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ORIGINAL PAPER

A reference genetic map of *Muscadinia rotundifolia* and identification of *Ren5*, a new major locus for resistance to grapevine powdery mildew

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Abstract *Muscadinia rotundifolia*, a species closely related to cultivated grapevine *Vitis vinifera*, is a major source of resistance to grapevine downy and powdery mildew, two major threats to cultivated traditional cultivars of *V. vinifera* respectively caused by the oomycete *Plasmopara viticola* and the ascomycete *Erisyphe necator*. The aim of the present work was to develop a reference genetic linkage map based on simple sequence repeat (SSR) markers for *M. rotundifolia*. This map was created using S1 *M. rotundifolia* cv. Regale progeny, and covers 948 cM on 20 linkage groups, which corresponds to the expected chromosome number for muscadine. The comparison of the genetic maps of *V. vinifera* and *M. rotundifolia* revealed a high macrosynteny between the genomes of both species. The S1 progeny was used to assess the general level of resistance of *M. rotundifolia* to *P. viticola* and *E. necator*, by scoring different parameters of pathogen development. A quantitative trait locus (QTL) analysis allowed us to highlight a major QTL on linkage group 14 controlling resistance to powdery mildew, which explained up to 58 % of the total phenotypic variance. This QTL was named 'Resistance to *Erysiphe Necator* 5' (*Ren5*). A microscopic evaluation *E. necator* mycelium development on resistant and susceptible genotypes of the S1 progeny showed that *Ren5* exerts its action after the formation of the first appressorium, and acts by delaying, and then stopping, mycelium development.

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Introduction

Grapevine is an economically important crop worldwide, and it has a central place in the cultural heritage of humanity. The common muscadine grape, *Muscadinia rotundifolia* [Michx.] Small (Weaver 1976; Bouquet 1980; Olmo 1986; Mullins et al. 1992), is closely related to *Vitis* species, to the point of being also referred to as *Vitis rotundifolia* [Michx.]. Indeed, there is much taxonomic controversy as to whether *Muscadinia* should be considered as a proper genus, or should be placed within *Vitis*. Small's (1913) classification will be used in this paper, considering *Muscadinia* as a genus. Muscadine grape is native to the southern United States of America, where it has been cultivated for more than 400 years, playing an important role in the history and sociology of this area. Although introduced in Europe in the late 19th century with most of the other American *Vitis* species, *M. rotundifolia* has not elicited any real interest from European growers, since the few attempts at cultivating it conducted at this time all failed (Bouquet 1983). From a genetic point of view the diploid chromosome number in *Muscadinia* species is 40 (2n = 40) in contrast to 38 (2n = 38) in *Vitis* grapes (Patel and Olmo 1955). Many morphological, anatomical and physiological characteristics also differ between *Vitis* and *Muscadinia*. Muscadine fruit has a

distinctive aroma and flavor, the juice being sweet in taste and typically low in acidity (Olien 1990). Muscadine grapes have a high degree of resistance to diseases that commonly occur on bunch grapes, including grey mold, anthracnose, nematodes, Pierce's disease, and downy and powdery mildews (Olien 1990).

Downy mildew caused by the Oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni (Dick 2002), and powdery mildew caused by the ascomycete *Erisyphe necator* (Schw.) Burr. (synonym *Uncinula necator*) are two important grapevine diseases. *Plasmopara viticola* and *E. necator* were introduced in France from North America during the 19th century together with accessions of American wild *Vitis* species and rapidly spread across Europe (Galet 1977). Today, they are found in all of the temperate regions where grapevines are cultivated. *Plasmopara viticola* and *E. necator* are both obligate biotrophs infecting all green tissues of the grapevine. *Plasmopara viticola* infects leaves, inflorescences and young bunches, leading to significant losses of productivity and quality (Lafon and Clerjeau 1988). *Erysiphe necator* produces whitish mycelia on the surface of leaves, stems, inflorescences and berries. Affected berries become russeted and often crack, causing significant reduction in yield and fruit quality. Grape powdery mildew epidemics can progress rapidly and cause serious economic losses (Pool et al. 1984; Gadoury et al. 2001; Calonnec et al. 2004).

In Europe, *Vitis vinifera* is the most widely cultivated grapevine species. All the traditional cultivars of *V. vinifera* are susceptible to downy and powdery mildew, although susceptibility varies among cultivars (Boubals 1959; Dubos 2002). Control of downy and powdery mildews on traditional European grapevine varieties requires regular application of chemicals, and growers tend nowadays to limit risk of epidemics by applying large amounts of fungicides. However, routine use of fungicides is becoming increasingly restrictive because of their cost, risk on human health and negative environmental impacts. Furthermore, fungicide-resistant strains of *P. viticola* and *E. necator* are now observed in the vineyard, decreasing the efficiency of these sprays (Gisi 2002; Wilcox et al. 2003; Chen et al. 2007; Gisi et al. 2007; Gisi and Sierotzki 2008; Baudoin et al. 2008; Furuya et al. 2010).

In this context, plant breeding for disease resistance appears to be an attractive way to control grapevine downy and powdery mildew effectively and in an environmentally friendly manner. Because of its high level of resistance to numerous grapevine pathogens, *M. rotundifolia* is an interesting source in which to study resistance factors. Accordingly, QTLs (quantitative trait loci) for downy and powdery mildew resistance have been identified from muscadine grape: *Rpv1* and *Rpv2,* located respectively on chromosomes 12 and 18, were found to be responsible for the resistance to downy mildew derived from *M. rotundifolia* cv. Trayshed (Merdinoglu et al. 2003; Wiedemann-Merdinoglu et al. 2006). A major QTL located on chromosome 12 and named *Run1*, was responsible for the resistance to powdery mildew derived from *M. rotundifolia* accession G52 (Pauquet et al. 2001; Barker et al. 2005). Recently, Riaz et al. (2011) identified on chromosome 18 two loci, named *Run2.1* and *Run2.2*, conferring resistance to powdery mildew from *M. rotundifolia* cv. Magnolia and *M. rotundifolia* cv. Trayshed, respectively. However, introgressing these resistance factors into traditional cultivated European species is challenging. On the one hand, hybridisation between *M. rotundifolia* and *V. vinifera* species is hampered by the difference in chromosome number between the *Vitis* and *Muscadinia* genomes (Bouquet 1983), which often brings along sterility problems in F1 hybrids and backcross generations, thus restricting interchange of genetic material between the two genera. On the other hand, the process of introgression of a resistance gene often results in the linkage drag of undesired traits from *M. rotundifolia* that may remain even after successive cycles of backcrossing.

Having a better understanding of the genetic and genomic differences between *M. rotundifolia* and *V. vinifera* becomes a central issue, in order to create new resistant grape varieties in an optimal way. Over the past decade, a large amount of molecular genetic information has become available to the grape research community for the genus *Vitis*, including genetic maps from a wide range of backgrounds (Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Doucleff et al. 2004; Adam-Blondon et al. 2004; Riaz et al. 2004; Fischer et al. 2004; Doligez et al. 2006; Lowe and Walker 2006; Welter et al. 2007; Di Gaspero et al. 2007; Troggio et al. 2007; Salmaso et al. 2008; Marguerit et al. 2009; Bellin et al. 2009; Moreira et al. 2010; Blasi et al. 2011), physical maps (Moroldo et al. 2008), and a completed grape genome sequence (Jaillon et al. 2007; Velasco et al. 2007). However, very little genetic information is available for *M. rotundifolia*. The lack of genetic studies concerning *M. rotundifolia* and the absence of a genetic map for this species limit considerably the opportunities to increase the efficiency of breeding programs by means of marker-assisted selection. It also restricts the exploitation of the resource provided by the genome sequence of *V. vinifera* (Jaillon et al. 2007) to understand the genetic and physiological bases of the traits of interest carried by *M. rotundifolia.*

Here, we report the construction of a *M. rotundifolia* genetic map using simple sequence repeat (SSR) markers and the identification of a major QTL for resistance to grapevine powdery, named *Ren5* and located on linkage group (LG) 14. Twenty LGs have been found for *M. rotundifolia*, which corresponds to the expected chromosome

number. The comparison of the genetic maps of *V. vinifera* and *M. rotundifolia* showed a high colinearity between the genomes of both species. These main results reported here will allow a more efficient use of the *Muscadinia* resources for the creation of high-quality grapevine resistant varieties.

Materials and methods

Plant material

In 2006, 669 seeds were produced from a selfing (S1) of *M. rotundifolia* cv. Regale, a hermaphrodite accession maintained at INRA Colmar, France. After seed germination, young plants were cultivated in stone wool substrate, and watered daily with a complete nutritive solution (4.8 % Norsk Hydro Hydrokani CPO, YARA). The mapping population consisting of 191 progeny from this selfing was maintained in greenhouse. Biological replicates between years were produced by pruning the plants to the basal two buds in winter and allowing them to re-grow in spring to ensure uniform shoot development.

V. vinifera cv. Cabernet Sauvignon clone 338, *V. vinifera* cv. Cinsault, *V. rupestris* cv. Rupestris du Lot, *V. riparia* cv. Riparia Gloire de Montpellier and *M. rotundifolia* cv. Regale were grown from green cuttings and maintained in greenhouse.

Evaluation of resistance to downy mildew

A strain of *P. viticola* collected from *V. vinifera* cv. Chardonnay in an experimental vineyard at INRA-Colmar (France) in 2006 was maintained on 6-week-old seedlings of *V. vinifera* cv. Muscat Ottonel placed in an opened cardboard box covered with a plastic bag. After 5 days of incubation in a growth chamber (21 °C, 100 % relative humidity, 50 µmol/m^2/s light intensity), sporangia were recovered from infected leaves by immersion in water and gentle shaking. The concentration of the *P. viticola* suspension was measured using a cell-counting chamber.

Evaluation was performed as described in Blasi et al. (2011). Two replicates were performed for each individual of the S1 population and nine replicates for each control. On 6 days post-inoculation (dpi), inoculated leaf discs were scored for the general level of resistance (OIV452) (Table 1). Two biological replicates were performed in 2009 and 2010, mean values of the two repetitions were considered in the analysis.

Evaluation of resistance to powdery mildew

The experiments were performed with a strain of *E. necator*, Chlo2b (biotype B) collected from *V. vinifera* cv. Merlot Noir at Pauillac (Gironde, France) in 2004 and maintained on detached juvenile leaves of greenhouse-grown seedlings from open pollination of 'Muscat Ottonel' or 'Cinsault'.

Inoculation was performed as described in Miclot et al. (2012), inside a vertical laminar flow station using dry inoculation with a settling tower. Four culture dishes were inoculated for each settling tower, containing one susceptible control ('Cabernet Sauvignon 338' or 'Cinsault') and one resistant control ('Regale'). Two replicates were performed for each genotype of the S1 population. Dishes were then sealed and incubated in a growth chamber at 25 °C and a photoperiod of 18-h light/6-h darkness. Inoculated leaves were scored everyday, 3–7 dpi, for the two semi-quantitative parameters described in Table 1 to measure the effect of resistance on the mycelium development of *E. necator* (MYC) and the intensity of sporulation (SPO), as described by Miclot et al. (2012). The 7 dpi scores gave the more discriminating results and were therefore the only time point considered in further analysis. Mean values of two biological replicates performed in 2010 and 2011 were considered in this study.

SSR marker analysis

DNA extractions and microsatellite analysis were performed as described in Blasi et al. (2011), with the following modifications. Amplifications were performed on a Perkin Elmer 9700 thermocycler programmed as follows: 5 min at 94 °C, 14 cycles of 20 s at 94 °C, 20 s at 65 °C with a touchdown (−1 °C per cycle) and 40 s at 72 °C, followed by 35 cycles of 20 s at 94 °C, 20 s at 50 °C and 40 s at 72 °C, and a final step of 7 min at 72 °C. Microsatellite fragments were resolved either on an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems) using a 36-cm capillary filled with the POP-4 polymer, or on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using 16 × 50 cm capillaries filled with the POP-7 polymer. Electropherograms were analyzed using Genescan™ 3.1 (Applied Biosystems). Alleles were identified using Genotyper™ 2.5.2 (Applied Biosystems) and their size was determined using the HD400-ROX internal size standard.

Genetic mapping

We used 451 primer pairs flanking microsatellite loci from marker sets VVS (Thomas and Scott. 1993), VVMD (Bowers et al. 1996, 1999), VrZAG (Sefc et al. 1999), VMC (Vitis Microsatellite Consortium, Agrogene, Moissy Cramayel, France), UDV (Di Gaspero et al. 2005), VVI (Merdinoglu et al. 2005), VrG (Regner et al. 2006), VVCS and SC8 (Cipriani et al. 2011), P2-298 (Pelsy, personal

Table 1 Variables scored to assess the resistance level to downy and powdery mildew

Pathogen	Variable name	Description	Scoring
Downy mildew	OIV452	Symptom-based semi-quantitative scoring of the downy mildew resistance adapted from the criteria of the Office International de la Vigne et du Vin (OIV; Anonymous 2009) http://news.reseau-concept.net/images/oiv/client/Code_descripteurs_2ed_FR.pdf)	From 1 (very susceptible) to 9 (totally resistant): 1 = abundant sporulation densely covering the whole disc area, absence of plant necrosis 3 = abundant sporulation present in large patches, absence of plant necrosis 5 = limited sporulation present in intercostal patches, plant necrotic flecks or speckles 7 = sparse sporulation, necrotic spots 9 = no sporulation, absence of necrosis or necrotic points
Powdery mildew	MYC	Visual semi-quantitative scoring of mycelium development on leaf surface	From 1 (very susceptible) to 9 (totally resistant): 1 = widespread mycelium densely covering the whole disc area 3 = widespread and locally dense mycelium 5 = widespread and sparse mycelium 7 = scattered and sparse mycelium 8 = rare and very short mycelium hyphaea 9 = absence of mycelium development
Powdery mildew	SPO	Visual semi-quantitative scoring of sporulation intensity	From 1 (very susceptible) to 9 (totally resistant): 1 = widespread sporulation with high density of conidiophores 3 = widespread with various density of sporulation 5 = scattered with various density of sporulation 7 = scattered with low density of conidiophores 8 = rare conodiophores 9 = absence of sporulation

communication), Chr7V003, Chr7V004 and Chr14V015 (Blasi et al. 2011) and the newly developed Chr14V041, Chr14V048, Chr14V054, Chr14V056 (Table S1).

All markers were screened for informative segregation on the parent *M. rotundifolia* and 8–12 randomly chosen individuals of the S1 population. One hundred and seventy-seven polymorphic SSR markers were used to analyze the entire mapping population. For mapping purposes, the same segregation pattern was assigned to all markers (<hkxhk>: locus heterozygous in both parents, two alleles), and genotypes were encoded (hh,hk,kk) for co-dominant loci and (h-,kk) for dominant loci, following JoinMap 3.0 data entry notation (Van Ooijen and Voorrips 2001).

Linkage analysis was performed with JoinMap 3.0 (Van Ooijen and Voorrips 2001), enabling the analysis of self-pollinated populations derived from a heterozygous parent and the construction of consensus linkage maps. Recombination fractions were converted into centimorgans (cM) using the Kosambi (1944) function. The threshold value of the logarithm of odds (LOD) score was set at 4.0 to claim linkage between markers with a maximum fraction of recombination at 0.45. The goodness-of-fit between observed and expected Mendelian ratios was analyzed for each marker locus using a χ^2 test. Markers showing segregation distortion were included in the final map if their presence did not alter surrounding marker order on the linkage group. Linkage groups were numbered according to internationally acknowledged grapevine reference genetic maps (Doligez et al. 2006; Di Gaspero et al. 2007).

QTL analysis

The genetic variance (σ_g^2) and experimental error variance (σ_e^2) required for heritability calculations were estimated using the statistical software R version 2.10.1 (The R Foundation for Statistical Computing) in the lmer function of the package lme4 by treating genotype as a random factor. Broad-sense heritability estimates were calculated on a genotype mean basis via the equation $H^2 = s_g^2/(s_g^2 + (s_e^2/r))$ (Gallais 1990), where the term r refers to the number of replicates for each genotype.

QTL analysis was carried out using MapQTL 6.0 software (Van Ooijen 2009). The significant LOD threshold for QTL detection at $P = 0.05$ for each linkage group was

determined by three independent permutation tests (1,000 permutations) of the phenotypic data. Non-parametric Kruskal–Wallis analysis and interval mapping (Mixture Model method) were performed on OIV452, MYC and SPO parameters. Maximum LOD values were used to estimate QTL peak positions, and the confidence intervals of QTL peaks were determined as the peak flanking regions in which LOD scores declined by two LODs. A manual cofactor selection was then used in Multiple QTL Model (MQM) analysis for powdery mildew phenotypic data.

Scanning electron microscopy

Observations were performed on a Hitachi TM1000 tabletop scanning electron microscope, with a 15-kV acceleration tension and a Backscattered Electron Detector. Samples correspond to 1-cm discs generated from leaves inoculated with the Chlo2b strain of *E. necator* using a settling tower. Resistant and susceptible genotypes of the S1 'Regale' population were inoculated with *E. necator* and observed 4 hpi (hours post-inoculation), 1 dpi and 2 dpi. Three genotypes were observed per class of resistance level.

Results

Muscadinia rotundifolia genetic map

451 SSR primer pairs were tested on the S1 population, from which 77 did not amplify or produced an unclear banding pattern, and 197 lacked polymorphism and were thus discarded. The remaining 177 primer pairs allowed us to detect 181 useful loci scored on the progeny, 145 being fully informative (hh,hk,kk) and 36 displaying a dominant pattern (h-, kk). Chi-square analysis among the 181 mapped markers indicated segregation distortion for 33 markers (22.3 %).

One hundred seventy-eight markers were mapped on 20 LGs (Fig. 1), and three markers were linked but unmapped due to weak linkages to other markers within the group. The observed linkage group number is consistent with the chromosome number in the *Muscadinia* genus (Bouquet 1983; Patel and Olmo 1955). Based on the latest published *V. vinifera* reference map (Doligez et al. 2006), LG20 of *M. rotundifolia* corresponds to the bottom part of *V. vinifera* LG7 (Fig. 2).

The total length of the map was 948 cM, with an average distance of 5.3 cM between markers. The largest group in terms of genetic distance, LG18, consisted of 12 mapped markers covering 80.4 cM, and the smallest, LG9, consisted of 10 mapped markers covering 11.8 cM. In terms of marker coverage, the largest group is LG14, including 16 markers, and the smallest is LG8, consisting of 5 markers. Overall, only 9 gaps were larger than 20 cM. The largest gap was on LG14, where the distance between marker VVC34 and VVIs70 was 35 cM. The marker order was consistent with the order determined from the *V. vinifera* genome sequence (Jaillon et al. 2007; http://www.genoscope.cns.fr/spip/Vitis-vinifera-sequencage.html) as well as with the *V. vinifera* reference maps (Adam-Blondon et al. 2004; Doligez et al. 2006), although in two genomic regions marker order was not syntenic in comparison to the reference maps [(LG6: 'VVIp28 to VVIm43'); (LG12: 'VMC4c10 to VVIv05')]. In these regions, little discrepancies in marker order are observed punctually, in particular for the positioning of VMC4g6 on LG6 and VVIm11 on LG12. Finally, some markers showed a multilocus pattern, and either they were located on several chromosomes or they mapped to chromosomes different from expected (VVIv61, VVIb19, VMCNG1d12, VVIp02 and VVCS1E043E23F1-1).

The *M. rotundifolia* map covers 56 % on average in genetic distance, compared to the reference map of Doligez et al. (2006). Nevertheless, the genetic distance ratio obtained using the common distal markers to align both maps is 70 %, suggesting that the recombination rate is, on average, much lower in the *M. rotundifolia* map than in the reference map (Table 2). Taking into account the lower recombination rate, the overall coverage of the map is estimated at 80 %.

Analysis of downy mildew resistance

The reliability of the downy mildew resistance test was assessed by scoring the OIV452 resistance parameter in control plants that represent a range of resistance levels from susceptible to highly resistant : 'Cabernet Sauvignon 338' (susceptible), 'Rupestris du Lot' and 'Riparia Gloire de Montpellier' (both partially resistant) and 'Regale' (the totally resistant parent of the S1 mapping population). All control plants as well as the parent of the S1 population behaved as expected for their level of resistance evaluated in this study (Table S2).

Resistance to downy mildew displayed little variation in the S1 population, which exhibits overall strong resistance to the pathogen. The distribution for OIV452 in the S1 population ranged from 7.0 to 9.0, the most susceptible individuals displaying strong partial resistance, whereas the most resistant were totally resistant, like the 'Regale' parent. More than 90 % of the individuals were totally resistant (OIV9), whereas the remaining part of the population showed strong partial resistance (Fig. 3a).

In the S1 population, the genotype factor had a highly significant ($P < 0.001$) effect on OIV452 scores. Broad-sense heritability, a direct measure of environmental

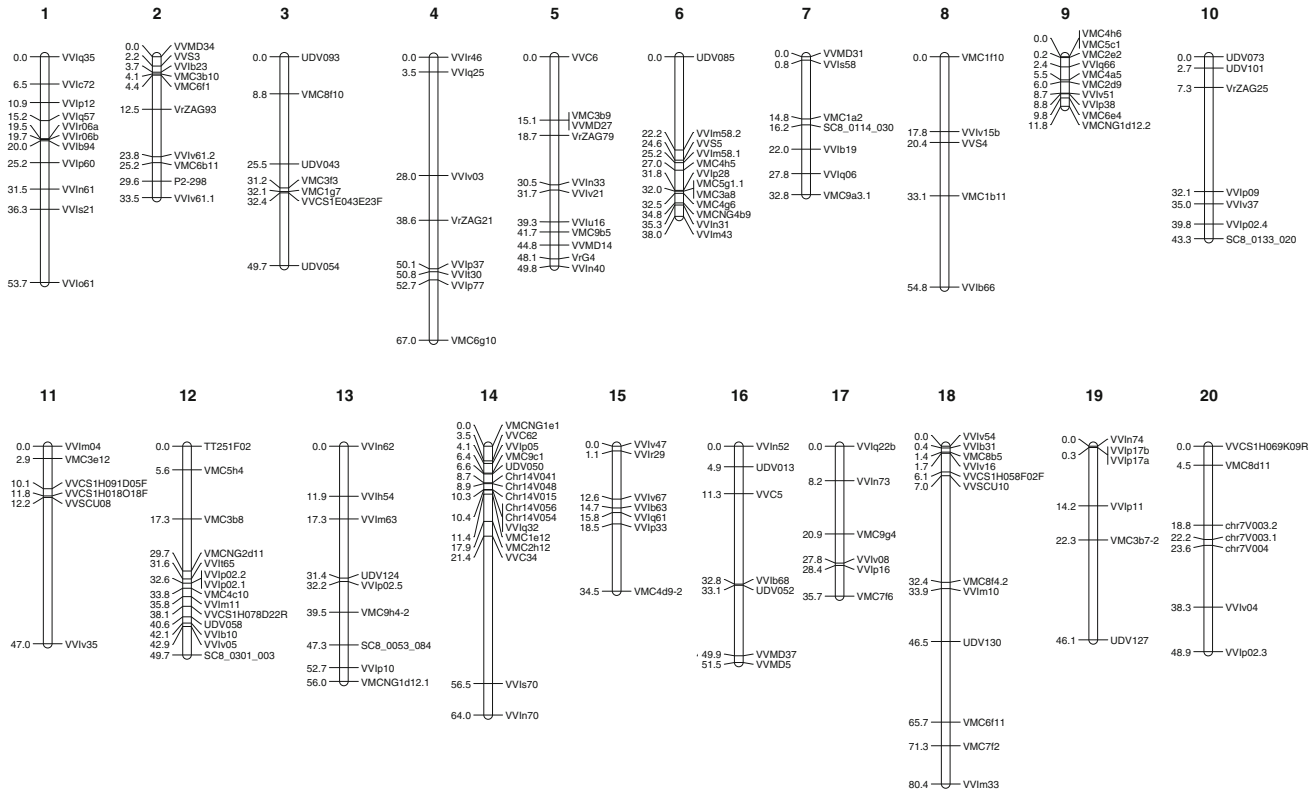

Fig. 1 *M. rotundifolia* genetic linkage map. The linkage groups were numbered LG1 to LG19 according to Adam-Blondon et al. (2004) and Doligez et al. (2006), and the remaining LG was named LG20

effects on phenotypic variance, was calculated for OIV452 using 146 genotypes. On a genotype mean basis, heritability for OIV452 was estimated at 0.53.

Analysis of powdery mildew resistance

The reliability of the powdery mildew resistance test was assessed by scoring the two resistance parameters, MYC and SPO, in the control plants 'Cabernet Sauvignon 338' or 'Cinsault' (susceptible genotypes), and 'Regale' (the resistant parent of the S1 mapping population). All control plants as well as the parent of the S1 population behaved as expected for the two resistance parameters evaluated in this study (Table S2).

Resistance to powdery mildew displayed a continuous variation in the S1 population and segregated as a quantitative trait, considering the mycelium development parameter. The distribution of MYC in the S1 population ranged from 3.5 to 9 (Fig. 3b), considering an average score of the two biological repetitions. The most susceptible individuals displayed partial resistance level, whereas the most resistant were totally resistant, similarly to the 'Regale' parent. The MYC scores displayed segregation patterns where roughly 50 % of the individuals were totally resistant, whereas the remaining part of the population showed various levels of partial resistance (Fig. 3b). The genotype factor had a highly significant ($P < 0.001$) effect on MYC and SPO scores in the S1 population. Broad-sense heritability for powdery mildew resistance was estimated for the two scored parameters using 126 genotypes. On a genotype mean basis, the estimate was 0.52 for SPO and 0.73 for MYC (Table 3).

QTL detection for downy mildew resistance

Despite the small phenotypic differences observed, four markers located on LG18 were significantly linked to the resistance for OIV452 using Kruskal–Wallis test ($P < 0.005$). Two of these markers, VMC6f11 and VMC7f2, showed the highest possible level of statistical linkage using the MapQTL software ($P < 0.0001$). Interval mapping analysis detected a QTL controlling resistance to downy mildew in the same chromosomal region as the Kruskal–Wallis non-parametric test (Table 4). This locus accounted for up to 24.7 % of the phenotypic variation for OIV452 (LOD score 6.06) and thus 46.6 % of the genetic variance, considering the broad sense heritability estimated at 53 %. This QTL was also detected for the two biological replicates performed taken separately (data not shown). It was located at a region covering a confidence interval of

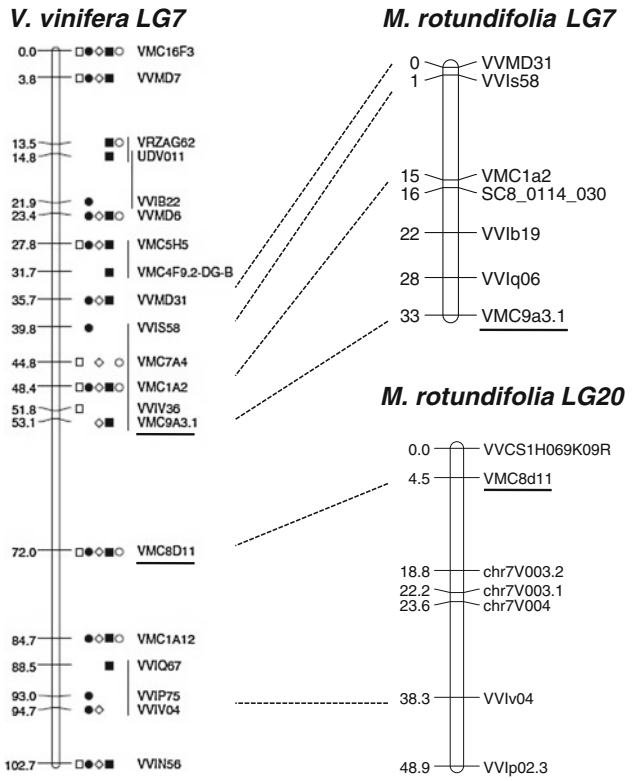

Fig. 2 Macrosyntenic comparison between *V. vinifera* LG7 and *M. rotundifolia* LG7 and LG20. All the markers located on *M. rotundifolia* LG20 are located on *V. vinifera* LG7 in the same genetic order. VMC9a3.1 and VMC8d11 (*underlined*) are separated by 18.9 cM on the *V. vinifera* reference map, but in the *M. rotundifolia* map they are located respectively the bottom extremity of LG7 and the upper part of LG20. *Dotted lines* link common markers between the two genetic maps. *Vitis vinifera* LG7 as in the reference map of Doligez et al. (2006)

19.2 cM, and it was placed between the SSR markers UDV130 and VMC6f11 (closer to VMC6f11) (Table 4).

QTL detection for powdery mildew resistance

Significant QTLs for powdery mildew resistance were obtained by interval mapping analysis, regarding mycelium development (MYC) and sporulation (SPO) of the pathogen (Table 4). A major QTL controlling resistance to powdery mildew was detected on LG14 for MYC and SPO. Analysis based on MYC gave the highest LOD score (23.73) and explained 58 % of the total phenotypic variance and thus nearly 80 % of the genetic variance, considering the broad sense heritability estimated at 73 %. The same region accounted for 11.4 % of the phenotypic variation (LOD score of 3.31) for SPO, and thus 22 % of the genetic variance, considering the broad sense heritability estimated at 52 %. The confidence interval for the QTL detected for MYC was located between the markers VVIp05 and Chr14V041 and the QTL peak was close to SSR marker VMC9c1 (Table 4, Fig. 4a). Kruskal–Wallis non-parametric tests confirmed these QTLs detected with interval mapping ($P < 0.0001$, data not shown). The locus at this position was named 'Resistance to *Erysiphe necator* 5' (*Ren5*). The genome region between the markers flanking the *Ren5* confidence interval covers a physical distance of 3.08 Mb and encompasses around 150 genes, according to the 12× grape genome sequence (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

A minor QTL was detected on LG20 for SPO (LOD 2.52) very close to VMC8d11 (Table 4). This QTL remained above the LOD threshold given by permutation test analysis (data not shown), and VMC8d11 achieved a fair statistical linkage to the disease QTL using Kruskal–Wallis test ($P < 0.01$).

Chosing VMC9c1 and UDV 050 as cofactors for further analysis with composite interval mapping (MQM mapping), the sporulation specific QTL on LG20 remained over the LOD threshold. Interestingly, a new minor QTL appeared on LG5 close to VVIv21 for both MYC and SPO (LOD 5.12 and 2.72), explaining 8.1 and 8.4 % of the phenotypic variation, respectively, thus 11.1 and 16.2 % of the genetic variance, considering the respective broad sense heritabilities (Table 4). All the QTL detected were found with the two biological replicates taken separately (data not shown).

Microscopic evaluation of the effect of *Ren5* on *E. necator* mycelium development

The marker VMC9c1 is the closest marker to *Ren5*. At this genetic locus, the allelic form 132 is associated with the resistance, whereas the allelic form 146 is associated with susceptibility. As shown in Fig. 4b, the distribution of MYC notations varies in groups of individuals based on their genotype at locus VMC9c1, with all homozygous 132/132 (resistant) individuals having a MYC score above 7, whilst the majority of homozygous 146/146 (susceptible) individuals show a MYC score of 7 or below. Heterozygous individuals show a wider distribution of MYC score and appear intermediate between the other two classes.

Scanning electron microscopy experiments were conducted to visualize the effect of *Ren5* on the early stages of *E. necator* mycelium development. Resistant (132/132 form of VMC9c1) and susceptible (146/146 form of VMC9c1) genotypes were inoculated with *E. necator*. Four hpi, both genotypes supported development of the first appressorium from the germinated conidia (Fig. 5), which corresponds to normal pathogen development, as described in Rumbolz et al. (2000). The presence of the appressorium structure is linked to the subsequent formation of a haustorium, which represents the first stage of the biotrophic phase of the fungus. Differences in mycelium growth

Table 2 Comparison of the genome coverage and genetic distances between *V. vinifera* reference linkage map (Doligez et al. 2006) and *M. rotundifolia* genetic linkage map

Linkage group	Markers common between maps		Genetic distance between common markers			Maximum genetic distance		
	Start marker	End marker	Reference map	*M. rotundifolia* map	Ratio	Reference map	*M. rotundifolia* map	Ratio
1	VVIq35	VVIo61	69.2	53.7	0.78	87.5	53.7	0.61
2	VVMD34	P2-298	48.2	29.6	0.61	79.7	33.5	0.42
3	UDV093	VMC1g7	45.2	32.1	0.71	70.3	49.7	0.71
4	VVIr46	VMC6g10	87.9	67.0	0.76	90.9	67.0	0.74
5	VVC6	VVIn40	79	49.8	0.63	83.4	49.8	0.60
6	UDV085	VVIm43	57.9	38.0	0.66	82.5	38.0	0.46
7	VVMD31	VMC9a3.1	17.4	32.8	1.89	102.7	32.8	0.32
8	VMC1f10	VVIb66	87	54.8	0.63	112.7	54.8	0.49
9	VMC5c1	VMC6e4	60.6	9.8	0.16	104.1	11.8	0.11
10	UDV073	VVIv37	56.7	35.0	0.62	83.7	43.3	0.52
11	VVIm04	VVIv35	64.5	47.0	0.73	75.1	47.0	0.63
12	TT251F02	VVIb10	72.9	42.1	0.58	81.9	49.7	0.61
13	VVIn62	VMCNG1d12.1	92.4	56	0.61	101.1	56.0	0.55
14	VMCNG1e1	VVIn70	94.8	64.0	0.68	94.8	64.0	0.67
15	VVIv67	VMC4d9.2	32.4	21.9	0.68	37.9	34.5	0.91
16	VVIn52	VVMD5	61.8	51.5	0.83	92.4	51.5	0.56
17	VVIq22b	VMC7f6	35.6	35.7	1.00	58	35.7	0.62
18	VVIb31	VMC7f2	94	70.9	0.75	131.5	80.4	0.61
19	VVIn74	UDV127	63.9	46.1	0.65	76.6	46.1	0.60
20	VMC8d11	VVIv04	22.7	33.8	1.49	–	48.9	–
Total			1,244.1	871.6	0.70	1,646.8	948.2	0.56

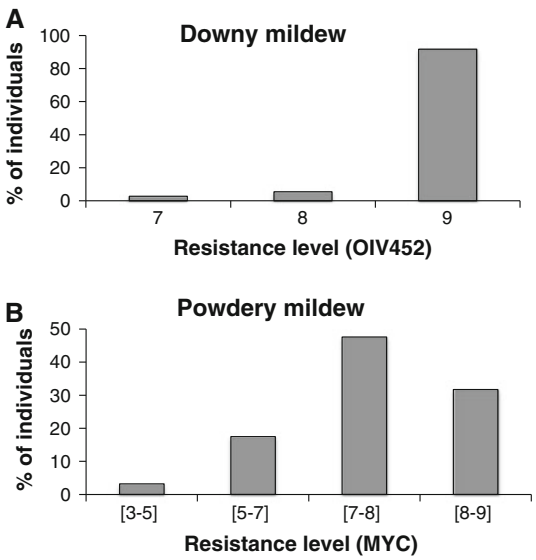

Fig. 3 Distribution of parameters for downy and powdery mildew resistance in the *M. rotundifolia* S1 population. Percentage of genotypes in the S1 *M. rotundifolia* cv. Regale population in each level of resistance for (**a**) downy mildew, using the OIV 452 parameter at 6 dpi. (**b**) powdery mildew, using the MYC parameter at 7 dpi. Individuals were classified in the different classes based on the mean value of two biological repetitions

between resistant and susceptible genotypes are visible 1 dpi; while resistant genotypes are still blocked at the first appressorium stage, primary and secondary hyphae begin to grow on susceptible genotypes. Two dpi, only primary hyphae are seen on resistant genotypes, while branched mycelium is observed on susceptible genotypes. The susceptible genotypes of the S1 population show less pathogen development that 'Cabernet Sauvignon 338', which is consistent with the macroscopic observations.

Discussion

Here, we report the first *M. rotundifolia* genetic linkage map, based on an intra-specific progeny and using SSR markers. Since SSR are easily transferable markers, there is a high level of reproducibility and polymorphism of *V. vinifera*-based SSR markers in non-*vinifera* species, even in a *M. rotundifolia* background. More than 80 % of the SSR markers tested on the S1 mapping population that were originally designed on *V. vinifera* amplified perfectly on *M. rotundifolia* DNA. The grape SSR marker system has been further used to manage *M. rotundifolia*

germplasm collections (Riaz et al. 2008) and our study confirms that it is a highly valuable genomic tool. The proportion of SSR markers with biased segregation observed in this study (22.3 %) was higher than that reported by Doligez et al. (2006) (9.2 %), Blasi et al. (2011) (11.3 %), Lowe and Walker (2006) (16 %) and Troggio et al. (2007) (20.3 %), and slightly equivalent than that of Grando et al. (2003) (22.4 %). Three clusters of markers with a distortion of segregation were found on LG6 (11 markers), LG8 and LG19 (6 markers each). The development of the new SSR markers Chr14V041, Chr14V048, Chr14V054 and Chr14V056 from the 12× grapevine genome sequence permitted the improvement of the construction of LG14, and especially enabled a more accurate detection and location of *Ren5*. Finally, 15 SSR markers (VVIr06, UDV054, VVIq25, VVIu16, VrG4, VVIm58, VVIq06, VVIv51, VMC5h4, VVIv47, VVIr29, VVC5, VVIb68, UDV130 and VVIm33), whose positions were unknown according to any previously published genetic linkage map, and the 12× grapevine genome sequence (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), were accurately located in this study on 10 different LGs.

The *M. rotundifolia* genetic map was composed of 178 markers mapped into 20 LGs. The total length of the map was 948 cM. Considering marker order, linkage group sizes and map length, the *M. rotundifolia* map built in this work is consistent with other published maps (Adam-Blondon et al. 2004; Doligez et al. 2006) and with the genome sequence [(Jaillon et al. 2007); http://www.genoscope.

Table 3 Descriptive statistical parameters for downy mildew and powdery mildew resistance parameters in the *M. rotundifolia* S1 population

	Downy mildew	Powdery mildew	
	OIV452	MYC	SPO
Average	8.89	7.62	8.95
Minimum	7.0	3.50	7.50
Maximum	9.0	9	9
Standard deviation	0.39	0.94	0.21
Heritability	0.53	0.73	0.52

Table 4 QTLs for resistance to *P. viticola* and *E. necator* detected in the *M. rotundifolia* S1 population

Pathogen	Trait	Linkage group	Nearest marker	Peak position	2-LOD interval	LOD score	Percentage of phenotypic variance explained	Trait heritability	Percentage of genotypic variance explained
Downy mildew	OIV	18	VMC6f11	55.5	47.5–65.5	6.06	24.7	0.53	46.6
Powdery mildew	MYC	14	VMC9c1	6.4	4.1–8.7	23.73	58	0.73	79.4
	MYC	5	VVIv21	31.7	21.7–44.8	5.12	8.1	0.73	11.1
	SPO	14	VMC9c1	6.4	0–21.4	3.31	11.4	0.52	21.9
	SPO	20	VMC8d11	0	0–23.6	2.52	8.8	0.52	16.9
	SPO	5	VVIv21	31.7	21.7–44.8	2.72	8.4	0.52	16.2

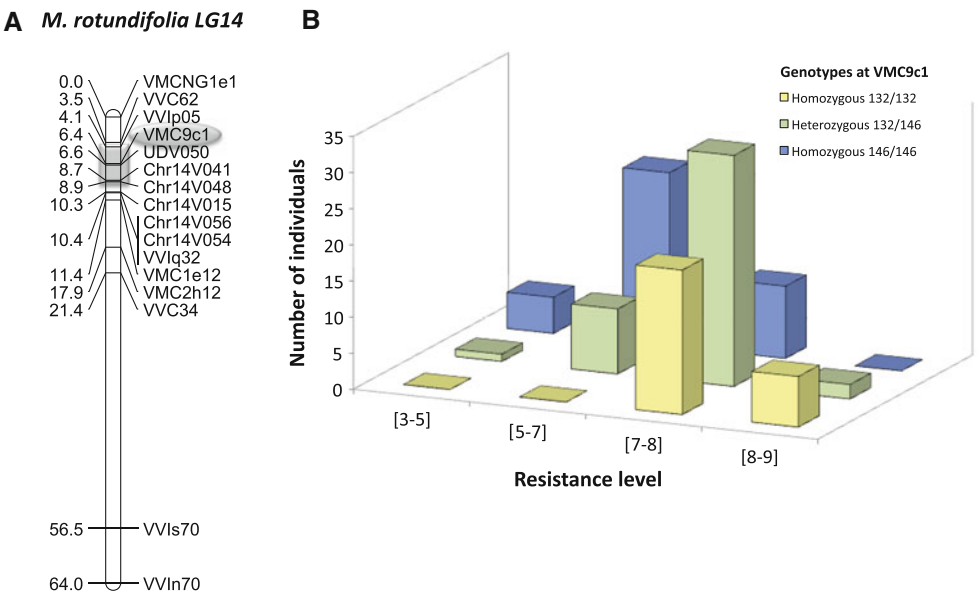

Fig. 4 Distribution of powdery mildew resistance based on the genotype at the *Ren5* locus (**a**) *M. rotundifolia* LG14 and confidence interval for *Ren5* (*grey box*), the marker VMC9c1 being the most strongly associated with *Ren5*. (**b**) *Yellow* homozygous resistant genotypes (132/132 form of VMC9c1); *green* heterozygous genotypes (132/146 form of VMC9c1); *blue* homozygous susceptible genotypes (146/146 form of VMC9c1) (color figure online)

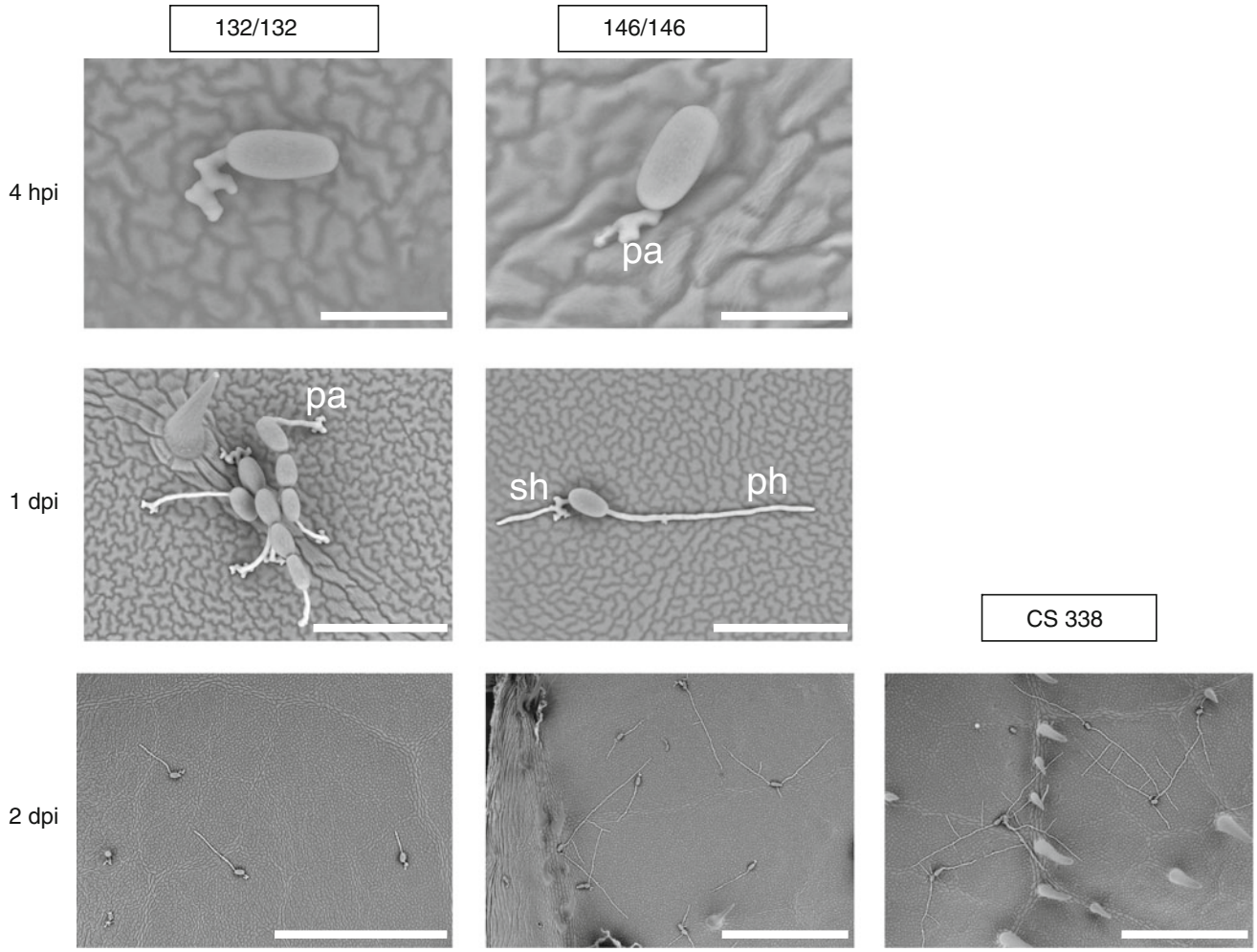

Fig. 5 Electron microscopy images of resistant and susceptible genotypes of the S1 population inoculated with *E. necator*. Resistant (132/132 at VMC9c1) and susceptible (146/146 at VMC9c1) genotypes of the S1 *M. rotundifolia* cv. Regale population, inoculated with *E. necator* and observed 4 hpi, 1 dpi and 2 dpi with a scanning electron microscope. Primary appressorium *pa*, primary hyphae *ph*, secondary hyphae *sh*. Data are representative of three independent genotypes per class. *V. vinifera* cv. Cabernet Sauvignon clone 338 (CS 338) is used as susceptible control. *Scale bars* represent 30 µm at 4 hpi, 100 µm at 1 dpi and 500 µm at 2 dpi

cns.fr/externe/English/Projets/Projet_ML/index.html] except for small discrepancies: two inversion sectors in marker order between the *M. rotundifolia* map and the reference maps, and a few cases where a marker was expected on a LG and was actually located on a different one due to multiloci alleles. One of the areas showing an altered marker order mapped to bottom part of LG12, in a region containing a large cluster of NBS-LRR genes, comprising *Rpv1* and *Run1*, two locus involved in downy and powdery mildew resistance in 'Trayshed' and 'G52'. NBS-LRR clusters are known to be subjected to frequent reshuffling and recombination events. The clustered organization is supposed to favor sequence exchanges, such as unequal crossing over and/or gene conversion events, which can give rise, in some cases, to new (nonparental) R specificities (Sudupak et al. 1993; Richter et al. 1995; Chin et al. 2001; Geffroy et al. 2009). Nevertheless, the high colinearity between the *M. rotundifolia* map and the previously published *V. vinifera* maps reveals a high level of global macrosynteny between both genomes. This finding will greatly facilitate the exploitation of the resource provided by the whole genome sequence of *V. vinifera* (Jaillon et al. 2007) to understand the genetic and physiological bases of the traits of interest carried by *M. rotundifolia.* Moreover, this framework *M. rotundifolia* genetic map will help processing comparative genetic analysis, especially to establish synteny for the regions that carry disease resistance genes, and understand the evolution of genomes.

Recombination rate was, on average, much lower in the *M. rotundifolia* map than in the latest *V. vinifera* reference map (Doligez et al. 2006). Such a similar result was obtained by Blasi et al. (2011) for a *V. amurensis* genetic map which led to the conclusion of lower recombination rates in the Asiatic species than in *V. vinifera*.

The *V. vinifera* LG7 corresponds to two separated LGs in the *M. rotundifolia* map, LG7 and LG20, *M. rotundifolia* LG20 corresponding to the bottom part of *V. vinifera* LG7. The markers VMC9a3.1 and VMC8d11, which are located 18.9 cM from each other in the *V. vinifera* reference map, respectively represent the bottom extremity of *M. rotundifolia* LG7 and the upper part of *M. rotundifolia* LG20. Considering the fact that the recombination rate in *M. rotundifolia* is lower than in *V. vinifera* (Table 2), we can assume that this distance should be even smaller in *M. rotundifolia*. If LG7 and LG20 of *M. rotundifolia* were part of the same chromosome we would then expect to find them assembled on the *M. rotundifolia* map. Our results thus strongly suggest that *V. vinifera* LG7 is splitted in two chromosomes in *M. rotundifolia*. A genetic map more with greater saturation in the VMC9a3.1–VMC8d11 interval, or Fluorescent In Situ Hybridization (FISH) experiments would help confirm this hypothesis.

QTL analysis demonstrated that downy mildew resistance derived from *M. rotundifolia* was determined by a QTL located on LG18 that explained nearly 46 % of the genetic variance. The confidence interval of the LG18 QTL for downy mildew resistance overlaps with *Rpv2* from 'Trayshed' (Wiedemann-Merdinoglu et al. 2006), which indicates that downy mildew resistance in muscadine grape cultivars Trayshed and Regale is at least partly governed by the same region on LG18. Nevertheless it is currently too premature to conclude that the same genetic factor is involved.

Resistance to powdery mildew displayed a continuous variation in the *M. rotundifolia* S1 population, where the most susceptible individuals displayed partial resistance. This suggests the existence of one or more homozygous non-segregating factors in the genetic background of 'Regale' that maintains residual resistance. Accordingly, QTL analysis demonstrated that powdery mildew resistance derived from *M. rotundifolia* was mainly determined by one major QTL located on LG14 that explained almost 80 % of the genetic variance for mycelium development. We named this locus *Ren5*, for *Resistance to Erysiphe necator 5*. This major QTL impacts on both mycelium development and sporulation intensity. A minor QTL detected on LG5 explaining up to 16 % of the genetic variation also plays a role in resistance. Moreover, a genetic factor specific to sporulation and located on the upper part of LG20 is involved in powdery mildew resistance in 'Regale'. The strong effect of *Ren5* together with the high level of genome coverage of the linkage map leads us to assume that no significant genetic factor involved in powdery mildew resistance other than those detected in this study segregates in the S1 progeny. Microscopic observations suggest that *Ren5* exerts its action after the formation of the first appressorium and acts by delaying, and then stopping, mycelium development.

Ren5 is located on the upper side of LG14, flanked by SSR markers VVIp05 and Chr14V041, on a genomic region never previously described to be controlling powdery mildew resistance in *M. rotundifolia* or in *Vitis* species. The locus Run1 has been identified to control resistance to powdery mildew in 'G52' on LG12 (Pauquet et al. 2001; Barker et al. 2005), and recently Riaz et al. (2011) identified in *M. rotundifolia* cv. Magnolia and 'Trayshed' two loci for resistance to powdery mildew named respectively *Run2.1* and *Run2.2*, both mapping on LG18. These results indicate that resistance to powdery mildew from *M. rotundifolia* may have different locations according to which cultivar is made use of. The *Ren5* locus is located in the confidence interval of *Rpv8*, the major QTL of resistance to downy mildew identified from *V. amurensis* (Blasi et al. 2011). Based on the latest version of the grapevine genome sequence (12×, http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), the region delimitated by markers VVIp05 and Chr14V041 is 3 Mb in size and contains around 150 predicted genes, which means that it is a bit premature to speculate on a putative candidate for *Ren5* function. Nevertheless, inspection of this region on the 12× version of the grapevine genome sequence revealed the presence of at least 7 predicted NBS-LRR genes as well as *V. vinifera* EDR1 a regulator of defense responses (Frye et al. 2001). Thus, we cannot discard that *Ren5* is a member of the NBS-LRR class of disease resistance genes.

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