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ORIGINAL PAPER

Genetic dissection of sex determinism, inflorescence morphology and downy mildew resistance in grapevine

Elisa Marguerit · Christophe Boury · Aurélie Manicki · Martine Donnart · Gisèle Butterlin · Alice Némorin · Sabine Wiedemann-Merdinoglu · Didier Merdinoglu · Nathalie Ollat · Stéphane Decroocq

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Abstract A genetic linkage map of grapevine was constructed using a pseudo-testcross strategy based upon 138 individuals derived from a cross of *Vitis vinifera* Cabernet Sauvignon × *Vitis riparia* Gloire de Montpellier. A total of 212 DNA markers including 199 single sequence repeats (SSRs), 11 single strand conformation polymorphisms (SSCPs) and two morphological markers were mapped onto 19 linkage groups (LG) which covered 1,249 cM with an average of 6.7 cM between markers. The position of SSR loci in the maps presented here is consistent with the genome sequence. Quantitative traits loci (QTLs) for several traits of inflorescence and flower morphology, and downy mildew resistance were investigated. Two novel QTLs for downy mildew resistance were mapped on linkage groups 9 and 12, they explain 26.0–34.4 and 28.9–31.5% of total variance, respectively. QTLs for inflorescence morphology with a large effect (14–70% of total variance explained) were detected close to the *Sex* locus on LG 2. The gene of the enzyme 1-aminocyclopropane-1-carboxylic acid synthase, involved in melon male organ development and located in the confidence interval of all QTLs detected on the LG 2, could be considered as a putative candidate gene for the control of sexual traits in grapevine. Co-localisations were found between four QTLs, detected on linkage groups 1, 14, 17 and 18, and the position of the floral organ development genes *GIBBERELLIN INSENSITIVE1*, *FRUITFULL*, *LEAFY* and *AGAMOUS*. Our results demonstrate that the sex determinism locus also determines both flower and inflorescence morphological traits.

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E. Marguerit
Université de Bordeaux,
UMR 1287 Ecophysiologie et Génomique
Fonctionelle de la Vigne (EGFV), 33175 Gradignan, France

C. Boury · A. Manicki · M. Donnart · A. Némorin ·
N. Ollat · S. Decroocq (✉)
INRA, UMR 1287 Ecophysiologie et Génomique Fonctionelle
de la Vigne (EGFV), Bât. ISVV, 210 Chemin de Leysotte,
CS 50008, 33882 Villenave d'Ornon cedex, France
e-mail: stephane.decroocq@bordeaux.inra.fr

E. Marguerit · C. Boury · A. Manicki · M. Donnart ·
A. Némorin · N. Ollat · S. Decroocq
Institut des Sciences de la Vigne et du Vin (ISVV),
UMR 1287 Ecophysiologie et Génomique Fonctionelle
de la Vigne, 33882 Villenave d'Ornon, France

G. Butterlin · S. Wiedemann-Merdinoglu · D. Merdinoglu
INRA, UMR 1131 Santé de la Vigne et Qualité du Vin,
68021 Colmar, France

Introduction

Grapevine is a crop of major importance worldwide. Within the large genetic diversity of the genus *Vitis*, subgenera *Euvitis Planch*. ($2n = 38$), generally only *Vitis vinifera* varieties have been cultivated for wine and table grape production. Since the second half of the 19th century the importance of the genetic diversity of other *Vitis* species has become apparent, because they are valuable sources of genes for resistance to diseases, insects and abiotic stresses (Mullins et al. 1992). The most successful breeding activity was dedicated to obtain grapevine rootstocks which permitted grapevine culture in the face of phylloxera (*Daktulosphaira vitifoliae*) attacks. Breeding grapevine varieties for fungal resistance, e.g. to powdery or downy mildew, has been less successful because most inter-specific hybrids tested so far present some organoleptic off-flavours. However, because of increasing environmental and human

health concerns related to chemical spraying of fungicides, a strong effort is currently underway to provide alternatives by genetic selection.

In woody plant species with a long life cycle such as grapevine, there is a great benefit in developing genetic maps to increase our knowledge of the genetic determinism of complex agronomical traits. This allowed us to identify molecular markers to assist breeding programs by early seedling selection. Among the 15 maps already published for grapevine, eight were produced from intra *V. vinifera* crosses (Doligez et al. 2002, 2006; Adam-Blondon et al. 2004; Riaz et al. 2004; Fanizza et al. 2005; Troggio et al. 2007). The other seven were produced from inter-specific crosses (Lodhi et al. 1995; Dalbó et al. 2000; Grando et al. 2003; Doucleff et al. 2004; Fischer et al. 2004; Lowe and Walker 2006; Salmaso et al. 2008). Only one was created with a large number of SSR markers (Lowe and Walker 2006). SSR markers are robust and highly transferable among grapevine genotypes; a large set of these markers is now publicly available [(Thomas and Scott 1993; Sefc et al. 1999; Di Gaspero et al. 2000, 2005; Decroocq et al. 2003; Adam-Blondon et al. 2004; Merdinoglu et al. 2005); NCBI UniSTS].

The published maps have permitted the identification of QTLs for several agronomical quantitative traits, such as berry color and seedlessness (Doligez et al. 2002), fruit yield components (Fanizza et al. 2005), phenology related traits (Costantini et al. 2008), and for disease resistance (Fischer et al. 2004) or for pest resistance (Xu et al. 2008). Up until now, very few reports on QTLs for resistance to grapevine downy mildew, a major grapevine disease caused by the oomycete *Plasmopara viticola,* have been published (Marino et al. 2003; Welter et al. 2007). From these studies, only a small number of molecular markers have been used for marker-assisted selection in grapevine. SCC8, a sequence characterized amplified region (SCAR) marker, was published and permitted the selection of seedlessness (Lahogue et al. 1998). The use of molecular markers to pyramid multiple resistance genes has already been successfully carried out in grapevine breeding (Eibach et al. 2007).

According to the literature, flower sex in grapevine is thought to be controlled by a single locus with three alleles, male, hermaphrodite and female, with the following hierarchy male > hermaphrodite > female (Levadoux 1946; Antcliff 1980). Using a small progeny and one of the first published molecular maps of grapevine, Dalbó et al. (2000) supported this monogenic determinism. Most of the wild *Vitis* species used as sources of resistance gene are dioecious. Populations obtained after crossing these genotypes may include M, H or F phenotypes. In order to quickly identify individuals according to their sexual characters, it could be very useful to use molecular markers.

The aim of our work was to improve our knowledge of the genetic determinism of some important traits, such as sex determinism and disease resistance, using a quantitative genetic approach. In this study, a map, essentially built with SSR markers, was based on a F1 population from an inter-specific cross between two heterozygous parents, *V. vinifera* × *V. riparia*. From this genetic map, this study investigated the linkage between the locus determining the sex of grapevine flowers and several morphological traits of inflorescences as well as localizing QTLs for resistance to downy mildew.

Materials and methods

Plant material and DNA extraction

The mapping pedigree used in this study consisted of 138 F1 individuals derived from the inter-specific cross of *V. vinifera* Cabernet Sauvignon (CS) × *V. riparia* Gloire de Montpellier (RGM). This F1 population, named CS × RGM1995-1, was developed at INRA Bordeaux (France).

DNA was extracted from 80 to 100 mg of young leaves of plants grown in a greenhouse using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

Choice of molecular markers

SSRs markers were mainly chosen from three larges series: VMC (Vitis Microsatellite Consortium, Agrogene SA, Moissy Cramayel, France), VVI (Merdinoglu et al. 2005) and UDV (Di Gaspero et al. 2005) in order to obtain a homogeneous coverage. Then individual markers were also chosen according to Sefc et al. (1999), Dalbó et al. (2000), Grando et al. (2003), Adam-Blondon et al. (2004), Riaz et al. (2004) and Doligez et al. (2006) in order to fill in the remaining gaps on the map. Other markers, EST SSR (Decroocq et al. 2003), were also amplified. As suggested by Lowe and Walker (2006), we consulted previously published maps and identified five or ten primer pairs per linkage group to test for polymorphism in order to quickly develop framework maps with 19 chromosomes.

Five new EST SSRs were obtained from the additional sequencing of the same EST SSR microsatellite enriched library (Table 1). Eleven new SSCPs markers are also reported (Table 2).

Microsatellite markers were first tested on the parents and two individuals in the population. Polymorphic markers were then run on the entire mapping population.

Table 1 cDNA microsatellite markers developed

Coded marker name	Primer name	Primer sequence	Map location
VVC 4	VVC 4 F	GATCACACCACAGAGAGAG	LG 11
	VVC 4 R	TACCGAGCCTGATGAGC	
VVC 9	VVC 9 F	TTAAGCTCAAAACAAGGTTCA	LG 12
	VVC 9 R	GCAAATGAGCAATGCAAG	
VVC 10	VVC 10 F	AGAAAGTCGATATTAAGCAACAGC	LG 7
	VVC 10 R	GAAATGCAGTCATGCCAGAG	
VVC 18	VVC 18 F	AAACCACTATTTAAAAGCCTGAAG	LG 11
	VVC 18 R	TCTGTTTGCCAAGAATAGATCC	
VVC 22	VVC 22 F	TGGGTTGGTAAGATGCAGT	LG 5
	VVC 22 R	CCAACAGCCAATGAACTTAG	

Table 2 SSCP markers developed

Coded marker name	Sequence name	Primer sequence	Map location
FRD3a	GSVIVT00024609001	AGGGTTCTGGTGAGAGACCATA	LG 6
		TGTGCTGGGGCTAGCATAG	
FROb	GSVIVT00026930001	CTCCCTCGCCAGTCTCTTA	LG 15
		GCAGGTGGAAAGAAAAGGA	
FROc	GSVIVT00011355001	CAATTGGCTAGATAATCCAACAT	LG 17
		GCTTTAGATGCCATTGTTCAG	
FROd	GSVIVT00011355001	TTAAGGTATGTTCAACTTTCAGCA	LG 12
		CGAGTGACATAAATCTGAATCTCA	
IRT1a	GSVIVT00032208001	AGGCGCACTCACTCTCAAAT	LG 4
		TCATAGTACTGGGTTCCGACG	
IRT1c	GSVIVT00024285001	TACCCTTTTGCCTTCATGCT	LG 6
		CAGGTTCCTCCATGCTTCTG	
IRT1d	GSVIVT00037540001	GATGCTTGTGCAACCTCTTAC	LG 3
		CTGAACAATGCAACCTCCAA	
IRT1e	GSVIVT00033350001	AGAAAAACGTGGGTTTGCTTAA	LG 19
		TCATAGATGCCACTACCCAGG	
IRT1f	GSVIVT00030117001	CCCAACTGCACTTATTGTTCAA	LG 1
		GAGAATGCAAAACTCCAATTCC	
IRT1 h	GSVIVT00032208001	GTTAGAAGATGGTGATGACGAAGA	LG 4
		TGCCAGCTCCAAGAAACAG	
IRT1i	GSVIVT00024060001	ATTTTCTGCGACAGGTTACGAT	LG 6
		TCGTCAACATGGGCATTG	

Amplification conditions and genotyping

Amplification and genotyping of all the PCR multiplexes were made as described by Merdinoglu et al. (2005). All other PCRs were performed by a single reaction with M13-tailed primer (Oetting et al. 1995) in 15 µl reaction volume containing: 6 ng of template DNA, 1.5 µl of 10× PCR reaction buffer, 0.2 mM of each dNTP, 0.4 U of *Taq* DNA polymerase (Sigma), 0.05 µM of M13 tailed SSR forward primer, 0.2 µM of SSR reverse primer, 0.2 µM of dye conjugated M13 primer. PCR reactions were carried out in a PE 9700 thermal cycler. Amplification conditions were the same for all primers pairs (5 min initial denaturation step at 94°C followed by 15 cycles (30 s denaturation at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C) followed by 30 cycles (30 s denaturation at 94°C, 30 s annealing at 50°C and 30 s extension at 72°C) then followed by 7 min final extension at 72°C). Visualisation was performed with a Ceq 8000 (Beckman Coulter). Up to ten microsatellites were precipitated and analysed in a single run. Scoring of each marker was double checked, and any ambiguous genotypes were re-run, re-amplified or left as unknown.

Phenotypic measurements

Resistance to downy mildew was assessed on leaf discs. Young plants were produced from green cuttings in the greenhouse. At 12-leaf stage, 16 leaf discs were sampled from the fifth and the sixth expanded leaves from the apex of the grape shoots, placed on wet filter paper in Petri dishes the abaxial side up and then artificially inoculated by spraying with a *P. viticola* suspension at ca 10^5 sporangia/mL. Two replicates were performed per individual. Samples were incubated in darkness overnight at 21°C. Petri dishes were then sealed and incubated in a growth chamber at 21°C and a photoperiod of 18 h light/6 h darkness. Six days post-inoculation, leaf discs were scored for their resistance level (resistance level) according to a visual semi-quantitative scale of notation, ranging from 1, for very susceptible, to 9, for highly resistant, based on criteria of the "Office International de la Vigne et du Vin" (Anonymous 1983). Resistance was also quantitatively assessed by counting the sporangia produced per unit of leaf area (sporangia number) using a Z2 Coulter cell counter (Beckman Coulter).

The determination of the sex of each plant was done in the greenhouse in 2002 and 2003; then verified in three other years. In 2003 inflorescences and single flowers were harvested and photographed with a digital camera and measured with the image analysis software ImageJ. The terminology used by May (2004) was chosen to describe inflorescence morphology (Fig. 1). Ovary length and width, stamen length, anther length, stigma width and flower pedicel length was measured along with measurements at inflorescence level: length of the hypoclade (AB), total length of the inflorescence (AD), lengths of the main branch (BC and CD) and on the wing (BE and EF). In the case of 31 genotypes, the wing did not bear any flowers. According to May (2004), this part was named tendril and its length was measured. The presence or the absence of flowers on the wing, the fruit set, the flowering date and the inflorescence number per shoot were also recorded.

Map construction

The double pseudo-testcross strategy was applied to produce the two parental genetic maps (Grattapaglia and

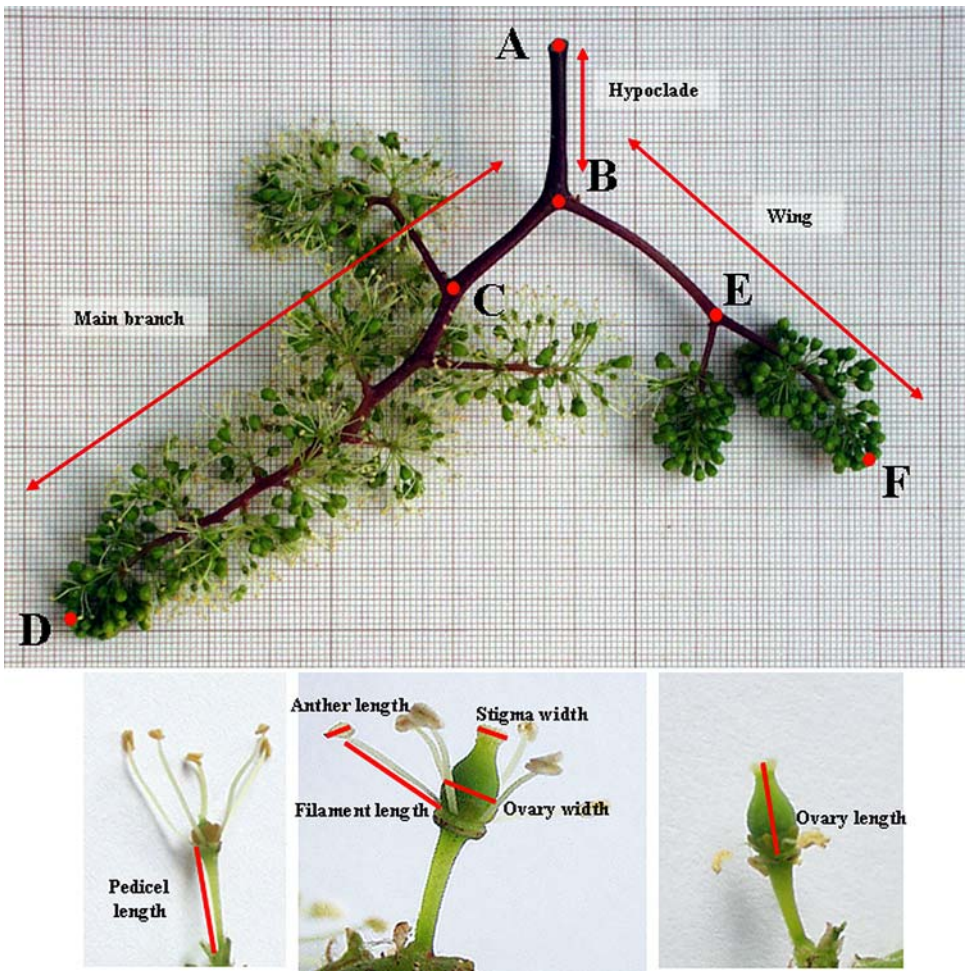

Fig. 1 Inflorescence pictures of one progeny of the studied cross detailing the different measurements made. Each *letter* corresponds to the tip of a measurement. For each cluster, different segments between dots were recorded and were named in the text by the letters located at its extremity

Sederoff 1994). For each marker, the goodness-of-fit of the observed segregation ratio to the appropriate expected ratio was tested using a χ^2 test. The two parental maps were constructed using the software CarthaGene (de Givry et al. 2005). They were constructed at a LOD value of 5.0 and at a maximum distance threshold of 45 cM. They were integrated in a consensus map built with the software JoinMap® 3.0 (Stam 1993). When the marker order obtained with the two types of software used was different, it was fixed with Carthagene and checked according to the previously published maps (Doligez et al. 2006; Lowe and Walker 2006) and the genome sequence [(Jaillon et al. 2007); http://www.genoscope.cns.fr/spip/Vitis-vinifera-sequencage.html].

For parental and consensus maps, the linkage groups were determined with a minimum LOD of 5.0, decreasing to a LOD of 3.0, to link 12, 8 and 2 markers for CS, RGM and consensus maps, respectively. The linkage groups were numbered LG 1–LG 19, according to Adam-Blondon et al. (2004).

QTL analysis

The normality of the phenotypic trait distributions was verified using the Fisher skewness and kurtosis coefficients whereby zero is considered as the ideal value. Deviations from normality were significant if they exceeded the double standard deviation of skewness and kurtosis coefficients, estimated by $(24/N)^{1/2}$ and $(6/N)^{1/2}$, respectively, with N being the number of individuals (Tabachnick and Fidell 1996).

QTL detection was performed with MapQTL 4.0 software (Van Ooijen et al. 2002) using different statistical methods: Kruskal–Wallis analysis, interval mapping, MQM mapping and permutation test. The maximum number of co-factors retained was four. The minimum LOD score retained for QTLs detection was three. The significant LOD threshold was calculated with $\alpha = 5\%$ for the linkage group through 1,000 permutations. The maximum LOD value was retained for QTL position and a ± 1 LOD interval for the confidence interval.

Results

Polymorphism of microsatellite markers

A total of 299 markers were tested to build these maps. Among all these markers, 87 were monomorphic or their amplification was difficult. Of the 212 polymorphic markers used for genotyping the progeny, 96 were fully informative (70 with four segregating alleles and 26 with three), 116 had two segregating alleles with, 76 and 36 informative in CS and RGM, respectively, and four segregated on both parental maps (Table 3). Chi square analysis indicated segregation distortion for a total of 11 markers at a significant threshold of 5%. Among these 11 markers, five showed a consistent linkage with the neighboring markers and consequently they were kept for the consensus map construction. The VMC8H10, VVIH02, VMC2B5, VMC3B12, VVIO52 and VVIV35 markers exhibited amplification at multiple loci. Distinct segregating loci pairs were scored as dominant markers with 7, 4, 3, 3, 2 and 2 sets, respectively. When multiple loci were amplified with the same primer pair, they were scored as dominant markers and the suffix 'a' 'b' 'c' etc. was added to the marker name. After checking the primer sequence, some markers were characterized as identical markers: VMC2G2/VMC2H9 on LG 6, VMC3D12/VVIC51 on LG 13 and VVIN74/VVIP17 on LG 19.

Parental and consensus map construction

A total of 174 markers were used to develop the female CS linkage map (Fig. 2). Five markers were unlinked, 167

Table 3 Genetic map characteristics for *V. vinifera* Cabernet Sauvignon × *V. riparia* Gloire de Montpellier inter-specific cross

Progeny number			138
Number of loci unlinked			3
Number of distorted loci at $\alpha = 1\%$ (% distorted loci)			11 (5%)
Linkage group included two or more distorted loci			8–9–13–16
Segregation type	Number of alleles in segregation	Informative for	Total 212 markers
	4	CS and RGM	70 (33%)
	3	CS and RGM	26 (12%)
	2	CS	76 (36%)
	2	RGM	36 (17%)
	2	CS and RGM	4 (2%)
Average number of individuals genotyped per loci (minimum–maximum)			129 (38–138)
Median number of individuals genotyped per loci			133

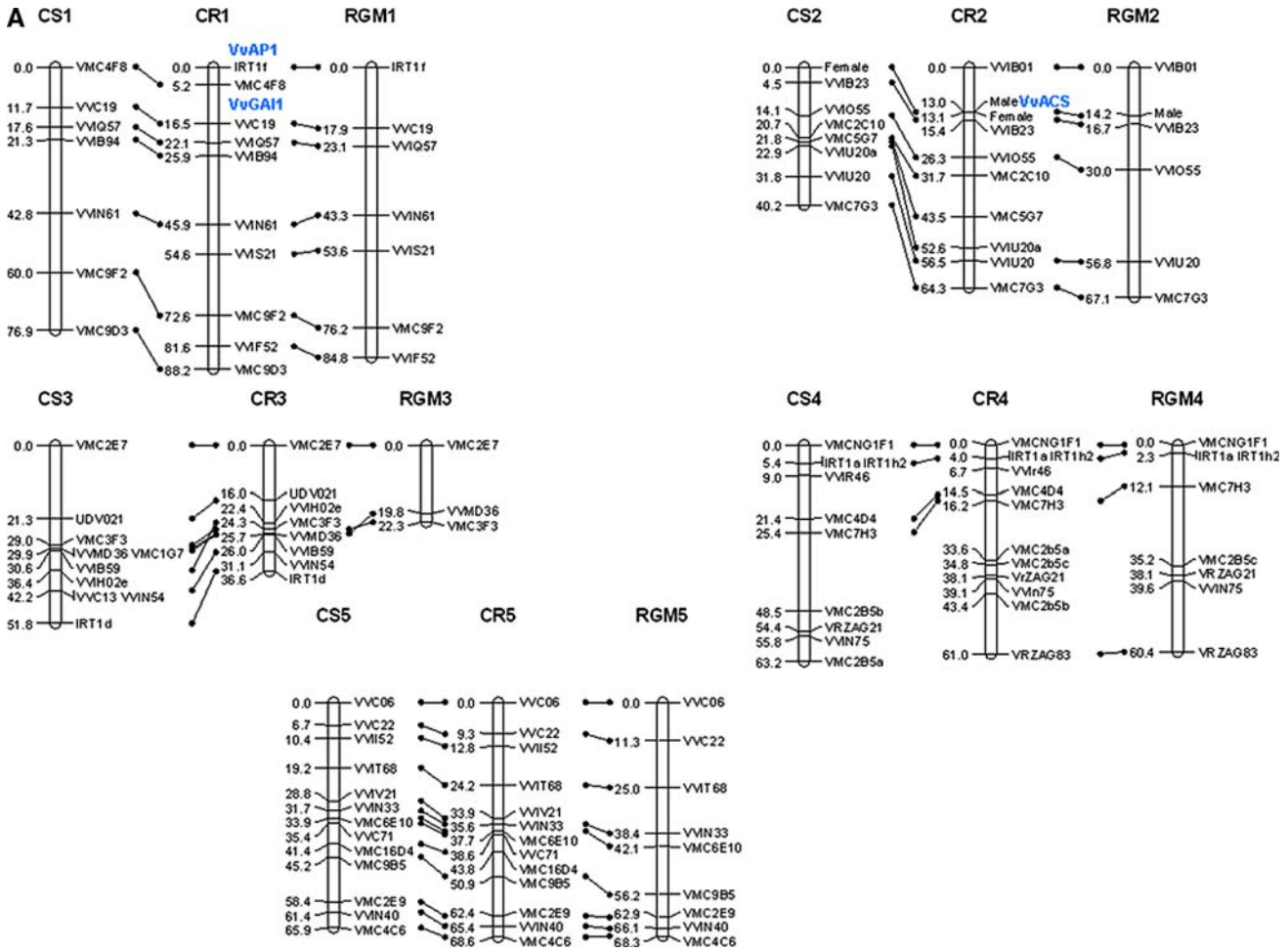

Fig. 2 Linkage maps of *Vitis vinifera* Cabernet Sauvignon, F1 population CS × RGM1995-1 and *Vitis riparia* Gloire de Montpellier. Linkage groups are named according to international consensus map. For each linkage group, the Cabernet Sauvignon map is on the *left* (*CS*), the Riparia Gloire de Montpellier map is on the *right* (*RGM*) and the consensus map is in the *center* (*CR*). Distances are in cM Kosambi. Markers showing segregation distortion are depicted with an *asterisk*. *Italicized markers* represent markers that are linked to the group but are unmapped. Six genes whose sequence was blasted on the *V. vinifera* genome sequence were localized: *V. vinifera APETALA 1* (*VAP1*), *V. vinifera GIBBERELLINS INSENSITIVE 1* (*VvGAI1*), *V. vinifera 1-aminocyclopropane-1-carboxylic acid synthase* (*VvACS*), *V. vinifera FRUITFULL* (*VvFUL*), *V. vinifera LEAFY (VvLEAFY)*, *V. vinifera AGAMOUS* (*VvAG*)

markers were mapped into 19 linkage groups and two markers were linked, but unmapped because of high mean square contributions or weak linkages to other markers within the group. The total length of the map was 1,269.8 cM with an average distance of 7.6 cM between markers (Table 4). There were 17 gaps larger than 20 cM and seven larger than 30 cM. The largest gap was on CS 14 where the distance between marker VMC8H10a and marker VMC8H10d was 38.6 cM. Linkage group sizes ranged from 21.1 cM (CS 19) to 107.8 cM (CS 14), with an average size of 66.8 cM (Table 4). Marker order on the female map was consistent with the consensus map and the male map with only small inversions (Fig. 2).

A total of 136 markers were set on the RGM male parental linkage map (Fig. 2). One hundred and twenty-eight markers were mapped into the 19 linkage groups; five markers remain unlinked. The map length was 1,410.1 cM with an average distance of 11.0 cM between markers (Table 4). There were 27 gaps on the male map with distances larger than 20 cM and five with gaps larger than 30 cM. The largest gap was on RGM 16, which presented a 42 cM distance between marker VMC1E11 and VMC5A1. This last marker was linked to this group with a LOD score of three. Linkage group sizes ranged from 22.3 cM (RGM 3) to 125.4 cM (RGM 16) with an average size of 74.2 cM (Table 4). Marker order on the male map was consistent with the consensus and the female maps (Fig. 2).

The consensus map was developed with 210 molecular markers and two morphological markers, male and female phenotype. The consensus map consisted of a total of 206 linked genetic markers ordered into 19 linkage groups (depicting the 19 *Vitis* chromosomes) with 186 mapped

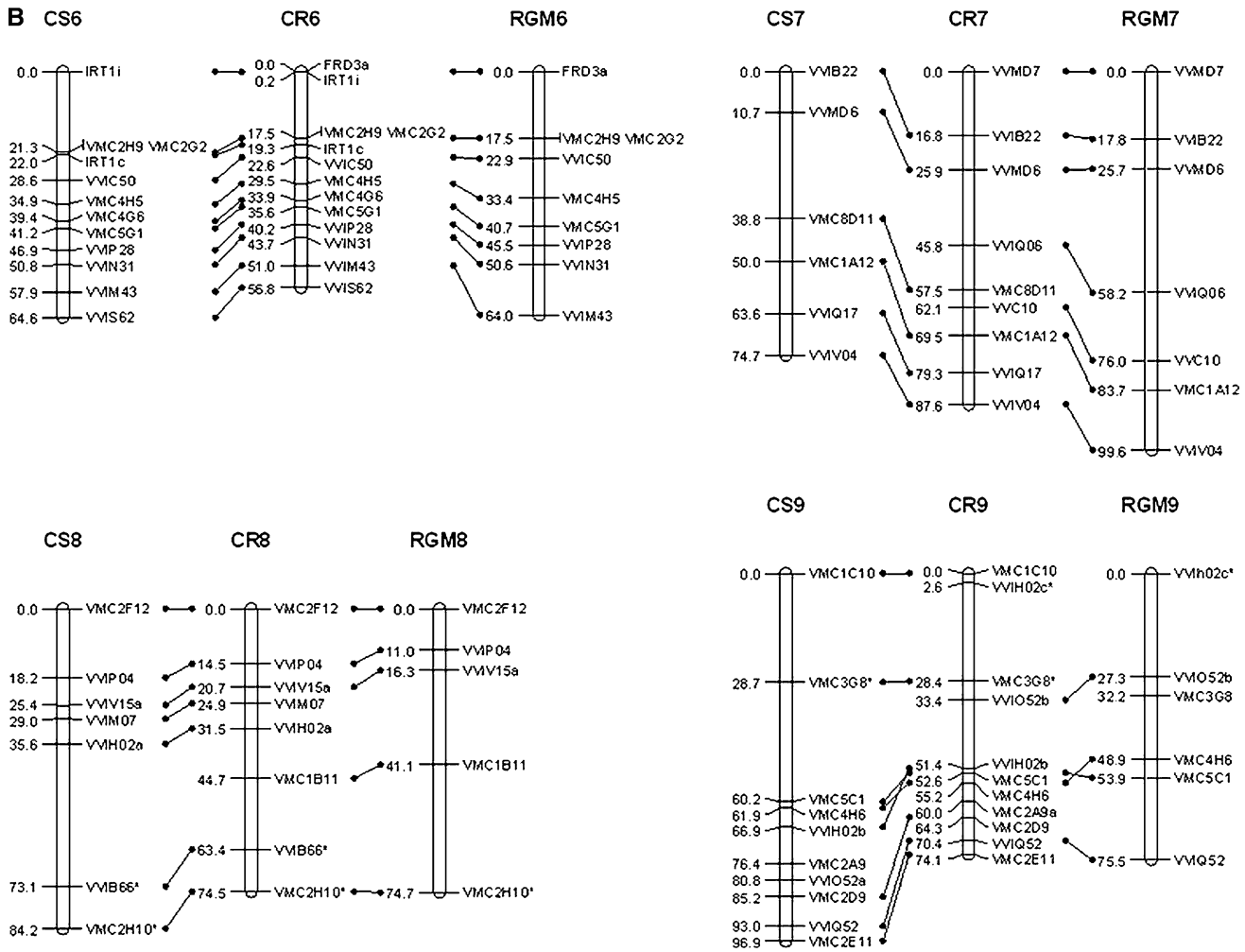

Fig. 2 continued

markers and an average of 9.8 markers per linkage group. Only one SSR marker, VVIC72 segregating in CS, and two markers segregating in both parents, VMC7A4 and VMC8D1, were unlinked. Among 17 unmapped markers, seven showed segregation for the female parent, six for the male parent and four segregated for two alleles in both parents. Only five unmapped markers showed segregation distortion. Even though VVIB66 and VMC2H10 were distorted, they were kept in the LG 8 map because they were correctly localized based upon the genome sequence. Linkage group sizes ranged from 36.6 cM (LG 3) to 94.0 cM (LG 18) with an average size of 65.7 cM. The CS × RGM1995-1 map covered 1,249.2 cM, with 6.7 cM on average between markers (Table 4). Marker order was generally consistent between parental and consensus maps. The marker order was also consistent with the order determined from the *V. vinifera* genome sequence of the French-Italian collaborative project [(Jaillon et al. 2007); http://www.genoscope.cns.fr/spip/Vitis-vinifera-sequencage.html].

Trait analysis

Resistance to downy mildew displayed a continuous variation in the CS × RGM 1995-1 population and segregated as a quantitative trait whatever the parameter used to measure it, either resistance level or sporangia number (Fig. 3), and both of the parameters are significantly correlated ($r^2 = 0.65$). The distribution of resistance level in the CS × RGM1995-1 population ranged from one, which means very susceptible, to five, which means partially resistant. The susceptible (CS) and the resistant (RGM) parents were scored at 3.0 ± 1.0 (susceptible) and 7.0 ± 0.0 (resistant), respectively. The distribution of sporangia number ranged from 12,950 sporangia per cm^2 of leaf for the most resistant genotype to 181,946 for the most susceptible, the susceptible (CS) and the resistant (RGM) parents displaying $63{,}721 \pm 19{,}043$ and $3{,}957 \pm 1{,}780$ sporangia per cm^2 of leaf, respectively. For both resistance parameters, the variation of the population resistance level is beyond

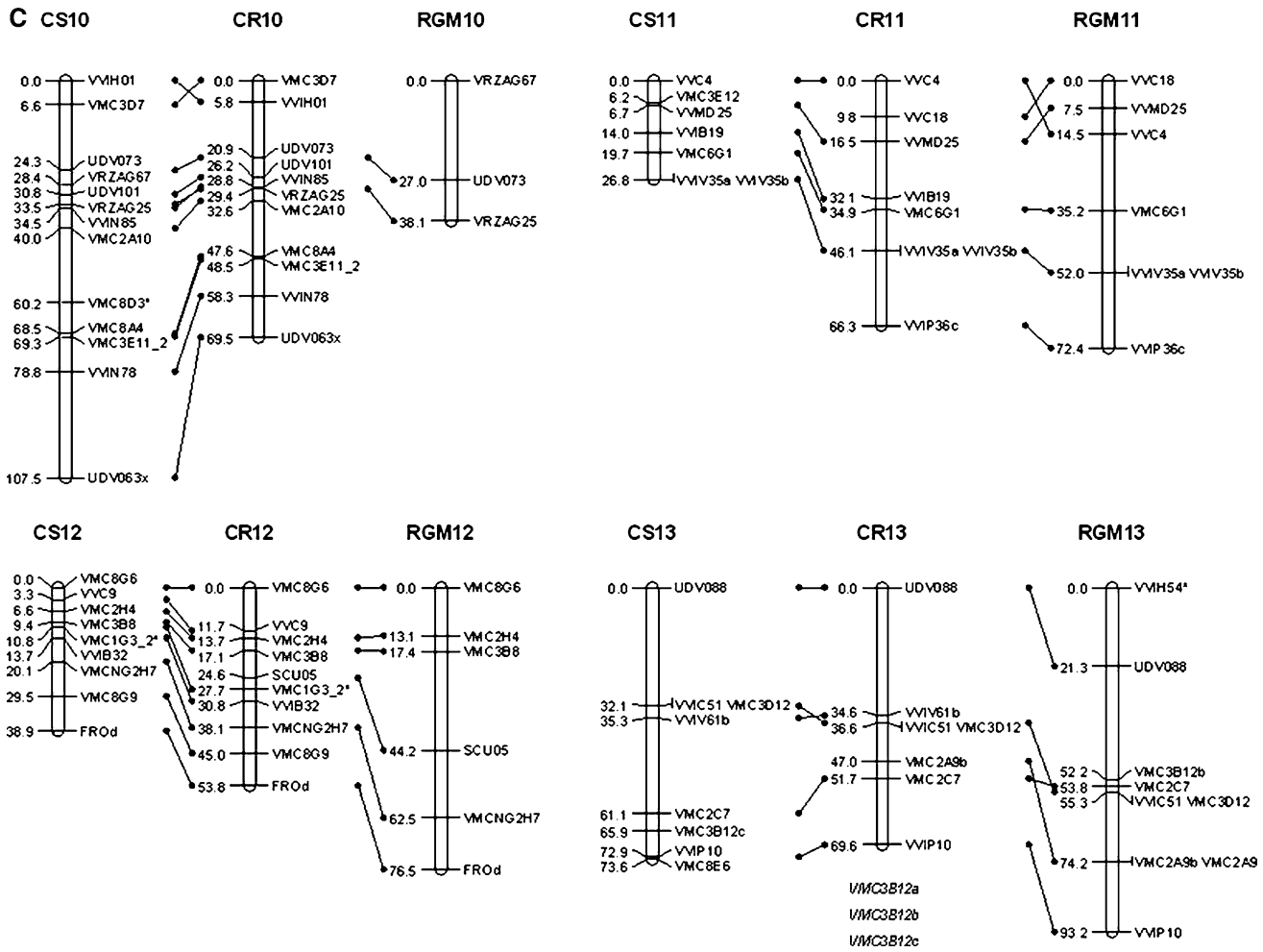

Fig. 2 continued

the range delimited by the parents, the major part of the individuals being more susceptible than CS and none of them reaching the resistance degree of RGM.

Among the different inflorescence traits quantified, significant correlations ($P \leq 0.01$) between the length AB and AC ($r^2 = 0.89$), BD and CD ($r^2 = 0.97$) and EF and the length of the wing/tendril ($r^2 = 0.65$) were found. This was not surprising because, for example, CD is a major part of BD. However, it may be underlined that length dedicated to flowers on the main branch (CD) and on the wing (EF) are highly correlated to the total length of the main branch ($r^2 = 0.97$) and of the wing ($r^2 = 0.65$), respectively. It is also interesting to note that the main branch length (BD) was correlated with the wing length (BF) ($r^2 = 0.86$). Correlations between EF and CD, and the CD:AD ratio and EF: length of the wing or the tendril ratio, were 0.65 and 0.64, respectively. Male inflorescences had significantly the longest inflorescences (AD and BD), the longest wings (BF), and the wing with the longest part of the flowers (EF) ($P < 0.01$).

Mapping of *Sex* locus

Among the 138 F1 plants studied, 35 were female, 60 male, 36 hermaphrodite and seven did not produce inflorescences during the period of study. Since the genetic determinism shows that the male allele is dominant over the hermaphrodite allele, and that this one is dominant over female allele, as discussed later, we decided to map this trait as a single locus on the RGM and CS parental map. For the RGM map, all individuals were scored as male versus female or hermaphrodite. On the female parental map, the male individuals were not scored and female individuals were scored versus hermaphrodite ones. Male and female markers were mapped close to VVIB23, for both parental and consensus maps. On the male map, the male marker was 2.5 cM away from marker VVIB23 whereas on the female map, the female marker was 4.5 cM away from marker VVIB23. On the consensus map, the male and female markers were 2.4 and 2.3 cM away from VVIB23, respectively. This marker segregated for both parents.

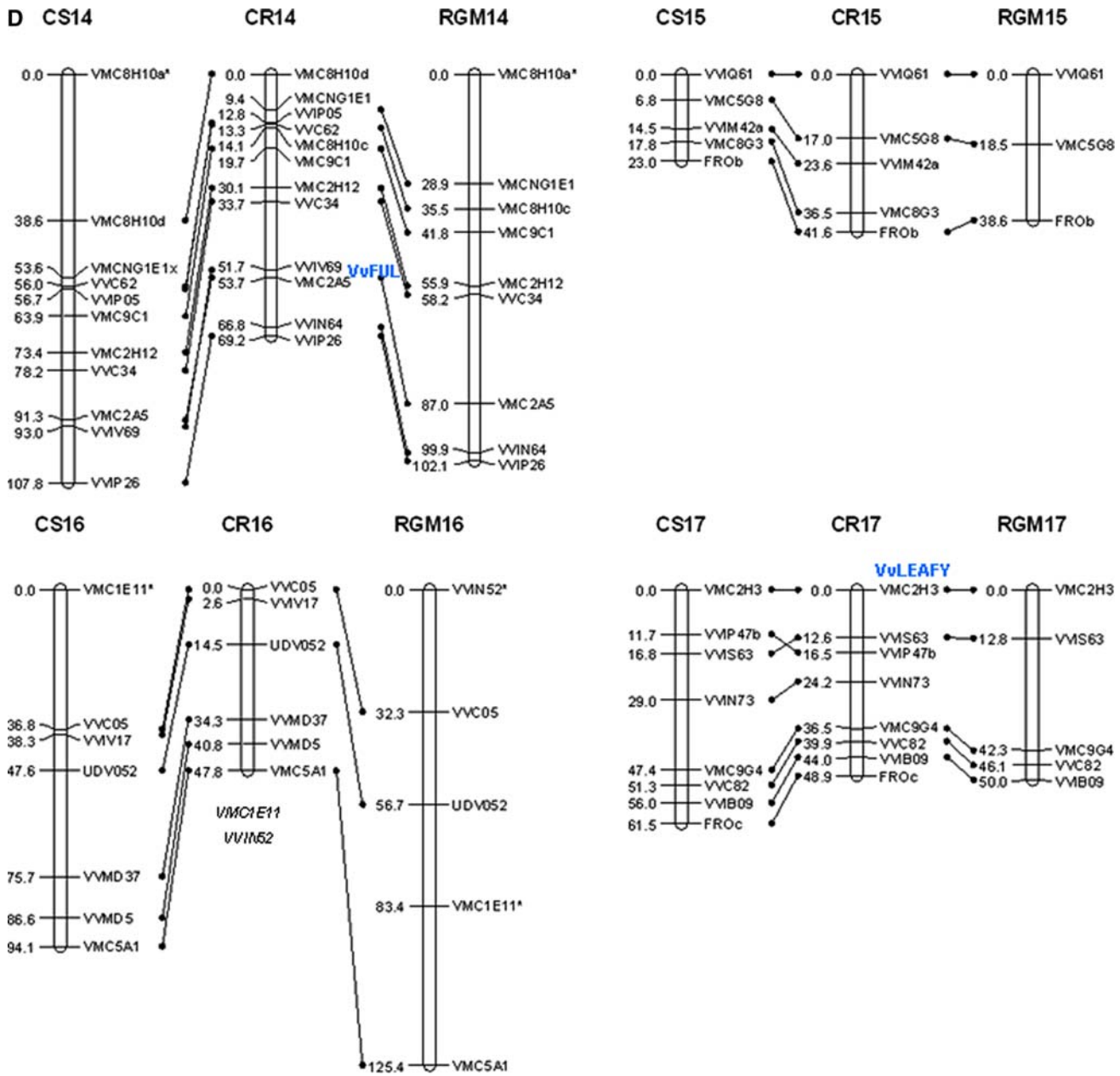

Fig. 2 continued

Close inspection of inheritance of alleles from CS ('a' or 'b') and RGM ('c' or 'd') showed that sex segregation was, as expected, in the parental genotype model HF × FM. There is a strong agreement between genotype and phenotype (Fig. 4). Individuals with a genotype that included the allele 'd' are characterized by a shorter ovary length but a longer stamen: this corresponded to a male phenotype. The individuals with the genotype 'bc' had a smaller stamen, thus it could be deduced that they presented a female phenotype (Fig. 4). Among five recombinants, only three with a genotype 'bc' did not exhibit a female phenotype. Phenotyping ovary width and stigma length also gave consistent results. The genotype 'ac' could be linked to the hermaphrodite phenotype.

QTL analysis

Significant QTLs were obtained for downy mildew resistance, flower and inflorescence morphology (Table 6).

Concerning resistance to downy mildew, two QTLs, located on linkage groups 9 and 12, were detected for resistance level and for sporangia number (Table 6). The LOD scores were slightly more significant for sporangia number than for resistance level. Individual QTLs for sporangia

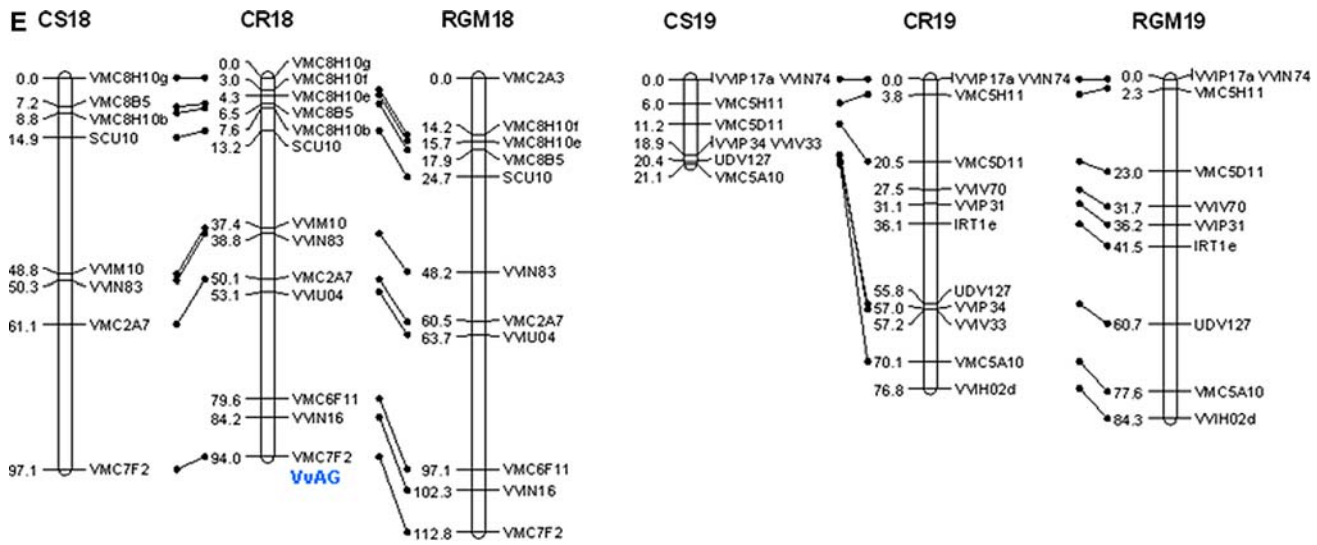

Fig. 2 continued

number accounted for 28.9–34.4% of the phenotypic variance and thus 38.6–46.0% of the genetic variance considering broad sense heritability estimated at 74.8%.

Several QTLs for inflorescence and flower morphology, number of inflorescences per shoot and flowering date were detected on LG 2. For some of them, the confidence interval of the QTL included the sex determinism locus, i.e.: AD, BF, distance BF or tendril length, CD, BD, number of inflorescences per shoot, date of flowering, ovary width, stigma width, filament length (Table 6). The QTLs detected on LG 2 explained from 14% of total variance of length AB and up to 70% of total variance of filament length.

Using co-factors in multiple interval mapping enabled additional QTLs to be found with respect to simple interval mapping. It was particularly true for the traits with a large part of the total phenotypic variation explained by the main QTL on LG 2 (Table 6). A QTL for AD on LG 10, close to the VMC2A10 marker, explained on average 12% of the total variance. Three QTLs for wing length (BE and the EF: length of the wing/tendril ratio) were located on LG 1 explaining from 11.5 to 19.3% of total variance. QTLs for rachis length were mapped on LG 14 (BC, BD; 6.9–20.8% of total variance) and on LG 17 (BC; 15.3% of total variance). A QTL for wing morphology (BF) explaining 16% of total variance, was localized on LG 18.

Discussion

Interest of the crossing

Considering the large phenotypic differences between *V. vinifera* Cabernet Sauvignon and *V. riparia* Gloire de Montpellier concerning flower sex, disease resistance and adaptation to soil characteristics, a population derived from a cross between them is likely to segregate for these traits which is the condition required for QTL detection. In addition, *V. riparia* is already known as an important donor of resistance traits in breeding so this map and the QTLs localized are of particularly high value. Moreover using pure species, rather than complex inter-specific hybrids, as parents allows us to produce clearer conclusions about the genetic determinism of some traits and to integrate the different origins of resistance in a breeding project.

Molecular markers

The high level of reproducibility and polymorphism of *V. vinifera* based SSR markers in non-*vinifera* species emphasizes the efficiency of the SSR marker system as a valuable genomic tool that provides a broader selection of markers than other non-*V. vinifera* mapping projects (Thomas and Scott 1993; Di Gaspero et al. 2000).

The proportion of SSR markers with distorted segregation observed in this study (5%) was lower than that reported by Troggio et al. (2007) (20.3%) and Grando et al. (2003) (22.4%). In addition, this proportion was also lower than the values reported by Lowe and Walker (2006) (16%) and Doligez et al. (2006) (9.2%). No evidence of marker segregation distortion clustering was found on this map.

Some SSCP markers, developed in a study of the genetic determinism of responses to mineral nutrition, were added because they improved significantly the covering of the map.

Female and male parental maps

Considering marker order, linkage group sizes and map length, the parental maps built in this work are consistent

Table 4 Characteristics of the *V. vinifera* Cabernet Sauvignon, CS × RGM1995-1, and *V. riparia* Gloire de Montpellier linkage maps

LG	Cabernet Sauvignon clone 337					CS × RGM1995-1					Riparia Gloire de Montpellier				
	Size (cM)	Markers	Distorted markers	Gaps (>20 cM)	Gaps (>30 cM)	Size (cM)	Markers	Distorted markers	Gaps (>20 cM)	Gaps (>30 cM)	Size (cM)	Markers	Distorted markers	Gaps (>20 cM)	Gaps (>30 cM)
1	76.9	7	0	1	0	88.2	10	0	1	0	84.8	7	0	2	0
2	40.2	8	0	0	0	64.3	10	0	0	0	67.1	6	0	1	0
3	51.8	10	0	1	0	36.6	8	0	0	0	22.3	3	0	0	0
4	63.2	10	0	1	0	61.0	12	0	0	0	60.4	8	0	2	0
5	65.9	13	0	0	0	68.6	13	0	0	0	68.3	9	0	0	0
6	64.6	12	0	1	0	56.8	13	0	0	0	64.0	9	0	0	0
7	74.7	6	0	1	0	87.6	9	0	0	0	99.6	7	0	1	1
8	84.2	7	2	1	1	74.5	8	2	0	0	74.7	5	1	2	0
9	96.9	10	1	2	1	74.1	11	2	0	0	75.5	6	1	2	0
10	107.5	13	1	2	0	69.5	11	0	0	0	38.1	3	0	1	0
11	26.8	7	0	0	0	66.3	8	0	1	0	72.4	7	0	2	0
12	38.9	9	1	0	0	53.8	10	1	0	0	76.5	6	0	1	0
13	73.6	8	0	2	1	69.6	7	0	1	1	93.2	9	1	2	1
14	107.8	11	1	1	1	69.2	12	0	0	0	102.1	9	1	2	0
15	23.0	5	0	0	0	41.6	5	0	0	0	38.6	3	0	1	0
16	94.1	7	1	2	1	47.8	6	0	0	0	125.4	5	2	4	2
17	61.5	8	0	0	0	48.9	8	0	0	0	50.0	5	0	1	0
18	97.1	8	0	2	2	94.0	13	0	2	0	112.8	11	0	2	1
19	21.1	8	0	0	0	76.8	12	0	0	0	84.3	10	0	1	0
Average	66.8	8.8	0.4	0.9	0.4	65.7	9.8	0.3	0.3	0.1	74.2	6.7	0.3	1.4	0.3
Total	1269.8	167	7	17	7	1249.2	186	5	5	1	1410.1	128	6	27	5
Average distance between markers	7.6 cM					6.7 cM					11.0 cM				

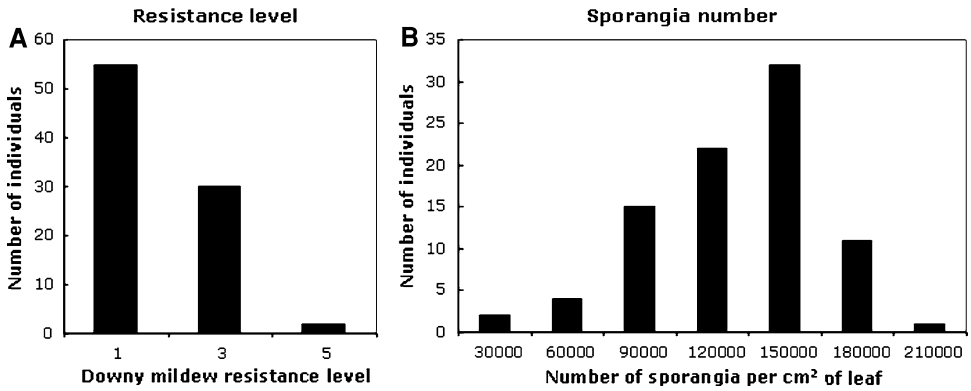

Fig. 3 Distribution of the level of resistance to downy mildew where "1" means very susceptible, "3" susceptible and "5" partially resistant, (**a**) and the number of sporangia per cm^2 of leaf (**b**) 6 days post-inoculation in the CS × RGM1995-1 mapping population

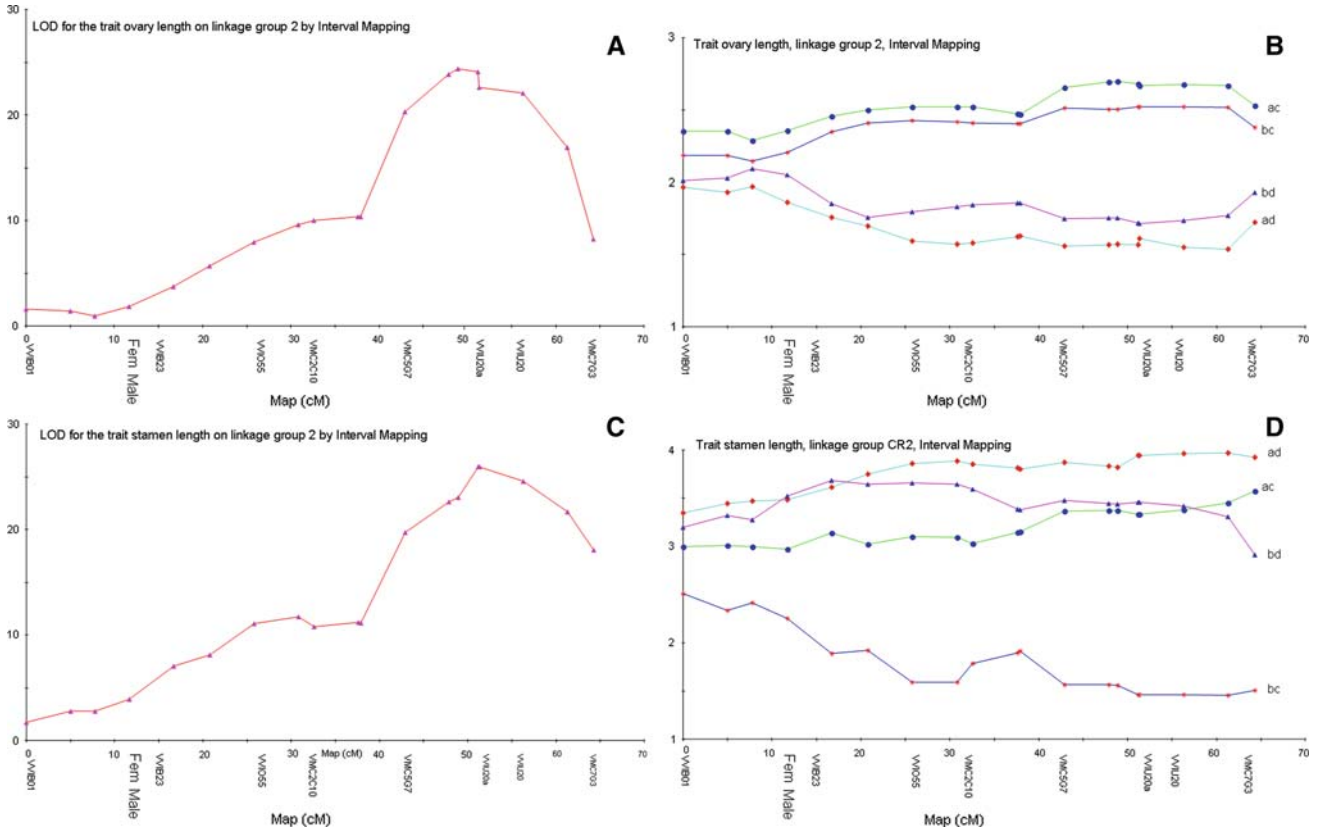

Fig. 4 The LOD score and the estimated mean of the distribution of the quantitative trait ovary length and stamen length associated with the 'ac', 'bc', 'ad' and 'bd' genotype on the LG 2 for the marker VVIB23. The alleles 'a' and 'b' are from CS. The alleles 'c' and 'd' are from RGM

with other published maps (Doligez et al. 2006; Lowe and Walker 2006), except for small discrepancies (two marker inversions in marker order between the consensus map and the genome sequence for linkage groups 10, 12, 13, 14, 17 and 19).

The higher level of homozygoty of RGM was probably the cause of some problems in mapping certain linkage groups. As reported by Grando et al. (2003) and Lowe and Walker (2006), it was difficult to map markers on LG 10 on the RGM map. Moreover, the distance between VRZAG25 and VRZAG67 markers on RGM 10 was 38 cM in our study whereas it was 5 cM in Doligez et al. (2006). Nevertheless it was still possible to detect QTLs with significant LOD and explaining around 10% of the total variance on this linkage group (Table 6). Finally the use of the marker VVC10 has permitted the improvement of the construction of RGM 7, which was split in two unlinked parts in the map of Lowe and Walker (2006).

In comparison with Doligez et al. (2006) (Table 5), the percentage of coverage is, in average, 76% for the CS map and 69% for the RGM one. The lower coverage for RGM could be explained by the fact that a *V. riparia* genetic map

Table 5 Percentage of coverage of the maps presented in Fig. 2 compared with the integrated map determined from five populations of *Vitis vinifera* (Doligez et al. 2006)

Linkage group	Percentage of covering for CS map	Percentage of covering for RGM map	Percentage of covering for concensus map
1	100	95	100
2	61	84	84
3	69	51	69
4	62	85	85
5	98	98	98
6	66	58	66
7	71	89	89
8	84	84	84
9	88	22	88
10	80	6	84
11	58	75	75
12	74	50	74
13	71	67	69
14	87	87	87
15	82	49	82
16	61	79	59
17	70	70	70
18	71	78	70
19	95	95	95

For this calculation, both end markers for each linkage group were reported on Doligez et al. (2006) corresponding groups. This allowed us to calculate the ratio of the length covered between our end markers on the overall length of Doligez et al. (2006) linkage groups

is compared to a *V. vinifera* integrated map. However, when compared to the RGM map published by Lowe and Walker (2006), the coverage clearly increased for 17 of the 19 linkage groups.

Comparison of CS × RGM1995-1 consensus map with other *Vitis* linkage maps and with the genome sequence

The marker order of CS × RGM1995-1 map was found to be consistent with the order found in the genome sequence [(Jaillon et al. 2007); http://www.genoscope.cns.fr/externe/English/Projets/Projet_ML/index.html]. Some markers characterized by an unclear position on a linkage group on the grapevine genome sequence were accurately located on our map i.e. VVIS21, VMC9F2, VVIO55, VVIQ06, VMC8D11, VMC3D7, VVIH01, UDV073, VVIB19, VVIV35, VMC2H12, UDV052, VVMD37, VVIN16, VMC6F11 and VVIN33 (Fig. 2). Among this list, VVIS21 (LG 1) and VVIQ06 (LG 7), which were previously unmapped, could be exactly located with our work. In comparison to the maps of Doligez et al. (2006), the CS × RGM1995-1 map covered up to 79% in average (Table 5).

Resistance to downy mildew

Downy mildew, caused by *Plasmopara viticola*, is a destructive disease and considered as the most important of European grapevine (*V. vinifera*) in humid climates. Grapevine downy mildew infects all green shoots tissues including leaves, tendrils, shoots, inflorescences and fruit bunches and significantly depresses productivity and quality (Lafon and Clerjeau 1988) The main method of prevention is fungicide application. As an alternative to fungicides, grapevine breeding programs targeting the development of new varieties resistant to fungal diseases (using hybrids between good quality European and resistant American species of *Vitis*) have been proposed (Fischer et al. 2004). Resistance to downy mildew from *V. riparia* was mainly determined by two QTLs located on LG 9 and LG 12 whatever method we used. The resistance QTL on LG 9 explained 26.0–34.4% of the total variance and the effect of the QTL on LG 12 was quantitatively similar, accounting for 28.9–31.5%. Considering the sporangia number parameter, the two QTLs of resistance accounted together for 84.6% of the genetic variance, which leads us to assume that a part of the genetic factors determining downy mildew resistance in RGM remains undetected. This assumption is in agreement with the detection of two additional QTLs on linkage groups 7 and 15, having LOD scores higher than two, but lower than the LOD threshold at $\alpha = 0.05$ and accounting for 13.8–16.4% of the phenotypic variance (data not shown). A more comprehensive study using a larger mapping population should be carried out to confirm the detection of the weak effect QTLs for downy mildew resistance located on the linkage groups 7 and 15.

To date very few reports on QTLs for resistance to grapevine downy mildew have been published (Marino et al. 2003; Fischer et al. 2004; Welter et al. 2007). Marino et al. (2003) detected two QTLs for resistance to grapevine downy mildew from *V. riparia* and located on linkage groups called 1 and 8 by the authors. According to the map of Grando et al. (2003), these linkage groups were actually the groups 12 and 4 with the international numbering (Adam-Blondon et al. 2004). As the map of Marino et al.(2003) was built with AFLP markers which were not reported on the map of Grando et al. (2003), it was not possible to confirm that the QTLs detected in our study are at the same location than those of Marino et al. (2003). Two QTLs for downy mildew resistance have been detected in Regent and located on linkage groups 4 and 18 (Fischer et al. 2004; Welter et al. 2007). The Regent pedigree potentially includes up to seven different wild *Vitis* species, which includes *V. riparia*, but the origin of the downy mildew resistance factors present in this cultivar is not yet known. The results presented here allow us to speculate that QTLs for downy mildew resistance detected in *V. riparia*

Table 6 QTLs detected

Traits	Linkage group (nearest marker)	LOD score	LOD threshold $\alpha = 5\%$ on the group concerned	LOD threshold $\alpha = 20\%$ on the whole genome	Confidence interval ±1 LOD in cM	Percentage of total variance explained by the QTL
AC	10 (VMC2A10)	3.5	2.7	3.4	29.4–42.6	10.3
	11 (VVMD25)	3.82	2.7		11.5–22	10.5
	13 (VMC3D12)	4.51	2.6		34.6–35.9	12.6
AD	2 (Male)	8.78	2.6	3.4	8–13.1	26
	6 (VVIC50)	3.30	2.6		19.3–27.6	8.6
	10 (VMC2A10)	4.40	2.8		29.4–42.6	12.4
AB	2 (VVI055)	3.96	2.7	3.4	16.4–31.7	13.6
	10 (VMC2A10)	2.99	2.6		29.4–42.6	10.4
BE	1 (VVIQ57)	4.9	2.7	3.4	21.5–22.1	19.3
	1 (VMC9D3)	4.33			81.6–88.2	18.2
	7 (VVIQ06)	4.03			30.9–50.8	18.7
BF	2 (Female)	9.38	2.8	3.6	13.1–15.4	27.9
	18 (VVIN16)	4.15	2.9		84.2–89.2	16
Presence or absence of tendril instead of a wing	2 (VVIB23)******					
EF	2 (VVIB23)	9.43	2.7	3.6	15.4–21.4	35.1
Distance BF or tendril length	2 (Female)	7.48	2.7	3.5	13.1–15.4	26.2
CD:BD ratio	2 (VVIB23)	7.79	2.6	3.4	15.4–21.4	28.3
EF:BF or tendril length ratio	1 (VMC4F8)	2.84	3.0	3.6	0–10.2	11.5
	2 (VVIB23)	9.15	2.8		15.4–16.4	37.8
CD	2 (Male)	12.57	2.5	3.5	13–13.1	35.3
BC	3(VVIB59)	2.9	2.3	3.3	21.1–25.7	7.9
	14 (VVIV69)	6.22	2.6		48.7–51.7	20.8
	17 (VVIN73)	4.21	2.6		21.5–29.2	15.3
BD	2 (Female)	11.30	2.7	3.5	13.1–15.4	29.2
	3 (VVMD36)	5.56	2.5		24.3–25.6	12.5
	14 (VVIV69)	2.97	2.7		43.7–51.7	6.9
CD:AD ratio	2 (VVIB23)	16.82	2.7	3.4	15.4–21.4	37.8
	3 (VVIH02e)	3.19	2.5		21.1–23.2	5.5
	12 (VMC2H4)	2.88	2.5		13.7–16.9	4.9
	13 (UDV088)	4.03	2.7		0–15	7.2
Number of inflorescences per shoot	2 (Female)	4.91	2.6	3.5	13.1–15.4	15.2
	18 (SCU10)	3.11	2.9		7.6–18.2	9.5
Date of flowering	1 (VVS21)	3.15	2.9	3.4	50.9–59.6	4.6
	2 (Male)	13.68	2.6		3–13	28.9
	7 (VVMD7)	4.79	2.8		0–15	13.4
	14 (VMC8H10c)	5.01	2.7		13.3–19.1	9.8
Presence or absence of fruit	2 (VVIB23)*******					
	12 (VMC2H4)***					
Flower pedicel length	1 (IRT1f)	2.75	2.7	3.5	0–5.2	7.5
	2 (VVIB23)	9.95			15.4–16.4	28.9
Ovary length	2 (VVIB23)	27.20	2.7	3.5	15.4–16.4	63.7
	10 (VMC2A10)	3.6	2.6		29.4–32.6	5.3
Ovary width	2 (Male)	8.63	2.7	3.4	8–13.1	27.3
	17 (VMC2H3)	3.23	2.6		0–5	9.6

Table 6 continued

Traits	Linkage group (nearest marker)	LOD score	LOD threshold $\alpha = 5\%$ on the group concerned	LOD threshold $\alpha = 20\%$ on the whole genome	Confidence interval ±1 LOD in cM	Percentage of total variance explained by the QTL
Ovary length: ovary width ratio	2 (VVIB23)	3.38	2.6	3.4	15.4–21.4	9.6
	6 (VVIC50)	2.98	2.6		22.6	8.4
	7 (VMC1A12)	3.62	2.7		67.1–69.5	9.9
	11 (VVC4)	3.07	2.6		66.2	9.6
Stigma width	2 (Female)	21.67	2.8	3.7	13.1–15.4	56.2
	10 (UDV073)	3.25	2.9		5.8–25.9	4.9
Filament length	2 (Male)	32.21	2.7	3.6	13–13.1	69.7
	6 (FROD3a)	5.65	2.7		0	9.4
	10 (VVIN78)	3.12	2.7		53.5–69.5	8.5
	11 (VVC18)	3.25	2.7		0–16.5	4
Anther length	6 (VVIP37)	3.70	2.9	3.4	43.7–46.6	11.3
	7 (VMC8D11)	4.13	2.7		50.8–57.5	13.1
	12 (VMC2H4)	4.09	2.5		13.7–16.9	11.6
	19 (VMC5H11)	4.36	2.9		0.3–13.8	11.7
Presence or absence of curved stamen	2 (From VMC2A10 to VVIB01) say on 31.7 cM					
Resistance level	9 (VVIO52b)	3.44	2.9	3.5	15.1–44.0	26.0
	12 (VMC8G9)	5.84	2.7		39–51.8	31.5
Sporangia number	9 (VVIO52b)	4.83	2.6	3.5	7.1–45.0	34.4
	12 (VMC8G9))	5.67	2.7		36.1–53.8	28.9

The marker name into brackets indicates the QTL position on the linkage group

The asterisks indicate that the statistical distribution was not normal. Kruskal–Wallis statistical test was used in these cases. Three, six and seven asterisks notified a significance of 1, 0.005 and 0.001%, respectively

The fourth column gives the value of LOD threshold being significant to 95% on the linkage group considered. The fifth column gives the value of LOD threshold being significant to 80% on the whole genome

are different from those of Regent and thus are not introgressed in this resistant cultivar.

Recently a new linkage map of grapevine displaying the locations of resistance gene analogs (RGAs) have been established (Di Gaspero et al. 2007). This map pointed out that, even if grapevine RGAs were found on most of the linkage groups, they were mainly clustered on seven of them, including linkage groups 9 and 12. More precisely, RGAs were identified in the vicinity of SSR markers VMC3G8 on LG 9 and VMC8G9 on LG 12. These markers are included in the confidence interval of the QTLs for downy mildew resistance found in *V. riparia*. This makes RGAs good functional candidates for these QTLs located on linkage groups 9 and 12.

Placement of sex expression locus and QTL analysis of flower and inflorescence morphology

Our work demonstrates that sex determinism and related traits are under the control of a single major region of grapevine genome located on LG 2, near the SSR marker VVIB23.

Previous studies in different backgrounds (Dalbó et al. 2000; Lowe and Walker 2006; Riaz et al. 2006) placed the *Sex* locus on LG 2 with a common linkage to the VVMD34 and VVIB23 markers. Our work is the first for which the segregation between male, hermaphrodite and female phenotypes has been reported together. Indeed, our cross between a male and a hermaphrodite resulted in the three sexes (male, hermaphrodite and female) segregating approximately 2:1:1, which is consistent with the single locus hypothesis (Antcliff 1980). The previous studies were based on crosses permitting the production of only two types of flower sex: female and male (Lowe and Walker 2006; Riaz et al. 2006) or hermaphrodite and male (Dalbó et al. 2000). The dominance relationship between the three alleles was clearly demonstrated in our data. Our data shed new light on sex determinism locus, which seemed to determine flower morphological traits such as ovary and stamen size, but also inflorescence size and number, inflorescence complexity (presence and size of the wing) and flowering time. For grapevine, Fanizza et al. (2005) determined one QTL for berry number per cluster on LG 2 close to

VVIO55 and localized at 13 cM from the position of *Sex* locus. Costantini et al. (2008) showed that QTLs for flowering time and mean seed number per berry are close to VVIB23, an other marker next to the *Sex* locus. All together these results support the hypothesis that this locus influences more characteristics than just the determinism of flower sex. Because of its high economical importance, sex determinism has been studied for a long time in papaya (Storey 1953) which is closely related to grapevine in the phylogenic tree based on dioecy and sex chromosomes (Charlesworth 2002). In the light of more recent advances in genetic linkage mapping tools, Sondur et al. (1996) suggested that sex determination in papaya is also controlled by a single gene with three alleles for male, hermaphrodite and female. In papaya and maize, flowers also displayed secondary sexual characteristics, such as modified ovary shape and peduncle length, that co-segregated with the *Sex* locus (Storey 1953; Dellaporta and Calderon-Urrea 1993).

Several genes involved in fertility control and inflorescence development have been studied (Boss et al. 2001; Boss and Thomas 2002; Calonje et al. 2004; Boualem et al. 2008). Using the information recently released from whole grapevine genome sequence [(Jaillon et al. 2007); http://www.genoscope.cns.fr/spip/Vitis-vinifera-sequencage.html], the putative location of these candidate genes was investigated.

The enzyme 1-aminocyclopropane-1-carboxylic acid synthase is involved in melon male organ development (Boualem et al. 2008). This protein sequence was blasted against the grapevine genome. After locating all the significant alignments on the grapevine genome, one significant alignment was found with the scaffold 112 on the LG 2, located in the confidence interval of all QTLs detected on this linkage group. Consequently, this gene could be considered as a putative candidate gene for the control of sexual traits in grapevine. Four other flowering-related genes map close to important regions controlling flower and inflorescence traits identified in this work: *V. vinifera GIBBERELLIN INSENSITIVE1*, which when mutated converts tendrils to inflorescences (Boss and Thomas 2002); *V. vinifera FRUITFULL*, which in grapevine is expressed in a fashion consistent with the role of its *Arabidopsis thaliana* homologue in floral transition and carpel and fruit development (Gu et al. 1998; Calonje et al. 2004); *V. vinifera LEAFY*, which probably has a role in flower meristem initiation and organization in most *LEAFY*-like genes studied in other species (Carmona et al. 2002; Carmona et al. 2008); and *Vitis vinifera AGAMOUS*, which along with other genes in *A. thaliana* is involved in the specification of stamens, carpels and ovules (Mizukami and Ma 1992; Ray et al. 1994; Boss et al. 2001). All these genes were located within the confidence intervals of the QTLs identified on linkage groups 1, 14, 17 and 18, respectively. It is also interesting to note that another floral identity gene *V. vinifera APETALA1* (Calonje et al. 2004) is localized next to the marker VMC4F8 on LG 1. The scale of QTL detection makes it impossible to establish a physical link between the QTLs detected and the position of these candidate genes. However, this work suggests that the study of these candidate genes would be of value.

Here we have demonstrated that the genetic map presented in this study is a useful tool to unravel the genetic determinism of some important traits. QTLs for downy mildew resistance, which were different than those previously detected on different crosses including Regent as a parent, were identified in this work. These results could shed new light on marker-assisted selection concerning downy mildew resistance. The fact that resistance to downy mildew may be under the control of non-homologous regions in different genetic backgrounds suggests that the use of several sources of genetic material could provide more sustainable resistance.

The position of the *Sex* locus on LG 2, close to VVIB23, was confirmed in this work. The gene encoding the enzyme 1-aminocyclopropane-1-carboxylic acid synthase, involved in melon male organ development and located close to the *Sex* locus, could be considered as a putative candidate gene for the control of sexual traits in grapevine. The *Sex* locus could also be involved in flower and inflorescence development. Some genes known to control these traits were found within the confidence intervals of several QTLs identified on other linkage groups. Our data support the monogenic determinism model of sex in grapevine. However, further work is necessary to assess whether it is a single gene or a regulon of multiple genes at this locus.

Considering breeding activities, grapevines are rather inconvenient as they are relatively large plants with a long generation time. In general grapevine seedlings typically do not flower until their second or third cycle. Marker-assisted selection of sex could be used to select individuals of the desired sex early in their life cycle so that only plants suitable for the next step of selection are propagated, saving time and space.

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