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ORIGINAL PAPER

Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for *R*-gene candidates

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Abstract Genetic maps functionally oriented towards disease resistance have been constructed in grapevine by analysing with a simultaneous maximum-likelihood estimation of linkage 502 markers including microsatellites and resistance gene analogs (RGAs). Mapping material consisted of two pseudo-testcrosses, 'Chardonnay' × 'Bianca' and 'Cabernet Sauvignon' × '20/3' where the seed parents were *Vitis vinifera* genotypes and the male parents were *Vitis* hybrids carrying resistance to mildew diseases. Individual maps included 320–364 markers each. The simultaneous use of two mapping crosses made with two pairs of distantly related parents allowed mapping as much as 91% of the markers tested. The integrated map included 420 Simple Sequence Repeat (SSR) markers that identified 536 SSR loci and 82 RGA markers that identified 173 RGA loci. This map consisted of 19 linkage groups (LGs) corresponding to the grape haploid chromosome number, had a total length of 1,676 cM and a mean distance between adjacent loci of 3.6 cM. Single-locus SSR markers were randomly distributed over the map (CD = 1.12). RGA markers were found in 18 of the 19 LGs but most of them (83%) were clustered on seven LGs, namely groups 3, 7, 9, 12, 13, 18 and 19. Several RGA clusters mapped to chromosomal regions where phenotypic traits of resistance to fungal diseases such as downy mildew and powdery mildew, bacterial diseases such as Pierce's disease, and pests such as dagger and root-knot nematode, were previously mapped in different segregating populations. The high number of RGA markers integrated into this new map will help find markers linked to genetic determinants of different pest and disease resistances in grape.

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Introduction

Linkage maps based on a skeleton of transferable markers are flexible tools for finding the chromosomal position of genes/QTLs for traits of interest and exploring the genetics underlying the observed phenotypic variation in natural germplasm and breeding lines. Candidate genes, QTLs and phenotypic loci positioned using one experimental cross can be tested for maintenance of their correlation in other genotypes, if the linked markers are of a suitable type for comparative mapping. Microsatellites or Simple Sequence Repeats (SSRs) are the best markers to fit into this scope. Hundreds of SSR markers were isolated in grape over the past decade (reviewed in Doligez et al. 2006) and were incorporated into several genetic maps. These maps were mainly aimed at identifying

the genetic determinants of sex, berry colour, seedlessness and disease-resistances (Lodhi et al. 1995; Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Doucleff et al. 2004; Fischer et al. 2004; Adam-Blondon et al. 2004; Riaz et al. 2004; Lowe and Walker 2006). A framework of a few dozen to 245 SSR markers, upon which to place other classes of markers (mainly AFLPs) that helped closing gaps, was adequate to achieve those goals and cover all linkage groups expected on the basis of the haploid chromosome number ($n = 19$). Recently, a consensus map entirely based on SSR markers has been constructed by merging segregation data from different crosses (Doligez et al. 2006). When further integrated with functional markers, SSR-based framework maps might guide one to the genomic region(s) responsible for the variation of a biological function and speed up the genetic evaluation of different phenotypes. For instance, functional markers related to disease-resistance, such as Resistance Gene Analogs (RGA) developed on DNA sequences that resembled plant resistance genes could assist breeding of disease-free grapevine varieties. In the past years, candidate genes that putatively translate into nucleotide-binding-site/leucine-rich-repeat (NBS–LRR) protein receptors have been isolated in grapevine (Di Gaspero and Cipriani 2002, 2003; Donald et al. 2002). NBS–LRR genes account for the majority of plant resistance genes. They have at least three structural domains, a C-terminal leucine-rich repeat region, a central nucleotide binding site and a *Toll*-interleukin1 receptor (TIR) domain or a coiled–coil (CC) motif alternatively present at the N-terminus, which allows the encoded proteins to function as antibody-like guards of pathogen-derived effectors and as activators of the defence mechanism (reviewed in Takken et al. 2006). Based on the N-terminal domain, they are classified as TIR– or CC–NBS–LRR genes. CC–NBS–LRR genes are present in all angiosperms, TIR–NBS–LRRs are present in dicotyledonous species (Bai et al. 2002) with one documented exception (Tian et al. 2004). Members of both groups have been found in grapevine (Di Gaspero and Cipriani 2003). This large gene family has several hundred members that operate and translate the cascade of events from pathogen perception to the hypersensitive plant cell death (Monosi et al. 2004). The members of this gene family are usually found into clusters. Their chromosomal positions are conserved among the taxa of a botanical family, albeit genotype-specific functionality is dependent on allelic variation.

Here we present the chromosomal localisation of RGA markers for NBS–LRR genes using a consensus linkage map based on the most recently updated skeleton of transferable microsatellite markers. This map is based on two full-sib populations of 46 individuals each (*Vitis vinifera* 'Chardonnay' × *Vitis* 'Bianca' and *V. vinifera* 'Cabernet Sauvignon' × *Vitis* 'breeding line 20/3'), both genotyped with the same set of markers. In the first cross, the segregation data of SSR markers already used for different aims (Di Gaspero et al. 2005; Doligez et al. 2006) has been substantially extended, while SSR genotyping of the second cross is completely novel. The aim of this paper is to assign to their sub-chromosomal region, RGA markers already developed for the major clades of NBS–LRR genes known in disease-resistant grapes (Donald et al. 2002; Di Gaspero and Cipriani 2003) as well as novel RGA markers for new NBS–LRR clades identified in BAC-end sequences (BES) of 'Cabernet Sauvignon'. These maps shall provide a tool for localising genomic regions that control the resistance to different pests and diseases in grape, paving the way to positional cloning of resistance genes.

Materials and methods

Plant material

Genomic DNA was extracted from young leaves (Doyle and Doyle 1990). Forty-six offspring of the cross *V. vinifera* 'Chardonnay' × *Vitis* 'Bianca' and 46 of the cross 'Cabernet Sauvignon' × *Vitis* 'breeding line 20/3' were used for segregation analysis. Plant material is maintained at the Experimental Farm of the University of Udine, Udine, Italy. 'Chardonnay' and 'Cabernet Sauvignon' are world-wide grown cultivars of *V. vinifera*. The male parents are breeding lines that originated from hybridisation between different *Vitis* species and have gained ~80% of *V. vinifera* genetic background following several cycles of backcrossing. 'Bianca' originated from a cross between 'Villard Blanc' (synonym of 'Seyve Villard 12-375'), which is a complex hybrid including *V. labrusca*, *V. rupestris*, *V. berlandieri*, *V. lincecumii* and *V. vinifera*, and *V. vinifera* 'Bouvier' (Csizmazia and Bereznai 1968). 'Breeding line 20/3' was selected from a cross between 'Bianca' and 'SK77-4/5' (Kozma 2000). 'SK77-4/5' was bred at the University of Novi Sad, Serbia and Montenegro (Cindric et al. 2000) by crossing 'Kumbarát', which originated from hybridisation of *V. amurensis* and *V. vinifera* (Koleda 1975), and *V. vinifera* 'Traminer'.

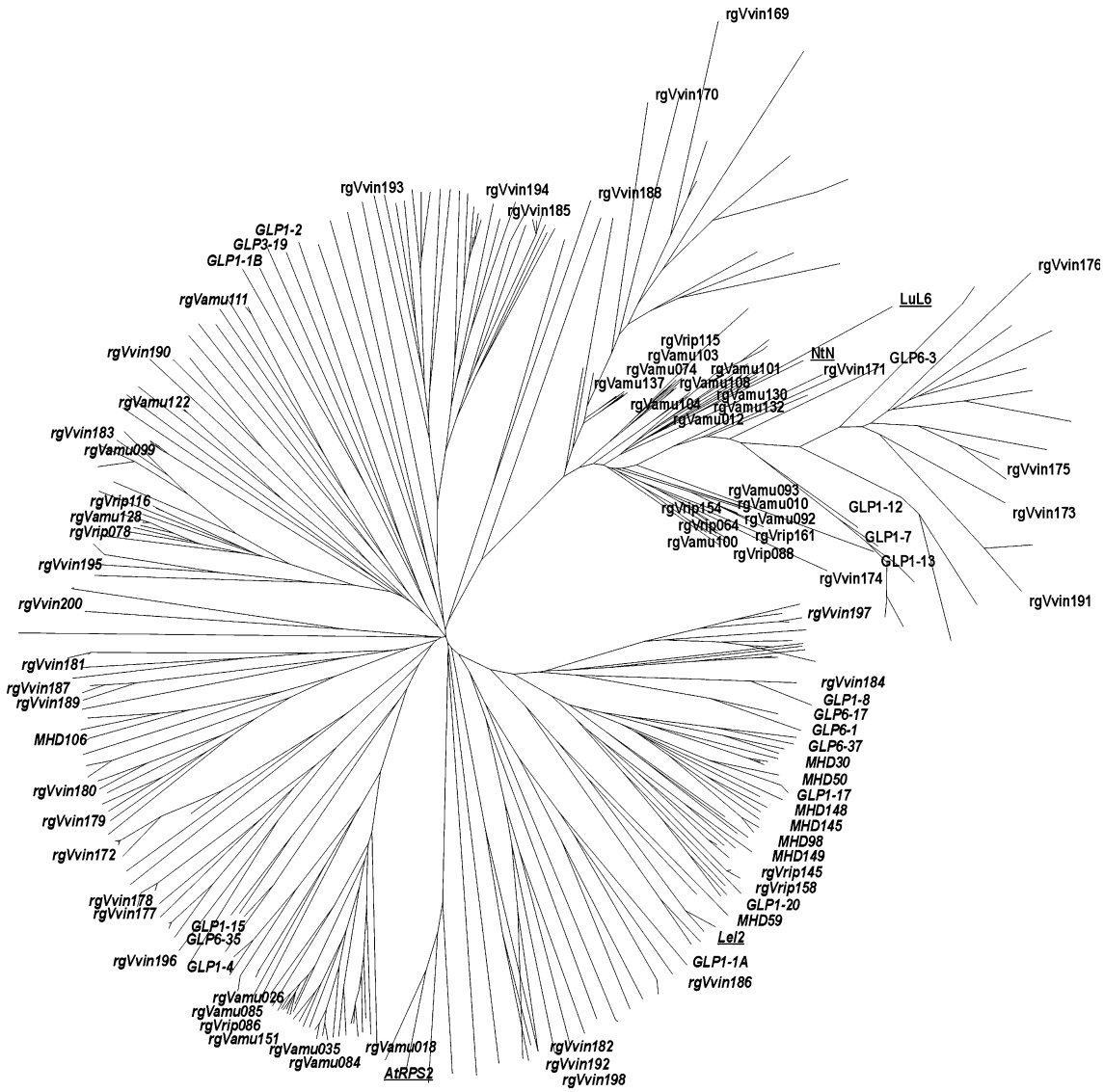

Fig. 1 Phylip tree of 366 NBS–LRR sequences from four grape accessions (*Vitis amurensis*, *Vitis riparia*, *Vitis vinifera* × *Muscadinia rotundifolia* 'BC5', *Vitis vinifera* 'Cabernet Sauvignon'). Sequence identifiers were omitted due to space limitation, except for the sequences used for marker development. Markers were designed on representative sequences dispersed over the major clades. Markers targeting NBS–LRR sequences of the coiled–coil type are in *italics*; those targeting the *Toll*-Interleukin1 receptor (*TIR*) type are in a *Roman face*. The NBS–LRR genes from *Arabidopsis thaliana*, *RPS2* (AAK38117, referred to as *AtRPS2*), *Lycopersicon esculentum*, *I2* (AAD27815, referred to as *LeI2*), *Nicotiana tabacum*, *N* (A54810, referred to as *NtN*), and *Linum usitatissimum*, *L6* (U27081, referred to as *LuL6*), are underlined and were included into the tree in order to draw the distance between grape sequences scaled to outgroup sequences

Development of new RGA markers for NBS–LRR genes

Nucleotide-binding-site/leucine-rich-repeat-like sequences were searched in 77,237 BES from a *V. vinifera* 'Cabernet Sauvignon' BAC library (Lamoureux et al. 2006). Known grape NBS sequences (GenBank accession nos. AY427077-135, AY427152-194, AF369813-37 and AF365879-81) were queried against the BES-database by tBLASTx. The threshold *E*-value was set at a relaxed stringency of 10 e^{-4} in order to avoid a biased identification of sequences closely related to the NBS–LRR clades used to query.

All previously known and the newly identified NBS–LRR sequences were aligned with ClustalW and arranged into a Phylip tree. Sequences were arbitrarily selected in order to cover all major branches of the tree (Fig. 1). Selected sequences were aligned again and PCR primers were designed using Primer3 (Rozen and Skaletsky 2000) on nucleotide sequence arrays that were specific to the different sequences.

BAC-end sequences that contained NBS–LRR-like genes were also searched for the presence of microsatellites using the software Repeats Search (http://depts.washington.edu/etyoung/microsats/repeats.htm). Dinucleotide microsatellites harbouring at least ten repeats, and trinucleotide and tetranucleotide with at least seven repeats were searched.

Progeny genotyping

The SSR primer pairs used for genotyping and their PCR conditions are provided as Electronic Supplementary Material (S1). PCRs were performed in a 10 µl volume containing 200 µM of each dNTP, 2.5 pmol of each primer (with one primer labelled with 6-FAM, HEX or TAMRA), 1 U of HotMaster *Taq* polymerase (Eppendorf, Milan, Italy) with 2.5 mM $MgCl_2$ buffer, and 10 ng template DNA. PCR products were separated by capillary electrophoresis using a MegaBace500 sequencer (GE Healthcare, Milan, Italy). Alleles were called and sized using Fragment Profiler v2.1 (GE Healthcare).

The RGA primer pairs and their PCR conditions are provided as Electronic Supplementary Material (S1). PCRs were performed in a 10 µl volume containing 200 µM of each dNTP, 5 pmol of each primer, 1 U of HotMaster *Taq* polymerase (Eppendorf) with 2.5 mM $MgCl_2$ buffer, and 30 ng template DNA. Single stranded PCR products were separated on 6% glycerol and 0.5 × MDE polyacrylamide gel (BioWhittaker Molecular Applications, Milan, Italy) run at room temperature for 16 h at 6 W and stained with silver nitrate. The SCAR marker STS-AA6 developed by Dalbó et al. (2001) was mapped as described for the RGA markers.

Genetic mapping

Linkage maps were constructed using CarthaGene 0.999R (de Givry et al. 2005). Individual maps were constructed for each parental genotype according to a two-way pseudo-testcross model (Grattapaglia and Sederoff 1994). Genotype data of the progeny were arranged into a F2 backcross-like dataset for each parent. All markers present in a heterozygous state ('*ab*') in a given parent were scored in the F1 progeny mimicking a '*ab* × *00*' segregation, irrespective of the allelic state of the second parent and with the following exceptions. Markers of '*ab* × *ab*'-type were scored in the progeny as '*aa*' = A, '*bb*' = H, '*ab*' = missing data. Markers of '*a0* × *ab*'-type were scored in the progeny as '*a*−' = missing data, '*ab*' = A, '*b0*' = H. In this category, markers were retained if linkage was maintained at LOD ≥ 4.0. In all cases when a marker yielded more than two amplicons per parent in any of the four parents, the marker was assumed to amplify duplicated loci. Non-allelic peaks were scored independently in the parents. Each parental locus was identified by the marker name, suffixed by the code of the parent (–CH for 'Chardonnay', –CS for 'Cabernet Sauvignon', –BI for 'Bianca', –H2 for 'breeding line 20/3') and a letter (–A, –B, –C, –D) standing for each non-allelic peak (band) segregating from a given parent.

Linkage groups were determined at a LOD of 4.0 and at a maximum distance threshold of 30 cM in the first round of grouping. Homologous LGs were compared with each other and to the reference consensus map of Doligez et al. (2006). In all cases when a reference LG split in a parental map due to the presence of large gaps, the fragmented groups were forced to stick together thus compelling the number of provisional LGs to match the grape haploid chromosome number. LGs were given the code LG 1–LG 19, according to Doligez et al. (2006). Marker order within each LG was heuristically determined using the 'build' command, which progressively places markers at the insertion points that lead to the final highest maximum likelihood. Marker order was then optimised, if a marker order with a higher log-likelihood occurred, using the algorithm 'taboo' driven by the command 'greedy 3 1 1 15' and locally refined, if a marker order with a higher log-likelihood was found, by computing the log-likelihood variation of all possible marker orders within a sliding window of five markers (command 'flips 5 2 1'). Map distances were calculated using the Kosambi function. Loci deviating from the first Mendel's law were identified by the χ^2 test of the observed allelic frequency for each locus of the parental data sets. Markers that showed a significant departure ($P = 0.05$) from 1:1 ratio were incorporated into the parental maps in the first round of ordering. Then, markers inconsistently positioned and markers that caused large increases in map distance between the adjacent loci in one parental map when compared to at least two other parental maps, were removed from that map.

A composite map was finally constructed using genotypic data of trimmed loci arranged according to the inferred parental phases. Loci amplified by multilocus markers were coded as in the parental maps and loci of each parental map were therefore treated as independent markers in the consensus map. Datasets from the two crosses were first merged using the command 'dsmergen' which calculates an overall recombination rate for each pair of markers, based on all available meioses, under the assumption of homoge-

neous recombination rate over the parents. LGs were constructed at a LOD of 4.0 and at a maximum recombination of 0.30. Under the same assumption, a framework map with marker order supported by a LOD of 2.0 was also built using the command 'buildfw 2 2 {} 1'. Datasets from the two crosses were then merged using the command 'dsmergor' which calculates a parent-specific recombination rate for any pairs of markers. Based on genotype-specific recombination, a consensus marker order was produced, without providing an integrated map distance. All integrated maps were then optimised and refined using the commands 'greedy 1 0 1 20' and 'flips 5 2 1'. A test for random distribution of single-locus microsatellites and RGA markers over the composite map was carried out by calculating the coefficient of dispersion (CD) as the ratio between the variance and the mean of the number of markers located within genetic intervals of 10 cM along the whole map (Cervera et al. 2001). All maps were drawn with MapChart (Voorips 2002).

Results

Generation of resistance-related markers

A set of 90 primer pairs were designed to amplify RGA markers covering all major clades of NBS–LRR gene-like sequences discovered in grape. NBS–LRR sequences came from three sources.

The first source was a batch of 103 sequences (GenBank accession nos. AY427077-135 and AY427152-194) spanning the NBS region and isolated from two wild *Vitis* species, *V. amurensis* and *V. riparia*, which are resistant against several fungal diseases (Di Gaspero and Cipriani 2002, 2003). From a previous work (Di Gaspero and Cipriani 2003), a set of 33 PCR-based RGA markers (series rgVrip and rgVamu) were selected in order to cover all the major NBS–LRR clades present in that batch of sequences.

The second source was a batch of 28 sequences (GenBank accession nos. AF369813-37 and AF365879-81) spanning the NBS region and isolated from a *V. vinifera* × *Muscadinia rotundifolia* BC_5 that is completely resistant to powdery mildew (Donald et al. 2002). The resistance-donor species *M. rotundifolia* has a repertoire of multiple resistances to fungal, bacterial and nematode-vectored viral diseases. In addition to the marker GLP1-12 developed by Donald et al. (2002), 25 new PCR markers (series GLP and MHD) were developed on the original RGA sequences (Electronic Supplementary Material S1). Two sequences (GLP1-3 and GLP6-38) were abandoned because they were too similar to others for designing selective primers.

The third source of sequences for marker generation were 235 NBS–LRR gene-like sequences identified in 77,237 BES from a *V. vinifera* 'Cabernet Sauvignon' BAC library (Lamoureux et al. 2006).

The complete set of 366 NBS sequences was grouped according to sequence similarities. A Phylip tree was constructed with ClustalW in order to identify major clades (Fig. 1). The in silico search in the BES provided further NBS–LRR sequences that generated new clades. A set of 31 new RGA markers (series rgVvin) were developed on a representative sequence for each clade not yet covered by any marker of the rgVamu, rgVrip, GLP and MHD sets.

The inspection of the tree topology revealed that clades representing CC–NBS–LRR sequences had longer terminal branches. This feature is indicative of an ancient origin and/or a higher diversification (Cannon et al. 2002). Fifty-six out of ninety RGA markers were intended to sample the gene diversity within the CC–NBS–LRR sub-class. Highly similar sequences occurred abundantly in the TIR–NBS–LRR clades, which expanded into a multitude of short terminal branches. The major clades of TIR–NBS–LRRs were covered by the remaining 34 RGA markers.

With regard to the origin of NBS–LRRs, the first (Di Gaspero and Cipriani 2002, 2003) and second (Donald et al. 2002) batch of sequences were independently obtained through a similar approach that used degenerate primers to target the same region within the NBS–LRR genes. Minor differences in the common procedure of isolation and the use of taxonomically unrelated genotypes yielded two non-redundant batches of sequences that were rarely included into the same clade. The batch of sequences gathered by in silico search was more comprehensive suggesting that a bias in the identification of certain clades slightly affected the isolation with degenerate primers.

A search for the presence of microsatellite repeats was performed using the 235 BES that showed similarity with NBS–LRR genes. Twelve sequences were found to contain a dinucleotide or a trinucleotide repeat alongside the NBS–LRR region and were used to design PCR primers that flanked the microsatellite (UDV-503 to UDV-522 in Electronic Supplementary material S1). These SSR markers are physically linked to RGA sequences and were generated because they may help facilitate a rapid cross-linking between different maps due to their co-dominant nature.

In summary, the entire set of 103 resistance-related markers included (1) 90 markers designed within NBS–LRR genes and one SCAR marker linked to powdery

Table 1 Main features of four parental maps (*CH* Chardonnay, *CS* Cabernet Sauvignon, *BI* Bianca and *H2* Breeding line 20/3) of the integrated map (I) and of the framework integrated map with marker order supported by a LOD of 2.0 (I-FW)

	CH	BI	CS	H2	I	I-FW
Mapped SSR markers	293	305	319	280	420	264
SSR loci[a]	302	313	324	286	536	266
Mapped RGA markers	27	33	45	55	82	16
RGA loci[a]	30	40	55	63	173	18
Total markers	320	337	364	335	502	280
Total loci[a]	332	353	379	349	709	284
Unlinked loci[b]	3	4	3	5	0	
Discarded loci	11	21	6	7	7	
Intra-parent inconsistency[c]	10	18	2	7	7	
Inter-parent inconsistency	1	3	4	0	0	
Segregation type of SSR markers[d]						
ab × aa	84	–	114	–		
aa × ab	–	100	–	76		
ab × ab (codominant)	18	13	17	16		
a0 × ab	7	8	2	3		
ab × a0	10	9	14	10		
ab × ac	125	126	93	96		
ab × cd	49	49	79	79		
Map features						
Total map length (cM)	1,210	1,425	1,254	1,418	1,676	1,428
Mean distance between loci (cM) (standard deviation)	5.9 (4.7)	7.0 (6.5)	5.8 (4.6)	6.9 (6.8)	3.6 (3.8)	5.4 (4.2)
Number of gaps >20 cM	4	5	6	8	1	3
Number of gaps >30 cM		3		4	1	
Number of distorted loci at $P = 0.05$ (% distorted loci)	12 (3.6)	47 (13.3)	50 (13.1)	11(3.1)		
LGs containing two or more distorted loci	5, 15, 19	1, 4, 5, 11, 14, 16	1, 8, 11, 14, 19	1, 3, 7, 13		

[a] Non-allelic peaks (bands) amplified by multi-locus markers in any of the parents were treated as independent markers in the integrated map

[b] At LOD = 4.0, $d = 0.30$

[c] Large increase of map length or order swapping of neighbouring markers

[d] Allelic state in the crosses 'CH × BI' and 'CS × H2'of the SSR markers that could be placed on the parental maps. Individual maps were constructed following the reduction of the actual allelic state listed below to a simulated allelic state of '*ab* × *00*' or '*00* × *ab*' that generated two sets of 'F2 backcross' data type per cross

mildew resistance (Dalbó et al. 2001) to be mapped by SSCP analysis and (2) 12 SSR markers physically linked to NBS–LRR genes to be mapped by length polymorphism.

Genetic maps

The four parental maps are provided as Electronic Supplementary Material (S2) and their main features are summarised in Table 1. Each individual map contained 320–364 markers. The number of LGs ranged from 22 to 30 in the individual maps. The LGs fragmented in one parent but known to belong to the same chromosome were merged by relaxing the LOD and/or increasing the maximum distance values. Map intervals where linkage was broken at the highest stringency are indicated by arrows in the parental maps of Electronic Supplementary Material (S2).

Total length of the parental maps varied from 1,210 to 1,254 cM for the *V. vinifera* parents and from 1,418 to 1,425 cM for the *Vitis* hybrids. Mean distance between adjacent loci was 5.8–5.9 cM for *V. vinifera* parental genotypes, and 6.9–7.0 for *Vitis* hybrids. The distribution of inter-locus distance between pairs of adjacent markers is shown in Fig. 2. LGs showed a general tendency to be longer in *Vitis* hybrids but the main contribution to map length increment was due to distance expansion in the middle of LG 1 and LG 17, and in the distal part of LG 16. Marker order among the four parental maps was well-conserved. Order swapping occurred only between pairs of tightly linked markers, affecting map intervals of 5.4 cM on average (Electronic Supplementary Material S3).

The composite map incorporated 502 markers and resulted in 19 LGs (Fig. 3). The integration of four parental maps containing complementary sets of segregating markers allowed to close most gaps present in the parental LGs. Moreover, the increased number of meioses analysed in the composite map provided a more precise calculation of marker order and map

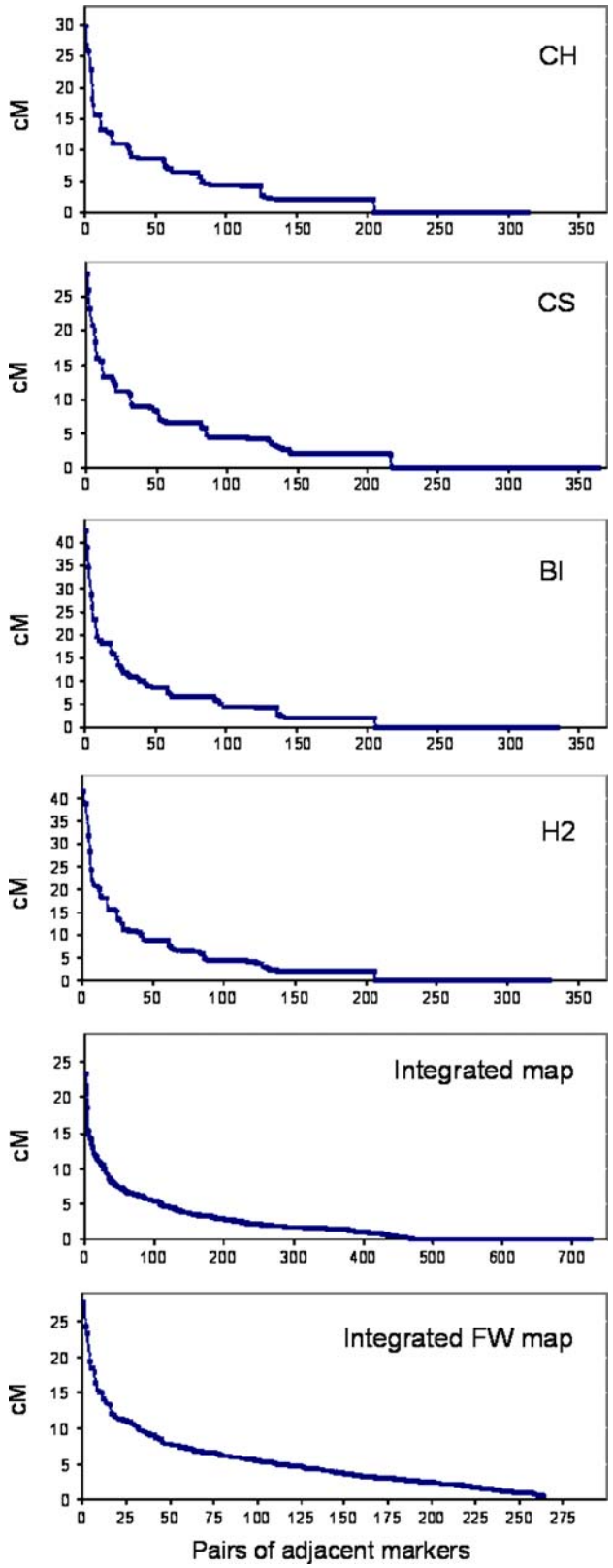

Fig. 2 Inter-locus distance (cM) between pairs of adjacent markers in four parental maps (*CH* 'Chardonnay', *CS* 'Cabernet Sauvignon', *BI* 'Bianca' and *H2* 'breeding line 20/3') and in the integrated complete and framework (*FW*) maps

distances. LGs were also consistent at a LOD = 6.0, with only five loci (VMC4c10–BI–B, rgVrip161–BI–B, GLP3-19–BI, GLP1-17–CH and GLP1-1B–CS–A) that were found unlinked to their LGs.

The microsatellite skeleton of the composite map consisted of 420 SSR markers that identified 536 loci. A set of 363 microsatellites were single-locus among which 56, 71, 113 and 123 were concurrently positioned on one, two, three and four parental maps, respectively. Test of random distribution proved randomness for genomic dispersion of single-locus SSR markers (CD = 1.12). The remainder SSR markers (13.5%) were multi-locus. Of these, 9.0% detected intra-chromosomal duplications and 4.5% amplified two or more loci that mapped to different LGs. The distribution of multi-locus SSR markers over the LGs is shown in Fig. 4. LGs 3, 9, 12, 13, 14, 16 and 18 were particularly rich in duplicated regions. LG 9 and LG 18 had predominantly multi-locus markers that identify intra-chromosomally duplicated loci. By contrast, LG 3 and LG 16 were rich in markers that detected at least one additional locus in a different LG.

A total of 82 RGA markers were positioned on the consensus map, which identified 173 RGA loci (Fig. 3). Three more RGA markers (Vvin170, Vvin172 and Vvin186) were only assigned to the corresponding LG (Electronic Supplementary Material S1) but not included in the composite map. Chromosome localisation of RGA markers showed a substantial departure from randomness (CD = 3.66). The presence of at least one RGA marker was found on 18 of the 19 LGs. However, most RGA markers showed a clustered arrangement into a few sub-chromosomal regions, with 83% of all RGA loci falling into seven LGs, namely 3, 7, 9, 12, 13, 18 and 19 (Fig. 5). Two chromosomes (LG 12 and LG 18) were particularly rich in RGA markers and they alone accounted for 43% of all mapped RGA loci.

Six of the 426 SSR markers reported in Electronic Supplementary Material (S1) could not be positioned on the consensus map because they caused major rearrangements of the neighbour loci. These markers were only assigned to the corresponding LG (Electronic Supplementary Material S1) but not included in the composite map. Twenty-six SSRs were homozygous in all four parents (data not shown). About 18 of the 103 resistance-related markers could not be assigned to any of the LGs because of lack of polymorphism or unscorable segregations.

The composite framework map with marker order supported by a LOD of 2.0 was made of 284 loci, which spanned 1,428 cM over 19 LGs, with a mean inter-locus

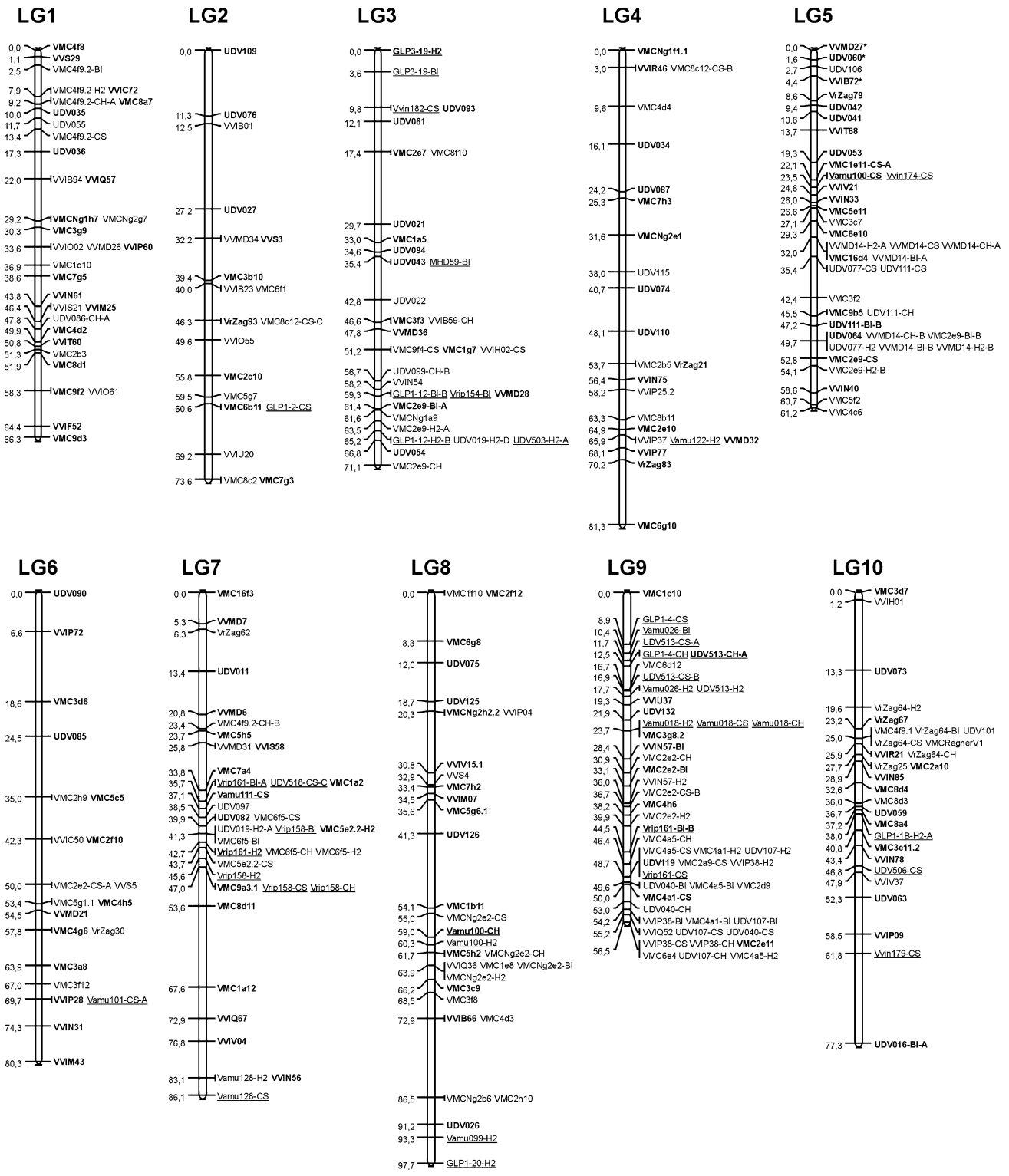

Fig. 3 Composite linkage map of grapevine based on the integration of four parental maps. Microsatellite markers belong to the series scu, UDV001 to UDV135, VMC, VrZag, VVI, VVMD and VVS. RGA markers (*underlined*) belong to the series GLP, MHD, Vamu, Vrip, Vvin, UDV503 to UDV522. *Bold* markers were included into the framework map with marker order supported by a LOD of 2.0. Swapping of marker order between the complete and the framework map is highlighted with *asterisks* (*). Multi-locus markers are identified by the *marker name*, suffixed by the code of the parent (*–CH* for 'Chardonnay', *–CS* for 'Cabernet Sauvignon', *–BI* for 'Bianca' and *–H2* for 'breeding line 20/3') and, when necessary, by a letter (–A, –B, –C, –D) standing for each non-allelic peak (*band*) segregating from a given parent. Map distance is expressed as cM Kosambi and represents an integrated distance calculated with the 'dsmergen' merging method

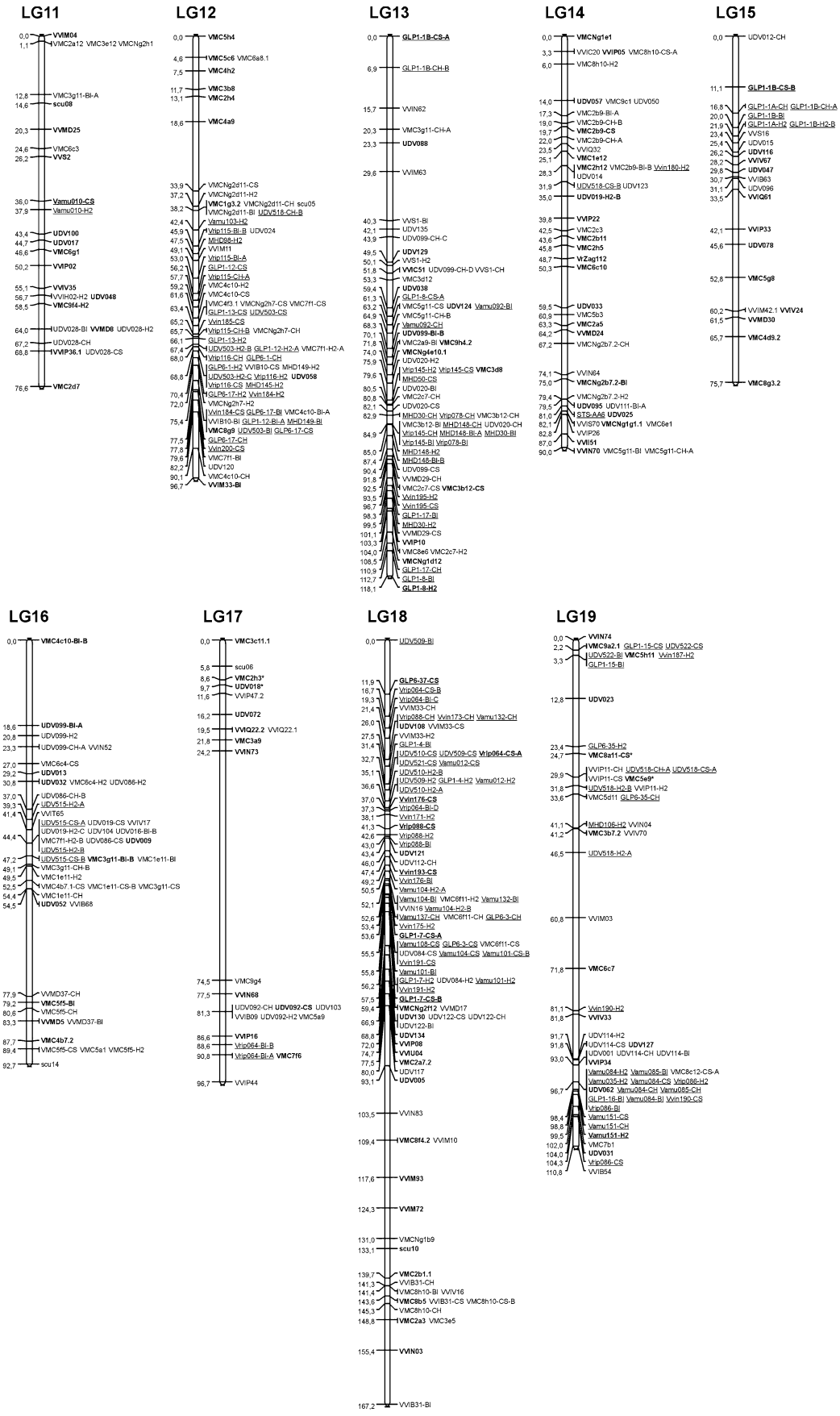

Fig. 3 continued

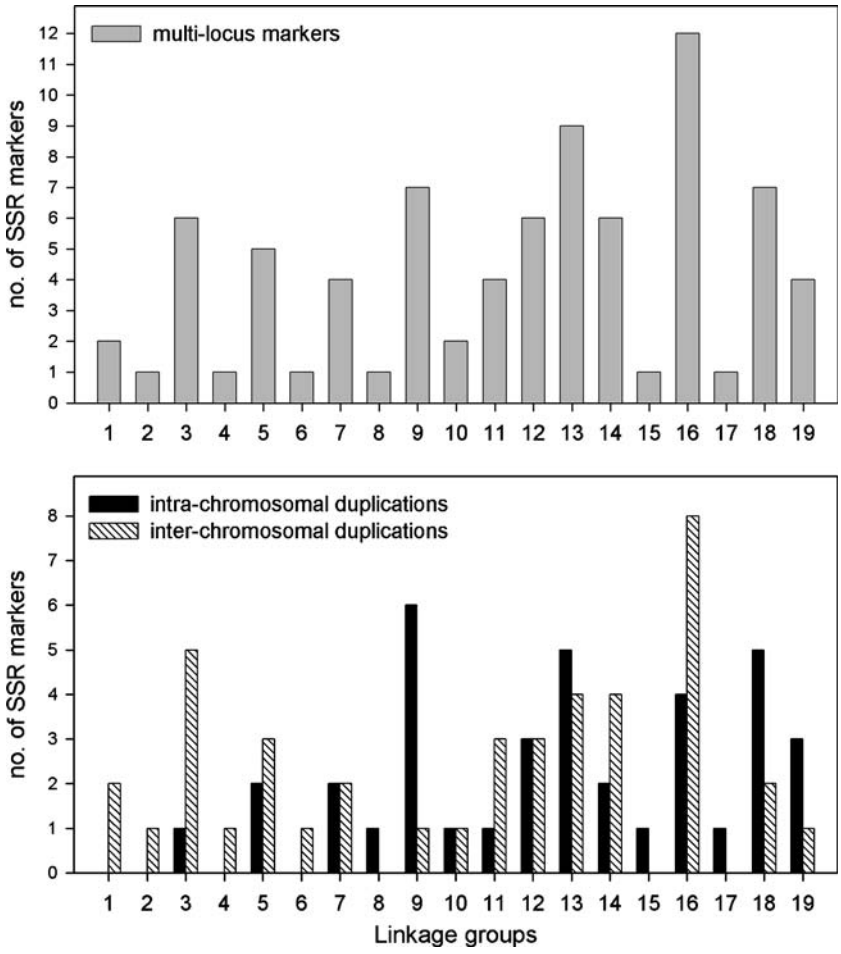

Fig. 4 Chromosomal distribution of duplicated loci amplified by 57 multiple-dose SSR markers (*above*). In detail (*below*), the number of SSR markers detecting intra-chromosomally duplicated loci is reported in *black*, the number of SSR markers amplifying at least one additional locus positioned on a different LG is in white

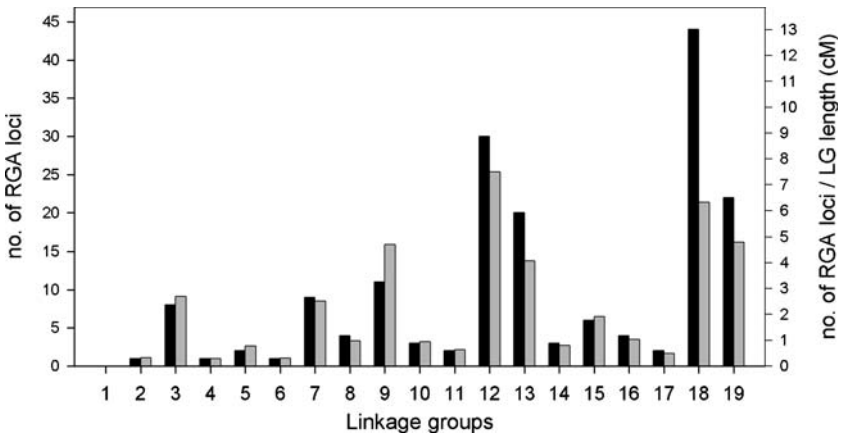

Fig. 5 Distribution of 173 RGA loci over the LGs. The number of RGA loci found per LG is reported in *black*, and the number of RGA loci related to the size of the corresponding LG is in *grey* and it is expressed as the ratio 'number of RGAs/LG total length (cM)'

distance of 5.4 cM. Locus order in the framework map was the same as in the complete composite map, except for two inversions of marker pairs on LG 5 and LG 17 (Fig. 3).

Comparison of marker order between the 'dsmergen' composite map and the most complete published map (Doligez et al. 2006) did not show relevant incongruities. Markers pairs with inverted linear order are reported in Electronic Supplementary Material (S3) and affected map intervals of 3.1 cM on average. Rearrangements involving more than two markers occurred on LG 6 (likely caused by the uncertain location of marker VMC5g1.1), LG 10 and LG 12 but remained confined into mean intervals of 9.3 cM on the different LGs (S3). Comparison of linear order of markers between the composite maps obtained with the merging methods 'dsmergen' and 'dsmergor' is reported in Electronic Supplementary Material (S4).

There were several cases of conflicting order between the two versions. Problems in the linear order was mainly confined to sub-chromosomal regions of LGs 7, 9, 12, 13, 14, 16, 18 and 19 that are particularly rich in multi-locus SSR or RGA markers.

Discussion

Genome coverage and robustness of marker order

The wild forms of *Vitis* species are dioecious and highly heterozygous. The domesticated forms mostly have perfect flowers that might be self-pollinated but they retained their primitive heterozygous nature, as reported in studies of genetic diversity (Aradhya et al. 2003) and preliminary sequencing efforts (http://www.genoscope.fr, http://www.appliedgenomics.org). Genome-wide homozygosity could not be achieved by coercive selfing due to inbreeding depression and dihaploid plants have not been attained so far. The lack of inbred lines hampers the development of standard F2 mapping populations. Genetic maps are therefore constructed using full-sib populations obtained by crossing heterozygous and distantly related parents. The key parameters of this mapping scheme are the heterozygosity of the parents which determine the number of markers that segregate and the percentages of markers that are alternatively heterozygous in either parent which causes gaps in marker coverage along the chromosomes of each parental map. We adopted the strategy of mapping with four parents and integrating segregation data from all parents, which allowed us to map an extraordinarily high proportion of markers (91%). Mapping with only one of the two cross populations would have resulted in only 75 and 80% markers segregating in either population.

The two mapping populations were of 46 individuals each, corresponding to 46–184 informative meioses when segregation data were integrated in the composite map, depending on the number of parents from which each marker segregated. Of the 363 single-locus microsatellites, which represent the SSR skeleton of the composite map, as few as 56 markers were heterozygous only in one parent, 71 segregated from two, 113 from three and 123 from four parents, thus raising the average number of informative meioses per marker to 130. The consistency of marker order in composite maps obtained by merging segregation data from independent crosses has been previously demonstrated for grapevine using different algorithms (Doligez et al. 2006). In the present work, marker orders were widely conserved for all LGs if compared to the most complete grapevine map published so far (Doligez et al. 2006). Even the length of our composite map (1,676 cM) was comparable to the one (1,646 cM) obtained by Doligez et al. (2006).

We experienced conflicts in fine marker order among individual maps, but these were not higher in number than those reported in maps obtained from larger populations (reviewed in Doligez et al. 2006). Most incongruities were restricted to genomic regions hosting multi-locus markers. Nevertheless, since they are not randomly distributed and discarding them would have depleted marker coverage of duplicated regions, we tried to integrate the multi-locus markers into the consensus map. In order to do this, non-allelic peaks (bands) in each parent as well as segregating peaks from different parents were treated as independent markers. In the case of an inter-chromosomal duplication of a given marker, this choice prevented two individual loci, that were heterozygous in different LGs in different parents, from bridging non-homologous LGs. In the case of intra-chromosomal duplications, we scored independently loci for which map positions were known and also loci for which information on map position was not available. In the first case which is exemplified by the marker VVMD14 on LG5, the alleles of individual parents scored as independent markers collapsed into the same positions in the integrated map and unambiguously identified a duplication occurring at a long distance along the chromosome. In the other case, which occurred more frequently, individual loci, amplified by the same primer pair and scored in different parents, were placed slightly apart from each other on an integrated LG. We considered three hypotheses: (1) the physical distance between the two loci alternatively scored in different parents was large enough to allow recombination; (2) the map distance was an artefact due to heterogeneous recombination rates with the surrounding markers in different parents; (3) alleles of the same size belonging to loosely linked loci and present in coupling on the same parental homolog were scored as a single deceptive marker (Frisch et al. 2004). In all such cases, the use of larger populations might not improve map precision and fine scale resolution should only be obtained with the support of a physical map (Castellarin et al. 2006).

Genotype-specific rate of recombination was likely to cause a map expansion over a few confined regions on both *Vitis* hybrid parental maps ('Bianca' and 'breeding line 20/3') or to cause a map distance compression on some LGs in the *V. vinifera* parents. For instance, LG 17 was substantially longer in *Vitis* hybrids (72.3–81.8 cM) compared to *V. vinifera* maps (43.8–59.3 cM). Total length of LG 17 in *V. vinifera*

'Chardonnay' and 'Cabernet Sauvignon' is comparable with that of other *V. vinifera* cultivars (47.2 cM in 'Syrah' and 53.6 cM in 'Grenache', Adam-Blondon et al. 2004), while the same LG is 96.0-cM long in the *Vitis* hybrid 'Regent' (Fischer et al. 2004). It is noteworthy that *Vitis* hybrid parents share one half-sib non-*vinifera* ancestor with 'Regent'. By contrast, LG 1 had a length of 35.5 and 48.3 cM in *V. vinifera* 'Chardonnay' and 'Cabernet Sauvignon' and of 82.4 and 90.1 cM in the *Vitis* hybrids. Other *V. vinifera* maps of LG 1 including the same distal markers had a length comprised between 97.6 and 103.0 cM (Adam-Blondon et al. 2004).

Genomic distribution of RGA markers

Nucleotide-binding-site/leucine-rich-repeat genes were found nearly in all LGs, with genes assigned to a given LG often clustered in small regions. Phyletically close sequences frequently mapped together to the same chromosomal region. As a result, the largest clusters predominantly consisted of one type of NBS–LRR genes, either CC–NBS–LRR or TIR–NBS–LRR. RGAs that mapped to LG 13 and 19 were exclusively of the CC–NBS–LRR type. By contrast, 18 out of the 20 mapped to LG 18 were of the TIR-type. Mixed clusters of TIR– and CC–NBS–LRR genes were present on LG 12 with a lack of predominance of either class. Such a clustered arrangement of closely related members of the NBS–LRR gene family has been already observed in *Arabidopsis* (Richly et al. 2002) and rice (Bai et al. 2002). By contrast, the occasional occurrence of mixed clusters was observed in barley (Madsen et al. 2003) and *Medicago* (Zhu et al. 2002). A mixed model of gene duplication and ectopic dispersion of segmentally duplicated blocks embedding NBS–LRR members should explain the observed genomic organisation of this gene family in grape. For instance, Baumgarten et al. (2003) found in *Arabidopsis* that almost all relocations of NBS–LRR gene copies among the chromosomes are due to duplication and rearrangement of a chromosome segment on which those genes reside. In grape we found a similar scenario at the genetic map level, with a high number of multiple-dose SSR markers on some LGs, i.e. 3, 7, 9, 12, 13 and 18, that are also rich in NBS–LRR gene clusters.

In the present work, many NBS–LRR genes were found by in silico search in genomic sequences of *V. vinifera* 'Cabernet Sauvignon' and all RGA markers that were previously developed in non-*vinifera* genotypes amplified homologous loci in the *V. vinifera* parents of the mapping populations. This confirms that NBS–LRR genes are widely present in cultivars of *V. vinifera* that are susceptible to most of the grape pathogens currently causing damage in vineyards. The failure to trigger the defence mechanism in *V. vinifera* is likely due to the fact that *R*-gene alleles did not develop into pathogen recognition specificities in absence of those pathogens that did not co-evolve with *V. vinifera*. NBS–LRR genes are reported to have undergone diversifying selection in plants (Meyers et al. 1998; Mondragon-Palomino et al. 2002) and allelic variation involving SNPs in functional domains (mostly the LRR) has proved to determine phenotypic divergence between resistant and susceptible genotypes (McDowell et al. 1998; Axtell et al. 2001). A high level of allelic variation at NBS–LRR genes is present between susceptible *V. vinifera* and resistant non-*vinifera* parents of our crosses as witnessed by the proportion (83%) of the RGA markers that were polymorphic and could be mapped.

Co-localisation of RGA markers and known phenotypic loci of disease resistance

Resistance gene analogs are functional markers that were frequently associated in plant species with QTLs or genes for disease resistance (see for instance Grube et al. 2000). We therefore revised the literature in order to detect possible co-localisation of QTLs/genes for disease resistance and RGAs markers on the integrated map, based on common SSR markers. RGA loci could be found in the vicinity of disease resistance loci reported in the literature for several fungal and bacterial pathogens as well as pests on LGs 7, 12, 14, 15, 18 and 19.

A major QTL for downy mildew resistance inherited from *Vitis* hybrid 'Regent' has been identified in the region close to SSR marker VMC6f11 (Fischer et al. 2004). VMC6f11 is the only microsatellite mapped in that region by the authors and it corresponds to the distal part of LG 18 in our maps, which is rich in RGAs. Moreover, the RGA marker rgVrip064 found to be associated with downy mildew resistance in some resistant genotypes (Di Gaspero and Cipriani 2002) is also located in the same region. Additional QTLs for downy mildew resistance inherited from *V. riparia* (Grando et al. 2003) and from the *Vitis* hybrids 'Gf.Ga-47-42' and 'Villard blanc' (Zyprian et al. 2005) were reported in the chromosomal region identified by SSR markers VMC8d11, VrZag62 and UDV-082. This region corresponds to the middle of LG 7 where three RGA markers are also present in our maps. A single QTL for powdery mildew resistance was mapped in *Vitis* hybrid 'Regent' on LG 15 (Fischer et al. 2004) distally to SSR markers VVIV67 and UDV-015 (Akkurt

et al. 2007). This region is cross-referenced to a chromosome end of LG 15 of the integrated map where a small cluster of RGAs is located. A different QTL for powdery mildew resistance was identified in an unrelated *Vitis* hybrid that has inherited the resistance trait from *V. cinerea* and *V. rupestris* (Dalbó et al. 2001). The marker STS-AA6 that is reported by the authors in linkage with that QTL, maps in the distal part of LG 14. In the same region, Krivanek and co-workers (2006) identified a major locus *PdR1*, responsible for the resistance against *Xylella fastidiosa*, the causal agent of Pierce's disease.

With regard to pest resistance, a natural defence from the dagger nematode *Xiphinema index* has been found in *V. arizonica* and the genetic determinant is thought of reside on LG 19 (A. Walker, personal communication). Some 13 RGA markers were positioned in different regions of LG 19. A major determinant for resistance to root-knot nematode (*Meloidogyne* spp.) has been studied in a cross between *V. champinii* and *V. riparia* and is being assigned to LG 18 (Lowe and Walker 2006; A. Walker, personal communication).

A disease resistance locus to powdery mildew (*Run1*) introgressed from *M. rotundifolia* into *V. vinifera* could be also aligned to the syntenic region of our map. The cluster of NBS–LRRs identified by markers GLP1-12, MHD98, MHD145 in the surroundings of *Run1* (Donald et al. 2002; Barker et al. 2005) and originating from *M. rotundifolia*, had a counterpart in the *Vitis* genome, which spanned the middle to one end of LG 12. Nearby the same region, a major determinant responsible for resistance against downy mildew has also been reported (Merdinoglu et al. 2003).

Conclusions

We presented in this paper a grapevine integrated genetic map, where 82 functional markers (Varshney et al. 2005) derived from analogs of resistance genes were placed together with 420 SSR markers recovered from the literature and the NCBI database. We offer such a map as a valuable tool for geneticists and breeders working in pest and disease resistance in grape, who can find, beside a large set of anonymous markers providing a dense genome coverage, a set of markers that identify a significant number of RGA clusters. It is likely that several of these clusters contain genetic determinants of resistance.

Our mapping populations have been designed to study the phenotypic segregation of resistance to grapevine mildews in two steps. The first one was oriented to rapidly produce a first draft of a genetic map, where as many SSR and RGA markers as possible were placed in the correct LGs, neglecting the fine order of those markers. This work has been carried out successfully by adopting the strategy of mapping with four parents and a limited number of progeny. This strategy has achieved the goal of yielding reliable linkage detection at minimal mapping cost. Now we have extended one of the mapping populations ('Chardonnay' × 'Bianca') to ~2,000 individuals and the phenotypic resistance is being mapped. As soon as we have identified the chromosomal region of interest we will refine and saturate that region with all markers tentatively placed nearby, thus opening the door to the positional cloning of the gene of interest.

This approach mimics the two-stage map-based cloning strategy adopted in *Arabidopsis* where, once a population segregating for a desired trait has been generated, some 50 plants are marker-genotyped and phenotypically characterised. Only once the trait of interest has been assigned to a sub-chromosomal region, 1,000–2,000 individuals of the same population are processed to pinpoint the position of the trait and the surrounding markers (Jander 2006).

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