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Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine

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Abstract A full-sibling F1 population comprising 153 individuals from the cross of 'Regent' × 'Lemberger' was employed to construct a genetic map based on 429 molecular markers. The newly-bred red grapevine variety 'Regent' has multiple field-resistance to fungal diseases inherited as polygenic traits, while 'Lemberger' is a traditional fungus-susceptible cultivar. The progeny segregate quantitatively for resistances to *Plasmopara viticola* and *Uncinula necator*, fungal pathogens that threaten viticulture in temperate areas. A double pseudo-testcross strategy was employed to construct the two parental maps under high statistical stringency for linkage to obtain a robust marker frame for subsequent quantitative trait locus (QTL) analysis. In total, 185 amplified fragment length polymorphism, 137 random amplified polymorphic DNA, 85 single sequence repeat and 22 sequence characterized amplified region or cleaved amplified polymorphic sequence markers were mapped. The maps were aligned by co-dominant or doubly heterozygous dominant anchor markers. Twelve pairs of homologous linkage groups could be integrated into consensus linkage groups. Resistance phenotypes and segregating characteristics were scored as quantitative traits in three or four growing seasons. Interval mapping reproducibly localized genetic factors that correlated with fungal disease resistances to specific regions on three linkage groups of the maternal 'Regent' map. A QTL for resistance to *Uncinula necator* was identified on linkage group 16, and QTLs for endurance to *Plasmopara viticola* on linkage groups 9 and 10 of 'Regent'. Additional QTLs for the onset of berry ripening ("veraison"), berry size and axillary shoot growth were identified. Berry color segregated as a simple trait in this cross of two red varieties and was mapped as a morphological marker. Six markers derived from functional genes could be localized. This dissection of polygenic fungus disease resistance in grapevine allows the development of marker-assisted selection for breeding, the characterization of genetic resources and the isolation of the corresponding genes.

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Introduction

The use of grapevine for fruit, juice and wine production is a tradition that goes back more than 8,000 years to ancient cultures in Mesopotamia. During this time, numerous cultivars have been selected for their quality and adaptation to different climatic conditions. During the 19th century, however, pathogens such as powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*) were carried to Europe from North America and became major risk factors for viticulture. Since *Vitis vinifera* does not carry any resistance to the mildew fungi, multiple fungicide applications per growing season became indispensable for traditional cultivars. Grapevine breeding to combine resistance, e.g., from American *Vitis* species, with good wine quality of *V. vinifera* became an important strategy to combat the fungi. Already around 1880, Millardet stated that a combination of resistance and quality must be possible (according to Alleweldt 1996). Despite many drawbacks in grapevine breeding over time, this goal seems to have been achieved by the end of the 20th century. Recently developed fungus-resistant varieties that can be cultivated under reduced protective treatments are a major achievement towards a more environmentally friendly and cost-efficient viticulture.

Many agronomically important traits are quantitatively inherited and thus difficult to control in grapevine breeding. The elucidation of inheritance of complex

characteristics can be addressed by establishing their association with linked molecular markers. Genetic factors involved in the variation of traits can be localized as quantitative trait loci (QTL) on the basis of a molecular map as introduced into plant genetics by Paterson et al. (1988) and later modified into QTL interval mapping (Lander and Botstein 1989). Efficient QTL analysis requires a densely covered genetic map with good saturation and an even distribution of markers. Once the correlation between a molecular marker and a specific phenotype has been established, the inheritance of a trait of interest can be scored in a progeny at very early stages of plant development (marker assisted selection; Lande and Thompson 1990). In addition, such markers may serve as anchors for intervarietal comparative studies, for the molecular characterization of genetic resources and for positional cloning of corresponding genetic regions. With these approaches, candidate genes can be identified and become available for the improvement of traditional grapevine cultivars after further functional characterization.

Model cross populations of numbers as large as possible are desirable for QTL mapping, but, with grapevine as with other woody crops, there are practical limits. Thus, the first grape mapping studies started on populations comprising between 50 and 80 individuals (Lodhi et al. 1995; Dalbó et al. 2000; Grando et al. 2003), involving American genotypes such as 'Cayuga White' and 'Aurore' (Lodhi et al. 1995), wild species native to China (Luo et al. 2001) or varieties that had their main focus on seedlessness (Doligez et al. 2002). Some investigations aimed at identifying resistance-correlated factors in *Vitis* ssp. (Luo et al. 2001; Di Gaspero and Cipriani 2002; Marino et al. 2003); however these studies were not performed in the context of QTL mapping on viticulturally important cultivars. None of the molecular maps described thus far for *Vitis* with relevance for European grapevine accessions has been correlated with QTL data for resistance and ripening.

To create a segregating population of 153 F1 individuals for mapping, the new fungus-resistant variety 'Regent' was crossed to the fungus-susceptible, traditional red wine cultivar 'Lemberger' (Ambrosi et al. 1998). The progeny were genotyped with a variety of dominant [random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR)] and co-dominant [single sequence repeat (SSR)] markers. Genetic maps of both parental types were developed separately by linkage and recombination analysis following the double pseudo-testcross strategy and aligned to each other and integrated into consensus linkage groups. The individuals were phenotypically investigated for their resistance levels to *P. viticola* and *U. necator*, as well as some additional agronomic traits in the field. Data from repeated evaluations over three or four vegetation periods were used for interval mapping of QTLs based on the maternal map.

Materials and methods

Plant material and DNA extraction

The grapevine variety 'Regent' was bred at the Institute for Grapevine Breeding Geilweilerhof and was released for quality wine production in 1996. It shows high-level resistance to both *P. viticola* and *U. necator* in the field (Anonymous 2000). Its reconstructed pedigree may include up to seven different wild species several generations back that potentially contributed resistance genes (*V. aestivalis*, *V. berlandieri*, *V. cinerea*, *V. labrusca*, *V. lincecumii*, *V. riparia* and *V. rupestris*). The origin of the resistance traits encountered in 'Regent' is not yet known.

The 153 individual plants of the mapping population from the controlled cross 'Regent'×'Lemberger' have been grown in the fields at the Institute for Grapevine Breeding Geilweilerhof since 1991. Young, healthy leaves were collected from the shoots during the early summer and stored after shock-freezing at −70°C until use. For preparation of genomic DNA, 2 g fresh weight leaf material was ground in liquid nitrogen to a fine powder and processed according to Thomas et al. (1993). The amount and integrity of the resulting genomic DNA was checked on 0.8% agarose gels prepared in TAE buffer.

Genotyping

Genotyping of the individual F1 plants was performed by applying a variety of PCR (polymerase chain reaction)-based marker technologies such as AFLP markers (Zabeau and Vos 1992; Vos et al. 1995), RAPD markers (Williams et al. 1989, 1993) and SSR (Akkaya et al. 1992; Thomas et al 1993) markers of the STMS type (sequence tagged microsatellite sites; Beckmann and Soller 1990). Most of the latter were developed for *Vitis* within an international consortium (VMC, *Vitis* microsatellite consortium) initiated by C.P. Meredith (University of California, Davis, USA) and organized by AGROGENE S.A. (Moissy Cramayel, France). In addition to these anonymous markers, we developed segregating markers from functional genes to start integrating these into the molecular map (see below).

For RAPD analyses, 10-mer primers from Kits A, M and N (Operon Technologies, Alameda, Calif. and Roth, Karlsruhe, Germany) were used in standard 50 μl PCR reaction mixtures according to Williams et al. (1993). The amplification products were separated by electrophoresis on 1.5% agarose gels in 0.5×TBE buffer at 8 V/cm. Banding patterns were visualized under UV light (λ=312 nm) after staining with ethidium bromide (0.5 μg/ml for 10 min) and documented with a Polaroid digital camera. Polymorphic RAPD bands were scored as either present or absent. In addition to the primer kits, the two 10-mers CS25 and BC356, found to produce markers correlating to *U. necator* resistance in a completely different genetic background as described by B. Reisch et al. (Dalbó 1998), were tested.

AFLP reactions were performed with the materials and following the instructions of the "AFLP plant mapping kit" (PE Applied Biosystems). Fifty to 150 ng of genomic DNA from F1 individuals and parental types were used. Preselective PCR was followed by second step amplification, where a selective "*Eco*RI"-primer 5′-labeled with one out of three possible fluorescing dyes (FAM, JOE, NED) was combined with an unlabeled "*Mse*I"-PCR-primer. Primers matched adaptor sequences plus two or three flanking nucleotides. Resulting fragments in the range between 30 and 500 bp were analyzed by automated capillary electrophoresis on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Selective amplification assays were carried out with 19 primer combinations over the mapping population. Two or three base overhangs on one side combined with three selective bases on the primer-flanked side produced between 6 and 64 well-defined peaks per primer combination with various proportions of segregating polymorphic fragments. Data were edited using Genotyper 2.0 (PE Applied Biosystems). AFLP profiles were checked for reproducibility in random replicate reactions.

For SSR (STMS) marker analysis, we used primers VVS1–5 (Thomas and Scott 1993), VVMD primer pairs (Bowers et al. 1996, 1999) and primers flanking loci VrZAG21, 25, 27, and 49 as communicated by H. Steinkellner and F. Regner prior to publication. Additional VrZAG primers used were described in Sefc et al. (1999). From the pool of SSR sequences characterized within the VMC, 101 SSR loci-flanking primer pairs were tested in a parental screening with 'Regent' and 'Lemberger', 87 loci were scored as segregating markers over the mapping population.

PCR over SSR loci was performed in standard reactions of 20 µl. Pre-screenings for segregating polymorphic allele combinations were analyzed on denaturing 6 or 8% polyacrylamide gels and silver-stained (Silver Sequence DNA Sequencing System, Promega, Madison, Wis.). Informative primer pairs applied in PCR reactions covering the complete mapping population were labeled with either ABI fluorescent dyes at the 5′-ends of "forward" primers and analyzed by capillary electrophoresis with automatic detection and sizing or visualized as non-labeled amplification products on silver-stained denaturing polyacrylamide gels.

For the development of CAPS (cleaved amplified polymorphic sequences; Jiang et al. 1997) and SCAR (sequence characterized amplified region; Naqvi and Chatoo 1996; Staub et al. 1996) marker sequences from an EST database prepared from 'Regent' cDNA collections of the Institute (Faes et al. 2003) and public information in databases such as that of the NCBI (National Center for Bioinformatic Information, http://www.ncbi.nlm.nih.gov) were used. Specific primers (Table 1) were derived using the Prime application from HUSAR (Heidelberg Unix Sequence Analysis Resources, Biocomputing Service Group, German Cancer Research Center, Heidelberg, Germany). CAPS fragments designated VR2 originate from a differential display assay where cDNAs from *Plasmopara*-infected versus uninfected resistant grapevine plants were investigated (Kortekamp, Vogt and Zyprian, in preparation). The SCAR marker Zy1/2 has been developed in previous work by sequencing an 800 bp anonymous RAPD fragment produced by RAPD primer OP-M10 containing an open reading frame of unknown function (Büscher and Zyprian, unpublished). *Vitis* sequences available in data bases were used after verification of the corresponding nature of PCR products by sequencing. Some SCAR primer sequences were kindly provided by Bruce Reisch (Cornell University, Geneva, NY).

PCR for CAPS and SCAR markers was carried out in standard assays in a total volume of 50 µl. Products were separated on 1.5–2% agarose gels. Some polymorphisms could be observed directly because SCAR markers produced segregating amplified products of various lengths. These were scored individually as dominant markers.

For the illustration of CAPS markers, 4-bp-cutter restriction endonucleases such as *Alu*I, *Hpa*II, *Mbo*I, *Rsa*I, *Taq*I and *Tsp*EI (New England Biolabs, Beverly, Mass.) were tested on monomorphic PCR products of parental DNA and a small subset of the progeny to detect segregating restriction site polymorphisms. The digestion of amplification products was performed by cutting nine microliters of PCR sample with 1–3 U of restriction endonuclease for 16 h at the appropriate temperature. Polymorphic patterns on 1.5–2% agarose gels were recorded. The glutamate dehydrogenase gene-derived fragments showed segregation with three alleles and were scored as co-dominant markers. All other segregating fragments were coded as individual dominant markers.

Marker designations indicate the parent of origin in the marker name with the initial letter(s) ("R" for 'Regent', "L" for 'Lemberger' or "RL" for both parents, respectively), followed by the primer code of the AFLP and the fragment size in bp. RL17367, for example, is a 367 bp marker occurring as a doubly heterozygous marker in both parents, 'Regent' and 'Lemberger', produced with primer combination 17 (*Eco*RI-AT/*Mse*I-CTT). RAPD markers were named similarly, giving the parent of origin (R, L, RL), the letter and number of the corresponding 10-mer primer, followed by the size of the PCR fragment in base pairs. CAPS fragment names consist of an acronym which is indicative of the encoded gene (see Table 1 for details).

Linkage/recombination analysis

Grapevine is recalcitrant to classical genetic analyses because homozygous parental lines are virtually impossible to obtain due to strong inbreeding depression. A special genetic approach, termed "double pseudo-testcross strategy", developed for highly heterozygous material has to be employed (Grattapaglia and Sederoff 1994; Weeden 1994). In this strategy, heterozygous genetic markers originating from either parent are followed in their segregation, linkage and recombination frequencies in a full-sibling F1 family. Maps are obtained separately for both parental types. They can be aligned to each other based on co-dominant or doubly heterozygous dominant markers present in both parental genotypes and integrated into a single map.

Linkage analysis was carried out with Joinmap 3.0 (Van Ooijen and Voorrips 2001) using the Kosambi function for the estimation of map distances (Kosambi 1944), and LOD (logarithm of the odds) score thresholds for the determination of linkage groups were deliberately set to 8.0, which is five orders of magnitude more stringent than the significance threshold of LOD 3 first suggested by Lander and Botstein (1989). The recombination fraction permitted was 0.4. Markers within the resulting groups were ordered relative to each other by automatic multipoint analyses using the default values of JoinMap 3.0 (Mapping threshhold LOD 1.0, Jump threshhold 5.0). Three rounds of mapping could be performed for 17 of the 20 linkage groups identified in 'Regent' and 11 of the 26 linkage groups identified in 'Lemberger'. Co-dominant markers and doubly heterozygous dominant markers were used to align the genetic maps obtained for both parental types. Homologous pairs were integrated into a partial consensus map.

Phenotypic evaluation

Agronomic traits were scored repeatedly during three or four growing seasons in the cross population according to criteria of the "Office International de la Vigne et du Vin" (OIV, International Wine Office, Paris, France; Anonymous 1983). Fungal disease resistances were scored in three vegetation periods omitting any fungicide protection for the population. Susceptibility or resistance to *U. necator* and *P. viticola* were evaluated separately on leaves and berries and classified according to the scale indicated in Table 2. During a cooperation with INRA Colmar, replicated plants of the 'Regent'×'Lemberger' progeny were placed in greenhouse conditions and tested for *P. viticola* resistance by applying a leaf disc assay test system (S. Wiedemann-Merdinoglu). The data revealed by these two different evaluation techniques showed a high degree of coincidence. Agronomic traits such as berry size, axillary shooting and the onset of ripening ("veraison") were scored in four growing seasons according to OIV scales (Anonymous 1983).

QTL analysis

QTL interval mapping (Lander and Botstein 1989; Young 1996) was performed using MapQTL 4.0 (Van Ooijen et al. 2000) based on the stringent maps for each parent in combination with the field evaluation data for resistance and agronomic traits. Phenotypic data from each year were used separately to analyze for QTL. Those QTL identifiable reproducibly through 3 or 4 years of investigation with a LOD score of $\geq$3.0 (as recommended by Van Ooijen 1999) were considered to be consistent.

Table 1 Sequence characterized amplified region (SCAR) markers mapped in the population 'Regent' × 'Lemberger'

SCAR/CAPS markers[a]	Primer sequences (5′–3′)[b]	T_A (°C)[c]	Reference	Enzyme[d]	Fragment size (bp)[e]	
					Reg.	Lem.
UDP-glucoseflavonoid 3-*O*-glucosyltransferase UFGT	UFGT-f: CGT TTA TAT GGT CCT AAG GG UFGT-r: TCA TTG CAT CCA AAC AGG TG	53	Sparvoli et al. 1994	*Hpa*II	-	530
Glutamate dehydrogenase GD	Gludehyd-f: GGA GAA GAG TCT TCT GAT AC Gludehyd-r: TAC CAC CTC TCT CAC CGA TG	55	Syntichaki et al. 1996	*Mbo*I	555 - 430 - 345 245 220	555 460 430 410 345 - 220
Alcoholdehydrogenase AD	Alkdehyd-f: GGA TCC TGA AGC AGG AGG GTA TGT GA Alkdehyd-r:AAG CTT GGC ATT GAC ATT TCC AGT GC	60	Sarni-Manchado et al. 1996	*Rsa*I	680 420	- -
Major allergen pru a1 Aller	Allerg1-f: TCA AGC TCT AAG ATC ATG GG Allerg1-r: GGT CCA CAC TTT GAC TGA TG	55	This work		1,225 915 725 680 580	- - - 680 -
Dehydration-induced protein RD22 RD22	RD22-f: CCC ATC CTT GCT CTT ATC TCT RD22-r CAC CTC CAG GTT TTC CAT TTC	57	This work		- 680 655 -	725 - - 640
Plasma membrane intrinsic protein 1 plasm	Plasmem-f: GCT CTT CTC TTT GCC TAG TG Plasmem-r: GCA AAT GGC TCC AAG ACA C	55	This work		1,355 -	- 1300
VR2	VR2-f: CCC AGA AGC GAT GAC AAA GG VR2-r :GCT GCT GCC TCC ACT CAT TC	58	A. Kortekamp et al. (unpublished data)	*HpaII*	400	-
VLG III BC 389b VLG	VLGIIIBC-f: CGC CCG CAG TTG ATG GTA VLGIIIBC-r: CGC CCG CAG TCG GTT GTA	60	B. Reisch, personal communication		- 860	930 -
Zy1/2	Zy-f: TCT GGC GCA CAT GAT ACA AAG Zy-r:TCT GGC CAC GAA TGA CGT TG	57	N. Büscher and E. Zyprian		800	800

[a] Designation of mapped SCARs
[b] Primer name (f=forward, r=reverse) and sequence
[c] Anealing temperature for each primer set
[d] Restriction enzyme used in the CAPS analysis
[e] Size of polymorphic fragments obtained per primer

Table 2 Description of the characteristics and classification of fungal disease resistances in field evaluations

Classification	*Plamopara* resistance (leaf) Descriptor OIV 452	*Uncinula* (*Oidium*) resistance (leaf) Descriptor OIV 455
9	Not limited, vast attacked patches or totally attacked leaf blades; strong fungus fructifications; pronounced and dense mycelium, very early leaf drop	Not limited and very vast attacked patches or totally attacked leaf blades; plenty of mycelium and ample fungus fructification
7	Vast, not limited attacked patches;very strong fungus fructification; numerous mycelia; leaf drop not as early as with note 9	Vast attacked patches, some of them limited; leaf blade partly attacked; plenty of mycelium and ample fungus fructification
5	Limited attacked patches 1–2 cm in diameter-more or less severe fungus fructification; irregular formation of mycelia	Attacked patches usually limited with a diameter of 2–5 cm
3	Less necrotic attacked patches; less fructifucation; few mycelia	Limited attacked patches of smaller extent; little mycelium and limited fungus fructification (presence of *Uncinula* is only indicated by a slight curling of leaf blade)
1	Punctuated necroses or no symptoms, neither fructification nor mycelium	Puntuated attacked patches or no symptoms at all; neither mycelium nor visible fructification (merely a slight curling of leaf blade)
Classification	*Plamopara* resistance (cluster/berries) Descriptor OIV 453	*Uncinula* (*Oidium*) resistance (cluster/berries) Descriptor OIV 456
9	More or less all clusters strongly affected or killed with consequences for the vintage	Very many berries of all clusters attacked (all clusters are more or less severely attacked);repeated cracked berries
7	A very high percentage of clusters strongly affected or killed	Many berries of most clusters attacked (some clusters moderately attacked; repeated cracked berries
5	20 to 30% of clusters attacked with distinct consequences for the vintage	Many attacked berries (up tp 30%), most clusters are moderately attacked, some, however, can be attacked severly; no or only a few cracked berries
3	Only some clusters slightly attacked without consequence for the vintage	Only a few berries out of all clusters are attacked (a few clusters only are slightly attacked)-no cracked berries
1	No attack	No attack

Results

Map construction

Calculation of the genetic maps by linkage/recombination analysis was performed with JoinMap 3.0. Following the double pseudo-testcross strategy, marker sets from either parent were processed separately and both maps aligned to each other by co-dominant SSR or doubly heterozygous dominant AFLP or RAPD markers (Table 3). A partial consensus map could be developed by integrating homologous linkage groups as indicated in Fig. 1. At LOD 8.0 in JoinMap 3.0, 265 markers originating from "Regent" and 164 markers from "Lemberger" could be linked and mapped on the basis of segregation data obtained from 153 individuals. This high stringency for linkage was chosen to construct a robust framework of markers. The relative contribution of the various marker types mapped in either one or both parental types is listed in Table 3. On the maternal map of "Regent" 20 linkage groups were obtained corresponding to one more than the number of chromosomes in the haploid set as recorded for grapevine (Husfeld 1932; Olmo 1937, as cited in Shetty 1959). The paternal "Lemberger" map consists of 26 linkage groups at the same stringency. The "Regent" map covers a total of 1,277.3 cM, with an average marker distance of 4.8 cM; the 'Lemberger' map extends over 1,157.7 cM, exhibiting an average marker distance of 7.0 cM. Out of the 20 'Regent' linkage groups, 18 could be homologized to corresponding 'Lemberger' linkage groups based on co-dominant SSR markers or doubly heterozygous dominant markers (Fig. 1).

During this alignment, however, two linkage groups originating from 'Regent' where aligned to the single linkage group 15 from 'Lemberger': 'Regent' linkage group 11 (based on doubly heterozygous AFLPs) and

Table 3 Contribution of the molecular marker types mapped in the "Regent" x "Lemberger" cross population

Contribution of the various marker types to the maps of "Regent" and "Lemberger"			
Marker type	mapped in "Regent"	mapped in "Lemberger"	mapped in both parents
AFLP	109	76	15
RAPD	98	39	14
SSRs	44	41	26
SCARs/CAPS	14	8	7
total	265	164	62

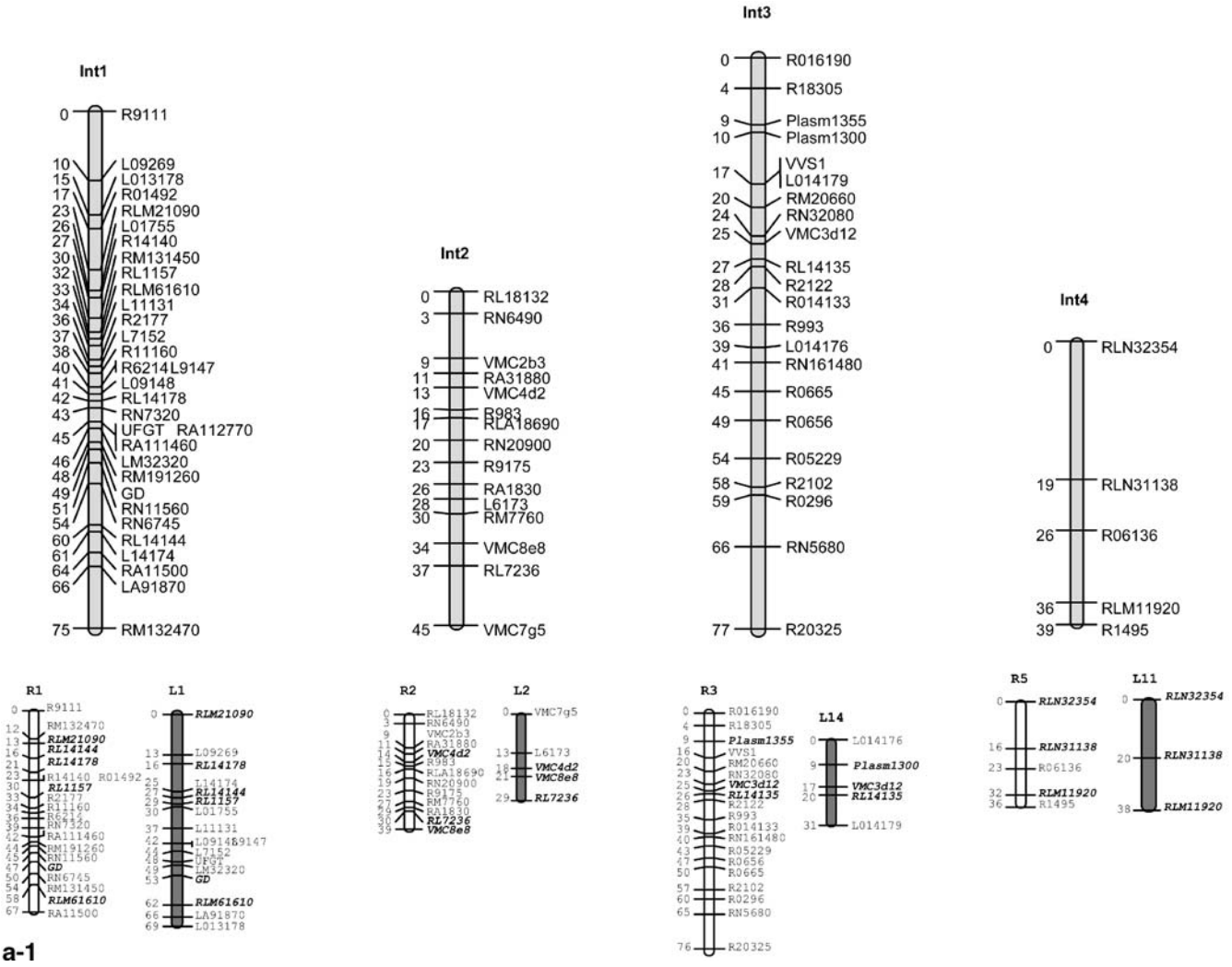

Fig. 1a–d Linkage groups as defined for 'Regent' and 'Lemberger'. **a** Integrated linkage groups of homologous linkage group pairs of the maternal parent 'Regent' (numbering starting with "R", *white bar*) and the paternal parent 'Lemberger' (numbers prefixed "L", *grey bar*) Aligments were done using codominant or doubly heterozygous markers shown in *bold/italic* print. Integration was performed with JoinMap 3.0. **b** Pairs of aligned linkage groups that could not yet be integrated due to insufficient linkage at high stringency. **c** Remaining individual (non-homologizable) linkage groups of 'Regent'. **d** Remaining individual (non-homologizable) linkage groups of 'Lemberger'

linkage group 12 (based on SSR loci). This alignment argues for a putative fusion that would reduce the current number of linkage groups from 'Regent' to 19, the actual number of chromosomes. The converse was true for linkage groups 17 and 18 from 'Lemberger', which were both aligned to linkage group 10 from 'Regent' based on a co-dominant and a doubly heterozygous SCAR, reducing the number of linkage groups identified in 'Lemberger' to 25. Homologous pairs of linkage groups could be integrated into 12 consensus linkage groups. Due to our strict threshold of linkage, this was not possible for all homologized sets. The linkage groups, their alignments and integrations are shown in Fig. 1. One small linkage group with two SSR markers linked 4 cM apart (VVMD5 and VMC5a1) was obtained unaltered for both parents and is not included in Fig. 1 ('Regent' linkage group 20 and 'Lemberger' linkage group 26). Markers derived from functional genes for glutamate dehydrogenase (GD, on linkage groups R1 and L1), a plasma membrane intrinsic protein (plasm1355 and plasm1300 on R3 and L14), alcohol dehydrogenase (AD on R9), a *Prunus* major allergen-like gene (*aller* variants on R10 and L18) and UFGT (UDP-glucose:flavonoid 3-*O*-glucosyltransferase on L1) were included in the maps. UFGT is involved in anthocyanine synthesis but mapped in a different group than berry skin color (on L13).

Marker distribution in the resulting maps seemed fairly even; pronounced clustering of any marker type was not evident. Nevertheless, large gaps of 20 cM distance or more between two markers still remain on six 'Regent' linkage groups and seven linkage groups identified from 'Lemberger'. These gaps seem to occur predominantly in terminal regions.

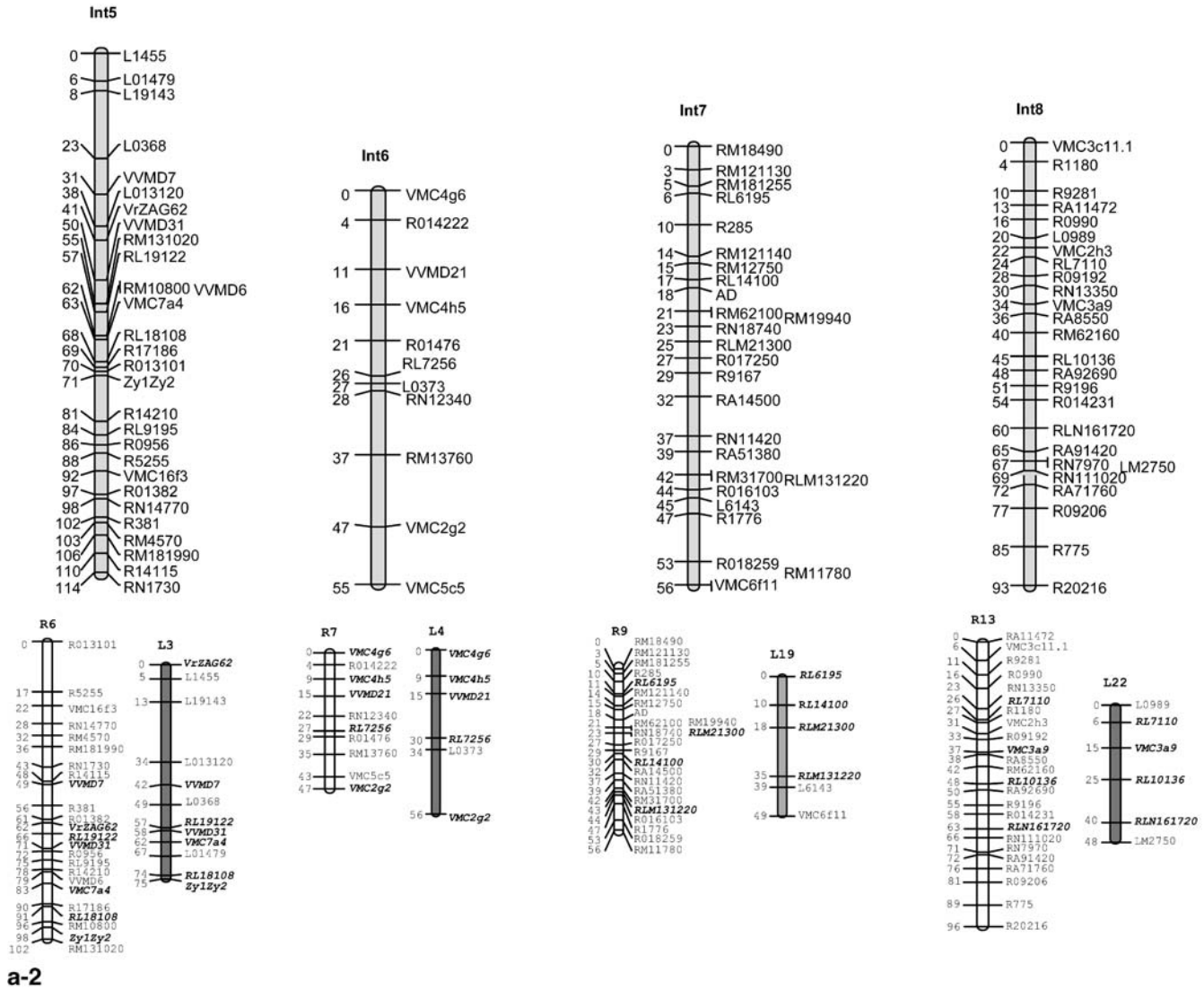

Fig. 1a–2

The individual linkage groups were compared to a microsatellite-based genetic map derived from the cross of 'Riesling'×'Cabernet Sauvignon' (C.P. Meredith, personal communication) wherever possible, based on the location of common SSR markers (Table 4). This map serves as a nomenclature reference as was determined in an international agreement achieved within the IGGP (International Grape Genome Program; http://www.vitaceae.org).

Phenotypic evaluation

The 153 siblings of the cross population 'Regent'×'Lemberger' show continuous variation of fungal disease resistances to *P. viticola* and *U. necator* on leaves and berries within the range delimited by the parents. In addition, the individuals of the mapping population show phenotypic variability with respect to other agronomic traits such as growth habitus (e.g., the degree of axillary shoot growth), the beginning of ripening (time of "veraison"; initiation of fruit softening) and fruit characteristics.

The levels of fungal resistance or susceptibility of the individuals were evaluated following the criteria of the OIV as outlined in Table 2. The phenotypic characterization was performed in 3 years (1995, 1999 and 2000), during which the plants were grown without the application of fungicide. The frequencies of observed resistance levels showed normal distribution as demonstrated in Fig. 2. Clear correlations within the same resistance characteristic were observed over the years of phenotypic investigation with average correlation coefficients of 0.52 for *P. viticola* leaf resistance, 0.37 for *P. viticola* cluster/berry resistance, and 0.49 each for *U. necator* leaf as well as berry/cluster resistance. In contrast, no correlation was found between the two different leaf resistances. From this result it can be concluded that resistances to the two

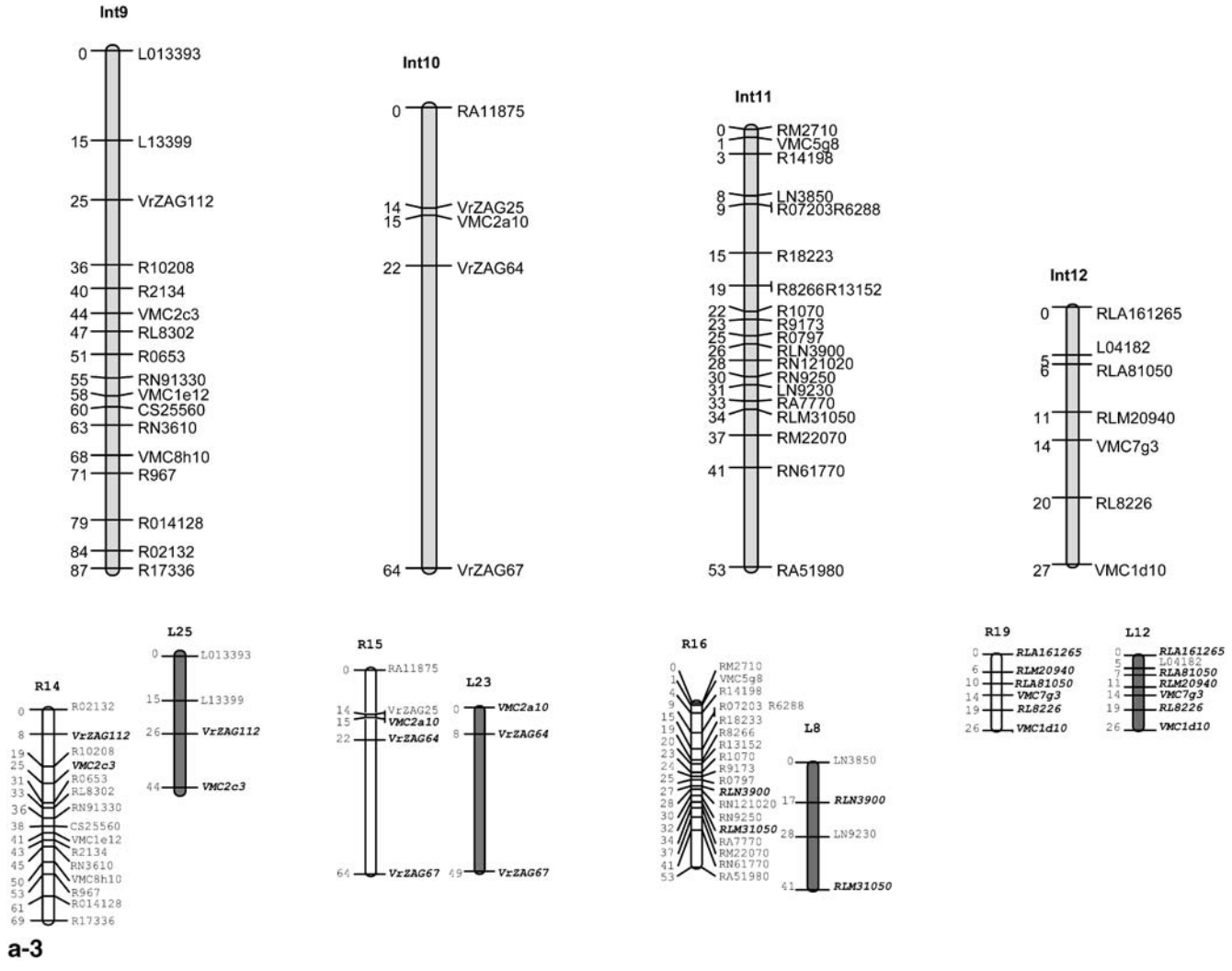

Fig. 1a–3

different fungal pathogens rely on independent genetic factors.

Segregating agronomic traits relating to phenological, growth and fruit characteristics were scored in the mapping population over four years following OIV descriptors. In particular, the growth of axillary shoots (OIV descriptor 352), berry size (OIV descriptor 220) and the beginning of berry ripening ("veraison"; OIV descriptor 303) showed clear phenotypic quantitative segregation, and were given scores of 1, 3, 5, 7 or 9. Berry color segregated as a simple qualitative trait in a Mendelian fashion from this cross of two red varieties (dark red/greenish-white 3:1 with χ^2 values of wellness of fit ranging from 0.018 to 2.24, depending on the number of scorable individuals each year), showing that both parental lines must be heterozygous for the corresponding locus. Berry color was thus included in the marker matrix as a morphological marker and mapped to linkage group 13 defined in the paternal 'Lemberger' map (Fig. 1).

QTL analysis of fungal disease resistances and agronomically important traits

The maternal map of 'Regent' was used preferentially to search for QTLs on the basis of interval mapping. Only QTLs consistent throughout the 3 or 4 years of field evaluation were considered significant.

Fungal disease resistance QTL

The clearest picture was obtained for resistance to *U. necator*. Factors correlating with endurance against this pathogen could be identified with high statistical significance (LOD values ranging from 8.4 to 20) on 'Regent' linkage group 16 in a densely marker-covered region including markers R9173 and RLN3900 (Table 5). The original scoring in the field for this trait was performed separately on leaves and berries over 3 years under natural pressure of fungus infection. QTL interval mapping placed both traits reproducibly and independently in

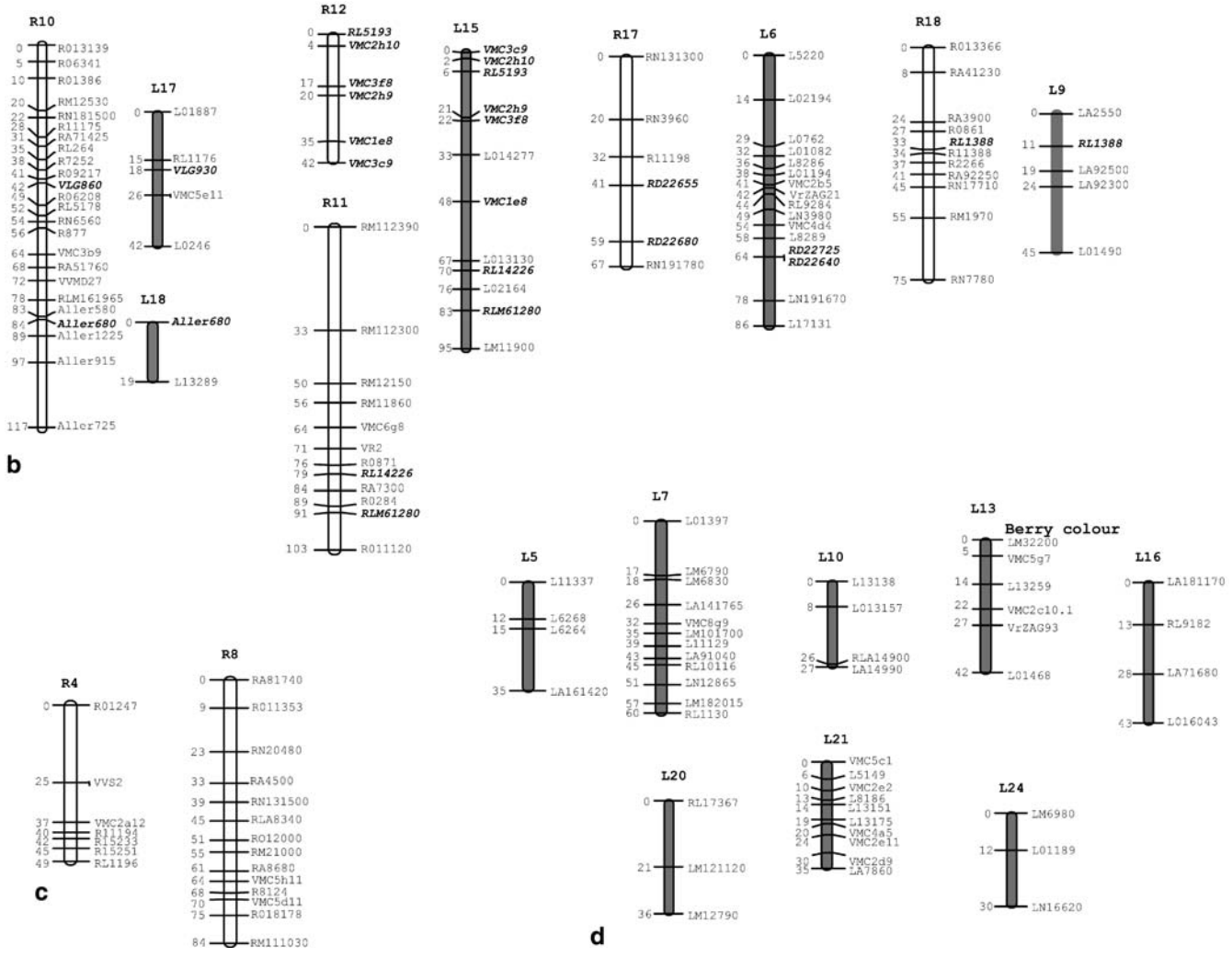

Fig. 1b–d

the same genetic region. Only one major QTL was detected, explaining up to 65% of phenotypic variation. Resistance to *U. necator* therefore seems to rely on that specific linkage group, probably introduced by introgression during the complex genealogy and selection history of the cultivar 'Regent'.

Analysis of QTL correlating with resistance to the downy mildew pathogen *P. viticola* yielded a different picture. Interval mapping identified two chromosomal regions of relevance: one major QTL was localized to 'Regent' linkage group 9 that could be identified by analyzing phenotypic data obtained over 3 years of scoring leaves and berries and using different modes of scoring for *Plasmopara* resistance (e.g., reporting the amount of necrosis caused by the fungus, the percentage of leaf surface damaged, employing the OIV scale, etc.). LOD values of leaf and berry resistance ranged from 12.3 to 20 and the peaks centered on the region encompassing marker RA14500 and RL14100 (Table 5). This region is well-saturated with molecular markers. In comparison to the *Uncinula* resistance QTL on linkage group 16, the area containing factors involved in *Plasmopara* resistance seems to extend over a larger area of the genetic map. The percentage of phenotypic variation explained by that major, wide QTL reached up to 70% in the peak region (Table 5). In addition, a less pronounced "minor" QTL for *Plasmopara* resistance of the leaves was detected on 'Regent' linkage group 10 in the terminal area limited and described by the placement of CAPS markers derived from the "*Prunus major allergen a1*" gene (Aller 915, Aller 725) and extending to the location of VVMD27. This minor QTL for *Plasmopara* resistance was also identified with data from 3 years of investigation and independent scoring of leaves and fruits with LOD peaks ranging from 2.8 to ca. 7.0.

Agronomic traits

Interval analysis using data from field evaluations localized one QTL correlating with axillary shoot formation to linkage group 3 of 'Regent' in the region between

Table 4 Homologization of linkage groups identified in 'Regent'×'Lemberger' to the consensus map chosen in the International Grape Genome Program (IGGP).

IGGP linkage group numbering[a]	Integrated linkage group numbering[b]	'Regent' linkage groups[c]	'Lemberger' linkage groups[d]	QTLs identified[e]
1	Int 2	2	2	-
2	Int 12	19	12/13	-
3	-	-	-	-
4	-	17	6	-
5	-	10	17/18	*Plasmopara viticola* resistance, berry size
6	Int 6	7	4	-
7	Int 5	6	3	Begin of ripening
8	-	11/12	15	Begin of ripening
9	-	-	21	-
10	Int10	15	23	-
11	-	4	-	-
12	-	-	7	-
13	-	-	-	-
14	Int 9	14	25	-
15	Int 11	16	8	*Uncinula necator* resistance
16	-	20	26	-
17	Int 8	13	22	-
18	Int 7	9	19	*Plasmopara viticola* resistance
19	-	8	-	-
20	-	3	14	Berry size, axillary shoot growth

[a] Numbering of linkage groups according to the map obtained from 'Riesling'×'Cabernet Sauvignon' (C.P. Meredith, personal communication)
[b] Numbering of integrated 'Regent'×'Lemberger' linkage groups
[c] Homologous linkage groups of 'Regent'
[d] Groups homologizable from 'Lemberger'
[e] QTLs identified in 'Regent'

Table 5 Details of the major quantitative trait loci (QTLs) identified for resistance to *Uncinula necator* and *Plasmopara viticola* with indication of linkage group (LG), map position (over 2 LOD support intervals), maximal LOD scores observed in the three years of investigation and % explained variance as well as delimiting markers

Statistic details and map positions for the major QTLs identified in 'Regent' for fungal disease resistances					
Fungal disease resistance trait	LG	Map position (2 LOD support intervals)	Max LOD	%expl.	Indicative marker/s
Uncinula resistance leaf 1995	R16	23.6 to 31.6 cM	11.74	52	RLM31050
Uncinula resistance leaf 1999	R16	27.3 to 27.8 cM	19.5	65	RLN3900, RN121020
Uncinula resistance leaf 2000	R16	19.6 to 27.8 cM	19.9	49.2	R9173
Uncinula resistance berry 1995	R16	22.6 to 27.8	11.1	32.3	R9173
Uncinula resistance berry 1999	R16	22.6 to 27.8	15.6	42.0	R9173
Uncinula resistance berry 2000	R16	22.6 to 30.4	8.4	22.9	R9173
Plasmopara resistance leaf 1995	R9	27.0 to 36.6	17.2	53.5	RL14100, RA14500
Plasmopara resistance leaf 1999	R9	29.1 to 36.6	19.7	56.0	RL14100, RA14500
Plasmopara resistance leaf 2000	R9	27.0 to 36.6	18.5	46.5	RL14100
Plasmopara resistance berry 1995	R9	27.0 to 36.6	13.2	60.2	RL14100, RA14500
Plasmopara resistance berry 1999	R9	29.1 to 36.6	12.3	51.3	RL14100
Plasmopara resistance berry 2000	R9	29.1 to 36.6 and 31.8 to 44.0	17.1 and 17	69.5	RL14100 and RLM131220

markers Plasm1355 and RN161480 (Table 4). QTLs affecting berry size were detected on linkage group 3 of 'Regent' in the region between markers Plasm1355 (plasma membrane intrinsic protein) and RN5229. From the current data it remains possible that there may be two separated QTLs in that area. An additional locus influencing berry size was identified on linkage group 10 around marker RN181500, spanning the region from R06341 to RA71425. Genetic effects related to the onset of berry ripening ("veraison") were identified in three different chromosomal regions on linkage group 1, linkage group 6 and linkage group 11 from 'Regent'. All of these QTLs for agronomic traits were repeatedly obtained in the same genetic regions by independently analyzing field data from four different years, however, with variable LOD scores in the range of 3 to 40, reflecting the environmental influences and climatic variations from year to year. These QTLs are summarized in Table 4 and Fig. 3.

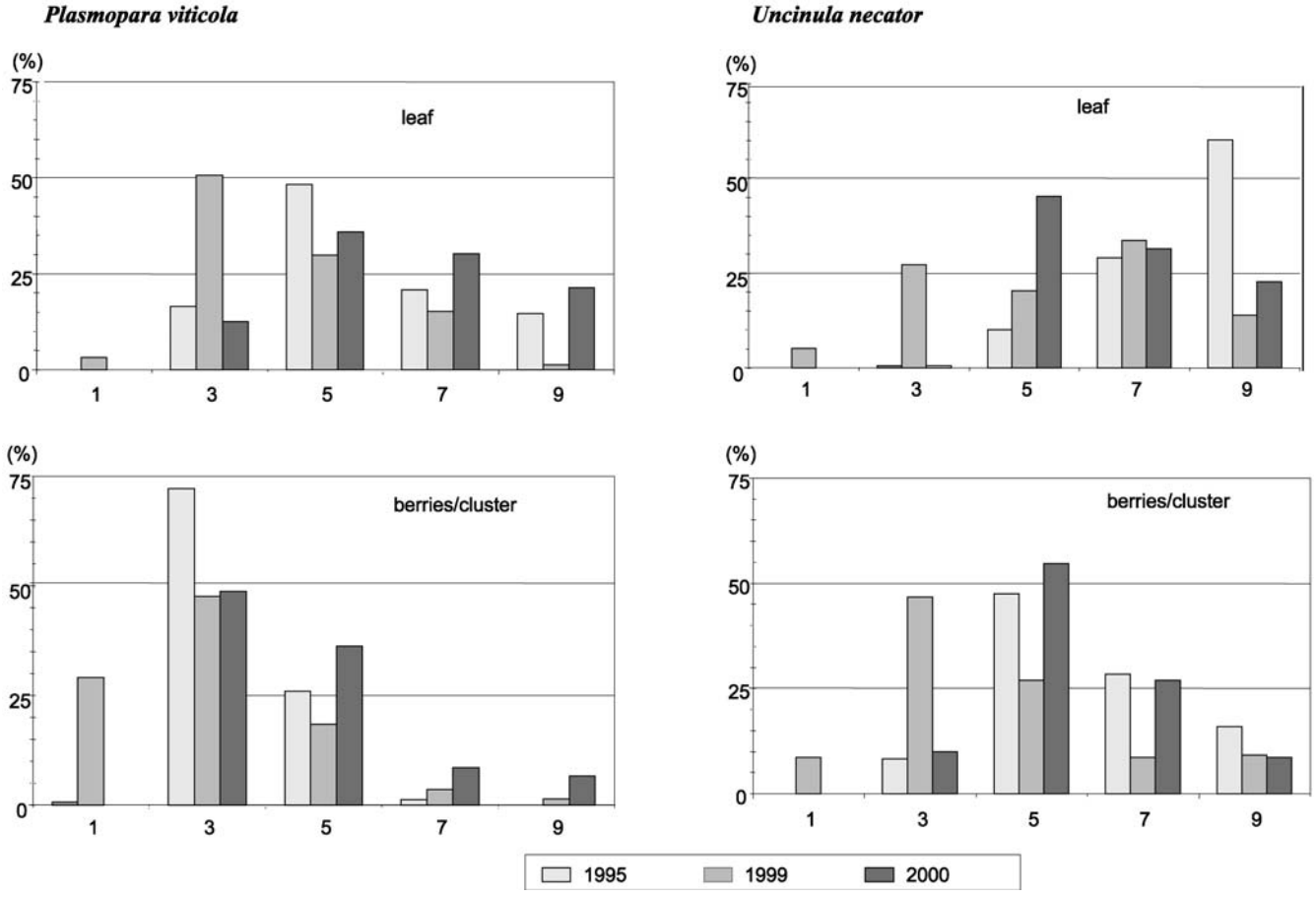

Fig. 2 Frequency distributions of the phenotypic scores obtained through field scorings of resistance to *Uncinula necator* and *Plasmopara viticola* on leaves and berries in the years of evaluation

Discussion

The molecular mapping of two high-quality European grapevine cultivars, 'Regent', a representative of new fungus-resistant varieties developed from resistance breeding at Geilweilerhof, and 'Lemberger', a traditional susceptible variety is described. The map of 'Regent' was used to initiate a dissection of genetic factors responsible for field resistance to downy and powdery mildew infections by QTL interval mapping.

Different types of molecular markers, predominantly RAPDs, AFLPs and SSRs were used in linkage/recombination analysis over a segregating population of 153 F1 individuals. In grapevine mapping approaches, evidence for clustering of AFLP markers has been reported (Doligez et al. 2002) and AFLP marker distribution in general has been discussed as being potentially biased by methylation patterns influencing the frequency and distribution of restriction enzyme target sequences in a given genome (Herbergs et al. 1999). Pronounced clustering of any marker type was not evident in the maps developed here. *Eco*RI/*Mse*I AFLP markers appeared to be evenly distributed. SSR-based markers proved to be the most useful due to their co-dominance and ability to be mapped in both parental maps resulting from the double pseudo-testcross strategy. They are the basis for homologization of linkage groups and comparisons to other grapevine maps as well as the international reference map (see below).

The 'Regent' and 'Lemberger' maps were deliberately constructed with very high statistical stringency (linkage threshold of LOD 8.0), which is much more stringent than in most other mapping studies in grapevine (Lodhi et al. 1995; Dalbó et al. 2000; Doligez et al. 2002) in order to construct a very robust and reliable framework for QTL analysis. As a consequence, we were able to map only about two thirds of scored markers because a considerable portion of the markers could not be reliably linked to the larger groups. The number of linkage groups we obtained for the already well-saturated marker map of 'Regent', however, is similar to the actual chromosome number.

Linkage and recombination analysis indicated 19 linkage groups for 'Regent' (the maternal variety) after homologization to the parental 'Lemberger' groups, which corresponds to the cytologically observed haploid number of *Vitis* chromosomes. For 'Lemberger', the paternal variety in the cross, fewer markers could be mapped and the number of linkage groups (25 after

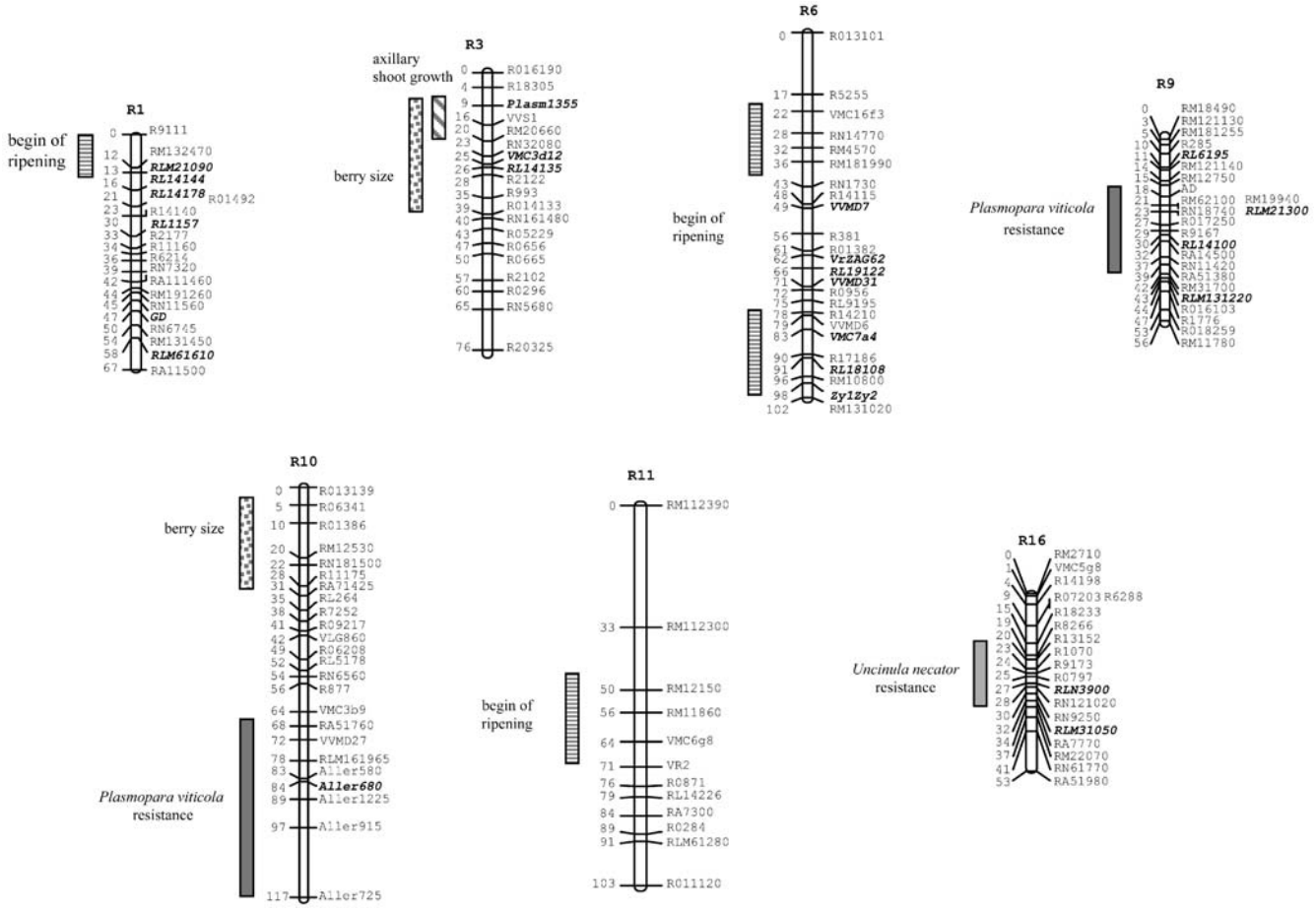

Fig. 3 Summary of quantitative trait loci (QTLs) for fungus resistances and agronomic taits identified in 'Regent'. The QTL positions are given where LOD scores exceeded the threshold level of 3.0: *P. viticola* resistance (*R9*, *R10*) and *U. necator* resistance (*R16*) with more details provided in Table 5; axillary shoot growth (*R3*)*, berry size (*R3*, *R10*)*, and the beginning of ripening (*R1*, *R6*, *R11*)*. *=See text for details

alignment to 'Regent') obtained still considerably exceeds the number of chromosomes. It is to be expected that the number of linkage groups will be reduced as more molecular markers are mapped for 'Lemberger'. The reason why the number of genetically localizable markers was different for both varieties is not understood, but may be related to a more complex genetic background in 'Regent' compared to 'Lemberger', carrying more loci in the heterozygous state and hence allowing the follow-up of their segregation for analysis.

The total genetic distance covered at this high stringency is 1,277.3 cM for 'Regent' and 1,157.7 cM for 'Lemberger', showing an overall similar frequency for both maternal and paternal meiotic recombination. Earlier mapping studies, using different varieties of grapevines and smaller segregating populations in some cases, resulted in 1,196 cM for 'Cayuga White' and 1,477 cM for 'Aurore' (Lodhi et al. 1995), 1,199 cM for 'Horizon' and 1,470 cM in 'Illinois 547–1' (Dalbó et al. 2000), 1,639 cM for "'Moscato bianco' and 1518 cM for *V. riparia* (Grando et al. 2003), 1,639 cM for 'Dattier de Beyrouth'×'75 Pirovano' and 1,908 cM for 'Alphonse Lavallée'×'Sultanine' (Doligez et al. 2002). JoinMap 3.0 software, which was used here with the Kosambi mapping function considering multiple crossovers and interference, has been reported to reduce map distances when values are compared to other mapping programs (Cai et al. 1994). Overall, the distance covered in the present study is in the range of those reported for grapevine. Of the 16 manually homologized pairs and triplets linkage groups identified in both parents, 12 could be integrated into consensus linkage groups at the high level linkage stringency employed.

Genetic mapping of grapevine is now recognized as an important step towards improved breeding strategies in all major viticultural countries. The aspects under investigation differ due to local requirements and preferences, resulting in different crosses chosen for segregation analysis. Comparison of the results requires standardization in the nomenclature of the resulting linkage groups. In this respect, transferable co-dominant SSR markers are the most valuable as anchors. An international initiative concerned with grapevine genetic analysis (IGGP) has therefore determined to use an SSR-based map resulting

from the cross of 'Riesling'×'Cabernet Sauvignon' (C.P. Meredith, personal communication) as a reference for the numbering of linkage groups. We used this nomenclature where possible (Table 4). Two of our 'Regent' (groups 5 and 18) and some of the 'Lemberger' linkage groups could not yet be assigned to any IGGP group due to lack of transferable markers. Comparing commonly-mapped SSR loci, a fairly good coincidence of the 'Regent' and 'Lemberger' maps with the marker order in the corresponding linkage groups of the IGGP-proposed grapevine consensus map was observed.

The IGGP-numbered consensus map currently contains 20 linkage groups, which is one more than the actual chromosome number (C. Meredith, personal communication). In the case of linkage group IGGP 3, for which we could not find any homologs in our mapping study of 'Regent'×'Lemberger', we were able to detect corresponding linkage groups in a different mapping population derived from the cross of breeding line 'Gf.Ga-47–42' with 'Villard blanc' (Zyprian et al. 2003). Linkage group IGGP 16 corresponds to the small group we observed both in 'Regent' (R20) and 'Lemberger' (L26) mentioned above, however, there is no equivalent to IGGP linkage group 13 as yet in any of our mapping studies, indicating that this linkage group may be the extra one in the 'Riesling'×'Cabernet Sauvignon' map that may be expected to be fused to one of the other linkage groups of that cross in the future.

Maps constructed used for interval mapping of QTLs for quantitative resistances

In 'Regent' we could identify a major QTL region for powdery mildew resistance on linkage group 16, as well as a major QTL for downy mildew resistance on linkage group 9 and a second one for resistance to that fungus on linkage group 10. A study of *P. viticola* resistance segregating from *V. riparia* in a cross with 'Moscato bianco' using data for leaf resistance resulted in QTLs identified on linkage groups 1 and 8 (Marino et al. 2003) of *V. riparia*. A genotype of this species could be one of the potential resistance donors involved several generations back in the breeding of 'Regent'. As in the case of 'Regent', *P. viticola* resistance has been localized to two different linkage groups in *V. riparia*. A preliminary study on a downy mildew resistance-segregating population derived from the cross of the fungus-resistant breeding line 'Gf.Ga-47-42' to resistant 'Villard blanc' indicates that a QTL for *P. viticola* resistance mapped to a linkage group from 'Villard blanc' that corresponds to linkage group 10 of 'Regent' (Zyprian et al. 2003). "'Villard blanc"' probably does not contain any genetic material from *V. riparia*, but most likely inherited its resistance from a pool of different *Vitis* species. It is thus tempting to speculate that *Vitis* species may carry their fungus resistance characteristics on a small number of "resistance" chromosomes. From the data available it may be concluded that *P. viticola* resistance relies on genetic factors on two different chromosomes. Future map integration and comparison will provide evidence as to whether the QTL identified in 'Villard blanc' will be confirmed and found to be in agreement with the resistance QTL location identified in 'Regent' and those of other resistant *Vitis* genotypes.

In contrast to this result for downy mildew, resistance to powdery mildew appears to be predominantly determined by a different and single linkage group from 'Regent', in accordance with the lack of correlation between both resistances observed. These results will have an impact on the intelligent design of pyramid strategies employing marker-assisted selection in the future. The complex pedigree of 'Regent' can be traced back to seven different wild species, and it is not yet clear from where its fungal resistance loci originate. The use of resistance-correlated molecular markers will help to elucidate this question in the near future.

Molecular labeling of resistance traits in grapevine has already been reported for a monogenic *U. necator* resistance gene termed *run*1, originating from *Muscadinia rotundifolia* (Pauquet et al. 2001), a genus not amenable to breeding use in grapevine due to its divergent chromosome number (20 chromosomes in the haploid set). Integration of the underlying molecular maps will allow to us to draw conclusions about common genetic factors involved in both cases. *P. viticola* resistance from wild species native to China (*V. quinquangularis*) has been labeled as a major resistance gene with a tightly linked RAPD marker that was converted into a SCAR (Luo et al. 2001). Asian resistance germplasm is probably not contained in the 'Regent" genealogy and our efforts to integrate that marker into the 'Regent' *P. viticola* resistance QTL regions have so far been unsuccessful. The marker described in *V. quinquangularis*, however, may be useful for the characterization of other genetic resources of *P. viticola* resistance in germplasm collections.

In addition to quantitative fungus resistances, QTLs for traits such as the tendency of axillary shoot growth, berry size and the beginning of ripening were identified. Another detailed QTL analysis for quantitative traits different from pathogen resistance in grape has so far only been reported for the characteristics of seedlessness and berry weight as traits important for table grape breeding (Doligez et al. 2002).

The average marker density of 4.8 cM for 'Regent' should allow the first attempts at physical isolation procedures for genes involved in QTL regions, because one cM is estimated to equal 300 kb in grapevine (Lodhi et al. 1995). The QTL for fungal disease resistances on linkage groups 9 and 16 of 'Regent' in particular have been identified in densely marker-covered regions. We are currently in the process of converting the QTL-linked RAPD and AFLP markers into sequence-characterized amplifiable markers to test their transferability within an extended cross population of the same parental types and other fungal disease resistance-segregating populations and germplasm material of resistant genotypes. This work

provides the basis for the establishment of marker-assisted selection procedures in grapevine breeding. It also allows an attempt at the physical isolation of candidate genes involved in resistance.

Further knowledge will be gained from the addition of more functional genes to our framework map in the near future. EST (expressed sequence tag) data from grapevine that are becoming available (Ablett et al. 2000; Davies and Robinson 2000; Driesel et al. 2003; Faes et al. 2003; VvGI Gene Index at TIGR http://www.tigr.org/tdb/tgi/vvgi) should facilitate the placement of markers derived from functional genes. In our study we were able to localize markers derived from the functional genes for glutamate dehydrogenase (GD, on linkage groups R1 and L1), a plasma membrane intrinsic protein (plasm1355 and plasm1300 on R3 and L14), alcohol dehydrogenase (AD on R9), a *Prunus* major allergen-like gene (*aller* variants on R10 and L18) and UFGT (UDP-glucose:flavonoid 3-*O*-glucosyltransferase on L1). Mapping of more functional genes will allow us to establish their putative role in determination of quality and defense reactions if they can be shown to co-localize with important QTLs. The alcohol dehydrogenase-derived marker was located close to the major QTL for *P. viticola* resistance on linkage group 9 of 'Regent' (R9), but the possible physiological impact of that finding requires further investigation. UFGT is the last enzyme in the biosynthetic pathway for anthocyanines, but has been localized to a different linkage group than berry color. It is known that UFGT expression in the cultivar 'Shiraz' is under special regulation during berry ripening compared to the genes for other enzymes involved in anthocyanine synthesis (Boss et al. 1996). The berry color locus may therefore represent a late-acting regulator.

Another means to investigate the genetic basis of resistance mechanisms is the approach of mapping resistance gene analogs (Donald et al. 2002; Di Gaspero and Cipriani 2003) and study their functional relevance in resistance. The addition of all this new information about the genetics of an ancient cultured plant will allow comparisons within grapevine varieties and accelerate the overall understanding of the physiological basis of traits desired in modern grapevine breeding. Finally, this work will help us to understand and make use of the mechanisms involved in disease resistance and quality.

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