Detection of downy and powdery mildew resistance QTL in a 'Regent' × 'RedGlobe' population

Carel J. van Heerden · Phyllis Burger ·
Abraham Vermeulen · Renée Prins

Abstract One hundred and eighty six F_1 plants from a 'Regent' × 'RedGlobe' cross were used to generate a partial linkage map with 139 microsatellite markers spanning all 19 chromosomes. Phenotypic scores for downy mildew, taken over two years, confirmed a major resistance QTL (*Rpv3*) against downy mildew in the interval VVIN16-cjvh to UDV108 on chromosome 18 of 'Regent'. This locus explained up to 62 % of the phenotypic variance observed. Additionally a putative minor downy mildew resistance locus was observed on chromosome 1 in one season. A major resistance locus against powdery mildew (*Ren3*) was also identified on chromosome 15 of 'Regent' in the interval UDV116 to VChr15CenGen06. This study established the efficacy of and validated the 'Regent'-derived downy and powdery mildew major resistance genes/QTL under South African conditions. Closely linked SSR markers for marker-assisted selection and gene pyramiding strategies were identified.

Keywords Genetic mapping · QTL mapping · Grapevine · Pathogen

C. J. van Heerden
Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

P. Burger · A. Vermeulen
ARC Infruitec-Nietvoorbij,
Private Bag X5026, Stellenbosch 7599, South Africa

R. Prins (✉)
CenGen (Pty) Ltd & University of Free State, 78 Fairbairn Street, Worcester 6850, South Africa
e-mail: cengen@cengen.co.za

Introduction

Most *Vitis vinifera* cultivars are susceptible to downy and powdery mildew infections and these infections cause large losses in production if left untreated. Treatment mainly comprises spraying with fungicides. Despite various studies aimed at better prediction of infection based on climatic conditions, these control measures are still costly and impact negatively on the environment. Additionally, the emergence of fungicide resistant or insensitive strains of *P. viticola* (Gisi 2002; Gisi et al. 2007) and *E. necator* (Erickson and Wilcox 1997; Savocchia et al. 2004; Baudoin et al. 2008; Furuya et al. 2010) emphasize the importance of durable host resistance. Genetic host resistance to both these pathogens are available in various cultivars, but these cultivars are mainly derived from non-*vinifera* species and resistance varies from complete resistance to partial resistance (Wang et al. 1995; Cadle-Davidson 2008).

Downy mildew

Downy mildew is caused by the biotrophic oomycete *Plasmopara viticola*. Infection occurs when hyphal tips from germinating zoospores, lying on the lips of stomata, penetrate the stomatal openings. An intercellular mycelial network with haustoria then develops inside the mesophyll tissue. Four to six days after infection, sporangiophores emerge through the stomatal openings. These sporangiophores produce sporangia which can then cause further infection (Gindro et al. 2003; Allègre et al. 2007; Rossi and Caffi 2007). It appears that infection by *P. viticola* interferes with the normal regulation of the guard cells of the stomata resulting in water loss (Allègre et al. 2007). It has also been shown that infection by *P. viticola* causes tissue damage and reduces the functional green area of the leaf as well as assimilation rates by the remainder of the leaf (Moriondo et al. 2005).

Genotypes that display a natural resistance to infection offer great advantage. *V. vinifera* have no known natural resistance to downy mildew infection (Staudt and Kassemeyer 1995; Cadle-Davidson 2008) with the exception of the minor *Rpv11* locus identified in 'Chardonnay' by Bellin et al. (2009). Genetic resistance to downy mildew infection is mainly confined to North American and Asian *Vitis* species like *V. aestivalis, V. berlandieri, V. cinerea, V. davidii var.* 'Cyanocarpa', *V. labrusca*, *V. lincecumii, V. pseudoreticulata, V. riparia, V. rupestris,* and *V. yeshanensis* (Alleweldt and Possingham 1988; Wan et al. 2007) as well as *Muscadinia rotundifolia* (Alleweldt and Possingham 1988). It is thought that the resistance to the pathogen co-evolved along with the pathogen, which is endemic to North America. Riaz et al. (2011) suggested that the resistance to *P. viticola* found in some Asian species like *V. amurensis* could have evolved as resistance to *P. cissii* and *P. amurensis*, which are endemic to Asia. Several breeding programmes strive to transfer resistance identified in other *Vitis* species to *V. vinifera*.

To date more than thirteen *P. viticola* major and minor resistance loci have been identified and mapped to chromosomes 4, 5, 7, 8, 9, 12, 14, 17 and 18 (Merdinoglu et al. 2003; Fischer et al. 2004; Welter et al. 2007; Bellin et al. 2009; Marguerit et al. 2009; Zyprian et al. 2009; Moreira et al. 2011; Schwander et al. 2012). Many of these loci were originally reported without specific gene names some of which

were later assigned names in the catalogue of mapped resistance loci (http://www.vivc.de), leaving a few reported loci unnamed. *Rpv1* on chromosome 12 (Merdinoglu et al. 2003) was identified in a cross between the susceptible *V. vinifera* 'Syrah' and the resistant '28-8-78' (derived from *M. rotundifolia*). This locus, which explained 73 % of the total phenotypic variance observed, was mapped to chromosome 12 of '28-8-78'. *Rpv2*, located on chromosome 18, also originated from *M. rotundifolia* (Wiedemann-Merdinoglu et al. 2006 as cited by Bellin et al. 2009 and Blanc et al. 2012) and explains 76 % of the observed phenotypic variance. This region was also identified in an S1 population of *M. rotundifolia* 'Regale' (Blanc et al. 2012). A cross between the resistant 'Regent' and susceptible 'Lemberger' yielded a resistance locus in the same region as *Rpv2* (Fischer et al. 2004; Welter et al. 2007), but since 'Regent' does not have any *M. rotundifolia* ancestors (Eibach and Töpfer 2003) this locus is assumed to be different from *Rpv2* and has therefore been designated *Rpv3*. Zyprian et al. (2009) reported a major resistance locus on chromosome 18 of 'Villard Blanc', which was also detected in a Villard Blanc-derivative, 'Gf.Ga-52-42' (Schwander et al. 2012). A resistance locus at the same position as *Rpv3* on chromosome 18 was also detected in a cross between 'Chardonnay' and 'Bianca' (Bellin et al. 2009). Since 'Bianca', the resistant parent, shares a common ancestry with 'Regent', this locus was also deemed to be *Rpv3*. Bellin et al. (2009) stated that the *Rpv2* and *Rpv3* disease response can be discriminated and that the BlastN projection of the marker sequences bordering the 'Bianca' *Rpv3*-interval and the *M. rotundifolia* *Rpv2*-interval on the PN40024 grape sequence (Jaillon et al. 2007) identifies two regions that are separated by approximately 1.5 Mbp on chromosome 18. An investigation into the *Rpv3* locus in a selection of North American *Vitis* species as well as cultivars known to be derived from North American species, revealed seven conserved haplotypes in the resistant accessions, while these haplotypes were absent from European cultivars produced before the spread of downy mildew (Di Gaspero et al. Di Gaspero et al. 2012). The authors concluded that the *Rpv3* locus found in resistant breeding lines had originated from multiple North American ancestors. Welter et al. (2007) detected a minor resistance locus (*Rpv4*) on chromosome 4. In a cross between *V. vinifera*

'Cabernet Sauvignon' and *V. riparia* 'Gloire de Montpellier' two resistance loci were identified on chromosomes 9 and 12 (Marguerit et al. 2009) and these were designated *Rpv5* and *Rpv6*, respectively. *Rpv5* linked to marker VVIO52, explained 26.0–34.4 % of the observed phenotypic variance. *Rpv6* is the second resistance locus (after *Rpv1*) to be identified on chromosome 12. The relative locations of the linked markers for *Rpv6* and *Rpv1*, together with the difference of origin, suggested that these two loci are indeed distinct. A minor resistance QTL explaining up to 12.7 % of the phenotypic variance observed, was identified on chromosome 7 (*Rpv7*) of 'Bianca' in a cross between 'Chardonnay' and 'Bianca' using various methods of phenotypic scoring (Bellin et al. 2009). An S1-selfed progeny of a hermaphrodite accession *V. amurensis* 'Ruprecht' revealed a major resistance locus on chromosome 14 linked to marker Chr14V015 located between markers VVIP05 and VVIP22 (Blasi et al. 2011). This locus, called *Rpv8*, explained up to 86 % of the observed phenotypic variance. Moreira et al. (2011) reported a resistance locus (*Rpv9*) on chromosome 7 using a cross between *V. vinifera* 'Moscato Bianco' and a *V. riparia* individual that showed resistance to downy mildew. This locus explained between 6.7 and 21.1 % of the phenotypic variance observed. Schwander et al. (2012) identified a resistance locus (*Rpv10*) on chromosome 9 of 'Solaris' that explained up to 50 % of the phenotypic variance observed. This locus was inherited from *V. amurensis*. In the study by Fischer et al. (2004) a minor resistance locus linked to VVMD27 on chromosome 5 was identified in three seasons. In contrast to Fisher et al. (Fischer et al. 2004), Welter et al. (2007) detected this locus only in one season, highlighting the importance of quantitative trait loci (QTL) validation. Schwander et al. (2012) detected the same minor locus in 'Solaris' by scoring intensity of sporangiophore formation using a leaf disk assay and proposed to designate this locus as *Rpv11*. A locus on chromosome 5 was also previously reported in 'Chardonnay' ($R^2 = 12.1$ %), a cultivar generally seen as susceptible to downy mildew (Bellin et al. 2009) when scoring mesophyll invasion while it remained undetected when a leaf disc assay was used. The catalogue of mapped resistance loci (http://www.vivc.de) cites this locus as *Rpv11* in 'Chardonnay' as well. The third major resistance locus originating from *V. amurensis* has been mapped to chromosome 14 associated with markers UDV014 and UDV370 (Venuti et al. 2013). This QTL, designated *Rpv12*, explained 78.7 % of the phenotypic variance observed. On the basis of differing phenotypic observation and the lack of contradictory marker information the authors concluded that *Rpv12* and *Rpv8* are probably not the same locus or allelic variants of the same locus. Moreira et al. (2011) reported *Rpv13* on chromosome 12, in a *V. vinifera* 'Moscato Bianco' × *V. riparia* population. *Rpv13* mapped close to the reported location for *Rpv1*. In addition to the above loci several unnamed minor loci have been reported on chromosomes 8, 12, 15 and 17 (Zyprian et al. 2009; Blasi et al. 2011; Moreira et al. 2011).

Powdery mildew

Powdery mildew is caused by the haploid heterothallic ascomycete *Erysiphe necator* (formerly *Uncinula necator*) (Braun et al. 2002). Infection occurs when germinating spores on the plant surface produce hyphal cells that grow across the plant surface and breach epidermal plant cell walls to obtain nutrients from the plant cells to grow and reproduce. Since infection is not limited by specific humidity and temperature conditions, this pathogen poses a global threat to grape production.

Genetic resistance to powdery mildew infection is mainly confined to North American *Vitis* species like *V. aestivalis, V. berlandieri, V. cinerea* and *V. labrusca*, and Asian species *V. amurensis, V. bashinica, V. davidii, V. liubanensis, V. piazezkii* and *V. romanetii* (Alleweldt and Possingham 1988; Wan et al. 2007). *Vitis vinifera* 'Kishmish vatkana' has also been found to be resistant to powdery mildew (Hoffmann et al. 2008).

As with downy mildew resistance, several powdery mildew resistance loci have been identified in different *Vitis* species and named either *Run* (resistance to *Uncinula necator*) or *Ren* (resistance to *Erysiphe necator*) loci. The first of these loci, *Run1*, was identified and mapped on chromosome 12 of *M. rotundifolia* (Pauquet et al. 2001; Donald et al. 2002; Merdinoglu et al. 2003; Barker et al. 2005). This locus was found to be closely linked to the *Rpv1* locus (Merdinoglu et al. 2003). The *Ren1* locus was identified on chromosome 13 of *V. vinifera* 'Kishmish vatkana' by Hoffmann et al. (2008). Riaz et al. (2011) identified several resistance loci on chromosome 18 in

various resistance sources i.e. *Run2.1* (*M. rotundifolia* 'Magnolia'), *Run2.2* (*M. rotundifolia* 'Trayshed') and *Ren4* (*V. romanetii* C166-043). Both *Run2.1* and *Run2.2* were inherited from *M. rotundifolia*, but the alleles associated with the two flanking markers differ for 'Trayshed' and 'Magnolia', while 32 % of the markers used in the study did not share any alleles. This suggests that 'Trayshed' and 'Magnolia' are not closely related. In short, *Run2.1*, *Run2.2* and *Ren4* all map to the same region on chromosome 18 as *Rpv3*. Dalbó et al. (2001) investigated a resistance locus in a 'Horizon' × 'Illinois 547-1' cross. The resistant parent, 'Illinois 547-1' is a hybrid between *V. rupestris* and *V. cinerea*. This locus was later designated *Ren2* and is reported to be on chromosome 14 (http://www.vivc.de/). *Ren3* was identified on chromosome 15 of *V. vinifera* 'Regent' (Fischer et al. 2004; Akkurt et al. 2007; Welter et al. 2007) and confirmed in 'Villard Blanc' (Akkurt et al. 2007). Blanc et al. (2012) identified and mapped the *Ren5* locus to chromosome 14 of *Muscadinia rotundifolia* cv. 'Regale'. Additional to this major locus, two minor loci were identified on chromosomes 5 and 20, but were not assigned specific names.

Several genetic maps have been constructed for grapevine (Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Adam-Blondon et al. 2004; Fischer et al. 2004; Riaz et al. 2004; Doligez et al. 2006; Lowe and Walker 2006; Di Gaspero et al. 2007; Troggio et al. 2007; Welter et al. 2007; Vezzulli et al. 2008; Bellin et al. 2009; Marguerit et al. 2009; Moreira et al. 2011; Blasi et al. 2011; Blanc et al. 2012). The first maps were constructed using mainly AFLP and RAPD markers. These maps were improved with the addition of microsatellite (SSR) markers which enabled the comparison of different genetic maps. The number of markers used to construct these maps varies dramatically and ranges from as little as 84 to more than 400. The reference map by Doligez et al. (2006) was constructed using 537 loci with an average intermarker distance of 3.3 cM as well as five individual mapping populations. However, between 200 and 450 markers are typically used in QTL mapping studies (Marguerit et al. 2009; Moreira et al. 2011; Blasi et al. 2011; Blanc et al. 2012). While most maps to date have been constructed for *V. vinifera*, Blanc et al. (2012) recently published an SSR based map for *M. rotundifolia* that showed a high degree of

similarity to the *V. vinifera* reference map of Doligez et al. (2006). These maps were used in QTL mapping of disease resistance, leaf morphology, seedlessness, veraison, and fruit quality. These QTL studies produced several flanking or linked markers that have since been applied in marker assisted selection (MAS) studies (Dalbó et al. 2001; Eibach et al. 2007).

Di Gaspero et al. (2012) showed that 'Villard Blanc', 'Bianca' and 'Regent' shared a common haplotype for markers linked to the *Rpv3* locus, indicating a common ancestral species origin, but with the advantage that they can be readily crossed with *V. vinifera* to transfer the resistance.

The objective of this study was (1) to determine the efficacy of the 'Regent'-derived downy and powdery mildew resistance under South African conditions, (2) to validate the reported QTL to both these traits, and (3) to identify markers closely linked to the QTL to aid in MAS and gene pyramiding strategies in the table grape breeding programme of the Agricultural Research Council Infruitec-Nietvoorbij (ARC). The ultimate aim is to combine reported host plant resistance found in wild species-derived cultivars with the desired fruit qualities of table grape cultivars.

Materials and methods

Plant material

'Regent' has a complex lineage, which includes *V. aestivalis, V. berlandieri, V. cinerea, V. labrusca, V. lincecumii, V. riparia* and *V. rupestris*, and is resistant to both downy and powdery mildew (Eibach and Töpfer 2003). 'RedGlobe' is a selection from an inbred cross [('Hunisia' × 'Emperor') × (('Hunisia' × Emperor') × 'Nocera')] and is classified as *V. vinifera* (http://www.vivc.de/). It is known to be susceptible to both downy and powdery mildew infection. The large berries produced by this cultivar make it a very desirable table grape. A segregating population consisting of 206 F_1 individuals originating from a 'Regent' × 'RedGlobe' cross were used as mapping population. These plants are maintained in a greenhouse at ARC Infruitec-Nietvoorbij. The plants have been cloned and these clones transferred into the vineyard at ARC Infruitec-Nietvoorbij for future evaluations of disease resistance and fruit quality.

Disease evaluation

Downy mildew: Spores were collected from natural downy mildew infections occurring in the vineyard and multiplied on surface sterilised *V. vinifera* cv. 'Chardonnay' (susceptible) leaves kept in a plastic bag in a growth chamber. The mapping population as well as 'Regent' and 'RedGlobe' were scored for resistance to downy mildew in 2005, 2006 and 2007 using a leaf disc assay (Brown et al. 1999). Ten one cm diameter discs were cut from the 5th and 6th mature leaves beneath the shoot apex, surface sterilised, then floated with the abaxial side up on sterile filtered water in two petri dishes (five disks per petri dish) and inoculated with 20 μl droplets of zoospore suspension (5×10^5 spores per ml). After inoculation the leaf discs were incubated in a growth chamber at 21 °C and a day length of 16 h for six days. After the first 24 h any remaining droplets were removed with blotting paper to prevent damage to the leaf discs. The level of infection by downy mildew was scored according to OIV descriptor 452-1 (Organisation Internationale de la Vigne et du Vin (OIV) 1984) as 9, 7, 5, 3 or 1 with 9 = very low, tiny necrotic spots, no sporulation nor mycelium; 7 = low, little sporulation or mycelium, sporulation smaller than droplet size; 5 = medium, more or less strong sporulation, sporulation as small as droplet size; 3 = high, strong sporulation and abundant mycelium, sporulation bigger than droplet size; 1 = very high, strong sporulation and dense mycelium, sporulation bigger than droplet size. For the 2005 (2005_01_28; 2005_02_25), 2006 (2006_04_02) and 2007 (2007_11_02) scores the two sets of five disks were scored together. For the 2007 season the two sets were also scored separately (2007_11_01-1; 2007_11_01-2).

Powdery mildew: Spores were collected from powdery mildew infections occurring naturally in the vineyard and multiplied on *V. vinifera* cv. 'Chardonnay' (susceptible) plants to provide sufficient inoculum. The infected 'Chardonnay' plants were placed among the 'Regent', 'RedGlobe' and the mapping population to allow for infection in 2009/2010 and 2011. After fourteen days the plants were scored according to OIV code 544 (Organisation Internationale de la Vigne et du Vin (OIV) 1984) using a scale of 9, 7, 5, 3 or 1 where 9 = no growth, 7 = little germination, 5 = germination and growth, 3 = little sporulation, and 1 = lots of sporulation). Three scores were done over two seasons (2009/2010 and 2011).

Subsequent to the 2009_11_24 score, the plants were left in the greenhouse without fungicide treatment for 50 days at which time the whole plant was scored again (2010_01_13) according to the same scale. An additional score was performed in the following season (2011_02_17).

All plants were scored according to the respective scales indicated above. In cases where no appropriate leaves could be found or where the test failed for whichever reason, the score was indicated as a missing value, indicated with a '*'. Spearman and Pearson correlations between the different phenotype scores as well as the significance test were calculated using R (R Development Core Team 2011). For one season (2007), the level of downy mildew infection was scored in duplicate (2007_11_01-1 and 2007_11_01-2) and the correlation between these two scores was also calculated. The frequency distributions of the different phenotypic scores were calculated. The mean phenotypic score for each of the possible genotypic classes was also considered in all five downy mildew scores.

Molecular analysis

DNA extraction was performed on young healthy leaves using the Macherey–Nagel Plant II DNA extraction kit implemented on a Tecan Genesis RMP200 liquid handler. The standard vacuum processing protocol described in the kit protocol was used. Extracted DNA was quantified using a Nanodrop spectrophotometer and the concentrations were standardised to fall within the range 25–35 ng/μl. SSRs were selected from the literature and the NCBI database (Thomas and Scott 1993; Bowers et al. 1996, 1999; Scott et al. 2000; Decroocq et al. 2003; Adam-Blondon et al. 2004; Di Gaspero et al. 2005; Merdinoglu et al. 2005; Doligez et al. 2006; Cipriani et al. 2008; http://www.ncbi.nlm.nih.gov/) with the aim to achieve comprehensive genome coverage and increased marker density for known minor and major mildew resistance QTL regions on chromosomes 5, 12, 15 and 18. A standard set of PCR conditions (1.8 mM $MgCl_2$, 0.75 U Supertherm Taq, 5 mM dNTP and 0.3 pmol/μl of each primer) was used for all reactions. The optimal annealing temperatures of the markers were determined and polymorphic markers with similar annealing temperatures were combined in

multiplex PCR reactions. New primers were designed for several markers to enable more effective multi-plexing and improve their performance. These markers were annotated by adding –cjvh to the original marker name (Online Resource 1).

Due to a lack of microsatellite markers on chromosome 15 distal of marker UDV116, eight new SSR markers were designed upon searching the Pinot Noir reference genome sequence (Jaillon et al. 2007) for dinucleotide repeats. These markers were given a CenGen designation (Online Resource 2). In total, 339 markers were tested on parental lines for multiplex development.

Once a multiplex was optimised the mapping population was typed. The multiplex PCR products were purified using the Machery-Nagel Nucleofast Post PCR purification kit implemented on a Tecan Evo150 liquid handler using the vacuum protocol provided with the kit. Two microlitres of the purified product separated on either an ABI 3130*xl* or an ABI 3730*xl* using Liz 500® internal size standard and a 50 cm capillary. The data scored using Genemapper® V4 and the called alleles were exported for formatting prior to mapping.

Linkage maps

JoinMap® 4.1 (Van Ooijen 2006; Van Ooijen 2011) and TMAP (Cartwright et al. 2007) were used to compute genetic linkage maps.

All the called alleles for all markers and individuals were collected in a single genotype file from which separate data input files for JoinMap® 4.1 and TMAP were prepared. For JoinMap® 4.1 the allele calls were coded according to the JoinMap® 4 manual (Van Ooijen 2006). The coding was done using logical arguments in Microsoft Excel and the coded data were saved as a new locus file to serve as data input file for JoinMap® 4.1. In the locus file the population type was set to 'CP'. For TMAP the GeneMapper® V4 raw allele calls were used.

With JoinMap® 4.1 the locus genotype frequencies were determined and markers displaying a significant deviation were noted for closer inspection during the mapping process. These markers were only discarded if the raw data were of low quality or resulted in a change in the marker order of the flanking markers. Markers with more than 20 % missing values were also removed. All individuals that were the result of

self-fertilization of 'Regent', and individuals with more than 10 % missing values, were removed prior to further analysis. The data were also tested for similarity of loci and similarity of individuals.

Integrated two-way pseudo-testcross

The two parental maps for 'Regent' and 'RedGlobe' were constructed using an integrated two-way pseudo-testcross (Grattapaglia and Sederhoff 1994). This method treats the F_1 as a doubled haploid population and scores each marker twice for each individual. For the first score only the inheritance of the 'Regent' alleles was considered and for these scores the marker names were amended with 'P1'. For the second score only the inheritance of the 'RedGlobe' alleles was considered and the marker names were amended with 'P2'. This is easily accomplished using the 'create maternal and paternal population nodes' function of JoinMap® 4.1. Data were then grouped using independence LOD starting from 2 and increasing the LOD by 1 up to a maximum of 10. Groups at an independence LOD value of 3 were accepted and unlinked markers were added to these groups, using prior information. Regression mapping was done using Kosambi's mapping function with a recombination frequency of less than 0.4 and a LOD higher than 3. Each of the parental maps contained only the markers that are polymorphic for the specific parent. The same parameters were used to construct the combined map. Maps supported by literature were used, in most cases the first or second round map.

For TMAP the phasing of the markers was first determined using the phasing script. This was followed by grouping the markers, using the grouping command with a minimum LOD (logarithm of the odds) of 5 and 40 cM as the maximum map distance. The consensus map order and distances were then determined for each separate group using the builder script. Separate maps for 'Regent' and 'RedGlobe' were generated by the split option in the builder script. Markers and individuals with missing values were not removed prior to mapping with TMAP.

QTL analysis

QTL mapping was performed using MapQTL® 6 (Van Ooijen 2009). The parental maps and locus file containing the duplicated marker set generated by

the integrated two-way pseudo-testcross were used together with the phenotypic data to identify possible resistance loci. Each phenotypic score was analysed individually. Kruskal–Wallis tests were performed on all markers as a non-parametric test to determine significant single marker-trait associations. Interval mapping (IM) using the parental maps was used to detect putative QTL using a step size of 1 cM. Markers close to the highest LOD position were then selected as a starting set for automatic cofactor selection and the markers selected by automatic cofactor selection were then used as cofactors in multiple QTL model mapping (MQM) to identify additional potential QTL and to determine the location of the QTL more precisely. The position of a particular QTL was estimated to be at the location of the maximum LOD as determined through IM and MQM. The process was repeated using the consensus map. The significant LOD threshold at $P = 0.05$ was estimated genome-wide as well as chromosome specific using 1,000 permutations of the phenotypic data.

Results

Disease evaluation

The Spearman rank and Pearson correlations were similar for all correlations performed (Table S3, Online Resource 3 and Table S4, Online Resource 4). For both downy and powdery mildew resistance scores moderate correlation between years were found. Correlations between downy mildew scores 2005_01_28 and 2005_02_25 were higher than the correlations between either of these scores and any of the 2007 scores. For the 2007_11_02 downy mildew score both sets of five discs were scored together while each set of five disks were also scored separately as 2007_11_01-1 and 2007_11_01-2 and can therefore be seen as replicates (Table S3, Online Resource 3). These three scores taken in 2007 showed a higher level of correlation than the correlations between different seasons. The 2006 (2006_04_02) score for downy mildew resistance showed less correlation with all other scores and this dataset was excluded from the analyses. None of the three powdery mildew infections were scored in duplicate. The Pearson correlations of the three powdery mildew infection scores are between 0.41 and 0.55 and the Spearman correlations

are between 0.48 and 0.57 and all three scores were retained in the analysis.

The downy mildew infection response of 'Regent' was as expected (either 7 or 9), while 'RedGlobe' scored in the susceptible range (either 3 or 5). Phenotypic scores for the population were distributed across all five response classes with a slight skewedness towards 1 (Fig. S1, Resource 3).

'Regent' behaved as expected for the powdery mildew infection response and was scored as 7 in all scores, while the susceptible 'RedGlobe' confirmed the reliability of the phenotypic scores with a score of either 1 or 3 (Fig. S3, Online Resource 4). The powdery mildew responses of the population were distributed across all five classes. The distribution was skewed towards 1 for 2010_01_13 and 2011_02_17 while it was skewed towards 9 for 2009_11_24.

Molecular markers

Of the 339 markers tested, 151 were arrayed in 28 multiplex PCR reactions of which the data of three markers were discarded prior to mapping. The number of markers per multiplex ranged from three to twelve with an average of 5.4 markers per multiplex. Of the 80 redesigned markers, 20 (25 %) were discarded due to poor amplification or complete failure to amplify, while 74 of the 250 (29.6 %) markers taken directly from the literature were discarded for the same reasons. The following markers were also removed due to a large number of missing genotypes for these markers: SCU08, UDV038, VMC6E4, VMC8H10-chr18-cjvh, VVIM04, VrZag29, VMC1G3.2-cjvh, VChr15CenGen01 and VVIN54. A total of 139 markers were therefore used for mapping using JoinMap® 4.1, whilst 148 were used for TMAP.

The microsatellite data revealed that six of the F_1 plants originated from self-fertilization events and contained only alleles originating from 'Regent'. These plants were genotyped together with the rest of the F_1 plants, but the data were removed prior to the linkage analysis. The removal of individuals with excessive missing values, along with the data for two plants that died, resulted in a final mapping population of 186 individuals.

Linkage maps

A set of four maps were constructed; the two parental maps generated using the integrated two-way pseudo-

testcross method using JoinMap® 4.1, the consensus map using JoinMap®4.1 and a consensus map using TMAP. The TMAP map was split using the 'split' option in the 'BuilderSplit' programme. The linkage groups were numbered according to the reference *V. vinifera* map (Adam-Blondon et al. 2004).

Parental maps

All 19 chromosomes were represented on the 'Regent' map, which consisted of 115 markers and had a length of 1020.2 cM (Online Resource 5). Chromosomes 2, 4 and 9 spanned only 11.5, 10.7 and 7.4 cM respectively. Furthermore, chromosomes 1, 6, 8, 14, 16, 18 and 19 had intermarker distances of more than 30 cM. In contrast, only 14 chromosomes, comprised of 86 markers, were represented in the 1054.9 cM 'Red-Globe' map. Chromosomes 3, 13, 14, 15 and 16 were not represented in the 'RedGlobe' map. The map also contained 13 intermarker distances of more than 30 cM distributed across all represented chromosomes except chromosome 17 and 19. Five markers including two chromosome 16 markers, VVMD5-cjvh and VVIN52, were unlinked in the 'RedGlobe' map, while only two markers were unlinked for 'Regent'. The marker order for 'Regent' chromosome 10 differed from that of 'RedGlobe' chromosome 10 and the consensus maps.

Consensus maps

Of the 148 markers, 47 (31 %) deviated from the expected Mendelian segregation $P < 0.05$ (Online Resource 6). These markers were not excluded from the map. Ten markers with more than 20 % missing genotypes were removed resulting in the utilisation of 138 markers for mapping with JoinMap® 4.1. Of these markers, 137 were arranged in a consensus map which spanned 1364.4 cM and represented all 19 chromosomes. Chromosomes 2, 4, 9, 10, 14, 16 and 19 contained intermarker distances of more than 30 cM. JoinMap® 4.1 failed to determine the phasing for markers VVIN52, UDV104 and VVMD5, but by excluding VVIN52 the remaining markers could be mapped. Therefore VVIN52 was not included in the consensus map for chromosome 16 despite being mapped to chromosome 16 for 'Regent'.

No markers were removed from the data set prior to mapping with TMAP and therefore the TMAP map

included several markers that were absent from the JoinMap® 4.1 map. This extended the map for chromosome 11 by 19 cM by including markers VVIM04 and SCU08. Similarly, VVIN52 extended the map for chromosome 16 by 14 cM. VVIV33 was the only unlinked marker in the TMAP map. The consensus map obtained using TMAP was made up of 147 markers and spanned 1047 cM covering all chromosomes and contains only 4 intermarker distances greater than 30 cM on chromosomes 4, 9, 14 and 16 (Online Resource 7). The TMAP-generated linkage map of chromosome 18 contains 23 markers, spanning 119.9 cM, while the JoinMap® 4.1 map for the same chromosome spans 159.9 cM (Online Resource 7). For chromosome 15, the TMAP-generated linkage map spans 33.5 cM, while the JoinMap® 4.1 map for the same chromosome spans 46.8 cM (Online Resource 7).

QTL mapping

Non-parametric Kruskal–Wallis (single marker regression) analysis revealed several markers on chromosomes 1 and 18 (*Rpv3*) to be associated with resistance to downy mildew (Online Resource 8). No other markers showed any significant association with downy mildew resistance. Permutation test with 1,000 permutations calculated the significance threshold at a LOD of 3.9 to 5.6 for the various phenotypic scores (Table 1). IM and MQM mapping confirmed the location of a major QTL explaining up to 62.1 % of the total phenotypic variance observed for downy mildew with a maximum LOD of 30.4 located between markers VVIN16-cjvh and UDV108 on chromosome 18 (Fig. 1). Automatic cofactor selection selected marker VMC7F2 as cofactor for this region in all scores. When this locus was treated as a cofactor, the potential resistance locus on chromosome 1 persisted with a LOD of 3.73, which is higher than the chromosome-specific significance threshold for chromosome 1. This locus explained 10.9 % of the phenotypic variance observed. Together, the locus on chromosome 18 and chromosome 1 therefore explains up to 52.4 % of the phenotypic variance observed.

The distribution of the mean phenotypic downy mildew scores across all five scores for the four possible genotypic classes showed clearly the effect of the major contribution of the *Rpv3* locus, whilst the genotypes containing both *Rpv3* and the chromosome

Table 1 The location, significance and confidence interval of QTL identified by MQM in 'Regent' for downy mildew resistance

LG[a]	QTL confidence interval	Nearest marker	QTL name		2005_01_28	2005_02_25	2007_11_02
18	VVIN16-cjvh-UDV108	VMC7F2	*Rpv*3	Max LOD	18.98	30.43	20.42
				%Var[b]	41.5	62.1	40.4
				LOD threshold—GW[c]	4.7	5.6	3.9
				LOD threshold—LG18	2.9	3.0	3.1
1	VVIM25-VVIF52	VMC9F2-cjvh[d]	Not named	Max LOD	3.73	ns[e]	ns[e]
				%Var[b]	10.9		
				LOD threshold—GW[c]	4.7	5.6	3.9
				LOD threshold—LG1	2.7	2.7	2.6

[a] Chromosome

[b] Percentage phenotypic variance explained

[c] Genome-wide

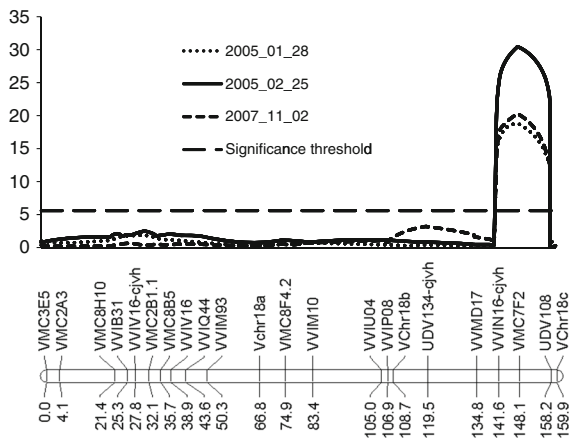[d] Identified with automatic co-factor selection

[e] Not significant

Fig. 1 LOD profiles for linkage group 18 after cofactor selection for resistance to downy mildew. The genome-wide significance threshold at $P < 0.05$ for the phenotypic score Pvit-2005_02_25 is shown
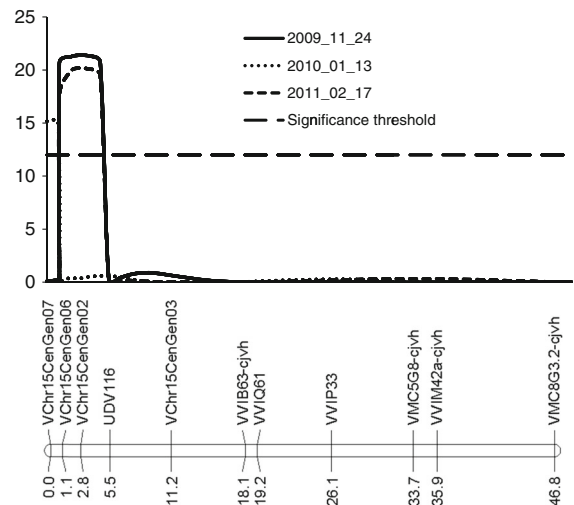

Fig. 2 LOD profiles for linkage group 15 after cofactor selection for resistance to powdery mildew. The significance threshold at $P < 0.05$ for the phenotypic score Enec-2009-11-24 is shown

1 (LG01) minor QTL had a slightly higher resistance response than plants containing only *Rpv3* (Fig. S2, Online Resource 3). As expected, the response of the LG01 minor QTL genotype class was in most cases in the same susceptibility range as the non-QTL carriers (Fig. S2, Online Resource 3).

Single marker regression analysis revealed markers on chromosome 15 (*Ren3*) to be associated with resistance to powdery mildew (Online Resource 9). Direct application of the IM and MQM algorithms resulted in a 'singularity error' as a result of the

complete homozygosity of all the markers on chromosome 15 for 'RedGlobe'. After re-coding the markers and re-classifying the population type as double haploid (DH), according to the MapQTL® 6 manual (Van Ooijen 2009), these algorithms both indicated the location of a major QTL located close to marker VChr15CenGen02, a marker 2.5 cM distal of marker UDV116 (Fig. 2). This QTL explained up to 43.9 % of the phenotypic variance with a maximum LOD of

Table 2 The location, significance and confidence interval of QTL identified by MQM in 'Regent' for powdery mildew resistance

LG[a]	QTL confidence interval	Nearest marker	QTL name		2009_11_24	2010_01_13	2011_02_17
15	UDV116-VChr15CenGen06	VChr15CenGen02	Ren3	Max LOD	21.39	15.17	20.17
				%Var[b]	43.9	32.3	42.5
				LOD threshold—GW[c]	12.0	5.3	7.8
				LOD threshold—LG15	1.5	2.6	1.5

[a] Chromosome

[b] Percentage phenotypic variance explained

[c] Genome-wide

21.39 (Table 2). Automatic cofactor selection selected either marker VChr15CenGen02 (2011_02_17 and 2009_11_24) or VChr15CenGen07 (2010_01_13) as cofactor for this region.

As expected, the mean powdery mildew phenotypic scores across all three scores for the *Ren3* genotypes were more resistant than that of the non-*Ren3* genotypes (Fig. S4, Online Resource 4).

Discussion

The leaf disc assay for downy mildew resistance proved successful in detecting QTL, especially those with major effects. However, it is a very labour intensive process, which is a limiting factor when the population size needs to be increased for fine-mapping. Phenotypic assessments could be improved by methods such as the semi-automated procedure described by Peressotti et al. (2011).

Of the 149 markers, 47 (31 %) deviated from the expected Mendelian segregation $P < 0.05$ (Online Resource 6). This is higher than that reported by Troggio et al. (2007) and Grando et al. (2003), but lower than the 40 % reported by Riaz et al. (2011). One region of distortion that stood out was chromosome 15 where all 13 markers showed significant segregation distortion as previously reported for 'Regent' in this region by Welter et al. (2007). Riaz et al. (2011) also reported segregation distortion for this chromosome in the '06708' population derived from 'Magnolia' (*M. rotundifolia*). This chromosome appears to be problematic as Lowe and Walker (2006) also could not construct a linkage group 15 for 'Riparia Gloire' (*V. riparia*), while the map for the other parent, 'Ramsey' (*V. champinii*), contained four markers spanning a region of 28 cM. Similarly, the map for 'Sirius' (an inter-species hybrid descendant from a cross of 'Bacchus' × 'Villard Blanc') was absent, despite the fact that the consensus map contained eight markers (Mandl et al. 2006). A map could also not be calculated for chromosome 15 in the 'VRH 3082 1-42' × 'SK77 5/3' population investigated by Moreira et al. (2011).

Several markers were found to have null alleles. Where null alleles were detected the null alleles were scored as alleles only if a clear distinction could be made between homozygous and heterozygous individuals. For instance, VVIN74-cjvh2 amplified only allele 188 for 'Regent' and alleles 186 and 198 for 'Red-Globe'. Multiple homozygous F[1] individuals displaying only allele 186 or 198 were found and the presence of a null allele can thus be deduced. F[1] individuals displaying a single peak for 186 or 198 were scored as 186:null and 198:null respectively since 186:186 is not a valid result. For marker VVIN54: 99:115 × 115:null, where 115:115 cannot be distinguished from 115:null as both were valid results, the data for such ambiguous individuals were scored as missing data. This led to a large number of missing genotypes for this marker and it was subsequently removed from the data. However, the scoring of null alleles in this way allowed for the scoring of 16 markers (Online Resource 10) that would otherwise have been excluded from the study. The accuracy of this scoring method was tested by redesigning the primers for one of the markers, VMC8E6, so that all four alleles could be amplified. This redesigned marker, designated VMC8E6-cjvh, was then scored and mapped. VMC8E6 and VMC8E6-cjvh map 0.6 cM apart. By inspecting the scoring for

these two markers it was found that VMC8E6-cjvh had only four missing values compared to the 17 for VMC8E6. Null alleles were found more often in 'Regent' than in 'RedGlobe'. Given that 'Regent' has both *V. vinifera* and non *V. vinifera* origins this is not unexpected. These markers are found on multiple chromosomes with chromosome 15 containing the most.

Previous reports on the mapping of grapevine (Riaz et al. 2008) suggested that TMAP produces maps similar to those produced by JoinMap® 3. The multipoint-likelihood maximisation method implemented by TMAP is robust when dealing with incomplete data sets as well as markers that are not completely informative (Cartwright et al. 2007) and proved to be useful to generate an integrated reference map for grapevine (Vezzulli et al. 2008). Since 46 % of the markers presented in this study are only informative in one of the two parents, TMAP was therefore used to verify the marker order obtained when using JoinMap® 4.1. Since JoinMap® 4.1 has the ability to employ the multipoint-likelihood maximisation mapping algorithm for cross pollination populations, it produced maps similar to TMAP in order, but with slightly longer intermarker distances (Online Resource 7).

The chromosome 18 map for detection of *Rpv3* is in agreement with recently published maps (Marguerit et al. 2009; Zhang et al. 2009; Blasi et al. 2011) as well as with the physical positions of these markers in the genome sequence (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis/, http://www.ncbi.nlm.nih.gov). Marker order inversions were observed only for closely linked markers. The total length of the map is higher than that published by Zhang et al. (2009). The JoinMap® 4.1 map for the same chromosome spans 159.9 cM (Online Resource 7). It is estimated that 1 Mbp is equivalent on average to 2.6 cM in *V. vinifera* (Troggio et al. 2007). With an estimated sequence distance of 28.8 Mbp between VMC3E5 and UDV108, a genetic distance of 74.88 cM is expected, but map distances of 118.6 cM (TMAP) and 158.2 cM (Join-Map® 4.1) were calculated. The reported location of *Rpv3* is represented by four markers, three of which is polymorphic in 'Regent'. Marker VMC7F2 was at the maximum LOD for *Rpv3* as determined by both IM and MQM.

Chromosome 1 is represented by nine markers and covers the entire chromosome (Online resource 5). The map is in agreement with the physical positions of these markers in the genome sequence (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis/, http://www.ncbi.nlm.nih.gov) and published maps (Doligez et al. 2006; Marguerit et al. 2009; Zhang et al. 2009; Blasi et al. 2011). There is a gap of 25 cM between VMC4F8 and VVIC72. Inspection of the parental maps shows that this gap is extended in 'Regent' while it is only 11.9 cM in 'RedGlobe'. For chromosomes 5 and 12 however, the 'Regent' shows shorter intermarker distances than 'RedGlobe'.

Minor downy mildew resistance loci derived from 'Regent' and *V. riparia* have previously been reported on chromosomes 4, 5 and 7. The *Rpv4* locus on chromosome 4 is situated in the interval between markers VMC7H3 and VMCNG2e1 (Welter et al. 2007). Neither of these markers was informative in this study, which resulted in a 48.2 cM gap in the combined map. Considering the minor effect of *Rpv4* it is thus not surprising that this locus could not be verified. The minor effect *Rpv11* locus on chromosome 5 (linked to VVMD27), have previously been detected with various degrees of success in 'Regent' (Fischer et al. 2004; Welter et al. 2007) and 'Solaris' (Schwander et al. 2012) using whole plant and leaf disk assays. This locus was only detected in 'Chardonnay' when evaluating mesophyll invasion by the mycelium and not when leaf disk assays were performed (Bellin et al. 2009). *Rpv11* was not detected in this study using a leaf disk assay, despite the inclusion of the linked marker VVMD27.

Two downy mildew resistance loci, originating from *V. riparia,* have previously been identified on chromosome 12. *Rpv6* was linked to marker VMC8G9 (Marguerit et al. 2009). This locus explained up to 31.5 % of the observed phenotypic variance. We did not detect any evidence that VMC8G9 was linked to downy mildew resistance in the 'Regent' × 'Red-Globe' population. Similarly, *Rpv13* also located on chromosome 12 of *V. riparia* were not detected in this study. Marker VMC1G3.2 was not included in the map due to a large number of missing values, but the map for chromosome 12 is represented by eight markers, including SCU5 and VMC4F3.1, two markers 8.5 cM apart that flank marker VMC1G3.2 and therefore represents the *Rpv13* location reported by (Moreira et al. 2011). Sufficient coverage of the region of importance was thus attained to allow detection of *Rpv13*.

The map for 'Regent' chromosome 15 is in agreement with the location as reported by genome sequencing efforts (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis/). Inspection of the marker distribution on chromosome 15 of the *Vitis* genome revealed that more than six million of the estimated 20 million bases that make up chromosome 15 lay distal of marker UDV116. Eight new primer pairs were designed to amplify dinucleotide repeats distributed in this region. Of these eight markers, only the five listed in Online Resource 2 were mapped to chromosome 15 and four of these five amplified null alleles. The additional five markers extended the chromosome 15 map in the region distal to UDV116 (*Ren3*). Chromosome 15 appears to be completely homozygous for 'RedGlobe'. All 19 markers tested were homozygous in 'RedGlobe' whilst 12 were heterozygous in 'Regent'. Four markers were monomorphic for both 'Regent' and 'RedGlobe' and three failed to amplify. 'RedGlobe' has a highly inbred lineage ('Hunisia' × 'Emperor') × (('Hunisia' × Emperor') × 'Nocera') (http://www.vivc.de/index.php), which explains the high level of homozygosity for chromosome 15 as well as for chromosomes 3 and 13. The coverage of the area carrying *Ren3* in the consensus map provided confidence in detecting this gene in the QTL analysis.

The non-requirement for recoding of allele calls and the ability to deal with markers and individuals with many missing values simplify the use of TMAP for linkage map construction. However, incorporating the TMAP results in MapQTL® 6 requires the incorporation of the phasing output of TMAP with the coded locus information, as well as editing of the map files which negates the advantage. In contrast, JoinMap® 4.1 can easily produce parental maps using the integrated two-way pseudo-testcross strategy and the incorporation of JoinMap® 4.1 results into MapQTL® 6 does not require additional editing steps. For this study, the JoinMap® 4.1 parental and consensus maps were used for QTL mapping.

The continuous nature of the downy and powdery mildew resistance phenotype in segregating populations necessitated QTL mapping as the most appropriate method of determining the chromosomal location and flanking markers of the resistance loci involved (Fischer et al. 2004; St. Clair 2010). Non-parametric Kruskal–Wallis (single marker regression) analysis revealed several markers on chromosome 18

to be associated with resistance to downy mildew with $P < 0.005$ (Online Resource 8). Permutation test with 1,000 permutations calculated the significance threshold at a LOD of 3.9–5.6 for the various phenotypic scores (Table 1). IM and MQM confirmed the location of a major downy mildew QTL explaining between 40.4 and 62.1 % of the phenotypic variance observed with a maximum LOD of 30.4 located between markers VVIN16-cjvh and UDV108 on chromosome 18 (Fig. 1). This location is similar to the location of the *Rpv3* resistance locus reported for 'Regent' and 'Bianca' (Fischer et al. 2004; Welter et al. 2007; Bellin et al. 2009) and distal of the reported location of *Rpv2* (Bellin et al. 2009). The 40.4–62.1 % variance explained is similar to the 46.5–69.5 % obtained for *Rpv3* in 'Regent' by Fischer et al. (2004) and higher than the 15.6–37.3 % obtained by Welter et al. (2007).

A putative minor downy mildew resistance locus on chromosome 1 (LG01) that has not been reported previously, explained 10.9 % of the phenotypic variance observed on chromosome 1 for one score (2005_01_28) only. While the LOD for this observation is lower than the genome-wide significance threshold, it is higher than the chromosome specific significance threshold for chromosome 1, and persisted when the major resistance locus on chromosome 18 was selected as cofactor. This lack of season-to-season repeatability has also been observed for other minor loci like *Rpv4* (Welter et al. 2007).

The most resistant phenotypic means across all scores belonged to the genotypic class (*Rpv3* + LG01) suggesting that it will be worthwhile to combine both the resistance loci in breeding efforts (Fig. S2, Online Resource 3).

The effectiveness of the *Ren3* locus on 'Regent' chromosome 15 was confirmed by three phenotypic scores taken over two seasons. Adding five new microsatellite markers including VChr15CenGen07, improved marker coverage of the *Ren3* region. The resistance linked allele for the marker (VChr15CenGen02) closest to the LOD maximum, is a null allele, which complicates the use of this marker in marker assisted selection. A similar situation was encountered for newly designed markers by Riaz et al. (2011). This failure to amplify using primers designed from *V. vinifera* sequences could indicate that the sequence in these areas differ substantially between *V. vinifera* and non-*vinifera* species. Automatic cofactor selection

varied depending which phenotypic score was analysed e.g. marker VChr15CenGen02 (2011_02_17 and 2009_11_24) or VChr15CenGen07 (2010_01_13). This is probably the result of a poor phenotypic score in the case of 2010_01_13 which gave the lowest correlation with the other two scores for powdery mildew resistance. It should also be noted that several of the markers in this region have null alleles (Online Resource 10) and show a high level of distortion. With this in mind, markers VChr15CenGen06 and UDV116 were identified as flanking markers for marker-assisted selection since they do not display null alleles in this population even though both these markers are highly distorted.

Recent evidence suggests that the resistance to *P. viticola* found in 'Regent' might be strain-specific (Cadle-Davidson 2008; Casagrande et al. 2011), illustrating the need to combine multiple resistance loci to increase the durability of these loci. Durability of resistance loci is particularly important in perennial crops (Dry et al. 2010; Katula-Debreceni et al. 2010).

This study validated the chromosome regions of the major components of downy (*Rpv3*) and powdery mildew (*Ren3*) resistance of 'Regent'. Closely linked markers for marker-assisted selection and gene pyramiding strategies were identified. This is the first report confirming the efficacy of *Rpv3* and *Ren3* in Sub-Saharan Africa. However, similar to Welter et al. (2007) the minor QTL (*Rpv11*) previously detected in 'Regent' on chromosome 5 remained undetected. The minor QTL detected for downy mildew resistance on chromosome 1 in this study, has not been reported before. A cloned F_1:'Regent' × 'RedGlobe' population was planted in the field at the ARC Infruitec-Nietvoorbij where they will undergo further screening for resistance and fruit quality traits, while the original plants are being maintained in a tunnel.

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