiology and berry characteristics are

often quantitative (Martínez-Zapater et al., 2009). Quantitative traits add complexity to the breeding process – in part because of the difficulty associated with combining multiple genes in the progeny, and because of the need for precise techniques to evaluate the quantitative phenotypic differences between genotypes segregating for those genes (Poland and Nelson, 2011; Poland et al., 2009).

Characteristics of breeding for disease resistance.

Disease resistance in perennial crops, such as grapevines, has to be designed to endure for long periods of time, under cyclic pressure of pathogen populations. Breeding for disease resistance in grapevines must consider the dynamic nature of the plant-pathogen interaction in order to promote durable resistance (Cadle-Davidson et al., 2011b; Peressotti et al., 2010; Ramming et al., 2011b). Evidence for races of powdery mildew (Cadle-Davidson et al., 2011a; Cadle-Davidson et al., 2011b; Ramming et al., 2011b) imposes new challenges to breeders. Single gene resistance conditioned by R-genes is not durable in systems where the pathogens have a high evolutionary potential (McDonald and Linde, 2002), as with *E. necator*. Vines carrying R-genes which show stable resistance at an experimental scale, like *Ren1* or *Run1* can be expected to become less effective once they are widely deployed. Even naïve isolates may be capable of overcoming specific R-genes (Cadle-Davidson et al., 2011b; Dry et al., 2010).

In order to obtain durable resistance to pathogens it is necessary to incorporate several sources of resistance in breeding programs (McDonald and Linde, 2002). In grapevines, pyramiding or stacking genes has been proposed but this approach has to deal with the problem of evaluating the presence of more than one genetic source that

leads to the same phenotype: resistance to the disease. Molecular markers and other genetic tools should be used in order to achieve this goal (Eibach et al., 2007).

Molecular markers, linkage maps and QTL analysis applied to grapevine breeding

A molecular marker is a protein or DNA sequence that shows polymorphisms between genotypes and can be associated to a position in the genome. Molecular markers by themselves have minor utility; they acquire their value either when they are positioned in relation with other markers, allowing the construction of genetic maps, or when they are linked to a gene or QTL that controls a trait. Molecular markers allow breeders to detect, through laboratory analysis, the presence of an allele of a given gene or locus. The characteristics of molecular markers used in grapevines and their progression will be discussed in more detail in the following sections.

Due to the long generation time and inbreeding depression in grapevines, the most common method for construction of genetic maps is the pseudo test-cross strategy (Grattapaglia and Sederoff, 1994) performed with an F_1 biparental population, where high heterozygosity and rapid linkage disequilibrium (LD) decay allow association analysis between markers and traits even with few recombination events. In this strategy, dominant markers that show heterozygosity only in one parent are used. Those markers segregate 1:1 in the population and allow the construction of 2 independent maps. Co-dominant markers heterozygous in both parents are used to combine the two parental maps (Dalbó et al., 2000; Mauro et al., 1992). This technique has allowed the construction of several genetic maps. The characteristics, uses and progression of genetic maps in grapevines will be discussed in more detail in the next section. Other population structures have also been used in grapevine; QTLs

for photoperiod-induced growth cessation in *V. riparia* were mapped by Garris et al. using an F_2 population (Garris et al., 2009). This F_2 approach is more desirable because the second round of recombination gives better resolution and allows the detection of recessive alleles.

Observation of phenotypic segregation combined with markers or linkage maps allows the localization of major genes or QTL. Monogenic traits can be mapped using bulked segregant analysis (Michelmore et al., 1991). This technique does not need genetic maps and allows the rapid identification of markers linked exclusively to a bulked group of vines that show the desired trait. Using this approach it has been possible to locate markers linked to candidate R-genes for powdery mildew resistance such as *Ren1* and *Run1* (Hoffmann et al., 2008; Pauquet et al., 2001). On the other hand, quantitative traits can be mapped using QTL statistical analysis. When several loci affect a trait, the contribution of each one on the observed phenotype may be variable. In a controlled cross, the segregation of the trait will follow a statistical distribution that, combined with a molecular linkage map of the population, can be used to estimate the position as well as the influence of each locus on the total variation for the trait (Lynch and Walsh, 1998).

One of the most useful applications of these techniques in grapevine breeding is Marker Assisted Selection (MAS), in which markers linked to genes or major QTLs can be used to assist the selection process. Using DNA markers, the presence of QTLs or single genes can be evaluated in seedlings, eliminating the need to wait long periods of time to assess the desired phenotype. Furthermore, discarding vines at the seedling stage allows enrichment for vines that carry the linked gene or QTL, increasing the probabilities of success (Dalbó et al., 2001). Moreover, MAS facilitates the combination of several genes in one genotype (pyramiding or stacking genes),

including genes that generate the same or similar phenotype (e.g., two disease resistance genes) (Eibach et al., 2006) or multiple traits of interest (e.g., disease resistance and seedlessness) (Mahanil et al., 2011). Further, MAS can be used to aid the introgression of genes or QTL into a cultivated background and facilitate the selection of parents for each cycle of breeding. Testing markers distributed along all chromosomes allows the removal of introgressed genome regions that are not related with disease resistance, and which can have a negative effects on other traits (Di Gaspero and Cattonaro, 2009).

A Chronology of the molecular genetics of disease resistance in grapevines

From molecular markers to linkage maps.

In grapevines, the application of molecular markers started in the 1970s and has proven increasingly useful over time. The first molecular markers, isozymes, were based on the comparison of electrophoretic profile of proteins with identical function (Loukas et al., 1983; Walter and Boursiquot, 1992; Weeden et al., 1988; Wolfe, 1976). Their application was limited due to a low number of proteins showing polymorphism. Isozymes were rapidly replaced when DNA markers were introduced being used for identification of cultivars (Striem et al., 1990) and in genetic diversity studies (Bourquin et al., 1993; Bowers and Meredith, 1996).

These early DNA markers included Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) markers. RAPDs are dominant molecular markers based on PCR amplification using short primers. Variation between alleles is due to differences in the priming site or in the length of the fragment within the amplified region. They are easier to implement than other markers, because no hybridization or previous

knowledge of the target sequence is needed. The main limitation of RAPDs is their sensitivity to changes in PCR conditions, which makes them hard to reproduce between laboratories. RFLPs are co-dominant markers based on the hybridization of a specific probe to DNA previously digested with a restriction enzyme. Variation between alleles is due to differences in restriction sites, and the specificity of the locus is due to the use of a probe. As co-dominant markers, RFLPs were useful to integrate maps, but a hybridization step and the need for existing sequences to create probes made them less numerous than RAPDs, more time consuming and expensive. AFLPs are usually dominant markers that combine digestion by restriction enzymes, ligation of an adaptor and amplification by PCR. Variation between alleles is due to differences in restriction sites and insertions or deletions between restriction sites, but not all bands obtained are allelic. No previous knowledge of the target sequence or hybridization is needed for AFLPs and they are more reproducible than RAPDs.

DNA markers allowed development of the first linkage maps and QTL analyses. RAPDs provided enough genome coverage, and co-dominant RFLPs were used to integrate parental maps and reduce the number of linkage groups (LG) to more closely match the chromosome number in bunch grapevines (n=19) (Mauro et al., 1992). A more dense linkage map in *Vitis* spp. was developed using 422 RAPDs, 16 RFLPs and one isozyme marker over a population of 60 seedlings derived from the cross of 'Cayuga White' (a hybrid of *V. vinifera, V. labrusca, V. rupestris* and *V. aestivalis*) by 'Aurore' (a hybrid of *V. vinifera, V. rupestris* and *V. aestivalis)*. The map for 'Cayuga White' comprised 214 RAPD and RFLP markers distributed in 20 linkage groups, covering 1196 cM. 'Aurore' was mapped with 225 RAPD and RFLP markers in 22 linkage groups, spanning 1477 cM. (Lodhi et al., 1995).

Later, a linkage map and QTL analysis of flower sex in grapevines was reported using 58 seedlings of an interspecific hybrid population derived from the cross of 'Horizon' ('Seyval' x 'Schuyler', descending from *V. vinifera, V. labrusca, V. rupestris* and *V. aestivalis*) x Illinois 547-1 (*V. rupestris* B38 x *V. cinerea* B9). In this work, a total of 277 RAPD, 25 SSR, 4 CAPS and 12 AFLP markers were used; 153 markers were used to create a map of 'Horizon' over 1199 cM distributed in 20 linkage groups, while Illinois 547-1 was mapped with 179 markers distributed along 1470 cM on 20 linkage groups (Dalbó et al., 2000). Several traits were analyzed in the 'Horizon' x Illinois 547-1 population, including powdery mildew resistance. A strong QTL was identified on what would later be denoted Chromosome 14 of Illinois 547-1, and 2 new markers, CS25b and AfAA6, were develop from previous markers linked to the resistant QTL (Dalbó et al., 2001). The use of these markers in breeding populations improved the selection efficiency when Illinois 547-1 or *V. cinerea* B9 were used as parents.

This study used two additional marker types for improved marker reliability, Cleaved Amplified Polymorphic Sequences (CAPS) and Simple Sequence Repeats (SSRs). CAPS are co-dominant molecular markers where a specific PCR product – such as sequence obtained from polymorphic bands of RAPD, RFLP and AFLP markers – is digested with an endonuclease. Variation between alleles is due to differences in restriction sites, and site specificity is due to PCR primers. SSRs are co-dominant markers based on the amplification of microsatellite regions in the genome. Variation between alleles is due to differences in the number of repeats, and the specificity of the locus is due to the use of primers targeting unique flanking regions. Currently, the most common molecular markers based on sequence amplification by PCR are SSRs. The first SSRs in grapevines date from 1993 (Thomas and Scott, 1993). SSRs were

used for germplasm characterization (Lamboy and Alpha, 1998), identification of varieties (Cipriani et al., 1994; Sefc et al., 1998a; Thomas et al., 1994) and parentage analysis (Bowers and Meredith, 1997; Meredith et al., 1999; Sefc et al., 1997; Sefc et al., 1998b). SSRs have also been used for linkage mapping and QTL analysis. Combined with RFLP, RAPD, AFLP and other sequence-specific markers, the inheritance of several traits has been studied including: disease resistance (Dalbó et al., 2001; Di Gaspero et al., 2007; Doucleff et al., 2004; Fischer et al., 2004; Grando et al., 2003; Riaz et al., 2006; Welter et al., 2007), seedlessness and seed weight (Cabezas et al., 2006; Doligez et al., 2002; Mejia et al., 2007), abiotic stress (Lowe and Walker, 2006), flavor (Riaz et al., 2004), and aroma (Doligez et al., 2006a).

Toward molecular marker standardization using Simple Sequence Repeats

SSR markers have several advantages over RAPD, AFLP or RFLP markers. As SSRs are PCR products from specific primers, they consume less time than hybridization-based markers and are more reproducible and specific than RAPDs. Characteristics such as co-dominance and high polymorphism make SSRs suitable for the study of heterozygous species. SSR markers developed from *V. vinifera* sequences could be useful for interspecific hybrids because they have a high degree of transportability between *Vitis* species (Di Gaspero et al., 2000; Lin and Walker, 1998). Transportability is necessary to identify linked genes or QTL in different genetic backgrounds, a feature necessary to combine different sources of resistance (Fischer et al., 2004). Compared to their predecessors, SSR markers can be located in physical maps, allowing anchoring within genetic maps (Doligez et al., 2006b) and facilitating the search for candidate genes closely located to resolved QTLs (Di Gaspero and Cattonaro, 2010). Because of these characteristics, it was desirable to develop high density maps, but the cost of accomplishing this goal for a single research group was

excessive (Riaz et al., 2004). For this reason, the international grapevine genetics community united efforts to develop a large number of markers in the Vitis Microsatellite Consortium (VMC). As result of this effort, 371 SSRs were cooperatively developed by 19 research groups coordinated by AgroGene S.A. (Adam-Blondon et al., 2004).

Lately, genetic maps that integrate VMC SSRs and other microsatellites previously published were developed to serve as a reference for the grapevine genetics community (Di Gaspero et al., 2005; Merdinoglu et al., 2005). First, the International Grape Genome Program (IGGP) adopted a map derived from 153 progeny plants of the cross of 'Riesling' x 'Cabernet Sauvignon'. This map was constructed with 152 SSRs and 1 polymorphic EST marker and comprised 1728 cM distributed in 20 LG (Riaz et al., 2004). A second reference map complemented this work, adding 123 more SSRs to a population derived from the cross of 'Syrah' x 'Grenache' and testing their heterozygosity in a selfed 'Riesling' population. This maps was constructed using 245 SSR markers and the number of LG was reduced to 19 (the number of chromosomes in the haploid genome) (Adam-Blondon et al., 2004). Recently, the integrated SSR map was updated with 515 markers from 5 mapping populations, with an acceptable marker order (Doligez et al., 2006b). This map was used as reference for the assembly of the first draft of the grapevine genome (Jaillon et al., 2007).

Even though SSRs have many advantages over their predecessors, the major drawbacks are low-throughput and high cost per marker. Some strategies to reduce this problem are multiplexing and multiloading, but neither allows high-throughput analysis (Merdinoglu et al., 2005).

Resistant Gene Analogs: Targets for the development of specific markers for disease resistance

Resistance gene analogs (RGA) are sequences with homology to known R-genes but whose function has not necessarily been proven. Nucleotide binding site (NBS) domains from R-genes are conserved among plants, and their sequences can be used to design degenerate primers to amplify, clone, and sequence homologous regions. Using this approach, RGAs were obtained from *V. amurensis* and *V. riparia* and then characterized among a panel of *V. vinifera* and other *Vitis* species. Sequences obtained showed high similarity to resistance proteins in other species (DiGaspero and Cipriani, 2002).

RGAs have been proven to be useful in the study of disease resistance genomics. RGAs obtained from a resistant BC_5 vine carrying the *Run1* locus were used to develop PCR-based markers. Combined with bulked segregant analysis, it was possible to locate three RGAs tightly linked to the *Run1* locus, which contains an RGA cluster (Donald et al., 2002). Similarly, RGAs were obtained from *V. cinerea* B9, *V. rupestris* B38 and the *V.* hybrid 'Horizon'. Their sequences were used to develop RGA-sequence tagged site (RGA-STS) molecular markers, and their segregation was studied in the 'Horizon' x Illinois 547-1 population, finding a correlation with a downy mildew resistance QTL for three of the markers (Mahanil et al., 2007).

A new consensus map was developed using 420 SSRs and 82 RGA markers distributed in two populations, 'Chardonnay' x 'Bianca' and 'Cabernet Sauvignon' x '20/3'. Combining SSR and RGA markers, 173 RGA loci were obtained and clustered in seven LG: 3, 7, 9, 12, 13, 18 and 19. Some of these RGA loci mapped in regions that were previously associated with disease resistance (Di Gaspero et al., 2007). While RGA markers provide a targeted candidate gene approach to identifying RGA clusters associated with disease resistance, this technique has some limitations. On one hand, degenerate amplification decreases the reproducibility of results. In addition, not all resistance genes will be associated with RGA clusters. Reproducibility can be improved by the use of SSRs as proxies for RGA clusters (Riaz et al., 2010), but still the discovery of novel resistance loci will require genotyping distributed across the genome.

SNPs: getting more information from a single nucleotide change

Single nucleotide polymorphisms (SNPs) are the smallest modification possible in the genome sequence: a change of a single base. SNPs are inherited in a co-dominant manner, are highly abundant, can be located in any region of the genome (including gene regions), and can be analyzed in a high throughput manner. Identified in genomic sequencing, expressed sequence tag (EST), and re-sequencing projects, SNPs are usually anchored to physical maps. In general a single SNP may be less informative than SSRs due to less polymorphism, but this drawback is compensated by their high density and the possibility of considering haplotype structures as a tag, in place of a single SNP (Rafalski, 2002).

In grapevines, the first studies of SNPs were realized by re-sequencing genes or EST fragments derived from a panel of *V. vinifera* and *Vitis* species, allowing the study of

haplotypes in *V. vinifera* (Owens, 2003) or in different *Vitis* species (Salmaso et al., 2005). SNP analysis was first scaled up to a high throughput manner in grapevine using the SNPlex™ platform. Using this technique, 80 validated SNPlex markers derived from EST sequences were analyzed across 368 accessions, with results typical of a highly polymorphic plant species with rapid LD decay. Because the SNPs were heterozygous at 30% of assayed loci, the authors estimated that 600 markers per parental map could be positioned from a set of 2000 SNPs, showing their potential usefulness in linkage analysis in grapevines (Lijavetzky et al., 2007).

Lately, information from the heterozygous 'Pinot noir' genome (Velasco et al., 2007), and the nearly homozygous grapevine reference genome (Jaillon et al., 2007), provided a useful source of information for SNP discovery. Electronic SNPs (eSNPs) obtained from the heterozygous 'Pinot noir' genome were used to develop a SNPlex assay and test 813 SNPs in a population derived from the cross of 'Syrah' x 'Pinot noir' (Pindo et al., 2008). The transferability of these SNPs was assessed through 69 grapevine accessions, validating their use within *V. vinifera*, but finding a limited application for non-*vinifera Vitis* species, where re-sequencing strategies are advised (Vezzulli et al., 2008). SNPlex™ technology has recently become obsolete, as Applied Biosystems no longer sells reagents for it; this is a problem not relevant to previous marker types.

Nowadays, the most comprehensive tool for SNP identification reported in grapevines is the Vitis 9K SNP array, containing about 9,000 SNPs developed by next-generation sequencing (NGS) of 17 *Vitis* accessions. Using this chip, it was possible to confirm the rapid LD decay in grapevines and distinguish between and within *V. vinifera* and *Vitis* species (Myles et al., 2010). The analysis of over 1,000 *V. vinifera* subsp.

vinifera and *V. vinifera* subsp. *sylvestris* accessions using the Vitis 9K SNP array aided in the examination of the domestication history and genetic structure of grapevine, showing a contra-distinction between the high diversity within *vinifera* and the low-diversity found among cultivars used for wine and table grapes (Myles et al., 2011).

Genotyping by sequencing (GBS)

Next-generation sequencing (NGS) platforms have revolutionized the application of genomics in plant genetics. Sequencing platforms vary in their technological approaches and data output, mainly in the number and length of reads. The Illumina Genome Analyzer (GA) has become the most widely used sequencing platform in grapevine genetics due to a relatively low-cost that generates hundreds of millions of short (currently 100 bp) reads. Single nucleotide polymorphisms can be identified from short reads generated by NGS, either by aligning to a reference genome or by *de novo* assembly (Nielsen et al., 2011). Using a reference genome based strategy, thousands of SNP markers have been discovered in crops of high value such as maize and soybean (Lai et al., 2010; Lam et al., 2010). In grapevines, NGS was used for SNP discovery to develop the Vitis 9K SNP Array described previously. The strategy included the establishment of Reduced Representation Libraries (RRLs) for 17 *Vitis* accessions by digestion using an endonuclease, followed by Illumina GA sequencing, alignment of reads to the grapevine reference genome (Jaillon et al., 2007), identification of single nucleotide polymorphisms, and statistical analysis to identify high-quality SNPs. The use of RRLs for genome complexity reduction allowed the enrichment of reads adjacent to the restriction site, improving the coverage of those areas to reduce the number of false SNPs (Myles et al., 2010).

Nowadays, GBS methodology provides a simpler but more robust procedure for SNP discovery using low-coverage genotyping of pooled samples. In this technique, a RRL from each sample is individually ligated to a unique barcoded adaptor, such that many uniquely barcoded samples (currently up to 384) can be pooled in one Illumina sequencing lane. Short reads sequences are then associated to each individual sample and analyzed for detection of polymorphisms (Elshire et al., 2011). The adoption of barcoded adaptors allows a dramatic reduction in the cost of each individual marker, while providing a high-resolution but low coverage genotyping, which should be sufficient to infer linkage on bi-parental populations and QTL mapping when a reference genome is provided (Davey et al., 2011). Low coverage will lead to missing data and errors when no reads are detected for a given site. To date, applications of GBS have been reported for homozygous crops as maize, barley and wheat where high-density maps of 34,000 and 20,000 SNPs were obtained for each (Poland et al., 2012). Due to its speed and lower cost, GBS seems to be a good strategy for the discovery of large numbers of SNPs on physical maps and the identification of loci affecting grapevine powdery mildew resistance from *V. rupestris* and *V. vinifera*.

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CHAPTER 2

PHENOTYPIC CHARACTERIZATION OF RESISTANCE TO ERYSIPHE NECATOR IN VITIS RUPESTRIS B38,'CHARDONNAY' AND ITS PROGENY

Introduction

The grapevine powdery mildew (PM) fungus, *Erysiphe necator,* is a major pathogen of cultivated grapevines (*Vitis vinifera)* and causes significant economic losses due to increased fungicide applications, and a decrease in yield and quality. Powdery mildew originated in the eastern United States, and spread to Europe and other grape-producing regions (Brewer and Milgroom, 2010). North American grapevines have co-evolved with this pathogen, and thus some species developed resistance to it. Several species, including *V. rupestris, V. riparia, V. aestivalis, V. cinerea* and *V. rotundifolia* have been used for production of resistant interspecific hybrid grapevines. For *V. rupestris*, the resistance mechanism of one *V. rupestris* genotype was classified as partial resistance (Feechan et al., 2010), and early studies suggested that PM resistance was controlled by a polygenic system (Boubals, 1961). However, foliar resistance to PM has been found to be variable among *V. rupestris* accessions (Cadle-Davidson et al., 2011a).

Individual PM conidiospores germinate, form an appressorium, and attempt to penetrate through the plant cuticle and cell wall. On a susceptible vine, the pathogen establishes an haustorium that obtains nutrients and secretes effectors that inhibit the immune response. Plant tissue is colonized by extension and branching of hyphae,

producing more appressoria and associated haustoria (Gadoury et al., 2012; Glawe, 2008). In contrast, resistant plants have developed mechanisms to prevent the invasion of biotrophic pathogens. Two layers of resistance are responsible for a majority of unsuccessful infection of non-host plants, but they also play a role in resistance of host plants. These two layers of resistance are based on the action of: 1) pre-formed physical and chemical barriers such as leaf surface wax and antimicrobial metabolites, and 2) Pathogen Associated Molecular Patterns (PAMP)-triggered immunity (PTI), (Jones and Dangl, 2006). PAMPs as chitin or other indicators of invasion induce defense responses that include the expression of pathogenesis-related (PR) genes, such as chitinases and β-glucanases (Giannakis et al., 1998), cytoskeleton rearrangements, and an increase in vesicle trafficking (Dry et al., 2009). As a result, induction of antimicrobial compounds and the reinforcement of the cell wall by papillae, callose, phenolic compounds and hydrogen peroxide are triggered (Nicholson and Hammerschmidt, 1992; Schmelzer, 2002; ThordalChristensen et al., 1997). In the vast majority of cases, this complex immune reaction is able to prevent plant colonization. Effector molecules released by fungal haustoria interfere with PTI and modify the interaction toward compatibility (Jones and Dangl, 2006). In response, plants have evolved a second layer of protection, effector-triggered immunity (ETI), characterized by a gene-for-gene interaction (Flor, 1942) mediated by R-genes that recognize a specific effector of PM, triggering a cascade of reactions. As such, attacked cells undergo programmed cell death (PCD), thus limiting pathogen growth and survival by eliminating biotrophic nutrient uptake (Jones and Dangl, 2006).

These mechanisms lead to two categories of disease resistance: 1) qualitative disease resistance, conditioned by a single gene that provides major effect protection, and 2) quantitative resistance due to the cumulative effect of several loci that contribute to resistance. While qualitative resistance is most commonly caused by the action of R-genes, the mechanisms of quantitative resistance are not well understood. Hypotheses for quantitative disease resistance can be classified as: 1) genes that regulate morphological or developmental phenotypes, 2) alleles of genes involved in basal defense, 3) chemical components of the plant-pathogen interaction, 4) loci involved in defense signal transduction, 5) weak forms of R-genes, or 6) unique forms of genes previously unidentified (Poland et al., 2009).

Loci encoding qualitative and quantitative resistance have been identified in grapevines. *Run1* (Barker et al., 2005; Bouquet, 1986; Pauquet et al., 2001), *Ren1* (Coleman et al., 2009; Hoffmann et al., 2008) and *Ren4* (Mahanil et al., 2011; Ramming et al., 2011a; Riaz et al., 2010) are dominant genes that provide qualitative resistance through restriction or slowing of hyphal, usually accompanied by PCD. Genotypes carrying those alleles rarely have macroscopic symptoms in field segregation tests, but some hyphal growth has been observed in laboratory assessments. Major QTL responsible for PM resistance have been identified, including *Ren2* from *V. cinerea* (Dalbó, 1998; Dalbó et al., 2001); *Run2.1* and *Run2.2* from *V. rotundifolia* (Riaz et al., 2010); and *Ren3* from the hybrid cultivar 'Regent', whose pedigree include *V. aestivalis, V. berlandieri, V. cinerea, V. labrusca, V. lincecumii, V. riparia* and *V. rupestris* (Fischer et al., 2004).

While qualitative genes are being characterized and utilized in grapevine breeding programs (Eibach et al., 2007; Katula-Debreceni et al., 2010), the resistance mechanisms associated with grapevine QTLs are still not fully understood. Moreover, quantitative traits add complexity to the breeding process, due to the difficulties of combining multiple genes, and because of the need for precise techniques to evaluate the quantitative phenotypic differences between genotypes that show segregation for those QTL (Poland and Nelson, 2011). In this work we sought to understand the genetics and mechanisms of PM resistance in *Vitis rupestris* B38 x *V. vinifera* 'Chardonnay' using natural infections in the field and single isolate inoculations in the screenhouse and on detached leaves, along with histological studies comparing the mechanisms of resistance in *V. rupestris* with the susceptible parent 'Chardonnay'.

Material and methods

Plant material

Seeds from the cross of *Vitis rupestris* B38 (resistant) and *Vitis vinifera* cv. 'Chardonnay' (susceptible) were obtained in 2008, which were stratified, germinated in a greenhouse, and planted to a field nursery (Geneva, NY) 0.46 m apart within rows and 1.52 m between rows in 2009. At the end of the growing season, vines were pruned and stored at 4 ºC in the dark over the winter. All 85 vines were planted in a single vineyard row in Geneva, NY in 2010. Vines were planted 1.2 m apart within rows and 2.74 m between rows. A control block was placed at the head of the row containing: *V. vinifera* cv. 'Chardonnay', 'Chancellor' (Seibel 5163 x Seibel 880), *V. rupestris* B38, 'Horizon' ('Seyval' x 'Schuyler'),the PM resistant genotype 'NY88.0514.04'. A susceptible control (*Vitis vinifera* cv. 'Chardonnay') was placed

after every 15 seedling vines. Downy mildew was controlled using the fungicide Captan 80WPG, which does not affect PM. A subset of the population was vegetatively propagated in 2010. Dormant cuttings were taken in early December, stored in a cold room for 5 weeks, and potted in a mixture of perlite:soil 3:1 with bottom heat (26 ºC). Vines were grown in a greenhouse under 16:8 (L:D) photoperiod at 27-30 ºC. Vines were pruned in order to assure uniform vegetative growth for experiments.

Powdery mildew isolates

PM isolates were provided by L. Cadle-Davidson from the sources described in Table 2-1. Leaves were surface sterilized with 1% sodium hypochlorite for 5 minutes, rinsed twice with sterile distilled water and placed in plates containing sterile 1% agar (Acros organics). PM isolates were propagated by tapping a leaf infected 2 weeks earlier over a newly plated 'Chardonnay' leaf. Plates containing infected leaves were sealed with parafilm and incubated at 25±2 ºC in a conditioned room with 16:8 (L:D) photoperiod.

Quantification of resistance to powdery mildew in the field

The study population was subjected to natural vineyard disease pressure. Symptoms on leaves were evaluated every year for three years. The first evaluation was conducted once in early October 2009 using a visual index tool that classified infected vines in only 3 categories (see Table 2-2). This system was replaced by a 1-9 scale established by the Organisation Internationale de la Vigne et du Vin (OIV) (IPGRI et al., 1997) (Table 2-3), in order to provide a better classification in the range where the differences between genotypes were observed. Moreover, use of the OIVscale allows standardizing field evaluations that are comparable between research groups (Hoffmann et al., 2008; Pauquet et al., 2001).

During 2010 and 2011, natural powdery mildew pressure in the vineyard was higher, and symptoms were observed earlier than in 2009. Field assessments were conducted between July and September in 2010 and 2011, using the OIV scale. In order to observe the progression of the disease during 2010, five field assessments were conducted, on August 9th, 20th and 30th and September 7th and 20th . By September 20th, susceptible checks were heavily infected. In 2011, two field assessments were conducted on July 12th and August 18th. Pearson's correlation coefficients (r) between assessments were calculated.

Table 2-1: Powdery mildew isolates: vine genotypes, resistance status, and location collected.

Name	Vine genotype	Vine resistance	Location
LNYM	*V. vinifera* 'Merlot'	Susceptible	Lockport, NY[1]
NY19	*V. vinifera* 'Chardonnay'	Susceptible	Burdett, NY[1]
NY90	*V. vinifera* 'Chardonnay'	Susceptible	Burdett, NY[1]
RoACS	*V. vinifera* 'Cabernet Sauvignon'	Susceptible	Hurdle Mills, NC[2]
Run1-137	Hybrid	*Run1* (Overcome)	Geneva, NY[3]
RoAwmus3	*V. rotundifolia* Wild type	Wild type	Hurdle Mills, NC[2]

References: 1 (Cadle-Davidson - unpublished), 2 (Frenkel et al., 2010), 3 (Cadle-Davidson et al., 2011b)

Table 2-2: Evaluation scale for powdery mildew on grapevine leaves (2009).

Score	*Symptom description*
0	Absent
1	Less than five small spots
2	Five to twenty spots, growing
3	Widespread PM, dense sporulation

Table 2-3: Evaluation scale (OIV) for powdery mildew in grapevine leaves as described in (IPGRI et al., 1997) (2010 and 2011)

Score	*Symptom description*
1	**Very low** (tiny spots or no symptoms, neither visible sporulation nor mycelium)
3	**Low** (Limited patches <2 cm in diameter, limited sporulation and mycelium; the presence of *Erysiphe* is only indicated by a slight curling at the leaf blade)
5	**Medium** (patches usually limited with a diameter of 2 – 5 cm)
7	**High** (vast patches, some limited, strong sporulation and abundant mycelium)
9	**Very high** (Very vast unlimited patches or totally attacked leaf blades, strong sporulation and abundant mycelium)

Quantification of resistance to a single isolate of powdery mildew in a screenhouse

Vines propagated from hardwood cuttings were used for screenhouse assays. From the 85 F_1 vines in the vineyard, only 53 were successfully propagated with at least 3 replicated vines each. A subset of 53 genotypes was randomly located in each of 3 replicated screenhouse blocks. Vines were infected with a single isolate of powdery mildew NY90 (Table 2-1) using heavily sporulating 'Chardonnay' vines as a source of inoculum. Distribution of conidiospores was achieved using a box fan for 2 min in each screenhouse. Disease progression was recorded as the percentage of leaf surface covered by PM at 7, 10 and 14 days post-inoculation. Scores for days 10 and 14 after inoculation were averaged and used for the analysis of resistance segregation.

Quantification of resistance to powdery mildew isolate LNYM on detached leaves

Leaves from a subset of 55 F_1 vines and parents grown in a greenhouse were used to quantify the segregation of resistance to the PM isolate LNYM (Table 2-1) under controlled *in vitro* conditions. Leaves at the 5^{th} position from two growing shoots per vine were labeled, detached, and placed in distilled water for transport to the laboratory. Leaves were surface sterilized with 1% sodium hypochlorite for 5 minutes, rinsed twice with sterile distilled water and placed adaxial side up in plates containing sterile 1% agar (Acros Organics). Inoculation was performed by spraying a suspension with 10^5 conidiospores/ml of 10-day old PM isolate LNYM (Table 2-1) in 0.001% Tween-20. Infected leaves were air-dried in a sterile hood before sealing with parafilm and incubated at 25 ± 2 ºC for 2 days in 16:8 (L:D) photoperiod . PM structures were stained using the Coomassie blue for visualization (Ramming et al., 2011a).

Penetration and microcolony success were quantified as follows. One hundred germinated conidiospores per leaf were observed with a compound microscope and classified as having: i) a primary hypha leading to an appressorium, ii) a single unbranched secondary hypha at least twice as long as the conidium, or iii) multiple or branching secondary hyphae. Penetration success rate was quantified as the number of conidiospores in categories (ii) and (iii) and microcolony success rate was quantified as the proportion of penetrating conidiospores (categories ii and iii) in category (iii). The experiment was repeated twice. Mean values were determined by ANOVA using vine genotype and experiment as predictors.

Characterization of the resistance response to multiple isolates of powdery mildew

The resistance mechanism of *V. rupestris* B38 and 'Chardonnay' was tested in response to 6 isolates of PM *in vitro*. Powdery mildew isolates were collected from *V. vinifera*, *V. rotundifolia* and hybrid grapevines (Table 2-1). Fully expanded leaves at the 4th position from *V. rupestris* B38 and 'Chardonnay' were collected from clean greenhouse vines and surface sterilized as described above. Leaf discs (1 cm^2) were obtained using a cork borer. From each genotype, 4 leaf discs were placed on 1% agar (Acros Organics) plates adaxial side up. For each PM isolate, 2 plates containing leaf discs from both parents were infected by touching 10-day old conidiating colonies to the adaxial leaf disc surface. Plates were incubated at 25$\pm$2ºC for 2 days in 16:8 (L:D) photoperiod. Hypersensitive response and fungal structures were evaluated by trypan blue staining (Feechan et al., 2010). Classification of 100 germinated spores per plate was done following the method described in the previous section. Additionally, for categories (ii) and (iii), programmed cell death (PCD) was recorded when grapevine cells bearing the fungal haustorium were completely stained (dead). Analysis of variance was performed using Vine genotype, PM isolate, and PM isolate * Vine

genotype as predictors. Least square mean differences were estimated using a Tukey HSD test.

Effect of leaf position on resistance response

Leaves from greenhouse vines were used for screening PM resistance *in vitro.* Leaf position was determined starting from the shoot tip with first unfold flat leaf designated as position one. Duplicate leaf samples from positions 2 to 5 from *V. rupestris* B38 and 'Chardonnay' were collected, surface sterilized and plated as described above. Inoculation was performed by spraying a solution with 10^5 conidiospores/ml of 10-day old PM isolate LNYM (Table 2-1) in 0.001% Tween-20. Infected leaves were dried in a sterile hood before incubation at 25±2 ºC for 2 days in 16:8 (L:D) photoperiod, PM structures were stained using the Coomassie dye (Ramming et al., 2011a). Penetration and microcolony success rate was determined following the technique described above. Analysis of variance was performed using vine genotype, leaf position and vine genotype * leaf position as predictors. Least square mean differences were estimated using a Student's t test.

Results

Segregation of PM resistance

Powdery mildew resistance segregated with a pattern of continuous variation whether tested: 1) under natural disease pressure in the field during 3 seasons (Figures 2-1A-C); 2) with single-isolate inoculations in a screenhouse (Figure 2-1D); or 3) under the single isolate laboratory inoculations (Figure 2-1E).

Under field conditions, *V. rupestris* showed no signs of infection during season 2010 and only low levels (OIV score of 3) by August 18th, while 'Chardonnay' became heavily infected early in the season with a median OIV score of 8 by August 20th in 2010 and August 1st in 2011) . In 2010 the progression of the disease was followed; differences between progeny vines were noticeable until mid August, but by September all genotypes were heavily infected, showing little variation between scores (Figure 2-2). Onset of symptoms was variable, becoming earlier every year: with differences among genotypes evident on October 2009, August 2010 and July 2011. Pearson's correlation coefficient (r) was 0.44 between 2009 (single data point) and early scores of 2010 (average of August 9th and 20th), 0.47 between early scores of 2010 and 2011 (average of July 12th and August 18th), and 0.15 between 2009 and 2011. Individual vines that showed low levels of PM during August 2010 also showed low values in August 2011. Among those, individual vines 03, 18, 35, 39, 44, 50, 66, 67, 70, 74 were also consistently lower than the progeny average later during the season.

In the screenhouse assay, mean PM leaf coverage at 14 days post inoculation confirmed the quantitative segregation of the trait among the progeny subset tested (Figure 2-1D). In the detached leaf assay, the resistance mechanism against a specific isolate of powdery mildew was assessed by microscopy as the ability to halt pathogen penetration and microcolony formation. Penetration success rate segregated in a quantitative manner within the population (Figure 2-1E), and microcolony success ratio values were largely located around 95-100%.

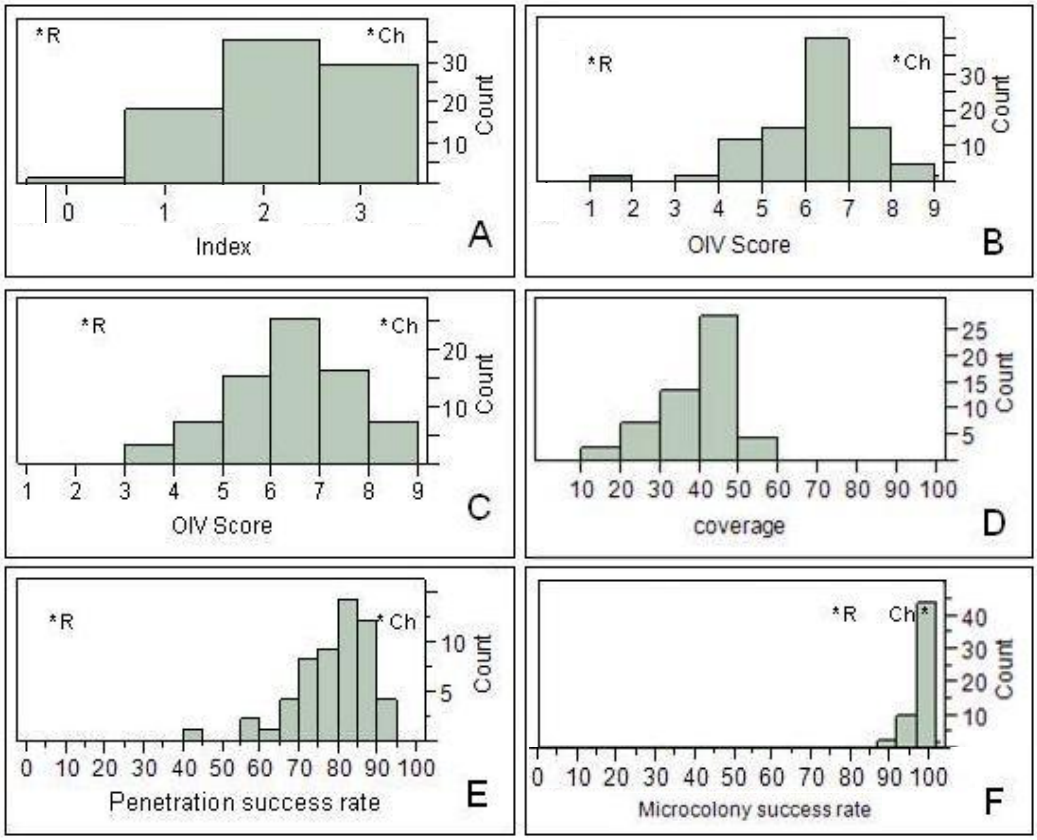

Figure 2-1: Frequency distribution of powdery mildew infection on the progeny of *Vitis rupestris* B38 (R) X *Vitis vinifera* 'Chardonnay' (Ch) : measured as A) Severity of naturally infected field vines in 2009 using a 0-3 severity index (Table 2-2), B) Mean severity of naturally infected field vines on 2 dates through August 20, 2010, using the OIV severity scale (Table 2-3), C) Mean severity of naturally infected field vines on 2 dates through August 18, 2011, using the OIV severity scale (Table 2-3), D) PM coverage of leaves in the screenhouse 14 days after inoculation by isolate NY90, E) Penetration success rate of isolate LNYM on detached leaves, and F) Microcolony success rate of isolate LNYM on detached leaves. Bars represent the number of F_1 individuals with values between the lower score (excluded) and higher score (included) on the x-axis.

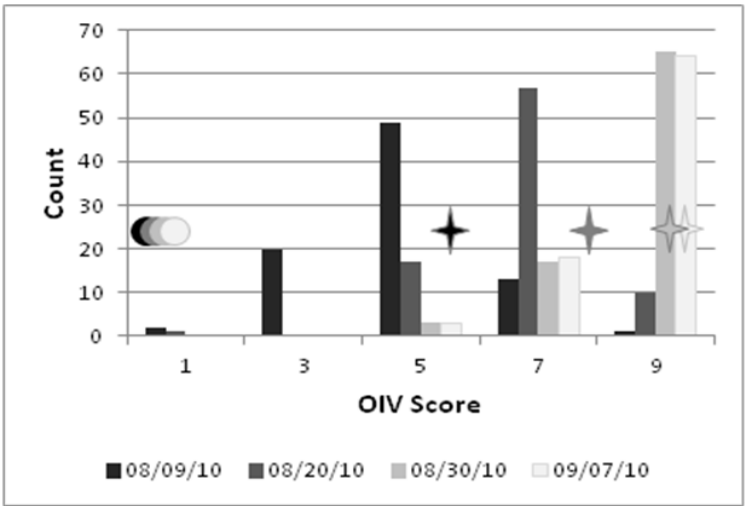

Figure 2-2: Disease progression following natural infections in a vineyard during 2010. Frequency distribution of powdery mildew scores (Table 2-3) on progeny at 4 time data points between August and September 2010. Parents (Circle *Vitis rupestris* B38 Star 'Chardonnay') are represented by the average values of control plants distributed in the vineyard.

Characterization of resistance of V. rupestris *B38 and* V. vinifera *'Chardonnay' to multiple isolates of Erysiphe necator.*

In order to test for race specificity, the resistance mechanism against six different isolates was characterized by microscopy in terms of: presence or absence of PCD, and infection stage progression on the resistant parent *V. rupestris* B38 and susceptible parent *V. vinifera* 'Chardonnay'. The overall response to *E. necator* isolates was significantly different between vine genotypes, PM isolate and vine genotype * PM isolate (p-value < 0.001 all). Within *V. rupestris* B38, penetration response varied significantly among isolates ($\alpha = 0.05$, Figure 2-3). While *V. vinifera* 'Chardonnay' showed no significant differences in susceptibility to the six isolates, the response of *V. rupestris* B38 showed significant differences. *V. rupestris* B38 was partial penetration resistance to *E. necator* isolates collected from *V. vinifera* sources, and strong penetration resistance to isolates collected from either *V. rotundifolia* or hybrid *Run1* vines. The response of the six *E. necator* isolates on *V. vinifera* 'Chardonnay'

showed penetration success rates ranging from 75% to 96% of germinated conidiospores, with 92% to 99%, being able to form microcolonies (Figure 2-3, top rows). *Vitis rupestris* B38 differential reactions to the PM isolates. Fungal isolates from *V. vinifera* sources were able to penetrate *V. rupestris* with percentages ranging from 41% to 68% of the germinated conidiospores, while an isolate obtained from *V. rotundifolia*, RoAwmus3, showed 24% penetration success rate. The least virulent *E. necator* isolate on *V. rupestris* B38 was Run1-137, collected from a hybrid vine with the *Run1* gene, with a 7% penetration success rate (Figure 2-3, lower rows). Microcolony success rate on *V. rupestris* ranged from 77% to 89%. PCD was not observed in any interaction (Figure 2-4).

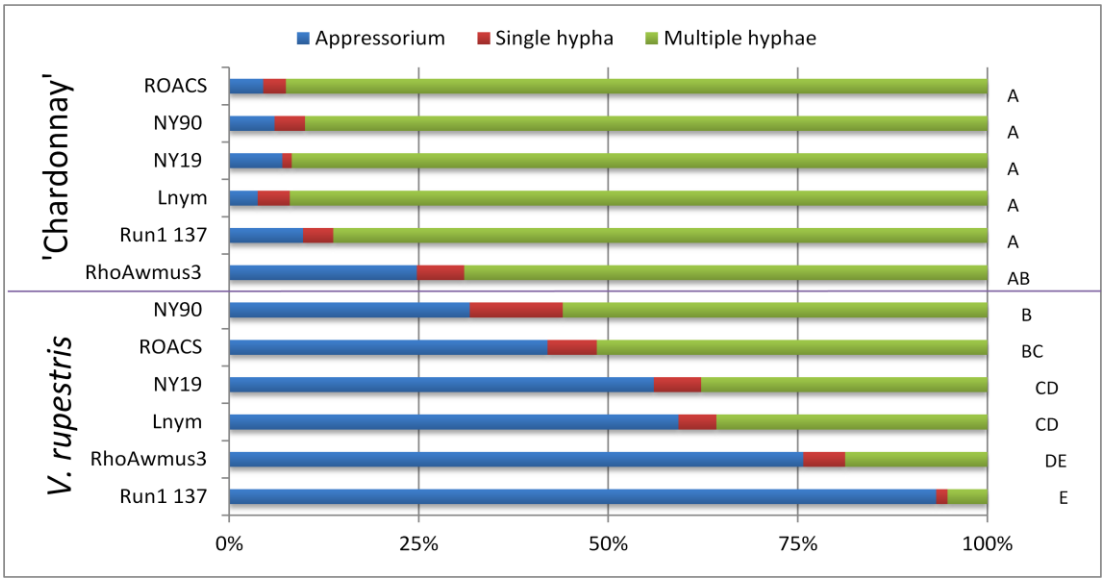

Figure 2-3: Quantification of the response of *Vitis vinifera* 'Chardonnay' and *Vitis rupestris* B38 to powdery mildew isolates ROACS, NY90, NY19, LNYM, Run 1-137 and RhoAwmus3. The ability of the vines to stop powdery mildew infection at 2 dpi was observed as the percentage of conidia that were able to progress from appressorium formation to the generation of a single hypha or the establishment of a microcolony with multiple hyphae. Non-overlapping letters to the right of the bars indicate statistically significant differences in penetration success rate at α=0.05.

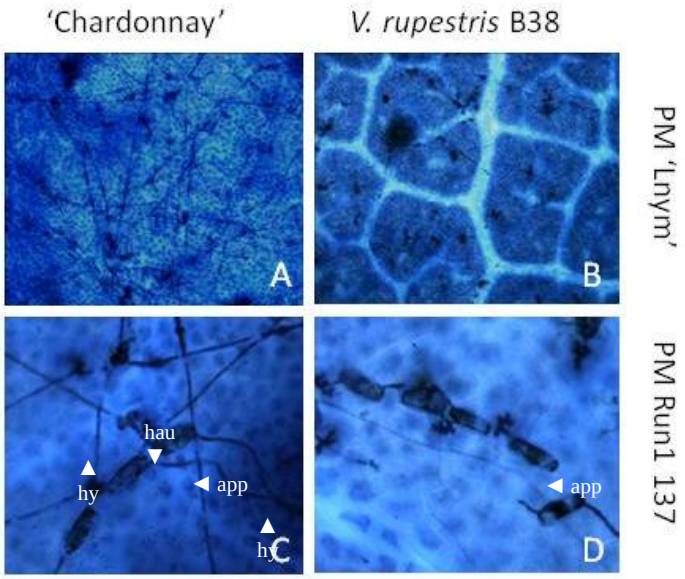

Figure 2-4: Interaction of powdery mildew isolates LNYM and Run1-137 on susceptible and resistant parents. A) Compatible interaction leading to microcolony formation and colonization of *V. vinifera* 'Chardonnay' by isolate LNYM, B) Partial penetration resistance, characterized by a lower frequency of microcolonies on *V. rupestris* B38 by isolate LNYM. C) Compatible interaction of isolate Run1-137 showing appressorium (◄), haustoria (▼) and multiple hyphae (▲) on *V. vinifera* 'Chardonnay'. D) Incompatible interaction of isolate Run1-137 and *V. rupestris* showing only appressorium formation (◄). PCD was not observed in any interaction.

Comparison of E. necator ontogenic resistance on V. vinifera 'Chardonnay' and V. rupestris B38 leaves

In order to determine the effect of leaf development on the resistance mechanism, the penetration success rate of *E. necator* isolate LNYM was assessed among the 2nd, 3rd, 4th and 5th leaves of *V. rupestris* B38 and *V. vinifera* 'Chardonnay' (Figure 2-5). At an early developmental stage (2nd, 3rd and 4th leaves) both parent genotypes showed indistinguishably high levels of penetration success rate. While penetration success rate was not affected by leaf development for both genotypes, ontogenic resistance became significant in the 5th leaf of resistant parent *V. rupestris* B38. Values for penetration success rate and microcolony success rate at each leaf position are given in Figure 2-5.

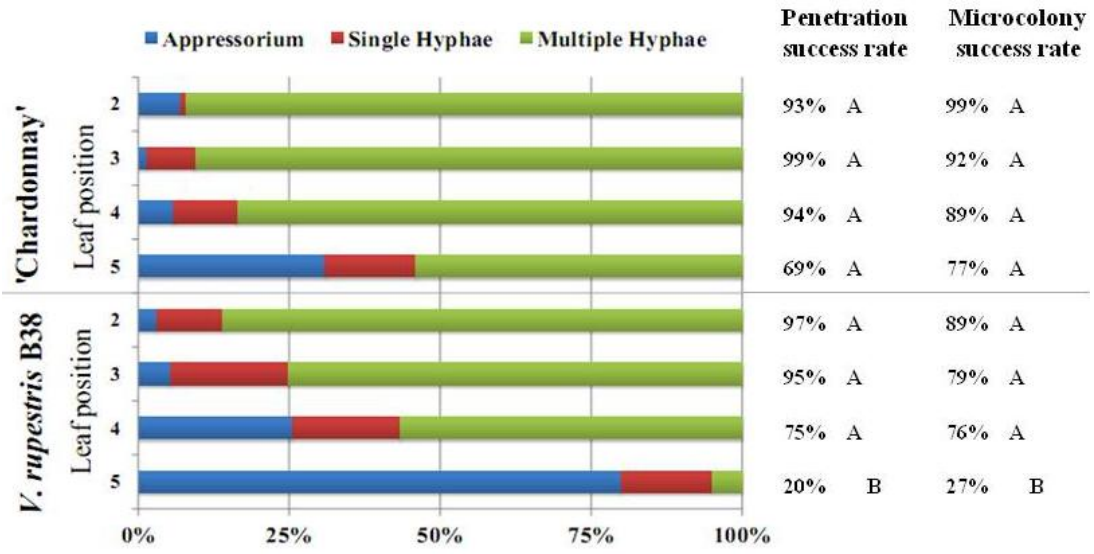

Figure 2-5: Effect of leaf position on powdery mildew LNYM development on parent genotypes *Vitis vinifera* 'Chardonnay' and *V. rupestris* B38. The ability of the vines to stop powdery mildew infection at 2 dpi was observed as the percentage of conidia that were able to progress from appressorium formation to the generation of a single hypha or the establishment of a microcolony with multiple hyphae. Penetration success rate was measured as percentage of conidia able to form a single hypha or multiple hyphae. Microcolony success rate was measured as the percentage of penetrating conidia that were able to form a microcolony. Non-overlapping letters to the right of the bars indicate statistically significant differences in penetration and microcolony success rate at α=0.05.

Discussion

We characterized *Vitis rupestris* B38 resistance to powdery mildew and found a pattern of continuous variation, supporting previous findings of polygenic inheritance (Boubals, 1961). Some factors involved in quantitative disease resistance were analyzed, showing evidence for enhanced penetration resistance, differential response to pathogen genotypes, as well as ontogenic resistance in leaves. Together, these findings allowed us to propose the hypothesis that *V. rupestris* resistance is due to the action of several mechanisms, making it an interesting source of resistance to powdery mildew.

Resistance in *V. rupestris* B38 x *V. vinifera* 'Chardonnay' progeny segregated in a quantitative manner. While the *V. rupestris* parent remained free of mildew or had low levels of mildew, the mean of the progeny showed high levels of infection by the end of the season, but lagged behind the susceptibility of 'Chardonnay' (Figure 2-2). Quantitative variation in the resistance response can be due to genetic and environmental effects. In the field, vines are subject to higher environmental variance. Compared with other crops where replicated field trials are often used, cost and labor requirements restrain the number of vines placed in a vineyard, limiting the use of replicate vines in favor of planting more genotypes. In this work powdery mildew resistance was evaluated over three years, observing a quantitative segregation pattern in every instance, with a reasonable correlation between seasons ($r = 0.44$ between 2009-2010 and 0.47 between 2010-2011). In order to reduce environmental variance, a subset of the segregating population was screened with a single isolate in a screenhouse and using detached leaf assays. Those results corroborated the continuous variation pattern found in field assessments.

In our experiments, *V. rupestris* resistance was strikingly different from the observed qualitative phenotypes of *Ren1*, *Ren4* or *Run1* vines. In field evaluations of *Ren1*, *Ren4* or *Run1* progeny, no macroscopic powdery mildew was observed on resistant vines, and 50% of testcross progeny were resistant (Hoffmann et al., 2008; Ramming et al., 2011a). Moreover, microscopic characterization of the *E. necator* interaction with vine genotypes bearing *Run1* or *Ren1* genes have shown the presence of programmed cell death (PCD) under appressoria associated with ETI responses triggered by R-genes. We did not find PCD for any isolates on *V. rupestris* B38. All these differences led us to reject the hypothesis that the *V. rupestris* B38 mechanism of

resistance to powdery mildew is controlled by a single R gene, and raised the question of which mechanisms contribute to its quantitative resistance.

Penetration resistance

Microscopically, the resistance mechanism on the 4th and 5th leaves of *V. rupestris* B38 reduced the penetration success rate of *E. necator* at 2 dpi. An increased number of conidiospores arrested at the stage of appressorium formation were observed (Figures 2-3, 2-4B and 2-4D), compared with 'Chardonnay' where the vast majority of germinated conidiospores were able to penetrate and create a microcolony (Figures2-3, 2-4A and 2-4C). Analysis of a subset of the population indicates that penetration success rate segregates in a quantitative pattern when analyzed against a single isolate at a specific leaf position, which may indicate the action of more than one allele related with this type of resistance. The method developed for the quantification of penetration success ratio facilitated the analysis of up to one hundred leaves under homogeneous conditions of infection with a single isolate showing a significant effect of genotype (p-value < 0.0001). Using this method it was possible to distinguish the 'Chardonnay' and *V. rupestris* B38 phenotypes with one order of magnitude of difference, from 92% in 'Chardonnay' to 9% in *V. rupestris* B38 (Figures 2-1E), which is similar to the range observed on the field.

Based on these results, we describe the *V. rupestris* B38 response on susceptible leaves as partial penetration resistance with no PCD observed, which is in overall agreement with (Feechan et al., 2010) where a genotype of *V. rupestris* was described as partially resistant, characterized by 44% of germinated spores failing to penetrate, and low levels of PCD. As opposed to other sources of resistance, like *Ren4*, the B38 penetration resistance was not complete, and a percentage of the conidiospores were

able to establish a compatible interaction that lead to formation of a microcolony (Figures 2-3 and 2-4B). Microcolony success rate on the 5th leaf was similarly high (80-90%) on Chardonnay and all progeny, but not on *V. rupestris* B38 (Figures 2-1 and 2-3). Also, microcolony success ratio segregating values were largely located around 95-100%, which correspond with the observation that microcolonies were determined once the isolate used in this analysis penetrated the plant's defenses.

Penetration resistance can be due to the effect of pre-formed barriers or PTI, being a component of non-host resistance. Quantitative variation of non-host resistance against powdery mildews has been previously shown in Arabidopsis (Ramonell et al., 2005). In *Vitis* species, the involvement of non-host resistance genes in the interaction with powdery mildew has been studied. At the transcriptome level, the susceptible *V. vinifera* responded with defense oriented reprogramming that failed to prevent PM infection, while the resistant *V. aestivalis* derived variety 'Norton' showed enhanced transcription of a few genes related with basal defense, which corresponded with a increased penetration resistance phenotype (Fung et al., 2008) . Other studies have focused on critical pathways for the non-host response such as actin cytoskeleton formation and vesicle trafficking. Both proved to play a dual role in powdery mildew interactions within *Vitis* species. While actin cytoskeleton formation and vesicle trafficking were required for non-host resistance, it was also shown that *E. necator* require those pathways to establish a compatible interaction with susceptible grapevines (Feechan et al., 2010). While we cannot hypothesize a mechanism for *V. rupestris* resistance without further experimental data, it is reasonable to expect that more than one mechanism is responsible for the quantitative segregation for penetration response observed in this population, with alleles from *V. rupestris* B38

and ‘Chardonnay’ contributing either to resistance or susceptibility to powdery mildew.

Race specificity

Variation in *V. rupestris* resistance to powdery mildew has been reported in previous investigations. In a detached leaf assay with *V. rupestris* B38 there was no infection at 21 days post inoculation with a single isolate, but natural infection was observed at two field locations (Cadle-Davidson et al., 2011a). In our experience, *V. rupestris* B38 showed no infection in the nursery (year 1) and in its first year of establishment in a vineyard (year 2), but low levels of infection in year 3 (Figure 2-1). Other explanations can account for the seasonal variation, such as maturity differences among the vines, locations, and canopy density. Some of the environmental variation observed may be explained by specialization or variation of pathogen populations. While the Cadle-Davidson et al.(2011) assessment was carried out in older vineyards that may host strains that had become specialized, our sources of inoculum were airborne in 2009 since we used newly propagated vines, but successful isolates could have overwintered on the experimental populations in each of the subsequent growing seasons. The changes in resistance scores over seasons, the evidence for races within powdery mildew populations (Ramming et al., 2011b), and the high genetic diversity found among isolates from NY (Brewer and Milgroom, 2010) justified the development of the single isolate assays that helped us to reduce the influence of experimental variables.

Although it was not possible to prove a classic gene for gene interaction of *V. rupestris* B38 with *E. necator*, at a microscopic level it was possible to observe significant differences in penetration success rate among PM isolates, PCD was not

observed. Isolates coming from *vinifera* sources were better adapted on *V. rupestris* B38 and showed significantly higher penetration success rates than those isolated from resistant vines *V. rotundifolia* or hybrid vines introgressed with the *Run1* gene (Cadle-Davidson et al., 2011b) (Figure 2-3). These results cannot be attributed to differences in the aggressiveness of the pathogen, as all powdery mildew isolates infected Chardonnay with similar success (Figure 2-3, group A). Moreover, the ease of infection on 'Chardonnay' leaves regardless of the isolate may indicate some susceptibility factor that allowed *E. necator* to overcome *vinifera* basal immunity at an earlier layer of defense than *V. rupestris*, where the relation with the pathogen showed a race-specific component, but with a minor effect on the overall level of resistance. It was also noticeable that a high level of resistance was observed in the interaction of *V. rupestris* B38 and the *E. necator* isolate from a vine carrying the *Run1* gene (Figure 2-3 and 2-4D), suggesting that a tradeoff may exist between the acquired ability to infect *Run1* vines and *V. rupestris*. A relevant application of these observations is that pyramiding *Run1* resistance with *V. rupestris* B38 resistant alleles may improve *Run1* durability.

R-genes have been related to quantitative disease resistance (QDR) in several crops, either by identification through cloning or co-localization of NBS-LRR Resistant Gene Analogs with QTLs (Poland et al., 2009). Defeated R-genes are still able to reduce the level of disease when compared with a genotype that does not carry the R-gene allele. This is exemplified in the *E. necator* - *Vitis* pathosystem by *Run1* (Cadle-Davidson et al., 2011b). This rapid overcoming of the resistance allele is an example of the dynamic interaction observed in this pathosystem. Even when we cannot be certain that R-genes are responsible for *V. rupestris* resistance without segregation tests or mapping of QTL, these results suggest that race-specific interactions between

V. rupestris B38 and *E. necator* may be playing a role in the quantitative resistance observed, and our results in controlled conditions support our field observations of reduced resistance over time, which could be due to selection for isolates with improved penetration efficiency.

Foliar ontogenic resistance

The ability of *E. necator* to penetrate and establish a microcolony was also influenced by leaf maturity, as measured by leaf position. Even though ontogenic resistance was observed for both genotypes (Figure 2-5), the reduction in successful penetration happened earlier in *V. rupestris* B38, reaching up to a 3-fold difference in penetration success rate when compared with 'Chardonnay'. This observation was useful to maximize differences in the detached leaf assay designed for this study.

Quantitative adult plant resistance has been widely used in wheat breeding. Its effect in *B. graminis* has been described as a slowing of its capability to infect adult wheat, propagate and sporulate, providing an incomplete resistance (Keller et al., 1999; Liu et al., 2001; Tucker et al., 2007). In grapevines, the effect of developmental stage on powdery mildew resistance has been reported: from broad observations on field assessments (Evans et al., 1996; Gadoury et al., 2001; Valdés-Gómez et al., 2011) to detailed characterization of the responses of grape berries (Ficke et al., 2004; Gadoury et al., 2003; Gee et al., 2008) and leaves (Doster and Schnathorst, 1985; Smith et al., 2008). Even though the mechanism responsible for the developmental effect on leaves and berries has not been elucidated, in both cases it has been shown that older tissues are increasingly more resistant to *E. necator* penetration. The fact that *V. rupestris* B38 showed this developmental effect earlier than 'Chardonnay' might play a role in

the quantitative disease resistance observed in the field as *V. rupestris* vines would have fewer susceptible leaves per shoot than 'Chardonnay'.

The powdery mildew resistance mechanism in *V. rupestris* can be described as a quantitative resistance with components including: penetration resistance, which reduces pathogen penetration rate without slowing hyphal growth or PCD; race specific interaction with powdery mildew isolates; and a developmental effect that raises penetration resistance on older leaves. Several studies have demonstrated the effect of host-derived secondary metabolites on grapevine powdery mildew (Feechan et al., 2010; Fung et al., 2008). These compounds have not been analyzed in this work, hence we cannot rule out a role for host secondary metabolites in reducing the progression of the disease in *V. rupestris* B38.

In order to dissect complex traits such as disease resistance, it is necessary to analyze the inheritance of the traits and the plant-pathogen interaction from different and complementary perspectives. Phenotyping and genotyping are two characterizations that allow mapping complex traits in the genome. In this work we presented a method that allowed us to quantify the response of the progeny against a single isolate, at a precise leaf position. In this sense, we think that our detached leaf assays can be useful as a quantitative screening system for the mechanism of powdery mildew resistance. This approach does not necessary mimic what happens in the field, and by itself may not be an effective tool for breeding selection for all resistance sources. The value of a race-specific quantification assays is that they may allow testing for segregation at an early stage and molecular mapping of specific QTLs that add effects to the overall trait, helping with the introgression of these traits by marker assisted selection.

On the other hand, for a crop like grapevine that is deployed in the field for 15 to 50 years, and a pathogen like *E. necator* that has been shown to have the ability to overcome R-gene resistance, penetration resistance – either by pre formed barriers or PTI – combined with R-genes may be a better long-term. The elements of the quantitative resistance from *V. rupestris* B38 described in this chapter segregated and lost their efficacy when combined with 'Chardonnay' alleles. Our single isolate observations suggest that 'Chardonnay' has enhanced susceptibility to all isolates tested, which may mask the effect of *V. rupestris* B38 alleles. Overall, the *V. rupestris* B38 resistance seems to be due to the action of several mechanisms which is interesting for long-term resistance, but they need to be combined with other sources to reach a desirable level. Based on the observations of the response to the powdery mildew isolates from *V. rotundifolia* and *Run1* vines, *V. rupestris* B38 alleles may be useful for combining with *Run1* in order to extend the durability of this resistant gene, but pyramiding 2 or more resistance genes cannot be obtained by phenotypic selection if resistance from two genes combined is similar to that from a single gene. Moreover, the introgression of quantitative traits must face the challenge of combining several QTL of small effects through breeding cycles that could take as much as 3 years. In this sense, the development of molecular tools such as molecular markers and genetic maps, and its combination with careful phenotypic analysis, would allow researchers to map and identify to introgress resistance alleles into a cultivated background.

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CHAPTER 3

GENOME WIDE ASSOCIATION ANALYSIS OF ERYSIPHE NECATOR RESISTANCE IN VITIS RUPESTRIS B38 AND VITIS VINIFERA 'CHARDONNAY'

Introduction

Powdery mildew resistance differs among and within Vitis species. While most European Vitis vinifera cultivars are susceptible to powdery mildew, North American species such as V. rupestris, V. riparia, V. aestivalis, V. cinerea and V. rotundifolia are generally considered to be resistant (Alleweldt et al., 1991; Pearson, 1988), as well as some Asian species such as V. romanetii and V. davidii (Wan, 2007; Wang et al., 1995). Studies on powdery mildew resistance in Vitis species and interspecific hybrids have found 2 main categories of resistance: dominant single locus and quantitative resistance. Single locus resistance has been identified, localized and characterized from different sources: Run1 introgressed from V. rotundifolia localized on chromosome 12 (Barker et al., 2005; Donald et al., 2002; Pauquet et al., 2001) and Ren1 from V. vinifera cultivars 'Kishmish vatkana' and 'Dzhandzhal kara' localized on chromosome 13 (Coleman et al., 2009; Hoffmann et al., 2008). Both have been co-localized with NBS-LRR sequences and their resistance mechanisms correspond with the action of one or more clustered R-genes. Another major gene, Ren4, introgressed from V. romanetii has been localized on chromosome 18, also near NBS-LRR sequences (Mahanil et al., 2011; Riaz et al., 2010), but confers non-race-specific penetration resistance (Ramming et al., 2011).

Early studies of the inheritance of powdery mildew resistance in V. rupestris and V. labrusca suggested that the trait was controlled by a polygenic system (Boubals, 1961). In grapevines some major quantitative trait loci (QTL) for powdery mildew

resistance have been localized, but the mechanisms of resistance still remain unknown. Some of these QTLs for powdery mildew resistance are: Ren2 on chromosome 14 from V. cinerea B9 (Dalbó, 1998; Dalbó et al., 2001), Ren3 on chromosome 15 in the hybrid cultivar 'Regent', whose pedigree includes the species V. aestivalis, V. berlandieri, V. cinerea, V. labrusca, V. lincecumii, V. riparia and V. rupestris (Fischer et al., 2004), and Run2.1 and Run2.2 on chromosome 18 from V. rotundifolia 'Magnolia' and 'Trayshed,' respectively (Riaz et al., 2010).

Dominant single loci and QTL control different mechanisms of plant-pathogen interactions. In grapevine powdery mildew, dominant single locus resistance is typically related to Effector Triggered Immunity (ETI) characterized by a gene-for-gene interaction between the grapevine R-gene and an effector molecule from the pathogen (Feechan et al., 2010; Jones and Dangl, 2006). This kind of resistance gives, in most cases, complete protection against specific races of the pathogen but also induces strong selective pressure over pathogen populations, which could potentially evolve to overcome resistance (Cadle-Davidson et al., 2011; Peressotti et al., 2010). However, quantitative resistance is described as less likely to be overcome as it is due to the accumulated effect of several loci that might play a role at different stages of the plant immune response, such as plant morphology and developmental phenotypes, basal immunity, chemical interactions, weak R-genes or defense signal transduction (Poland et al., 2009).

The introgression of these traits into a cultivated background must overcome biological and genetic barriers related to the breeding process. Wild species harbor an assortment of undesirable flavors and aromas, and thus introgression of powdery

mildew resistance alleles is usually accompanied by off-flavors and traits that are not always appreciated by consumers. Besides the quality issue that affects grapevine breeding for fruit production, the genetic nature of the resistance mechanism also adds complexity, as quantitative traits may need the introgression of several minor loci in order to reach the desired level of resistance. Use of major R-genes is then an easier choice for achieving resistance in the short term, but it is associated with short durability (McDonald and Linde, 2002). For a vineyard that is expected to be productive for 15 to 20 years, durable resistance is desired. Marker assisted selection helps to overcome these constraints (Dalbó et al., 2001). Molecular markers linked to the powdery mildew resistant allele of interest can be used to select resistant genotypes, avoid susceptible alleles, combine traits, reduce linkage drag and stack several loci, while markers distributed along the genome may help to recover the cultivated background (Dalbó et al., 2001; Di Gaspero and Cattonaro, 2009; Eibach et al., 2007; Mahanil et al., 2011). In order to achieve this goal, it is necessary to develop a set of informative markers distributed along the genome as well as markers tightly linked to resistance loci that can be identified by bulk segregant analysis, QTL mapping or Genome-wide Association Study (GWAS).

The relevance of molecular markers to grapevine genetics has driven the development of a common set of markers and genetic maps (Adam-Blondon et al., 2004; Doligez et al., 2006; Riaz et al., 2004). Physical maps have also been developed, such as the Vitis vinifera reference genome for a nearly homozygous selection, PN40024 (Jaillon et al., 2007), 'Cabernet Sauvignon' (Moroldo et al., 2008) and the heterozygous cultivar 'Pinot noir' (Velasco et al., 2007). Nowadays, the International Grape Genome Program refers to an integrated map containing more than 400 SSR markers (IGGP, 2012) in addition to a dense genetic linkage map anchored to the ‘Pinot noir’ genome

(Troggio et al., 2007). Lately, Next-Generation Sequencing (NGS) has been employed for the construction of a dense SNP chip with an array of about 9,000 SNPs derived from 10 cultivated V. vinifera varieties and 7 wild species which have allowed the characterization of the genus Vitis (Myles et al., 2010) and the study of its genetic structure and domestication history (Myles et al., 2011).

Whole genome sequencing and NGS have boosted genomic research in several plant species, where the most commercially valuable crops have lead the application of these tools, widening the set of techniques available for genomic research (Deulvot et al., 2010; Morrell et al., 2012; Poland et al., 2012; Xie et al., 2010). In grapevines, the Illumina Genome Analyzer platform has become the most widely used sequencing tool (Myles et al., 2010; Picardi et al., 2010; Zenoni et al., 2010) due to a relatively low-cost for the generation of hundreds of millions of short (currently 100 bp) reads. Single nucleotide polymorphisms (SNP) markers can be identified from short reads generated by NGS, either by aligning to a reference genome or by de novo assembly (Nielsen et al., 2011). The adoption of techniques, such as Reduced Representation Libraries (RRLs) (Barbazuk et al., 2005; Van Tassell et al., 2008; Wiedmann et al., 2008) to lower the genome complexity, and bar-coded adapters to allow pooling hundreds of samples in a single sequencing lane, has significantly reduced the cost per marker (Elshire et al., 2011).

Nowadays, Genotyping-by-sequencing (GBS) provides a simple and robust procedure for SNP discovery through pooled barcoded RRL, Illumina sequencing and SNP calling based on alignment of short reads. As a result, low coverage genotyping is obtained (Elshire et al., 2011), which should be sufficient to infer linkage in bi-parental populations and for QTL mapping (Davey et al., 2011). Due to its speed and

lower cost, GBS seems to be a good strategy for discovery of SNPs suitable for developing dense map in a grapevine segregating population in a short period of time. The goals of the current study were to use GBS for map development in an F1 grapevine population and to combine the results with phenotypic quantification to identify loci associated with powdery mildew resistance. The results may allow identification of candidate genes and help grapevine breeding process through marker assisted selection.

Methods

Plant material

A bi-parental population of 85 individuals was obtained from the cross of *V. rupestris* B38 (resistant) and *V. vinifera* 'Chardonnay' (susceptible) as described in chapter 2.

Powdery mildew isolates

Powdery mildew isolates were obtained and maintained as described in chapter 2.

Rating and quantification of powdery mildew resistance

Powdery mildew resistance was evaluated in the field under natural conditions or on inoculated detached leaf assays as described in the chapter 2.

DNA preparation

DNA was extracted from two unexpanded leaves (less than 1 cm^2) from each parent and progeny vine using either the DNeasy® 96 Plant Kit (Qiagen) or the DNeasy® Plant Mini Kit (Qiagen) and quantified using Quant-iT™ PicoGreen ® dsDNA Kit (Invitrogen). Whole genome amplification (WGA) was performed using 10 ng of

DNA and the Illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare). Amplified DNA (1.0 µg) was plated and dried using a vacuum centrifuge.

Library preparation

Dried DNA was resuspended and digested at 75ºC for 2 hours using a 10 µl mix containing 4 units of *ApeKI* restriction endonuclease (New England Biolabs, Ipswich, MA) and 1 µl of 1X NEBuffer 3, then cooled on ice. Dried barcoded adapters (Elshire et al., 2011) were resuspended by pipeting 40 µl of a ligation mix containing 4 units of T4 DNA Ligase (Promega) in 2X rapid ligation buffer (Promega). Resuspended barcoded adaptors were mixed with cooled digested DNA. Ligation was performed at room temperature for 60 minutes, heated to 65ºC for 30 minutes to inactivate the enzyme and finally cooled on ice prior to the next step. Ligation products were purified using 90 µl of Agentcourt AMPure (Beckman Coulter) beads per the manufacturer's instructions and eluted in 35 µl of EB Buffer (Qiagen). PCR was performed by adding the following to 10 µl of the eluted ligation product: 2 µl of dNTPs (10 mM), 5 µl of Primer mix (5' AATGATACGGCGACCACCGAGATC ACTCTTTCCCTACACGACGCTCTTCCGATCT 3' and 5' AAGCAGAAGACG GCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT 3', 5 µM each), 10 µl of 5X buffer and 0.5 µl of Phusion DNA polymerase (Finnzymes). Amplifications were performed by initial denaturation at 98ºC for 30 s, 18 cycles of 10 s at 98ºC, 30 s at 65ºC and 30 s at 72ºC and a final step of 72ºC for 5 min. PCR products were purified using Agentcourt AMPure beads and EB Buffer as described above. Libraries were quantified using Quant-iT™ PicoGreen ® dsDNA Kit (Invitrogen). Libraries with concentration lower than 10 ng/µl were repeated either by starting from new leaf tissue or repeating the PCR step in triplicate and pooling 3 PCR products during the last AMPure elution step. Quality and size of a representative

sample of libraries were determined using an Experion™ Automated Electrophoresis System (Bio-Rad).

Pooling and Sequencing

Two pools of up to 48 samples were prepared using barcodes as described previously (Elshire et al., 2011). Samples were quantified by Quant-iT™ PicoGreen ® dsDNA Kit (Invitrogen) and normalized before pooling. Both pools were sequenced on Illumina instruments at the Cornell University Life Sciences Core Laboratories Center using single-end 100bp: the first pool on an Illumina Genome Analyzer III (GA3) and the second on an Illumina HiSeq 2000.

SNP calling and mapping

Hapmap files were created by analyzing and filtering raw sequence data using the TASSEL 3.0 Genotyping-by-Sequencing (GBS) pipeline (Glaubitz et al., 2012). Reads were converted to tags (roughly representing an allele) by performing the following actions: 1) Keeping reads that match one of the barcodes; 2) trimming reads to 64 bp, cutting off the barcode, cut site remnant and bp excess; and 3) truncating reads that have an internal cut site in order to discard reads that were partially digested, DNA chimeras or restriction fragments shorter than 64 bp. SNPs were called by aligning tags to the 12X grapevine reference genome (Jaillon et al., 2007) for chromosomes 1 to 19 (complete and random) and random chromosome 20 using standard Burrows – Wheeler Aligner (BWA) (Li and Durbin, 2009) parameters. Tags located at the same position on the reference genome were aligned against each other, allowing identification of SNPs. Standard parameters were used for filtering as follows: 1) Reads with greater than two SNPs relative to the reference were discarded; 2) Reads aligning to multiple locations on the reference genome were discarded; and 3) small

insertions and deletions (INDELS) were discarded. Finally, the following parameters were altered to match population characteristics: minimum locus coverage (proportion of vines with a genotype) of 0.4; number of alleles 2; and minimum minor allele frequency (mnMAF) was set to 0.15 and maximum minor allele frequency (max MAF) to 0.35, due to the expectation of minor allele frequency of 0.25 in markers AB:AA. These filters resulted in a 42k SNP set.

Map analysis and Genome-Wide Association Study (GWAS)

More stringent filters were used for GWAS. Sites with less than 20% missing data (ie. minimum locus coverage of 0.8) were retained and about 4% of the SNPs were inferred to heterozygosity when only the minor allele was sequenced. Sites were further filtered out to retain SNPs with MAF 0.25 +/- 0.05. These filters resulted in a 16k SNP set.

The 16k SNP set was used for several analyses. A bin analysis was used to estimate the SNP density across the genome, by counting the number of SNPs in contiguous windows of 500 kb. Linkage disequilibrium of markers coming from both parents was analyzed using the rapid permutations option of TASSEL 3.0 within the full matrix of SNPs on each chromosome. Calculations of p-values used a two-sided Fisher's exact test. A General Lineal Model was used to test association between genotype and each phenotype data set in TASSEL 3.0, which runs permutation tests (Anderson and Braak, 2003).

Results

Phenotypic quantification of powdery mildew resistance

Resistance to powdery mildew in the population under study was evaluated for 3 years in the field under natural disease pressure and in a single isolate detached leaf assay. In all cases, powdery mildew resistance followed a quantitative variation pattern with the distribution skewed toward the susceptible parent when evaluated in the field. Methods and detailed results are presented on chapter 2.

Genotyping: Sequencing, SNP calling and SNP selection.

Averages of 712,400 reads per vine (n= 46) and 2,787,000 reads per vine (n= 42) were obtained per sequencing batch, for Illumina GA3 and HiSeq, respectively. The distribution of the number of reads obtained per vine sample is shown in Figure 3-1. SNP calls were based on alignments to the 12X grape reference genome (Jaillon et al., 2007), obtaining 42,172 biallelic SNPs with minor allele frequency (MAF) of 0.25 $\pm$ 0.10 (42K set). Of the 42K SNP set, 39,802 SNPs (94%) were located on assembled chromosomes 1 to 19, with a Pearson's correlation coefficient (r) of 0.90 between marker number per chromosome and physical chromosome size. After discarding sites with more that 20% missing data and inferring heterozygous calls (4%), the number of selected SNPs was reduced to a set of 16,834 SNPs (16K set) with MAF 0.25 $\pm$ 0.05. From the 16K SNP subset, 15,938 SNPs (95%) were located on assembled chromosomes 1 to 19, with Pearson's correlation coefficient (r) of 0.82 between marker number per chromosome and physical chromosome size. The numbers of SNPs per chromosome for 42K and 16K sets are show on Figure 3-2.

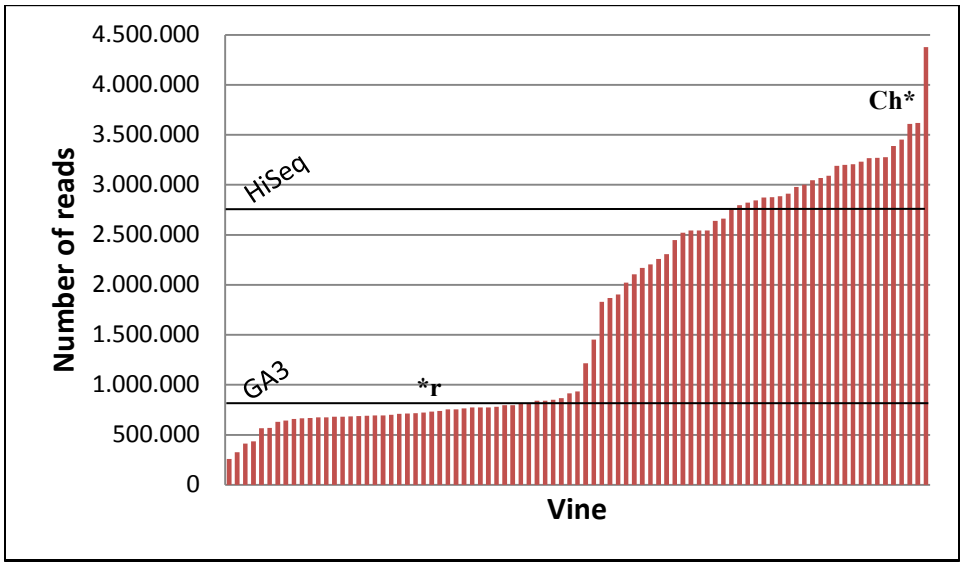

Figure 3-1: Distribution of number of reads per vine. The numbers of reads obtained per vine are shown in ascending order. Numbers of reads obtained for parents *Vitis rupestris* B38 (r) and *Vitis vinifera* 'Chardonnay' (Ch) are also indicated. Sample average values for each pooled library are indicated by a horizontal line.

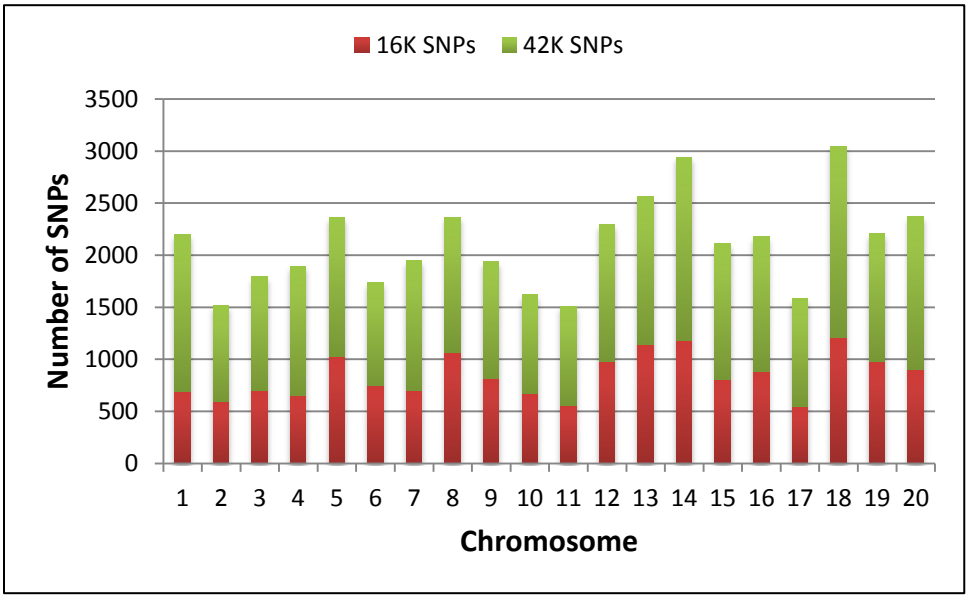

Figure 3-2: Distribution of 42K and 16K SNP sets per chromosome. The direct output from TASSEL 3.0 pipeline was a set of 42K SNPs (green bars) with minor allele frequency (MAF) of 0.25± 0.1. After selecting for maximum missing data of 20%, MAF of 0.25 ± 0.05 and inferring that rare allele homozygote are actually heterozygous, a subset of 16K SNPs (red bars) were chosen for further analysis.

SNP distribution, density and LD pattern

Chromosomes averaged similar marker densities, ranging from one SNP per 36 kb on chromosome 4 to one SNP per 21 kb on chromosome 8. In order identify local increase or decrease in marker density, the grapevine reference genome was divided into 863 bins of 500 kb and the number of SNPs within each bin was determined. Distribution analysis of the 16K SNP set (Figure 3-3) showed a mean value of 18 SNPs per 500 kb bin with standard deviation of 11.6 SNPs per 500 kb bin.

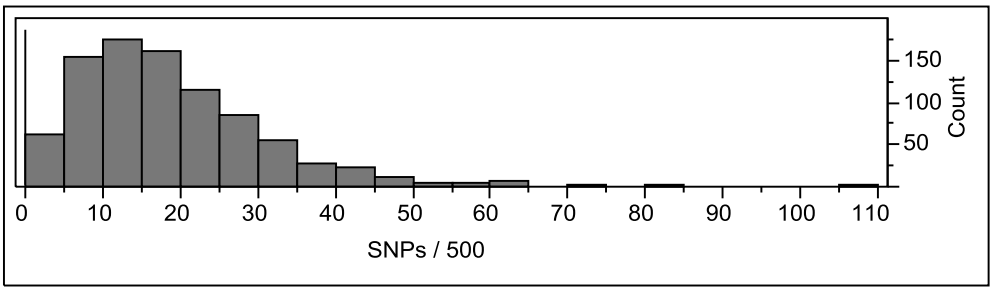

Figure 3-3: Distribution of number of SNPs located within each bin of 500 kb. Position of the 16K SNPs set was based on the 12X grape reference genome (Jaillon et al., 2007).

While 606 (70%) of the 500 kb bins had a moderate number (10-50) of SNPs (Figure 3-4), there were 240 500 kb bins (28%) with fewer than 10 SNPs (Figure 3-4, green), 7 500 kb bins with zero SNPs (Figure 3-4, grey) and 10 500 kb bins with higher SNP density, from 51 up to 106 SNPs (Figure 3-4, red).

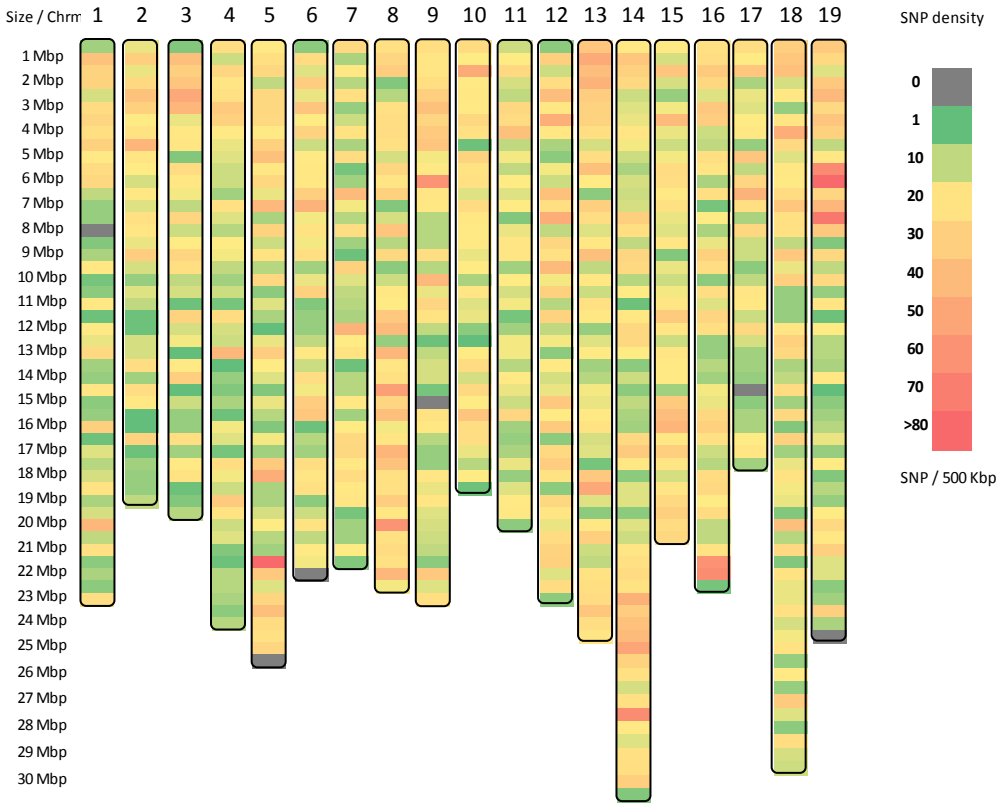

Figure 3-4: SNP density across *Vitis vinifera* 12X reference genome. From 16K SNP set, 95% of markers were distributed among assembled chromosomes 1 to 19, with a mean value of 17 SNPs / 500 kb (yellow). Each block represents consecutive bins of 500 kb from the *Vitis vinifera* 12X reference genome. Color scale on right represents the numbers of SNPs located within each 500 kb bin. Areas with no markers are depicted in grey color. Correlation (r) between chromosome size and number of markers per chromosome was 0.82.

Linkage disequilibrium analysis showed significant correlation (up to D' = 1 and p-values lower than 0.001, red regions in Figure 3-5) between markers within assembled chromosomes (1 to 19). The extent of linkage disequilibrium was variable among chromosomes and some markers did not correlate with any other markers located on the same physical chromosome (D' lower than 0.01 and p-values higher than 0.01, light blue and white regions in Figure 3-5).

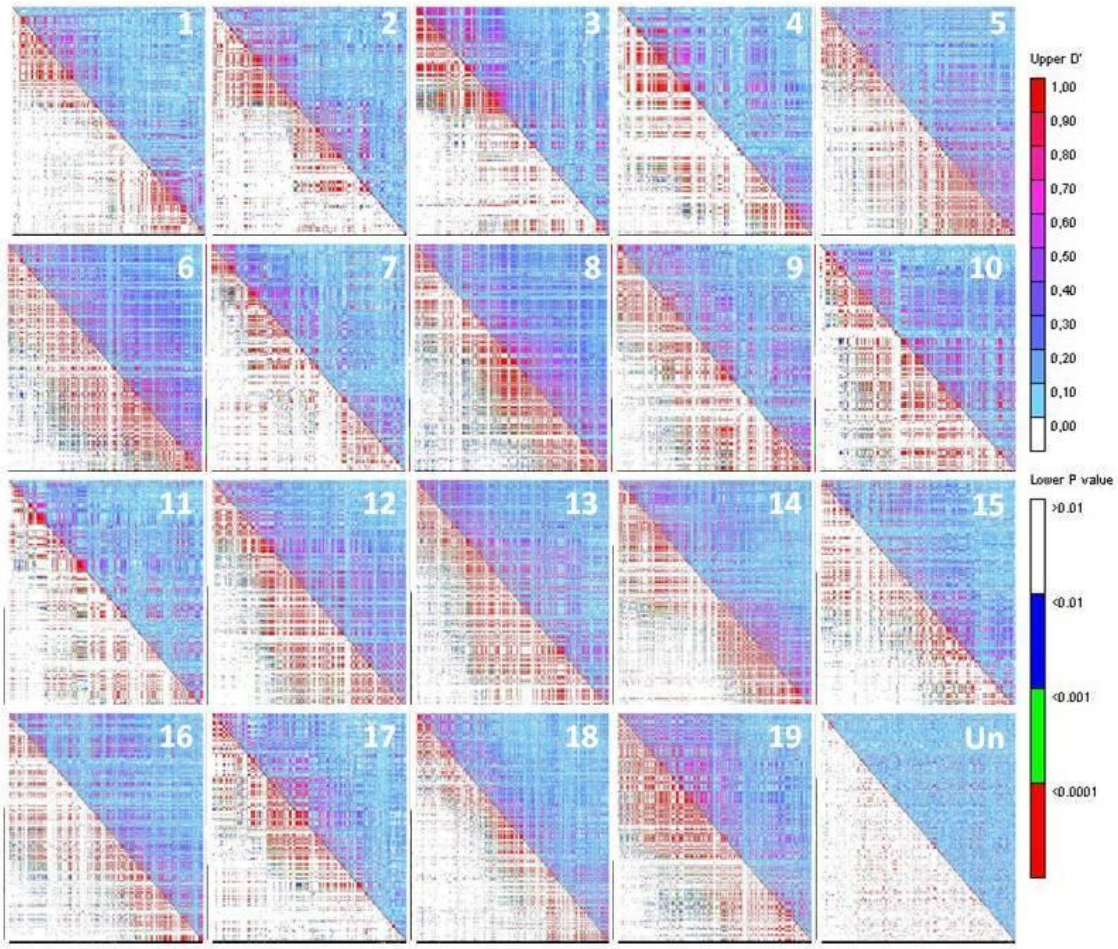

Figure 3-5: **Linkage disequilibrium (LD) pattern for Chromosomes 1 to 19 and unassembled chromosome (Un).** LD of markers from both parents was analyzed together on TASSEL 3.0. The upper scale represents D` in the upper right-hand of each graph, while the lower scale shows p-values calculated by two-sided Fisher's exact test in the lower left-hand of each graph.

GWAS analysis

The strongest association was found between SNP S20_23819354 and PM resistance evaluated on 07-12-2011, with a p-value of $3.8 \cdot 10^{-6}$. However, under a conservative multiple test correction (Bonferroni threshold was $2.97 \cdot 10^{-6}$ at a significance level of $\alpha = 0.05$), no association was significant. Therefore, for further analysis we established criteria for considering SNPs that: i) showed significance under a relaxed threshold of $5.0 \cdot 10^{-4}$ and ii) were significant in more than one phenotypic evaluation (field or detached leaf assay). A total of 116 markers met the $5.0 \cdot 10^{-4}$ threshold; of these, 25

markers were significant in multiple evaluations and were selected for further analysis (Table 3-1). 'Chardonnay' contributed 16 markers located on chromosomes 4, 5, 9, 13, 15 and 17, whereas 9 markers were from *V. rupestris* B38 on chromosomes 7, 11, 16 and the unassembled 'chromosome' 20 (Table 3-1). Individually, variance explained by each marker ranged from 0.14 to 0.26 (Table 3-2). The estimated effect of alleles coming from 'Chardonnay' always contributed positively to disease severity, with values between 4.6% and 17%. In contrast, the contribution of *V. rupestris* B38 alleles always reduced disease severity by -9.5% to -19.1%

Table 3-1: Statistical significance of marker associated with powdery mildew resistance or susceptibility in *Vitis rupestris* B38 and *Vitis vinifera* 'Chardonnay'. Genome-Wide Association Study was performed on powdery mildew assessments either in the field (2009 to 2011) or by detached leaf assay. Criteria of selection for significant marker were i) threshold of $5.0 \cdot 10^{-4}$ and ii) significance in more than one assessment.

SNP Name	Chrom	Field							Detached leaf		
		2009	08/09/10	08/20/10	08/30/10	09/07/10	09/20/10	07/12/11	08/18/11	Microcolony	Penetration	
S4_21739173/76	4				4.2E-04		3.4E-05		1.1E-04			'Chardonnay'
S5_12925192	5						1.5E-04		1.3E-04			
S9_10379981/82	9						2.3E-04		9.7E-05			
S9_10380022/23	9						3.4E-04		2.1E-04			
S9_10531863	9						1.3E-04		4.2E-05			
S9_13661499	9						1.0E-04		3.0E-05			
S9_17940130	9				5.0E-04				1.4E-04			
S9_18099474	9						2.2E-04		3.0E-05			
S9_18392869	9						3.7E-04		4.3E-04			
S9_21527229	9	2.3E-04				4.9E-04						
S9_22612745/53	9	2.5E-04				2.6E-04						
S13_8723867	13						5.3E-05		3.3E-05			
S15_5224226	15						3.6E-04		1.7E-05			
S17_10740891	17			2.5E-04	1.4E-04							
S17_15941498	17			1.4E-04	6.0E-05							
S17_16004459/76	17									1.7E-04	2.8E-04	
S11_11750531/34	11		2.2E-04					2.1E-04				*V. rupestris*
S16_7421749	16		3.4E-04					1.9E-04				
S18_17981498	18						5.0E-04		4.9E-04			
S20_8530574	Un						4.5E-04	4.2E-04				
S20_23796628	Un		3.1E-04					1.8E-05				
S20_23819240	Un		2.4E-04					1.1E-05				
S20_23819354	Un		7.5E-05	3.6E-04				3.8E-06				
S20_32360020/31	Un		2.4E-04					3.5E-05				
S27_1104742	7_Un		1.6E-04	2.8E-04				2.5E-05				

Table 3-2: Summary statistics for marker associated with powdery mildew resistance or susceptibility. For coefficient of determination (R^2) and estimated allele effect s (%), Min and Max represent the minimum and maximum value obtained at each locus across significant phenotypic evaluations. Loci with 2 SNPs from the same read were considered a single locus, with the last two position digits for the second SNP listed after a '/' (eg, two SNPs were identified in S4_21739173/76, separated by two nucleotides). Alleles are listed using standard IUPAC nucleotide codes.

SNP Name	Chrom	R^2		Estimate (%)		Allele (IUPAC)	Parent
		Min	Max	Min	Max		
S4_21739173/76	4	0.15	0.20	6.9%	13.9%	K / R	Chardonnay
S5_12925192	5	0.16	0.19	10.3%	15,0%	R	Chardonnay
S9_10379981/82	9	0.15	0.19	4.6%	15.1%	Y / Y	Chardonnay
S9_10380022/23	9	0.14	0.17	9.7%	14.3%	R / R	Chardonnay
S9_10531863	9	0.16	0.21	10.5%	15.8%	R	Chardonnay
S9_13661499	9	0.18	0.23	10.9%	17.0%	S	Chardonnay
S9_17940130	9	0.17	0.22	10.7%	16.7%	Y	Chardonnay
S9_18099474	9	0.17	0.23	10.9%	17.0%	Y	Chardonnay
S9_18392869	9	0.14	0.16	9.7%	14,0%	Y	Chardonnay
S9_21527229	9	0.14	0.16	7.0%	9.3%	W	Chardonnay
S9_22612745/53	9	0.16	0.16	7.2%	10.2%	Y / W	Chardonnay
S13_8723867	13	0.18	0.21	10.9%	16.2%	Y	Chardonnay
S15_5224226	15	0.14	0.23	9.7%	16.6%	Y	Chardonnay
S17_10740891	17	0.16	0.17	10.6%	12.9%	Y	Chardonnay
S17_15941498	17	0.17	0.19	11.2%	13.4%	Y	Chardonnay
S17_16004459/76	17	0.24	0.26	8.3%	9.6%	M / S	Chardonnay
S11_11750531/34	11	0.18	0.21	-17.0%	-14.5%	R / W	*V. rupestris*
S16_7421749	16	0.15	0.18	-15.2%	-13.1%	Y	*V. rupestris*
S18_17981498	18	0.15	0.17	-14.2%	-9.9%	R	*V. rupestris*
S20_23796628	Un	0.15	0.23	-17.8%	-13,0%	R	*V. rupestris*
S20_23819240	Un	0.17	0.26	-18.4%	-14.5%	Y	*V. rupestris*
S20_23819354	Un	0.15	0.28	-19.1%	-12.6%	R	*V. rupestris*
S20_32360020/31	Un	0.15	0.21	-17.1%	-13.2%	R / R	*V. rupestris*
S20_8530574	Un	0.14	0.16	-14.6%	-9.5%	Y	*V. rupestris*
S27_1104742	7_Un	0.15	0.22	-17.0%	-12.1%	R	*V. rupestris*

The significant QTL in Tables 3-1 and 3-2 represent at least 10 different linkage groups based on the physical map. To test whether they genetically map to fewer loci, a marker correlation analysis was conducted (Figure 3-6). We found some markers located on different physical chromosomes that were highly correlated ($r > 0.9$) in both parents. In 'Chardonnay', markers S13_8723867 and S15_5224226, located on chromosomes 13 and 15 respectively, were highly correlated with a cluster of markers in chromosome 9 (S9_10379981/82, S9_10380022/23, S9_10531863 and S9_13661499). Marker S4_21739173/76 located on chromosome 4 was highly

correlated with markers S13_8723867 in chromosome 13. In *V. rupestris*, markers located on unassembled chromosome 20 and unassembled chromosome 17 (S20_23796628, S20_23819354, S20_32360020/31, S27_1104742) were highly correlated with marker S16_7421749 located on chromosome 16.

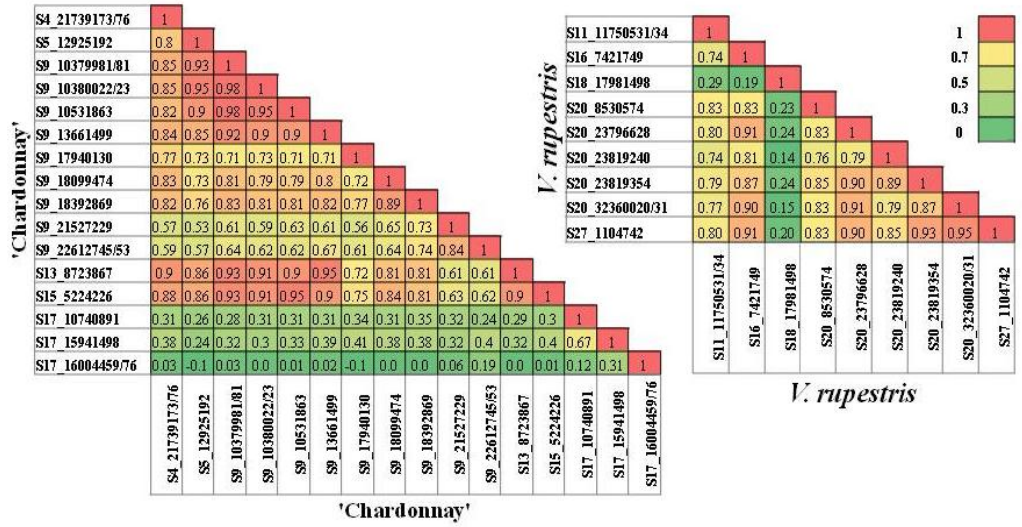

Figure 3-6: Pearson's correlation analysis between markers located on different chromosomes, within 'Chardonnay' (A) or *Vitis rupestris* (B). Marker position was determined based on homology with the grape reference genome (Jaillon et al., 2007)

Discussion

We identified 16,834 high quality SNPs distributed across the grapevine genome, analyzed their association with powdery mildew resistance in a segregating population of 85 individuals, and found evidence for resistance alleles from *V. rupestris* B38 as well as susceptibility alleles from *V. vinifera* 'Chardonnay'.

SNP distribution and localization

In this work we present the first application of next generation sequencing for genotyping in grapevine. SNPs generated by GBS were filtered based on their segregation ratio among the offspring and percentage of missing data to develop a

stringent set of reliable markers for GWAS. Data analysis included the study of SNP distribution, localization on the reference genome and association analysis with phenotype, as well as LD patterns across chromosomes.

In this study, SNPs derived from NGS data were prone to errors associated with the sequencing technology and the characteristics of the GBS protocol, and thus reliable markers had to be filtered from among the initial collection of SNPs. Pooling samples in one sequencing lane lowers the read coverage for each SNP marker when compared with other genotyping strategies that use a single lane of NGS per sample (Myles et al., 2010), leading to an increased in number of missing data. The application of GBS has been successfully reported in GWAS of homozygous lines of maize and barley (Elshire et al., 2011; Poland et al., 2012) but the implementation of this technique in a F1 progeny of two heterozygous parents brought new challenges. On the one hand, heterozygosity increases the error rate as heterozygous marker can be called as homozygous when only one allele has been read. Also, in order to create maps for each parent and impute missing markers among sites it is necessary to phase haplotypes on each parental chromosome. Our attempts to utilize BEAGLE (Browning and Browning, 2009) software for phasing did not meet the expected results (data not shown) indicating the need for new algorithms suitable for data sets with low coverage and a high percentage of missing data.

One strategy to select SNPs that are good molecular markers is to determine the parental genotypes based on deep sequencing of their libraries (Davey et al., 2011) and then use this information to select SNPs that segregate at the expected ratio. As an alternative approach, we conducted shallow sequencing of the progeny and followed a strategy of selecting SNPs based on the segregation ratio among the progeny. In this

work we only selected SNPs with MAF of 0.25 (± 0.05), and as a consequence several SNPs that could have become informative molecular markers, as biallelic heterozygous markers in both parent or tri-allelic and four-allelic SNPs, were not considered at this stage. These informative SNPs could be included once phasing and genetic linkage mapping is completed.

The selection strategy used in this work proved to be sufficient to give a robust set of 16K markers with good coverage of the grapevine reference genome (Figure 3-4). Distribution analysis of the number of SNP markers located within a bin of 500 kb showed a pattern of continuous variation with some outlier 500 kb bins with high numbers of SNPs (Figure 3-3) and some 500 kb bins with few or no SNPs. Continuous distribution of the counts indicates that the RRL created with enzyme *ApeKI* succeeded to reduce the complexity of the genome without introducing a significant bias on the localization of the SNPs at the resolution analyzed here. Outlier 500 kb bins could be a minimal source of error and may be explained by several factors, including: errors in the physical map of the reference genome, differences between the reference genome and parental genomes, and local enrichment of repetitive DNA for which reads would have been discarded (Figure 3-3).

The 16K set of markers were distributed across the grape reference genome, covering the whole length of each chromosome, with an average density of 36 SNPs/Mbp (Figure 3-4). This is the most densely-saturated map created to date for a biparental population in grapevines, where the biggest gap (less than 1 Mbp) is several-fold smaller than the maps published until now (Adam-Blondon et al., 2004; Di Gaspero et al., 2007; Doligez et al., 2006; Mahanil et al., 2011; Myles et al., 2010), and thus presents an improvement over current grapevine genetic mapping standards. Analysis

of the LD pattern of the selected SNPs confirmed linkage disequilibrium between markers located within chromosomes 1 to 19 (Figure 3-5). Chromosome 20 constitutes unassembled contigs or scaffolds, and as expected, SNPs located in this chromosome showed a random pattern and shorter extent of linkage disequilibrium. Within assembled chromosomes, it is possible to observe variation in the LD pattern; while some chromosomes like 6, 8, 12 and 13 showed a longer extent of LD, chromosomes 1, 2 and 7 had SNPs correlated at the extremes of the chromosomes. A possible explanation could be translocations on the arms of some chromosomes, either on the reference genome or in *V. rupestris* B38 related with *V. vinifera* 'Chardonnay'. Several mapping projects have found translocations and discrepancies (Cadle-Davidson, Pers. Comm.) with the reference map, which is currently undergoing revision (Adam-Blondon, Pers. Comm.). LD analysis also showed SNPs that are not correlated with neighboring markers on the chromosome where they have been physically mapped (white or blue sectors in Figure 3-5) indicating that some markers are incorrectly positioned and a genetic map specific to this population may provide a better indication of marker location and order than the physical map of the reference genome presented in this work.

Although the results presented here are just a portion of the information that can be distilled from the data generated in this study, we considered that this set of molecular markers was suitable for discovery of loci associated with powdery mildew resistance/susceptibility on *V. rupestris* B38 and *V. vinifera* 'Chardonnay' through GWAS. Subsequent analyses including phasing, imputation of missing markers and construction of genetic maps through linkage analysis are currently underway. Markers and maps generated in this study have further applications. As an example, they could be used to improve the reference genome sequence, by localization of

SNPs found on random and unaligned chromosomes or by generation of genetic maps that could help to resolve translocations on chromosome arms.

GWAS analysis

In this work, we found that powdery mildew severity on individuals of the segregating population, *V. rupestris* B38 x 'Chardonnay', was associated with several markers with small effects from both parents that have an antagonist effect upon disease severity, possibly acting at different developmental stages. While all marker from *V. rupestris* B38 contributed resistance to powdery mildew early in the season, QTLs from 'Chardonnay' conferred susceptibility with the effect becoming apparent later in the season (Tables 3-1 and 3-2).

Alleles from *V. rupestris* B38 always reduced powdery mildew severity with estimated allele effects between -9.5% to -19.1% (Table 3-2). No major genes for resistance were found in this analysis, consistent with the quantitative segregation patterns discussed in Chapter 2. *Vitis rupestris* alleles located on chromosomes 7, 11, 16 and 20 were significant early in seasons 2010 and 2011, but their effects were lost once the disease progressed (Table 3-1), which could be an indicator of a developmental effect on the powdery mildew resistance mechanism in *V. rupestris* B38, or of selection for virulent isolates as the season progressed. On the other hand, markers from 'Chardonnay' showed an opposite response. All 16 associated sites had a positive effect on the severity of the disease and thus contributed to susceptibility with estimate allele effects between 4.6% and 17.0%. The timing when markers from 'Chardonnay's

became significant was more variable than with *V. rupestris* B38 with markers becoming significant late in the seasons 2010 and 2011. Markers located on

chromosome 17 were significant only at the midpoint of season 2010 or in a single isolate detached leaf assay (see chapter 2) two days after inoculation.

Single associated markers from 'Chardonnay' were located on chromosomes 4, 5, 13 and 15, but two clusters of 9 and 3 markers each were located on chromosomes 9 and 17, respectively. Single markers at chromosome 4, 5, 13 and 15 have Pearson's correlation coefficients of r = 0.8 to 0.95 with clustered markers S9_10379981/82 to S9_18392869 on chromosome 9, suggesting that it is likely they would genetically map to this chromosome. Markers located on chromosome 17 showed low correlations with the cluster located on chromosome 9 and with each other. Similarily, in the *V. rupestris* correlation analysis, it was found that the single marker S16_7421749 was correlated with markers located on the unassembled chromosome 20 as well as the unassembled portion of chromosome 17. This suggests a possible chromosome assignment for chromosome 20, as well as possible errors in the current version of the reference genome sequence. The single marker located on chromosome 18 did not correlate with any other associated marker from *V. rupestris*.

Even though none of the *V. rupestris* B38 or 'Chardonnay' alleles were significant enough to pass a conservative Bonferroni multiple test correction, there are several factors that may have contributed either to an overestimation of the significance threshold value or to lowering of the statistical power of our approach: (i) redundant markers included in the 16K SNP set, (ii) small population size (85), (iii) insufficient numbers of phenotyping replicates, or (iv) using average severity rather than a complete statistical model. The effect of small population size is exemplified by our single isolate detached leaf assay, which had a subset of just 55 progeny and only one significant marker.

Overall, we estimate that QTLs from *V. rupestris* B38 described in this work constitute novel sources of powdery mildew resistance as they were located on chromosomes where no resistance marker have been described previously or on contigs that have not been assembled (chromosome 20). It is also possible that the number of marker contributed by *V. rupestris* B38 has been understimated, as marker located in regions of *V. rupestris* B38 with lower homology to the reference genome were discarded from this analysis since the SNP calling strategy required an alignment step with the reference genome. This factor may have contributed to the relatively lower number of significant marker from *V. rupestris* B38 detected in this analysis.

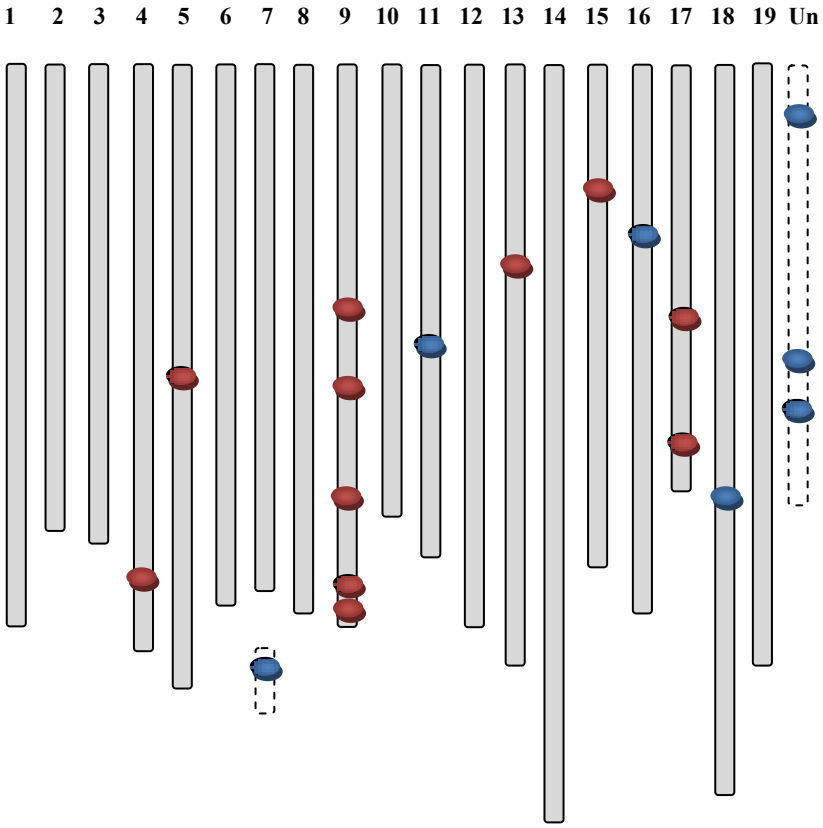

Figure 3-7: Localization and direction of effect of significant markers on the *Vitis vinifera* reference genome. Susceptibility markers were found from *V. vinifera* 'Chardonnay' (red) and resistance markers were found from *V. rupestris* B38 (blue). Dotted lines indicate unansembled contigs on chromosome 7 and 20.

In chapter 2, observations of a quantitative segregation pattern and the characterization of the powdery mildew resistance response led a rejection of the hypothesis of resistance controlled by a single dominant locus and suggested that the powdery mildew resistance mechanism in *V. rupestris* is a quantitative trait with components including: penetration resistance, race specific interaction with powdery mildew isolates, and a developmental effect that increases penetration resistance on older leaves. The direction of effect of each marker (improving resistance when from *V. rupestris* and increasing susceptibility when from 'Chardonnay'), the presence of multiple QTL with small effects in both directions, and the timing according to disease progression helps to support the hypothesis raised in chapter 2. In this sense, we have found no evidence for single gene, qualitative powdery mildew resistance in *V. rupestris* B38 (Figure 3-7), but instead several quantitative loci. Additionally, this work provides a new saturated SNP map in grapevine and contributes with relevant information about susceptibility alleles present in *V. vinifera* that can be either introduced or avoided in grapevine breeding programs. These newly reported resistancet alleles could be used to introgress resistance in hybrid crosses with *V. rupestris* B38, while avoiding susceptible alleles in crosses with *V. vinifera* cultivars.

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