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ORIGINAL PAPER

Rpv10: a new locus from the Asian *Vitis* gene pool for pyramiding downy mildew resistance loci in grapevine

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Abstract A population derived from a cross between grapevine breeding strain Gf.Ga-52-42 and cultivar 'Solaris' consisting of 265 F1-individuals was genetically mapped using SSR markers and screened for downy mildew resistance. Quantitative trait locus (QTL) analysis revealed two strong QTLs on linkage groups (LGs) 18 and 09. The locus on LG 18 was found to be identical with the previously described locus *Rpv3* and is transmitted by Gf.Ga-52-42. 'Solaris' transmitted the resistance-related locus on LG 09 explaining up to 50% of the phenotypic variation in the population. This downy mildew resistance locus is named *Rpv10* for resistance to *Plasmopara viticola*. *Rpv10* was initially introgressed from *Vitis amurensis*, a wild species of the Asian *Vitis* gene pool. The one-LOD supported confidence interval of the QTL spans a section of 2.1 centi Morgan (cM) corresponding to 314 kb in the reference genome PN40024 (12x). Eight resistance gene analogues (RGAs) of the NBS–LRR type and additional resistance-linked genes are located in this region of PN40024. The F1 sub-population which contains the *Rpv3* as well as the *Rpv10* locus showed a significantly higher degree of resistance, indicating additive effects by pyramiding of resistance loci. Possibilities for using the resistance locus *Rpv10* in a grapevine breeding programme are discussed. Furthermore, the marker data revealed 'Severnyi' × 'Muscat Ottonel' as the true parentage for the male parent of 'Solaris'.

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Introduction

Pest management became one of the main tasks for European viticulture after the introduction of the fungal pathogens causing powdery and downy mildew in the second half of the nineteenth century. Nowadays considerable amounts of fungicides sprayed periodically during the vegetation period are necessary to prevent crop loss and to ensure the harvest of healthy grapes. In addition to powdery mildew (*Erysiphe necator* Schwein. 1834), the downy mildew caused by *Plasmopara viticola* [(Berk. & M.A. Curtis) Berl. & De Toni 1888] is the major pathogen threat. As an obligate biotrophic oomycete, *P. viticola* invades the highly susceptible European grape cultivars (*Vitis vinifera L.*) with motile zoospores through the stomata and colonises the intercellular space of the mesophyll (examples given in Burruano 2000; Kiefer et al. 2002; Kortekamp et al. 1998). Heavy infections can cause extreme defoliation up to total loss of leaves. Furthermore, especially infections of the inflorescence prior to or during flowering may result in total yield loss (Mohr 2005; Nicholas et al. 1994).

Sources of resistance against the mildews were identified in a range of American wild species (Alleweldt and Possingham 1988; Eibach et al. 2010). In the past, grape breeders tried to use these sources and undertook a lot of efforts to combine the resistance traits from the American wild species with the quality of *V. vinifera* cultivars. Meanwhile, these empirically based breeding programmes have resulted in a range of newly developed cultivars with

high-quality features and considerable mildew resistance characteristics (Bundessortenamt 2008). Recent research has led to the genetic identification of different resistance loci. Molecular markers linked to these loci allow a marker-assisted selection (MAS) in the offspring of cross combinations in an early seedling stage, resulting in an acceleration of the breeding process (as reviewed in Töpfer et al. 2011). Furthermore, MAS allows the selection of genotypes with combined resistance loci in order to build up sustainable resistance. This is of special interest because the implementation of a single resistance locus may not be sufficient for a durable resistance as shown by Peressotti et al. (2010). An example for such a combination ("pyramiding") of different resistance loci by the application of MAS was recently described by Eibach et al. (2007).

Next to the American wild species, Asian wild species are also known to carry resistance features against the mildews (e.g. Jürges et al. 2009; Wan et al. 2007). The utilization of these resources started in recent decades and resulted in new cultivars like 'Solaris' with the Asian species *V. amurensis* in its parentage. Thus, 'Solaris' confers downy mildew resistance accompanied by necrosis (Boso and Kassemeyer 2008), callose deposition (Gindro et al. 2003) and stilbene accumulation (Gindro et al. 2006; Pezet et al. 2004) as activated defence mechanisms. The investigations described in this paper were carried out in order to genetically characterise this source of resistance and to develop molecular markers for their application by MAS in breeding programmes.

Materials and methods

Mapping population

Investigations were carried out on the progeny of the cross between Gf.Ga-52-42 ('Bacchus' × 'Villard blanc') and 'Solaris' ('Merzling' × Geisenheim 6493). Gf.Ga-52-42 is a genotype derived from the breeding programme of the Institute for Grapevine Breeding Geilweilerhof. It shows a high degree of resistance against downy mildew. 'Solaris', also highly resistant against downy mildew, is a protected new cultivar registered in the German variety list, available for commercial plantings. The cross was performed in 2008 and seeds were germinated in the greenhouse in spring 2009. The whole population comprises 265 individuals.

Phenotyping

Leaf disc tests were used to determine the leaf resistance against downy mildew. For standardising the physiological stage, two leaves from the third and fourth apical insertions were used to excise four leaf discs with an 18-mm-diameter cork borer. The leaf discs were placed upside-down in Petri dishes containing 0.8% water agar. Each disc was artificially infected with 40 µl of a *P. viticola* sporangia suspension (20,000 sporangia per ml). Sporangia were collected from leaves of non-sprayed field-grown plants of different cultivars showing fresh sporulation after incubation overnight in a wet chamber. The infected discs were incubated for 5–7 days at 25°C with a photoperiod of 12 h and high relative humidity.

In total, four test series were performed in 2009 and 2010 using all individuals of the population as well as the parents. Infected leaf discs were examined with a stereo microscope at tenfold magnification. This rating on low magnification allowed screening of a large number of leaf discs with a resolution adequate to observe single sporangiophores. The degree of infection was estimated based on the intensity of sporangiophore formation (9: no, 7: one to five, 5: six to twenty, 3: more than twenty, 1: dense sporangiophore carpet). This method is similar to the evaluation protocol given by OIV descriptor 452 (OIV 2009). QTL analyses were performed using the mean overall infection values of the four test series. To check the reliability of the different series, the mean values of the four leaf discs of each assay were separately analysed in addition.

DNA extraction

Small leaf pieces of about 1 cm^2 from the third apical inserted leaf were collected in pre-cooled 96 deep well plates (ABgene, Epson, UK). After lyophilisation and grinding with the TissueLyser mill (Qiagen GmbH, Hilden, Germany) the DNA was isolated using the Qiagen® DNeasy 96 Plant Kit (Qiagen GmbH, Hilden, Germany).

Genotyping

For marker analyses, microsatellites, also called simple sequence repeats (SSR), were used. Initial mapping was performed using the sets of SSR flanking primer pairs from SCUvv (Scott et al. 2000), UDV (Di Gaspero et al. 2005), VChr (Cipriani et al. 2008), VMC (Vitis Microsatellite Consortium, managed through AGROGENE, Moissy Cramayel, France), VrZAG (Sefc et al. 1999), VVI (Merdinoglu et al. 2005), VVMD (Bowers et al. 1996, 1999) and VVS (Thomas and Scott 1993). Additional primer pairs were newly developed based on the grapevine genome sequence of PN40024 (Jaillon et al. 2007) available at the Grape Genome Browser (http://www.genoscope.cns.fr/vitis). The sequence in the region of interest was scanned for microsatellites using WebSat (Martins et al. 2009, http://wsmartins.net/websat/) and primers were developed using the imbedded Primer3 programme (Rozen and

Skaletsky 2000). According to a standardised denomination these markers were labelled with "GF" for "Geilweilerhof", the LG they were associated with and a following consecutive number (e.g. GF09-04). Primer sequences for SSR markers located on LG 09 and LG 18 are given in Suppl. Table 1. Newly developed primer sequences on the other LGs will be published by Fechter et al. (in preparation).

For fragment length determination on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany), all forward primers were 5′-labelled with a fluorescent dye (6-FAM, HEX, TAMRA or ROX). The combination of markers with different labels and diverse fragment lengths allowed performing the polymerase chain reaction (PCR) as multiplexes of up to 12 markers. The Qiagen® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany) was used to set up 10 µl reaction mixtures containing master mix, 1.5 – 5 pmol of each primer and about 3 ng of template DNA. ABI 9700 thermal cyclers (Applied Biosystems, Darmstadt, Germany) were used for the amplification starting with 15 min initial denaturation at 95°C, followed by 30 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 60 s. A final extension was performed at 72°C for 20 min. 1 µl of the PCR product was used for fragment length determination and the results were processed with GeneMapper® 4.0 software (Applied Biosystems, Darmstadt, Germany).

Genetic mapping and QTL analysis

The two parental as well as the integrated maps were constructed according to the description in Zhang et al. (2009) using the JoinMap® 4 software (Van Ooijen 2006). Estimated genome length calculations were performed according to Hulbert et al. (1988).

QTL analyses were calculated using MapQTL® 5 (Van Ooijen 2004). Identification of QTL areas and trait-linked markers was carried out by Interval mapping (IM) in 1 cM intervals on the three maps. Closely flanking markers were selected as cofactors and QTL detection analysis was repeated in multiple QTL mapping (MQM) in 0.5 cM intervals (Jansen 1993). Analyses with varied cofactors and cofactor combinations were performed to get the most reliable results. The genome-wide and LG-specific LOD (logarithm of the odd) thresholds at $\alpha = 0.05$ (5%) were calculated by at least 1,000 permutations.

Results

Phenotyping

Tests with artificially infected leaf discs were performed to determine the leaf resistance level against downy mildew.

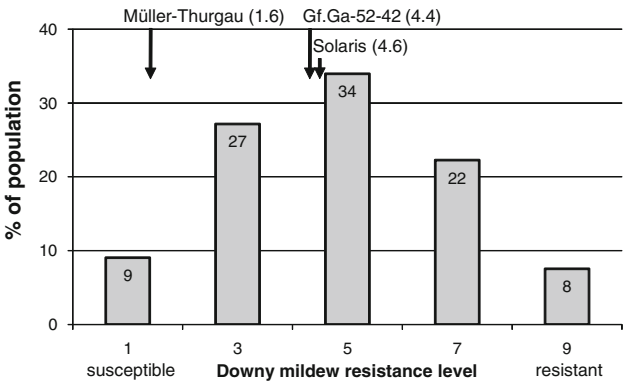

Fig. 1 Frequency distribution (%) for the degree of infection of *P. viticola* in a population derived from the cross of Gf.Ga-52-42 × 'Solaris' ($N = 265$). The intensity of sporangiophore formation was rated (9: no, 7: one to five, 5: six to twenty, 3: more than twenty, 1: dense sporangiophore carpet) and classified according to the OIV descriptor 452 from susceptible (1) to resistant (9) using the average of four independent leaf disc tests. Average resistance levels of the controls are 4.4 for Gf.Ga-52-42, 4.6 for 'Solaris' and 1.6 for the susceptible cultivar 'Müller-Thurgau'

The rating of sporangiophore formation permits to evaluate the success of the pathogen to infect, colonise and reproduce on leaf tissue from the individual plants of the F1-progeny. The classified results based on mean values of four independent screening series are presented in Fig. 1. Data roughly follow a normal distribution pattern with 8% of the plants showing high resistance and 9% showing high susceptibility, respectively.

Genetic mapping

In a first step, a core genetic map with an average of five publicly available SSR markers per LG equally distributed throughout the genome was constructed. After a first QTL analysis based on this preliminary integrated map, the marker coverage was mainly increased on LG 09 which showed considerably elevated LOD (logarithm of the odd) scores for downy mildew resistance. For further improvement of the map, markers next to the ends of the LGs were added to cover the exterior parts of the chromosomes.

In total, 208 SSR markers were used to set up an integrated map with 19 LGs (Table 1, Fig. 2). LOD score thresholds equal or greater than ten were used for the determination of the LGs. LG 09 was covered most densely with 45 markers, whereas LG 17 showed the least coverage with eight markers. This unbalance was mainly due to the targeted mapping of the observed QTL on LG 09 in the preliminary map. To some extent, experimental factors in multiplex sets like the combinability of amplicons due to fragment length or failure of amplification of certain markers also contributed to this pattern. 157 markers are included in the maternal map of Gf.Ga-52-42,

Table 1 Main characteristics of linkage groups in the integrated, the maternal Gf.Ga-52-42 and the paternal 'Solaris' maps

LGs	Integrated map			Map of female parent Gf.Ga-52-42			Map of male parent 'Solaris'		
	Covered length (cM)	No. of markers	Average distance (cM)	Covered length (cM)	No. of markers	Average distance (cM)	Covered length (cM)	No. of markers	Average Distance (cM)
1	62.66	14	4.48	68.36	12	5.70	56.48	10	5.65
2	34.35	7	4.91	33.82	5	6.76	31.81	4	7.95
3	51.06	8	6.38	57.37	7	8.20	48.41	5	9.68
4	64.35	7	9.19	48.79	5	9.76	50.64	5	10.13
5	68.18	10	6.82	47.63	6	7.94	64.11	6	10.69
6	48.45	8	6.06	45.56	5	9.11	47.40	6	7.90
7	71.63	10	7.16	78.20	9	8.69	65.24	8	8.15
8	68.54	11	6.23	74.32	7	10.62	60.09	8	7.51
9	57.58	45	1.28	66.57	32	2.08	49.44	30	1.65
10	63.98	8	8.00	62.22	5	12.44	64.28	5	12.86
11	49.95	7	7.14	62.33	6	10.39	46.21	7	6.60
12	60.33	12	5.03	68.85	8	8.61	42.87	9	4.76
13	58.79	13	4.52	59.25	12	4.94	9.26/10.81	3/5	3.48/3.15
14	65.22	9	7.25	71.36	8	8.92	59.21	7	8.46
15	42.07	7	6.01	44.89	6	7.48	31.74	5	6.35
16	40.99	7	5.86	6.96/9.55	3/2	2.32/4.78	40.35	6	6.72
17	48.73	6	8.12	48.08	5	9.62	48.02	6	8.00
18	85.23	10	8.52	40.99	7	5.86	76.83	8	9.60
19	55.20	9	6.13	52.52	7	7.50	40.74	8	5.09
O(G)	1,097.28	208	6.27	1,047.61	157	7.59	943.95	151	7.22
E(G)	890.93			1,281.85			1,178.47		

O(G) observed genome length (sum length of all linkage groups)

E(G) estimated genome length, calculated by the moment estimator $E(G) = M(M-1)X/K$ (Hulbert et al. 1988) (M = number of marker; X = maximum observed map distance; K = number of locus pairs over LOD threshold)

ranging from 5 to 32 markers (LG 09) per LG. The five markers on LG 16 are split into two discrete groups of three and two markers. On the paternal 'Solaris' map (151 markers), LG 09 again exhibits the best coverage with 30 markers. LG 02 shows the lowest coverage with four markers while LG 13 is split into two parts of three and five markers (Table 1). Calculation of the estimated genome length according to Hulbert et al. (1988) indicates adequate genome coverage. For the integrated map, the observed genome length even exceeds the estimated value (Table 1).

QTL analysis

IM was performed to locate QTLs for *P. viticola* resistance. QTLs with LOD scores higher than the LG- and genome-wide LOD thresholds were observed on LG 09 and 18. Further analyses using full MQM were performed as a more sensitive approach. This resulted in the additional identification of a minor QTL on LG 05.

On LG 09 of the integrated map, the QTL reaches a maximum LOD value of 39.9 in IM and even 47.9 in the MQM analysis and explains up to 50.0% of the phenotypic variance observed in the population. Using the one-LOD supported confidence interval, a region of 2.1 cM can be ascertained for both mapping methods (Table 2; Fig. 3). This locus was named *Rpv10*. Looking at this LG on the 'Solaris' map (LOD_{max} 38.7, 49% explanation), the same QTL can be found. In the map of Gf.Ga-52-42 the LOD stays below the significance threshold for the entire LG 09 (Table 2).

The second QTL, found on LG 18, explains up to 15.5% of variance with maximum LOD values of 8.4 (IM) and 16.8 (MQM) in the integrated map. The one-LOD support interval covers 5.9 cM at IM and 4.4 cM at MQM (Table 2; Fig. 3). In the Gf.Ga-52-42 map, the QTL (LOD_{max} 7.9, 13.6% explanation) is also found, whereas no significant QTL can be detected on LG 18 in the 'Solaris' map (Table 2).

The minor QTL on LG 05 was only identified using MQM with cofactors GF09-46 and GF18-06. With a maximum LOD value of 4.8, this minor QTL explains 3.4% of the phenotypic variance in the integrated map. VCHR05C as the nearest marker was inherited paternally

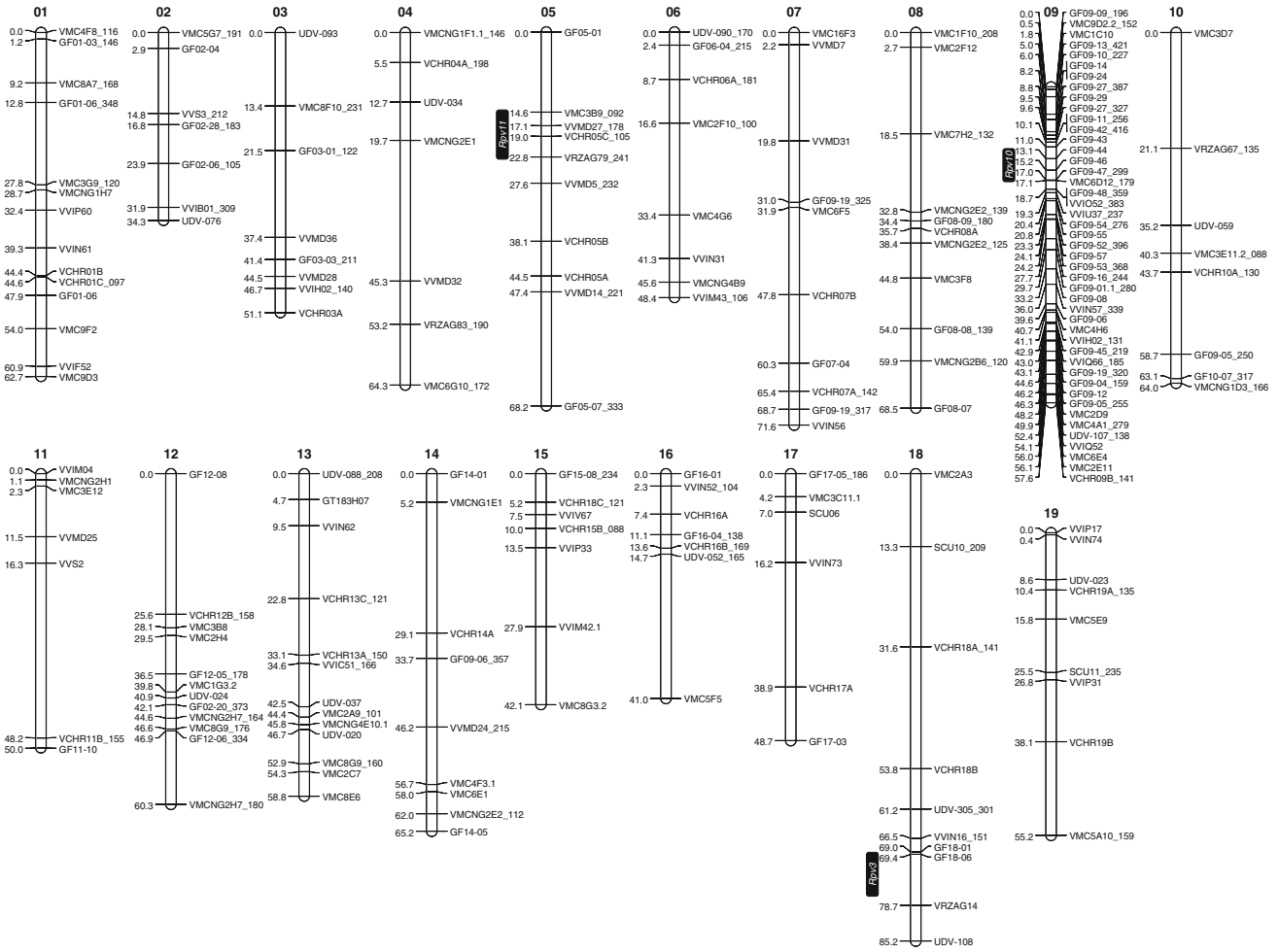

Fig. 2 Integrated genetic map of the cross population Gf.Ga-52-42 × 'Solaris' with an offspring of 265 individuals. Distances are given in cM using Kosambi's map function. Additional digits behind the marker names give the coded fragment lengths (bp) of markers heterozygous in only one parent

and thus the QTL was also found in the 'Solaris' map (LOD_{max} 1.6, 1.4% explanation), exceeding the LG-specific threshold of 1.3.

The QTL position in cM differs between the integrated and the paternal map. This is due to the differences in the total length of the LGs. In case of LG 18 where the maternal map is missing the first three markers of the integrated map, the positions differ strongly. Nevertheless, the QTL is flanked by the same markers.

Pyramiding effect

The effects of pyramiding the resistance loci derived from both parents can be followed by comparing the resistance levels in distinct genotypic classes of plants in the offspring. Figure 4 indicates the frequency distribution for the individuals of the population separated for presence or absence of the resistance-related alleles of the neighbouring markers GF09-46 on LG 09 and GF18-06 on LG 18, respectively. When both resistance-related alleles are absent, there is a clear shift towards a higher frequency of individuals with high susceptibility, whereas the presence of the resistance-related alleles from both parents leads to a shift towards the classes with a higher downy mildew resistance level. The presence of only one resistance-related allele of one parent results in an intermediate distribution.

The mean values for the degree of resistance within the different segregation pattern groups reflect the different frequency distributions. While the mean resistance level is lowest for the group with no resistance-related alleles ($x = 2.6 \pm 1.2$), the maximum value is reached for the group of individuals with both resistance-related alleles ($x = 6.7 \pm 1.2$). Intermediate values can be found for the groups of individuals with one resistance-related allele, either from the female or from the male parent (values indicated in legend of Fig. 4). The analysis of variance results in significant differences between the groups with P values below the statistical threshold of $P \leq 0.05$.

Table 2 Summary of QTL characteristics for *Plasmopara* resistance identified on LG 05, LG 09 and LG 18

Map	LG[a]	Mapping type	LOD_{max}	LOD threshold specific LG[b]	LOD threshold genome wide[b]	LOD_{max} position (cM)[c]	Confidence interval (cM) [$LOD_{max} - 1$]	Variance explained (%)	Cofactor
Consensus	09	IM	39.92	3.0	4.2	15.2	14.1–16.2	50.0	
		MQM	47.94	3.0	4.2	15.2	14.1–16.2	49.1	GF09-46
	18	IM	8.36	2.6	4.2	70.9	69.0–74.9	15.5	
		MQM	16.76	2.6	4.2	70.8	69.0–73.4	14.3	GF18-06
	05	MQM	4.79	2.6	4.2	19.0	14.6–22.8	3.5	
Gf.Ga-52-42	09	IM	–[d]	1.8	2.9	–	–	–	
		MQM	–[d]	1.8	2.9	–	–	–	
	18	IM	7.91	1.4	2.9	17.5	16.4–21.5	13.6	
		MQM	7.91	1.4	2.9	17.5	16.4–21.5	13.6	GF18-06
	05	MQM	–[d]		2.9	–	–	–	
'Solaris'	09	IM	38.73	1.6	2.7	14.79	13.9–15.8	49.0	
		MQM	38.73	1.6	2.7	14.79	13.9–15.8	49.0	GF09-46
	18	IM	–[d]	1.5	2.7	–	–	–	
		MQM	–[d]	1.5	2.7	–	–	–	
	05	MQM	1.53	1.3	2.7	17.0	7.0–27.0	1.4	

[a] Linkage groups according to the International Grape Genome Program (IGGP) nomenclature

[b] Estimated value using a permutation test with 1,000 permutations at $\alpha = 0.05$

[c] Positions in the context of markers are identical between maps. Differences result from different map lengths

[d] LOD for the entire LG below significance level

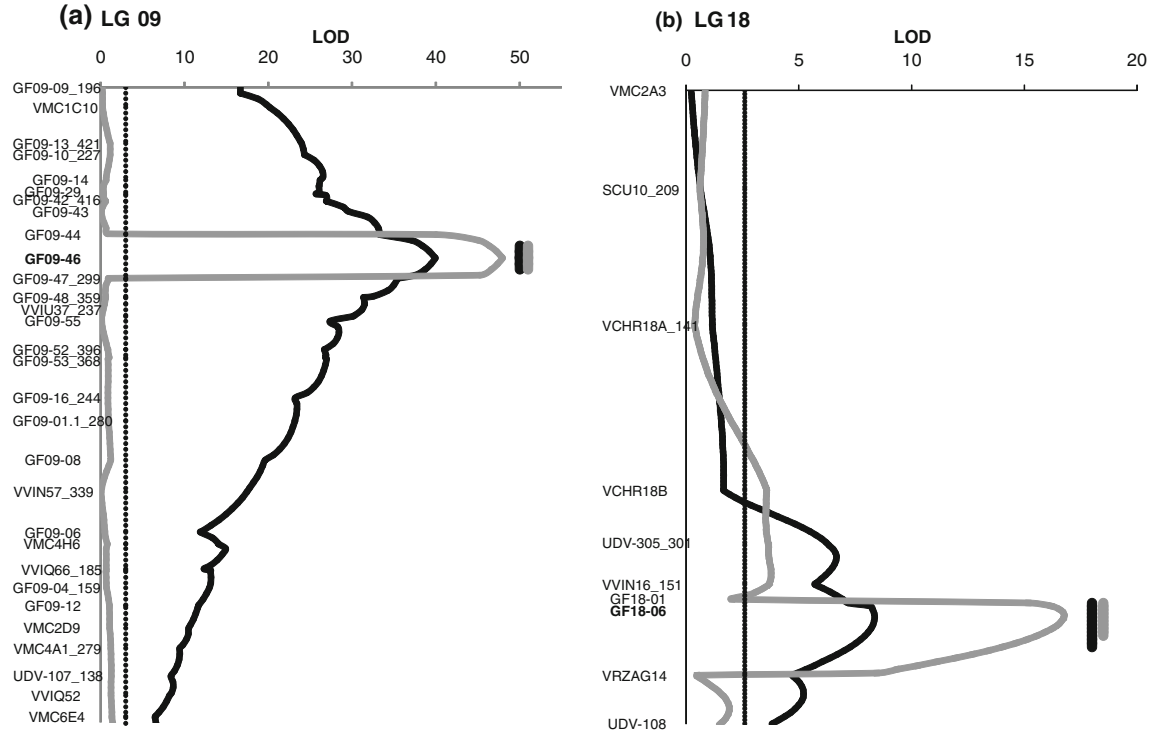

Fig. 3 Quantitative trait loci for *Plasmopara viticola* resistance identified on LGs 09 (**a**) and 18 (**b**) of the consensus map by Interval Mapping (*black*) and MQM (*grey*, cofactors in *bold*). One-LOD support interval is delimited by bars. Linkage group specific statistical threshold values are indicated by the *dotted line*. Indicated SSR markers were reduced to get a clear arrangement

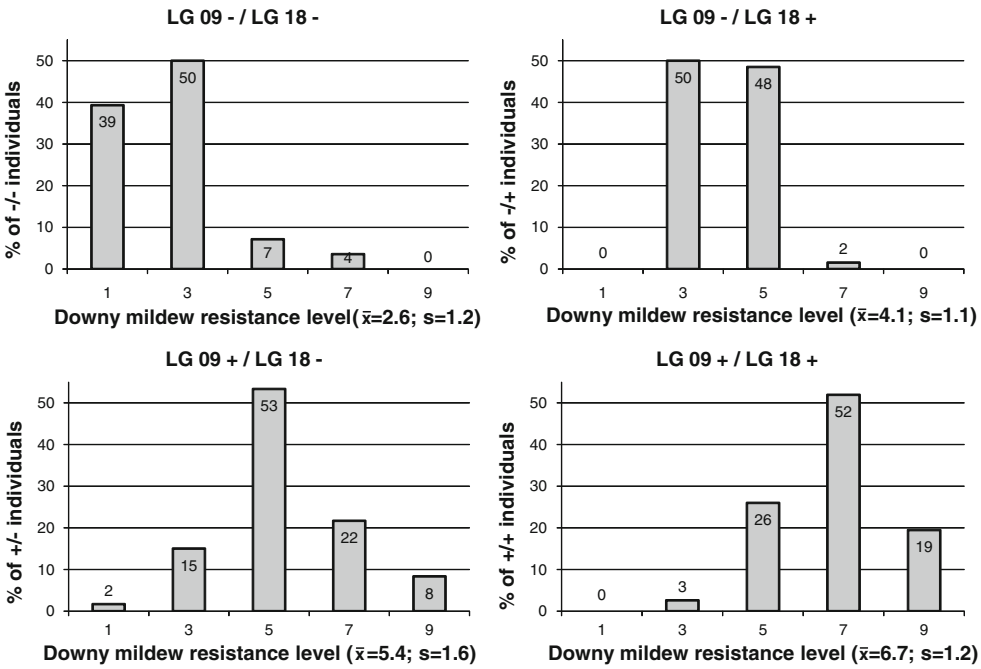

Fig. 4 Frequency distribution (%) of *Plasmopara viticola* resistance separated for the presence (+) or absence (−) of the resistance correlating alleles of GF09-46 on LG 09 and GF18-06 on LG 18. Mean of the downy mildew resistance level in this group ($\bar{x}$) with standard deviation (s) in brackets

Analysis of the genomic sequence of PN40024 corresponding to the *Rpv10* locus

The annotated genomic sequence of PN40024 offers the opportunity to search for gene position and function via the Genewise Uniprot database (http://www.uniprot.org). Therefore, the corresponding sequence of the extended QTL region of *Rpv10* on chromosome 09 of PN40024, delimited by the markers GF09-14 (2.4 Mb) and GF09-16 (5.9 Mb), was screened for genes associated with resistance (Fig. 5). Twenty-six resistance gene analogues (RGAs) of the NBS–LRR type were predicted in this region. Most of them were arranged in three clusters with 3, 7 and 13 genes within regions of 31, 102 and 404 kb, respectively (Fig. 5b). Genes of the first cluster, located upstream of the one-LOD confidence interval next to GF09-42, show homology to RGAs isolated from *V. riparia* (Di Gaspero and Cipriani 2003) and *V. bryoniifolia* (Wang and Wang 2006). Cluster two, flanked by the markers GF09-44 and GF09-46 being next to the LOD_{max}, consists of four *V. vinifera* RGAs (Donald et al. 2002) and three further NBS–LRR homologues. Thirteen *V. amurensis* resistance protein candidates (Di Gaspero and Cipriani 2003) were organized in the larger third cluster located between GF09-55 and GF09-52. Three, four and six of the repetitive copies are related to the three *V. amurensis* RGAs rgVamu075, rgVamu149 and rgVamu150, respectively. Besides these clustered RGAs there are three further single RGAs interspersed between the clusters: (1) One copy related to *V. riparia* (Di Gaspero and Cipriani 2003) is located between GF09-46 and GF09-47; (2) downstream of VVIU37, a predicted protein shows similarity to an NBS–LRR type disease resistance protein from *Poncirus trifoliata*; (3) in the vicinity of GF09-16 a gene is described coding for a protein with similarity to a *Solanum trilobatum* resistance protein.

In addition to the NBS–LRR proteins, a high number of putative receptor-like kinases including serine/threonine protein kinases and phosphatases can be found in the analysed region. Between the markers GF09-44 and GF09-47 delimiting the central part of the *Rpv10* corresponding region, homologues to the Avr9/Cf-9 rapidly elicited protein, a class III HD-Zip protein, a GATA transcription factor, an ankyrin-like protein, an ethylene-responsive transcription factor and a VHS domain-containing protein were mapped (Fig. 5c). Nine further predicted proteins were of unknown function.

Pedigree analysis

In addition to the individuals of the cross population, the DNA of the available next ancestors was analysed with the SSR markers used for mapping. The data confirm 'Bacchus' and 'Villard Blanc' as parents of the breeding line Gf.Ga-52-42 and 'Merzling' as the true mother of 'Solaris'. However, the DNA of the pollen donor Geisenheim 6493 is not available since it describes a pollen mixture of a cross population, but not of an individual genotype. Two different possibilities for the ancestry to *V. amurensis* were given in the "Vitis International Variety Catalogue" (http://www.vivc.de) according to different references (Becker 1981; Hillebrand et al. 2003; Lott et al. 2010; Staatliches

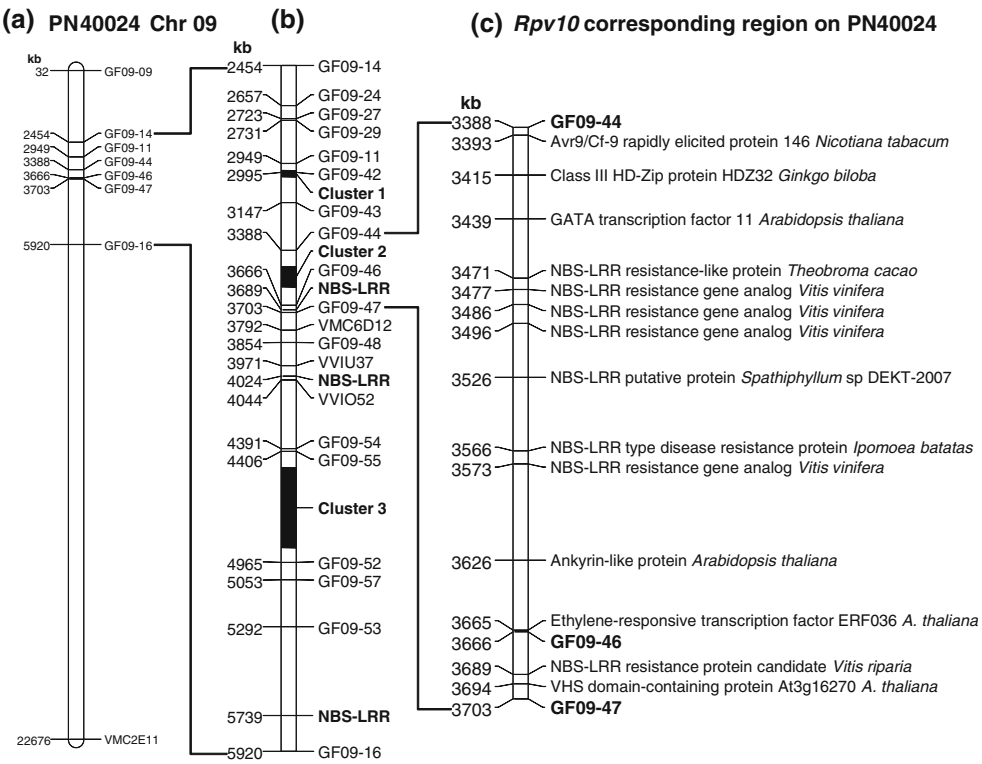

Fig. 5 **a** Linkage group 09 of the reference genome PN40024, **b** the extended QTL region with the NBS-LRR-clusters and **c** the *Rpv10* corresponding region on PN40024 with the predicted proteins between the flanking SSR markers

Weinbauinstitut Freiburg 2010). One version indicates the population of Geisenheim 6493 as a cross between 'Zarya Severa' ('Seyanets Malengra' × *V. amurensis*) and 'Muscat Ottonel', whereas 'Saperavi Severnyi' ['Severnyi' ('Seyanets Malengra' × *V. amurensis*) × 'Saperavi'] was given as an alternative maternal possibility for Geisenheim 6493 (Suppl. Fig. 1).

The DNA of 'Muscat Ottonel', 'Zarya severa', 'Severnyi' and a cultivar assumed to be 'Saperavi Severnyi' were analysed in order to uncover the real maternal parent. The marker data revealed that 'Severnyi' and the variety labelled as 'Saperavi Severnyi' had the same allele pattern for all tested markers. Ampelographical investigations identified both cultivars as 'Severnyi' as described in Ampelografija of the USSR (1955). The allelic pattern of 'Solaris' was checked to clarify the genetic descent. Interestingly, the paternal allele of 'Solaris' always matched to one of the alleles of either 'Severnyi' or 'Muscat Ottonel'. This finding could be demonstrated for 57 SSR markers inherited from 'Severnyi' and 37 SSR markers from 'Muskat Ottonel', respectively. The remaining markers could not be clearly assigned due to homozygous alleles, null alleles or missing data. The strong disequilibrium in the quantity of markers is due to the high marker density in the QTL region on LG 09 which has been inherited from 'Severnyi'.

Additionally, 'Zarya Severa' was analysed using 58 of the SSR markers to get a clear exclusion as a grandparent of 'Solaris'. In seven cases (approx. 1/8), 'Solaris' carries fragment lengths of alleles that are paternally inherited (not in 'Merzling') but absent in 'Muscat Ottonel' and 'Zarya Severa' while they are present in 'Severnyi' (as exemplarily illustrated in Suppl. Fig. 2). This excludes 'Zarya Severa' and additionally confirms 'Severnyi' as real ancestor of 'Solaris'. The ratio of the marker differences and the data of the analysis confirm the reported full-sibling relationship between 'Zarya Severa' and 'Severnyi'. The resulting pedigree of the Gf.Ga-52-42 × 'Solaris' cross population with the revised parentage of 'Solaris' is indicated in Fig. 6.

To gain further insight into the locus on LG 09, nine flanking SSR markers were used for screening different cultivars of interest (Table 3). For 'Zarya Severa', the results are in accordance with those reported from the 58 SSR marker set. Three nearby flanking markers (GF09-44, GF09-47 and GF09-48) possess the resistance-related allele lengths while they are absent in the six other cases, including the centred GF09-46. The *V. riparia* selection 'Gloire de Montpellier' lacks the resistance-related alleles for eight markers. Only for GF09-52, located 8.1 cM downstream of the maximum LOD position, an identical fragment length was observed. The test also included the cultivars 'Rondo' (selection of Geisenheim 6494 reported as 'Zarya Severa' × 'Saint Laurent') and 'Bronner' ('Merzling' × Geisenheim 6494)

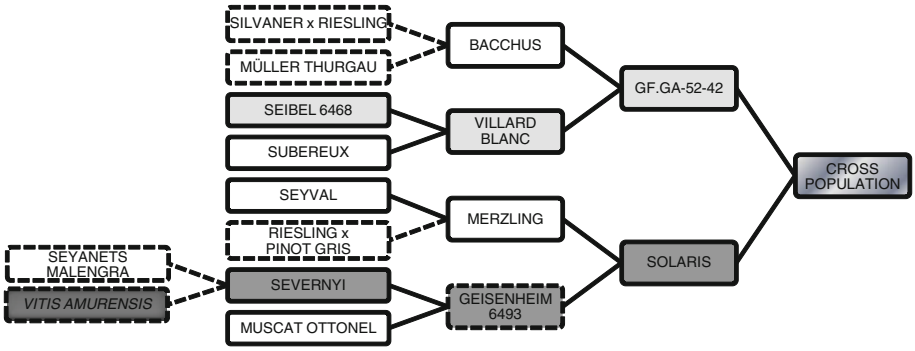

Fig. 6 Pedigree of the cross population Gf.Ga-52-42 × 'Solaris'. *Continuous lines* indicate SSR marker confirmed linkages between tested cultivars (*continuous boxes*). Cultivars in *dashed boxes* and *dashed lines* are related predecessors according to the "Vitis International Variety Catalogue" (http://www.vivc.de). *Light grey* represents presence of *Rpv3* and *dark grey* the presence of *Rpv10*

Table 3 Allele lengths of *Rpv10* flanking SSR markers for cultivars of interest

					Rpv10													
Marker name	GF09-11		GF09-43		GF09-44		GF09-46		GF09-47		GF09-48		GF09-55		GF09-52		GF09-57	
Resistance related allele length	256		428		230		416		299		359		249		396		303	
Gf.Ga-52-42	254	280	422	426	242	245	423	425	296	296	349	349	238	264	393	393	347	366
Solaris	256	280	422	428	230	245	416	425	296	299	349	359	249	264	393	396	303	366
Merzling	277	280	422	430	245	-	406	425	296	-	349	356	262	264	393	394	360	366
Severnyi	248	256	428	-	230	244	416	423	296	299	349	359	249	257	393	396	303	360
Muskat Ottonel	254	-	425	450	236	245	425	-	292	296	349	-	237	264	393	395	365	-
Zarya Severa	260	-	426	462	230	242	407	423	296	299	349	359	260	-	393	399	301	345
Riparia Gloire de Montpellier	273	276	418	447	229	-	407	-	289	-	351	364	248	252	393	396	354	360
Bronner	256	280	422	428	230	245	416	425	296	299	349	359	249	264	393	396	303	366
Rondo	256	-	424	428	230	-	416	423	296	299	349	359	249	264	393	396	303	366
Saint Laurent	286	-	424	-	230	245	423	425	296	-	349	-	237	264	393	-	347	366

Resistance-related allele lengths with *grey* background; homozygous or null alleles are indicated by -

which have all of the resistance-related alleles. This excludes 'Zarya Severa' as their ancestor and revealed, with regard to the allelic pattern of 'Saint Laurent', that 'Severnyi' is also the real ancestor of these cultivars. Thus, 'Rondo' and 'Bronner' carry the *Rpv10* locus.

Discussion

Phenotyping

Performing leaf disc infection tests is a well-established method to get reliable data for *P. viticola* leaf resistance. Comparisons with data obtained from artificially infected potted plants, naturally infected plants in the field or from infection tests on detached leaves show good correlations (Bellin et al. 2009; Boso and Kassemeyer 2008; Boso et al. 2006). Furthermore, according to Bellin et al. (2009), the environmental variance can be reduced by using greenhouse plants for leaf disc tests resulting in a restriction of the QTL region.

The method enables the screening of a large number of individuals and their replicates with reasonable efforts. In the cross population a roughly normal distribution is observed (Fig. 1). This shape of the total phenotypic distribution is obtained by the summing of the four genotypic distributions shown in Fig. 4. An additive effect on the downy mildew resistance level by combining the resistance loci *Rpv3* and *Rpv10* indicates that these loci are applicable for pyramiding of resistances in MAS.

Genetic mapping and QTL analysis

The establishment of a genetic map based on a cross population is a common procedure for the localisation of segregating target characteristics within the genome. A non-exhaustive list of mapped populations can be found on 'http://www.vivc.de' (Database search: Data on breeding and genetics) with a number of 33 discovered

trait loci so far. Use of a core set of markers per LG permits a calculation of a preliminary integrated map. QTL analysis carried out with the preliminary map with four to five markers per LG and the first phenotypical data already gave a strong hint for significant QTLs on LGs 09 and 18. This map provided the basis for very efficient genotyping by setting the focus on these LGs. In particular, for LG 09 new targeted markers were developed and mapped, given that this QTL was inherited from 'Solaris'. The availability of the grape genome sequence of the 'Pinot Noir' inbred line PN40024 (Jaillon et al. 2007) allowed the quick and easy designing of new SSR markers specific to the region of interest. On the other hand, markers next to the LG ends were used to cover the major part of the genome in order to reduce the possibility to miss regions of interest (Table 1). This approach of reference genome-based chromosomal mapping proved to be extremely successful for creating a map focused on the trait of interest which subsequently can be expanded for other traits. The relative order of the markers on LG09 was checked against the PN40024 reference genome to verify the synteny. Only one exchange of marker order was observed, namely between markers VVIO52 and VVIU37, while all other 43 markers exhibit identical order. The genome coverage of 1,097 cM equals that of maps with similar marker quantity (Moreira et al. 2011; Zhang et al. 2009) and represents about 65% of the size of more expanded maps (Di Gaspero et al. 2007; Doligez et al. 2006; Welter et al. 2007).

Two major QTLs were detected in the Gf.Ga-52-42 × 'Solaris' F1 population. The QTL on LG 18 was inherited from the maternal Gf.Ga-52-42 and is identical with the one already found in 'Regent' (Fischer et al. 2004; Welter et al. 2007). It is described as *Rpv3* in 'Bianca' (Bellin et al. 2009). Resistance-linked alleles of Gf.Ga-52-42 originate from 'Villard Blanc' which is an ancestor of 'Bianca' and a full sibling of S.V. 12-417, a grandparent of 'Regent'. The pedigree of 'Villard Blanc' includes accessions of five American wild *Vitis* species as the possible donor of resistance. It was shown in 'Bianca' that the resistance is co-localised with formation of necrotic spots as the result of a hypersensitive response (HR) (Bellin et al. 2009). A comparison of the QTL characteristics between the cross populations has to take into consideration the different genetic background of the mapping populations as well as the presence of two major QTLs for the same trait in this study. Although the observed maximal LOD values for *Rpv3* are lower than the top values given in the other studies, they are significant for the different years and phenotyping methods. The apparent reduction in the percentage of total variance explained by this QTL can be mainly announced to the second major QTL in this F1 progeny possessing a more distinct genetic influence in defense against *P. viticola*. Furthermore, other genetic factors segregating in this specific cross population that has a genetic background different from the other populations studied will affect the explained variance.

On LG 09, a very strong QTL with a LOD score of 47.9 in MQM mapping was found, explaining up to 50% of the variance in IM. The resistance correlated alleles were inherited from 'Solaris' and trace back to a 'Seyanets Malengra' × *V. amurensis* cross done at the All-Russia Research Institute of Viticulture and Winemaking in 1936 (http://www.vivc.de). Some genetic information for downy mildew resistance in *V. amurensis* is meanwhile available in literature. A cross population of two breeding lines with *V. rotundifolia* and *V. amurensis* in the pedigree was investigated and QTLs going back to *V. amurensis* were detected on LG 06 and LG 01 (Moreira et al. 2011). Luo et al. (2001) established a SCAR marker linked to downy mildew resistance in wild grapes native to China, including *V. amurensis*. A Blast search with the given primer sequences against the 12× PN40024 genome matched in a region on LG 01 at the position of 10.9 Mb. The linkage map of a *V. amurensis* S1 progeny was published recently identifying a locus on LG 14 that confers total resistance to downy mildew (Blasi et al. 2011) designated as *Rpv8*.

Marguerit et al. (2009) described in a 'Cabernet Sauvignon' × 'Gloire de Montpellier' mapping population a downy mildew resistance QTL on LG 09 named as *Rpv5* (http://www.vivc.de). This QTL found on LG 09 exhibits broad one-LOD confidence intervals of at least 28.9 cM that include the region found in this work. 'Gloire de Montpellier' as a selection of the American species *V. riparia* has a completely different genetic background compared with the Asian species *V. amurensis*. The difference was proved by analysing a set of nine SSR markers flanking the QTL region. The observed lack of the resistance-related alleles confirms the independency of the QTLs (Table 3). In contrast, the cultivar 'Bronner' was also screened with these markers and showed the same allelic pattern as found for 'Solaris'. This indicates that the QTL for downy mildew resistance on LG 09 could be identical with that of a 'Bronner' progeny that was recently reported to account for 52% of the phenotypic variation (Blasi et al. 2010). We propose to name the newly identified locus for downy mildew resistance introgressed from *V. amurensis* as *Rpv10*.

Apart from both major QTLs, a minor QTL was identified on LG 05 explaining 3.4% of the variance. A QTL showing small effects for downy mildew resistance in this section of LG 05 was already described by Fischer et al. (2004) showing LOD scores of the same level. A gene encoding a Ca^{2+}-binding protein associated with the hypersensitive reaction was reported for this locus (Salmaso et al. 2008). In regard to the repeated appearance

in different mapping populations we propose to name it *Rpv11*. However, with regard to the minor effect to the downy mildew resistance level the suitability of the *Rpv11* locus for utilisation in a breeding programme is rather limited.

Genomic sequence of PN40024 corresponding to the *Rpv10* locus

Disease RGAs in *V. vinifera* cv. 'Pinot Noir' were identified by Velasco et al. (2007) who reported 37 RGAs on LG 09. The major group consists of 19 genes of the CC–NBS–LRR-type mainly located within the first six mega bases. LG 09 thus carries the largest CC–NBS–LRR cluster of the genome which includes the region of the QTL determined. NBS–LRR proteins in plants functionally act within pathogen detection. They provide a nuclear binding site (NBS) and a leucin-rich repeat (LRR). CC–NBS–LRRs additionally have α-helical coiled-coil-like sequences in their amino-terminal domain (DeYoung and Innes 2006; Ingle et al. 2006). A number of NBS–LRRs are reported to be linked with resistance in grapes (Barker et al. 2005; Di Gaspero and Cipriani 2003; Di Gaspero et al. 2007; Donald et al. 2002; Kortekamp et al. 2008). In addition, a homologue of the pathogenesis-related protein *PDF2* and four homologues of the powdery mildew non-host resistance-related gene *PEN3* are located on LG 09. Three of the *PEN3* homologues providing a non-host penetration resistance have been annotated to the extended QTL region (Stein et al. 2006; Velasco et al. 2007).

The high number of NBS-LRRs is also confirmed by the genomic sequence of PN40024. A screen of the broader QTL region identified 26 RGAs of the NBS-LRR type of which eight are located within the one-LOD confidence interval. Additionally, further analogues of resistance-related proteins were found between the flanking markers (Fig. 5). A stress-activated ethylene-responsive transcription factor and an ankyrin-like protein are tightly linked to the central marker GF09-49. Ethylene is an endogenous plant hormone that among other things influences the plant response to biotic and abiotic stress (Van Loon et al. 2006). Proteins containing ankyrin repeats like the NPR1 play a key role in the salicylic acid-depending systemic acquired resistance (SAR). SAR mediates plant immunity to a broad spectrum of pathogens (Cao et al. 1997). Functional analysis of two NPR1 orthologs isolated from *V. vinifera* cv. 'Chardonnay' showed that they are likely to control the expression of SA-dependent defence genes (Le Henanff et al. 2009).

Although the sequenced 'Pinot Noir' cultivars exhibit structures playing a role in pathogen defence, they are susceptible to several pathogens, including downy mildew. In addition, a high level of allelic divergence between resistant and susceptible phenotypes is suggested (Hvarleva et al. 2009). Nevertheless, the presence of resistance-related proteins underlines the importance of this region for resistance. Functional differences may be related to various expression levels that remain to be investigated.

Pedigree analysis

Marker analyses reveal that the assumed ancestry of 'Solaris' is not accurate. 'Severnyi' and 'Muscat Ottonel' were identified and confirmed as true parents of the pollen donor for the cross with 'Merzling'. Trueness to type was verified ampelographically for 'Severnyi'. Investigations using nine *Rpv10* flanking markers additionally identified this locus in 'Rondo' and 'Bronner' suggesting them also to be descendents of 'Severnyi' progeny. According to the marker data, 'Zarya Severa' is a full-sibling of 'Severnyi'. Interestingly, 'Zarya Severa', which is described as downy mildew resistant (Ampelografija of the USSR 1954), does not exhibit all the resistance-related alleles of *Rpv10* as given in Table 3. Therefore, it is unlikely that the downy mildew resistance of 'Zarya Severa' is caused by exactly the same region as in 'Severnyi'. Moreover, the resistance of 'Zarya Severa' might be due to other resistance-related loci inherited from the initial *V. amurensis* genotype in the pedigree of both cultivars. The reported reduction of downy mildew resistance in leaf disc tests of 'Solaris' in comparison with *V. amurensis* provides an indication for a loss of additional resistance factors leading to a reduction during colonisation (Boso and Kassemeyer 2008). In addition, the finding of a downy mildew resistance locus on LG 14 (Blasi et al. 2011) supports this assumption.

'Merzling' as the maternal parent of 'Solaris' also provides resistance to downy mildew (Becker 2005) inherited from 'Seyval', which is different to *Rpv3*. This additional resistance could either be the minor QTL on LG 05 or was lost during the crossing steps to 'Solaris'.

Conclusions with special regard to strategic grapevine breeding for resistance

For an effective resistance management in the frame of grapevine breeding programmes it is very important to identify and localise new resistance loci within the genera *Vitis* and *Muscadinia*. Plants providing resistance against the powdery and downy mildews have the potential for a significant reduction of fungicide application. For grapevine, which is grown in the vineyard for 30 or even more years, these resistances have to be stable over long periods. Therefore, the breeding strategies for grapevine require a design supplying the best possible protection against a breakdown of resistance. Examples of overcoming resistance barriers in cultivated plants are given by McDonald and Linde (2002). For grapevine, Peressotti et al. (2010)

recently showed that the race-specific downy mildew resistance locus *Rpv3* has been overcome by a Czech *P. viticola* isolate. To prevent the loss of laboriously introgressed resistance loci, the breeding process should be targeted to design elite lines with combined resistances ideally based on different defence mechanisms. Therefore, marker-assisted selection (MAS) is a powerful tool to identify new cultivars showing different resistance loci pyramided. Process of pyramiding can be further optimised by strategic parental selection using molecular markers. For the mildews, a range of resistance loci has already been described which are accessible for grape breeders (http://www.vivc.de [Database search: Data on breeding and genetics]). Breeders have to reflect on the number of resistance loci per pathogen to be pyramided. The absolute number of resistance loci is definitely not the only matter. However, a minimum of two, better three resistance loci per pathogen should be used as a general rule. Moreover, this decision is also influenced by the degree of resistance contributed by the different loci. For example, two pyramided strong resistance loci based on different resistance mechanisms may lead to the same or even to a higher sustainability of resistance than three or more loci exhibiting less resistance or which are based on analogous resistance mechanisms. Not knowing the exact resistance mechanisms currently breeders at the best can use resistance loci from different sources: e.g. *Rpv1* (*Muscadinia rotundifolia*) and *Rpv3* (American spec.) as pyramided in Eibach et al. (2007) plus *Rpv10* (*V. amurensis*). However, knowing the mechanisms of resistance becomes more and more important to build on durability. A further part of a strategy for achieving sustainable resistance is to develop different new cultivars with various combinations of resistance loci. It is likely that the widespread use of a broad range of cultivars whose resistances are based on only a few resistance loci favours the selection of the pathogen for overcoming the genetic resistance barriers. For this reason, cultivars with various combinations of resistance loci should be grown in viticultural areas.

Taking these considerations into account, the resistance locus *Rpv10* described here provides an excellent additional source for developing new cultivars with sustainable resistance. It provides a high resistance level to downy mildew, which according to these investigations seems to be even superior to the *Rpv3* locus. The identified SSR markers closely linked to *Rpv10* allow grape breeders to use this resistance source in MAS. Tracing back of the locus to the Asian gene pool of *V. amurensis* and further studies on pathogen–plant-interactions (Boso and Kassemeyer 2008; Gindro et al. 2003, 2006; Pezet et al. 2004) let assume that the mechanisms differ from the ones already known from *Vitis* spp. The observed effect on the resistance level to downy mildew has proven the suitability of the *Rpv3* and *Rpv10* loci for use in pyramiding of resistance.

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