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ORIGINAL PAPER

Mapping of crown gall resistance locus *Rcg1* in grapevine

Anett Kuczmog · Anikó Galambos · Szabina Horváth · Anikó Mátai · Pál Kozma · Ernő Szegedi · Péter Putnoky

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Abstract Agrobacteria are efficient plant pathogens. They are able to transform plant cells genetically resulting in abnormal cell proliferation. Cultivars of *Vitis vinifera* are highly susceptible to many virulent *Agrobacterium* strains but certain wild *Vitis* species, including *Vitis amurensis* have resistant genotypes. Studies of the molecular background of such natural resistance are of special importance, not only for practical benefits in agricultural practice but also for understanding the role of plant genes in the transformation process. Earlier, crown gall resistance from *V. amurensis* was introgressed into *V. vinifera* through interspecific breeding and it was shown to be inherited as a single and dominant Mendelian trait. To develop this research further, towards understanding underlying molecular mechanisms, a mapping population was established, and resistance-coupled molecular DNA markers were identified by three different approaches. First, RAPD makers linked to the resistance locus (*Rcg1*) were identified, and on the basis of their DNA sequences, we developed resistance-coupled SCAR markers. However, localization of these markers in the grapevine genome sequence failed due to their similarity to many repetitive regions. Next, using SSR markers of the grapevine reference linkage map, location of the resistance locus was established on linkage group 15 (LG15). Finally, this position was supported further by developing new chromosome-specific markers and by the construction of the genetic map of the region including nine loci in 29.1 cM. Our results show that the closest marker is located 3.3 cM from the *Rcg1* locus that may correspond to 576 kb.

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A. Kuczmog · A. Galambos · S. Horváth · A. Mátai · P. Putnoky (✉)
Faculty of Sciences, Institute of Biology,
University of Pécs, Ifjúság u. 6., 7635 Pécs, Hungary
e-mail: putnoky@ttk.pte.hu

P. Kozma
Faculty of Sciences, Institute of Viticulture and Oenology,
University of Pécs, Pázmány Péter u. 4., 7634 Pécs, Hungary

E. Szegedi
Research Institute for Viticulture and Enology,
P.O. Box 25, 6001 Kecskemét, Hungary

Introduction

Most agrobacteria are efficient pathogens capable of infecting and genetically transforming a variety of plants. Natural transformation of plant cells by T-DNA (transferred DNA) of agrobacteria results in unregulated phytohormone (auxin, citokinin) production causing abnormal cell proliferation and the development of crown gall or hairy root. T-DNA integrated in the plant chromosome also directs the production of opines, specific amino acid and sugar conjugates. These compounds can be utilized mainly by the invading *Agrobacterium* strain and thus the infected plant becomes a specific "niche" for the pathogen (Dessaux et al. 1998; Gelvin 2010; Pitzschke and Hirt 2010; Tzfira and Citovsky 2008). Although efficient, high through-put plant biotechnology techniques benefit from and have consequently aimed at extending the range of plant species prone to *Agrobacterium*-mediated genetic transformation, these gram-negative soil bacteria may cause serious damage in economically important plantations, and therefore, there is an increasing need for *Agrobacterium* or crown gall resistant cultivars in agriculture (Escobar and Dandekar 2003; Otten et al. 2008).

When natural resistance gene sources are available, breeding of crown gall resistant cultivars is possible by conventional crosses as well as by transgenic techniques. Both modern genetic mapping and map-based cloning require the development of molecular (DNA) markers tightly linked to the gene of interest. Crown gall-resistant cultivars have been reported for woody plants including apple, peach, plum, aspen, roses and grapevine (Beneddra et al. 1996; Bliss et al. 1999; Mahmoodzadeh et al. 2004; Moriya et al. 2008; Reynders-Aloisi et al. 1998; Szegedi et al. 1984; Süle et al. 1994; Zoina and Raio 1999). Genetic mapping and isolation of the resistance gene are essential steps to understand the molecular background and physiological bases of natural crown gall resistance.

Crown gall of grapes occurs in most parts of the world where grapes are grown. Infected plants may remain symptomless until they are injured by freezing, pruning, grafting and by other mechanical treatments employed in maintaining the vineyard. As the gall forms, vascular bundle tissues become highly disorganized and lose their ability to transfer water and photosynthetic products. Large galls girdle the stem and result in significant grape decline and may even lead to plant death. Grapevines crown gall is caused mainly by *A. vitis* and occasionally by *A. tumefaciens*. Like other plant pathogens, the *A. vitis* is very much diverse and may harbor octopine/cucumopine, nopaline or vitopine-type tumor-inducing plasmids (Ti plasmid, pTi) (Burr et al. 1998; Paulus et al. 1989; Ridé et al. 2000). Most of *A. tumefaciens* isolates that naturally occur on grapevines have either an *A. vitis*-type octopine/cucumopine or a nopaline pTi (Szegedi et al. 2005).

While *Vitis vinifera* cultivars are highly susceptible to *Agrobacterium* infections and crown gall formation, wild *Vitis* species such as *V. labrusca* and *Vitis amurensis* have resistant genotypes (De Cleene and De Ley 1976). A few decades ago, crown gall resistance from *V. amurensis* was introgressed into *V. vinifera* through interspecific breeding and it was shown to be inherited as a single and dominant Mendelian trait. This locus inherited stably through four generations (F1, F2, BC1, BC2) and provided a wide spectrum of resistance against the three types of *A. vitis* and the *A. tumefaciens* with a nopaline-type pTi (Szegedi and Kozma 1984; Szegedi et al. 1984).

During the last decade, *Vitis* genomics have undergone substantial development. From the first published molecular marker based on the genetic map of grapevine (Lodhi et al. 1995), several genetic maps were constructed to promote marker-assisted selection and map-based cloning and localization of several economically important traits like seedlessness, berry weight and disease resistances has been started (Akkurt et al. 2007; Adam-blondon et al. 2004; Barker et al. 2005; Blasi et al. 2011; Di Gaspero and Cipriani 2002; Doligez et al. 2002; Fischer et al. 2004; Grando et al. 2003; Hoffmann et al. 2008; Pauquet et al. 2001; Riaz et al. 2008; Welter et al. 2007). Recently, a reference-integrated map with more than 1,000 molecular markers including 283 microsatellite or simple sequence repeat (SSR) markers was published (Vezzulli et al. 2008). Moreover, draft genome sequences are also available (Jaillon et al. 2007; Velasco et al. 2007) which substantially aid in developing additional molecular markers and gene cloning.

Taking advantage of recently available molecular biology techniques, we report the localization of a crown gall resistance locus originated from *V. amurensis* in the *V. vinifera* genome. Several RAPD markers were identified and SCAR markers were developed that are coupled to this resistance locus. Moreover, SSR markers linked to the resistance were also identified and a draft genetic map of the region was constructed.

Materials and methods

Plant material and crown gall test

Vitis sp. "Kunbarát" (A6/1) is a crown gall resistant BC1 hybrid originated from the cross of a resistant F2 hybrid (28/19) of *V. amurensis* 115 × *V. vinifera* crossing and the susceptible *V. vinifera* cv. Italia (Koleda 1974). *V. vinifera* cv. "Sárfehér" is a Hungarian cultivar susceptible to agrobacteria and unable for self-pollination. The mapping population (BC2) was established by crossing "Kunbarát" and "Sárfehér" and 272 seedlings were grown up and propagated for crown gall tests and DNA extractions. DNA of *V. amurensis* 115, ancestor of Kunbarát, was used as control in all experiments while DNA of *V. vinifera* cv. Pinot Noir was used as control in the development of LG15 specific markers (see below).

Phenotypic screenings for *Agrobacterium* resistance were performed with artificial inoculation tests in the greenhouse. The *A. tumefaciens* strain C58 (nopaline pTi, Hooykaas et al. 1980), *A. vitis* strains Tm4 (octopine/cucumopine pTi), AT1 (nopaline pTi) and S4 (vitopine pTi, Szegedi et al. 1988) were used for inoculation. The bacteria were cultured on YE agar containing 1 % glucose, 0.5 % yeast extract supplemented with AB salts (Lichtenstein and Draper 1986) and 1.5 % agar at 28 °C for 2 days and were suspended for $OD_{590} = 0.3$ that is about 10^8 CFU/ml. The infection was carried out by wounding the stems at the nodes with a sterile needle dipped into the bacterial suspension. Each progeny was vegetatively propagated and three plants of each genotype were tested. Plants were inoculated at two to three nodes at 4–6 leaf stage. Six weeks later, inoculation sites were visually evaluated to determine whether crown galls had formed. Based on the

symptoms, the plants were grouped as symptomless or resistant and susceptible (Fig. 1). The inoculation test was repeated twice in two consecutive years.

DNA extraction and RAPD experiments

Total DNA was extracted from young leaves (1.0 g) by the method of Arnedo-Andres (Arnedo-Andres et al. 2002) and diluted to 10 ng/µl for amplifications. Initially, DNA samples of five resistant and five susceptible progeny and of the parents were used in PCR experiments. PCR amplifications were carried out according to the method described in Williams et al. (1990). The final reaction volume (12 µl) contained 10 ng of genomic DNA, 20 mM of Tris–HCl (pH 8.4), 50 mM of KCl, 2 mM of $MgCl_2$, 100 µM each of dATP, dGTP, dCTP and dTTP (Fermentas, Vilnius, Lithuania), 0.5 µM of each primer and 0.2 units of *Taq* DNA polymerase (Dream Taq, Fermentas, Vilnius, Lithuania). The reactions were performed in a Bio Rad S1000 thermocycler, using the following procedure: 1 cycle of 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 36 °C and 1 min at 72 °C. Finally, the samples were incubated for 2 min at 72 °C. Amplification products were separated by gel electrophoresis in 1.5 % (w/v) agarose in 1× TBE buffer, stained with ethidium bromide at 50 ng/µl.

Single operon decamer primers (Operon, Alameda, CA, USA) and primer pairs were used for exploring polymorphisms. Markers were also searched by tecMAAP PCR experiments (Caetano-Anollés et al. 1993), where DNA samples were first digested with different restriction enzymes (*Eco*RI, *Pst*I, *Hin*dIII), precipitated and solved in distilled water in 10 ng/µl concentration for amplification.

Development of SCAR markers

Standard cloning procedures, including DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation were performed using conventional methods (Sambrook et al. 1989) or as recommended by the suppliers. RAPD fragments showing

Fig. 1 Test for crown gall resistance. **a** Formation of crown gall at the inoculation sites on clone No. 24 (a representative of susceptible progeny). **b** No crown gall formation at the inoculation sites (*black arrows*) on clone No. 36 (a representative of resistant progeny). Plants were inoculated by *A. vitis* AT1 and gall formation was evaluated 6 weeks later

Table 1 Primer sequences used in this work

Name	Sequence	Reference
SCAR primers		
OPT17sc	GTTGCGGTTCCCATTTTTCACC	This work
	AGCCAACTTGCGCCCCTTAG	This work
OPX05sc	GTTTCTTGATTCCATTGTTTGTA	This work
	TTAGGAACTTCGACAACATTG	This work
OPQ15sc	AGTAGAGATAATGATGGTGTAG	This work
	GCACATGATACAAAATCTGT	This work
6M1sc	AACCCGATTAAAGGCTCTA	This work
	TGGGAGAATCAAGTGGAC	This work
9M3-3	CAAGTGGCTCTTCTCCATA	This work
	GGTGTGATGTAGAGTGAAAC	This work
11M1ig	CTACTAGGCCAAGGCTTAC	This work
	ATGGAGGCAGAATATGTAGC	This work
SSR primers		
VVIV67	AACTTGATTGAACAAAGGCCTA	Vezzulli et al. (2008)
	TATTATGCCTATCCAGTTTCGA	
VVIV67-2	ATTCTCATTTGGGTTCTCAC	This work
	TTCAGTAGTCACTCTCAAC	
VVS16	TCAAACTATTATTCAAACCAAAGTACG	Doligez et al. (2006)
	TCGATTTCAACAAATTTAGAAATATG	
UDV015	TGCACATTTCCCTCCTTAG	Doligez et al. (2006)
	CGGGTTACTGGGAAGGGTAT	
UDV047	TGTATGATAATCCATAATGTGC	Doligez et al. (2006)
	TAGGCATGCTTGACTTATTC	

linkage to the resistance were isolated from the agarose gel and cloned into pBluescript II SK(+) (Agilent Technologies, Inc., Santa Clara, CA, USA.) or into the positive selection cloning vector pJET1.2 (Fermentas, Vilnius, Lithuania). DNA sequences of the cloned fragments and their subclones were determined using vector specific primers by the BigDyeTerminator kit on an Applied Biosystems 373A sequencer. After assembly for sequence comparisons, BLAST servers at the NCBI (blast.ncbi.nlm.nih.gov) or at GENOSCOPE (www.genoscope.cns.fr) were used.

When at least one part of the determined sequence showed similarity to a unique contig or chromosomal locus of the grapevine genome sequence two specific oligonucleotides (each 18–26 nt long with Tm = 56–58 °C) were designed close to the ends of the fragment. Characteristics of the primers were controlled by the PCR Primer Stats program (www.bioinformatics.org/sms2/pcr_primer_stats.html). These primers were tested on DNA samples of five resistant and five susceptible progeny in stringent PCR experiments to determine whether they result in the appearance of a resistance-coupled SCAR marker. Sequences of three SCAR primer pairs (OPT17sc, OPQ15sc, OPX05sc) that we used later in genetic mapping are listed in Table 1.

SSR marker analysis

For SSR marker analysis, we retrieved primer sequences from the NCBI UniSTS database (www.ncbi.nlm.nih.gov/unists). There are six series of SSR primers: VVS (Thomas and Scott 1993), VVMD (Bowers et al. 1996; Bowers et al. 1999), VrZag (Sefc et al. 1999), VMC (Vitis Microsatellite Consortium coordinated by Agrogéne, Moissy Cramayel, France), VVI (Merdinoglu et al. 2005) and UDV (Di Gaspero et al. 2005). Based on the integrated reference map of cultivars of *V. vinifera* (Vezzulli et al. 2008), SSRs representing all linkage groups were selected and the appropriate primer pairs were tested on the parent DNA samples. Amplification conditions were optimized individually for each marker. PCR products were separated either on a 4 % MetaPhore (Lonza Group Ltd., Basel, Switzerland) agarose gel or on a denaturing polyacrylamide sequencing gels (8 %) and were visualized by ethidium bromide or silver staining. Primers with detectable differences between the parents were tested further on the mapping population. In order to detect the VVIV67 locus more reliably on a 2 % agarose gel, a new primer pair was designed (VVIV67-2, Table 1). These primers resulted in an approximately 550 bp long PCR product instead of a 360 bp one.

Development of LG15 specific markers

Using the 8× WGS (whole-genome shotgun) and later the 12× WGS database (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis/?name=chr15) we designed altogether 78 primer pairs for amplification of intron and intergenic sequences of LG15. Primer pairs, each 18–26 nt long with Tm = 56–58 °C, were designed about 2 kb far from each other and their characteristics were controlled as described above.

Construction of the genetic map of the *Rcg1* region

The order of the markers was established first by a non-mathematical color mapping procedure as described elsewhere (Kiss et al. 1998). This preliminary marker order was verified by calculating the genetic distances. First, the recombination frequencies were calculated pairwise between nine loci of the region. Map units were derived from the Kosambi mapping function (Kosambi 1944), and the markers were arranged according to the calculated distances. Finally, to get a more precise genetic distance for outside markers of a given central marker, occurrences of double crossing overs were counted and the genetic distance for the outside markers was recalculated.

Results

Segregation of *Agrobacterium* resistance

The monogenic and dominant nature of crown gall resistance was previously determined in a variety of crosses (Szegedi and Kozma 1984; Szegedi et al. 1984). In order to establish a larger BC2 population for genetic mapping, the susceptible *V. vinifera* cv. Sárfehér and the heterozygous resistant BC1 hybrid Kunbarát were crossed for the present study. Altogether 272 seedlings were vegetatively propagated and tested in inoculation experiments.

In the first screen, 27 progeny were infected by strains *A. tumefaciens* C58, *A. vitis* Tm4, AT1 and S4. All progeny fell into two distinct groups as they were either resistant or susceptible to all four *Agrobacterium* strains tested. Taking advantage of this uniform response, the rest of the progeny were tested only for resistance to *A. vitis* Tm4 (octopine) and AT1 (nopaline) isolates. Again, we found no plant that showed different susceptibility to the two pathogen strains. The locus responsible for crown gall resistance was designated as *Rcg1* (resistance to crown gall).

In 153 progeny, the resistance clearly appeared while 119 seedlings were susceptible and developed crown galls within 6 weeks after inoculation (Fig. 1). The segregation ratio of this mapping population (153 vs. 119) slightly differed from the expected 1:1 ratio towards the resistant class ($\chi^2 = 4.25$).

Screens for parent-specific RAPD markers

In conventional RAPD experiments, of the 520 decamer primers, 232 resulted in at least one specific DNA fragment that appeared only in one of the parents, either in the Kunbarát (110) or in the Sárfehér (122) reaction. In order to detect, more differences between the parental genomes modified versions of the RAPD method were also applied (see Materials and methods).

First, the DNA samples were digested by a restriction enzyme before RAPD reactions (tecMAAP PCR). In this way, 497 additional polymorphisms (398 Kunbarát specific) were detected in 1,560 different reactions. Secondly, the decamer primers were applied two by two in 1,038 combinations resulting in the detection of additional 387 polymorphisms (180 Kunbarát specific). Altogether 688 polymorphisms (unique fragments) present only in the resistant Kunbarát cultivar were identified.

Identification of *Agrobacterium* resistance-coupled RAPD and SCAR markers

As a first screen for resistance-coupled polymorphisms in subsequent RAPD experiments, we used DNA samples from five resistant and five susceptible progeny. When the presence of a RAPD fragment was characteristic for the majority of the resistant but not for the susceptible individuals, a second screen was carried out using additional progeny and finally all of the progeny were tested for the presence of the polymorphism. Of the 688 resistant parent (Kunbarát)-specific polymorphisms, the presence of nine correlated with the resistance (detected with primers OPD10, OPJ06, OPQ15, OPT17, OPU07, OPU10, OPW15, OPX05, OPB01/OPJ17).

In order to determine the DNA sequence of the resistance-coupled DNA fragments and develop SCAR markers, the appropriate bands were cloned and sequenced. Unfortunately, two or more different sequences were determined from the majority of the isolated fragments. Close to the ends of each sequences, primer pairs were designed and tested on progeny in stringent PCR experiments whether they result in resistance-coupled single bands. In this way, five sequences derived from five different RAPD bands could be identified that represented crown gall resistance-coupled SCAR markers (Table 1).

In order to obtain information on the genomic locations of these resistance-coupled sequences, BLAST program were used to search for homologous regions in the grapevine genome sequence. According to these analyses, only OPT17.1 sequence showed homology to a unique contig

Table 2 Segregation of markers closely linked to the *Rcg1* locus

Marker	Allele in progeny	Resistant	Susceptible	cM	LOD
OPT17sc	574 bp[a]	136	11	10.4	42.7
	Null	17	108		
6M1sc	Null	141	5	6.3	54.3
	1600 bp	12	114		
OPX05sc	563 bp[a]	146	8	5.5	56.7
	Null	7	111		
OPQ15sc	483 bp[a]	147	7	4.8	59.2
	Null	6	112		
UDV015	300 bp	147	7	4.8	59.2
	280 bp	6	112		
9M3-3	500 bp	148	4	3.3	64.7
	Null	5	115		
VVS16	260 bp	147	8	5.2	57.9
	300 bp	6	111		
VVIV67-2	519 bp[a]	142	17	10.1	43.7
	567 bp[a]	11	102		
11M1ig	1300 bp	137	15	11.6	40.0
	2000 bp	16	104		

[a] DNA sequences of the marked alleles were determined, in other cases, size of the alleles were estimated by agarose gel electrophoresis

with an unknown chromosomal location (AM435764). The other four sequences (OPX05.1, OPQ15.1, OPW15.1, OPB01/OPJ17.1) showed homology to more loci mainly representing retroviral-like sequences. Although we could successfully identify five resistance-coupled SCAR markers their chromosomal location remained unclear.

A search for crown gall resistance-coupled SSR markers

Beside the RAPD experiments, the presence of several SSR markers (Doligez et al. 2006) representing the 19 linkage groups were tested on the parent samples. Of the 41 SSR primer pairs, 20 resulted in a specific allele characteristic for the resistant parent (Kunbarát). Distributions of these alleles were preliminary tested on 10 resistant and 10 susceptible progeny. The Kunbarát specific allele of VVIV67 appeared preferentially in the resistant progeny suggesting a linkage between VVIV67 and *Rcg1* loci. Using new primers (VVIV67-2, see Materials and methods), all of the progeny were tested and a strong linkage between VVIV67 and *Rcg1* was detected (10.3 cM, Table 2).

The VVIV67 locus is located on LG15 of *V. vinifera*; therefore, we tested the coinheritance of the *Rcg1* locus with several additional SSR markers of this linkage group. Of the 12 SSR primer pairs (Doligez et al. 2006), 4 gave a unique Kunbarát specific allele. The others were not useful in our mapping population.One of the Kunbarát-specific SSR allele (UDV116, 120 bp) appeared in 72 % of the susceptible progeny suggesting the presence of this marker on the homologous chromosome that carries no resistance (*rcg1* allele). In the case of three additional SSR loci (VVS16, UDV015, UDV047), we could detect a unique allele specific for the resistant parent that appeared mainly in the resistant progeny (96, 96 and 65 % of the resistant progeny, respectively) suggesting a linkage of these markers to the locus. The presence of VVS16 and UDV015 markers was tested in all individuals of the mapping population to establish their map distances to the resistance locus (Table 2). These results suggest that the crown gall resistance locus is located in LG15 of the grapevine genome.

Development of new SCAR markers specific to LG15

Development of locus-specific markers was based on the continuously growing public *V. vinifera* genomic database. Using the 8× WGS (whole-genome shotgun) and later the 12× WGS database, we designed primer pairs for amplification of intron and intergenic sequences of LG15. Considering that grapevine cultivars as many plants exhibit high heterogeneity between homologous chromosomes, we supposed that some primer pairs would detect polymorphic regions in the parental DNA samples and that these new markers would be useful for fine mapping of the *Rcg1* locus.

Our mapping data above on SSR markers suggested the location of the resistance locus in the middle part of LG15. This region corresponds to the LG15 sequence from 4 to 12 Mb (12× WGS); therefore, within this segment, altogether 78 primer pairs were designed and tested first on DNA samples of the parents (Fig. 2) and subsequently on

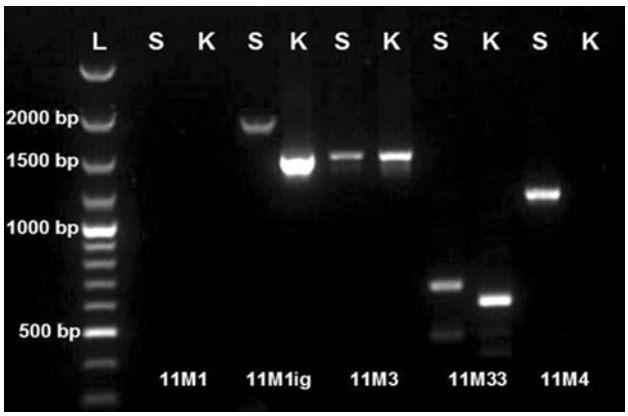

Fig. 2 Primer pairs designed for the sequence of LG15 often detect polymorphic regions between crown gall resistant (K Kunbarát) and susceptible (S Sárfehér) parents. 11M1, no product; 11M1ig, 11M33, 11M4, differences found; 11M3, no difference was detected. In further experiments, distribution of the polymorphic regions in the progeny was established. L, 100 bp ladder control

selected samples of the progeny. In this way, 14 new polymorphic regions were detected. Four of them showed a linkage to the *Rcg1*, six to the *rcg1* locus. Four additional primer pairs resulted in PCR products that were present in the susceptible parent (Sárfehér) and in all of the progeny therefore, they were not useful in this mapping. Finally, we could not detect any difference between the parents in additional 38 primer pairs while 26 of the 78 did not work at all on our DNA samples.

In a further step, the presence of three closely linked markers (6M1sc, 9M3-3, 11M1ig) were tested on the whole mapping population and the coinheritance of the new markers with the crown gall resistance was detected (Table 2). The closest marker we could identify was 9M3-3 that located 3.3 cM from the resistance gene.

Genetic map of the *Rcg1* chromosomal region

We identified 24 DNA markers linked to the *Rcg1* locus and we established the molecular genotype of 272 individual homologous chromosomes around this locus. In these experiments, *V. amurensis* 115 DNA was involved in as control and all of the markers coupled to the *Rcg1* locus were also detected in this sample (data not shown). This result indicates that the resistant parent of our mapping population (Kunbarát) may harbor a substantial part or the whole chromosome 15 of *V. amurensis* that carries the *Rcg1* allele.

The order of the markers was established by using a haplotype chart (color mapping) and by calculating the genetic distances between each locus (see Materials and methods for details). Genotypes of several recombinant chromosomes are shown in Fig. 3, which presents the distribution of crossing-over events around the *Rcg1* locus. On the right side of the *Rcg1* locus, the exchange of chromosomal regions with different length could be detected, while on the left side, one can see a preferred crossing-over point close to the resistance locus. Interestingly, on the left side we have found many recombinant chromosomes carrying only one single locus from the homologous chromosome (Fig. 3) suggesting that the heterogeneity between the homologous chromosomes in this region may be more extended and this may influence the distribution of crossing overs.

The genetic map and the calculated genetic distances for eight tightly linked molecular markers around the *Rcg1* locus are shown in Fig. 4. The resulting genetic map was compared to the corresponding physical map (sequence) of grapevine. In Fig. 4, we show that the two maps are colinear, although both recombination frequencies and physical distances may fluctuate in the region. Around the *Rcg1* locus, a 29.1 cM region of the genetic map overlaps with a 5.08 Mb sequence of the 12× WGS sequence of the grapevine genome. Assuming that the length of the *V. amurensis* sequence in this region is not significantly different from the known genome sequence, we can calculate that an average map unit corresponds to 171 kb and that our closest marker (9M3-3) may locate 576 kb (3.3 cM) far from the *Rcg1* locus.

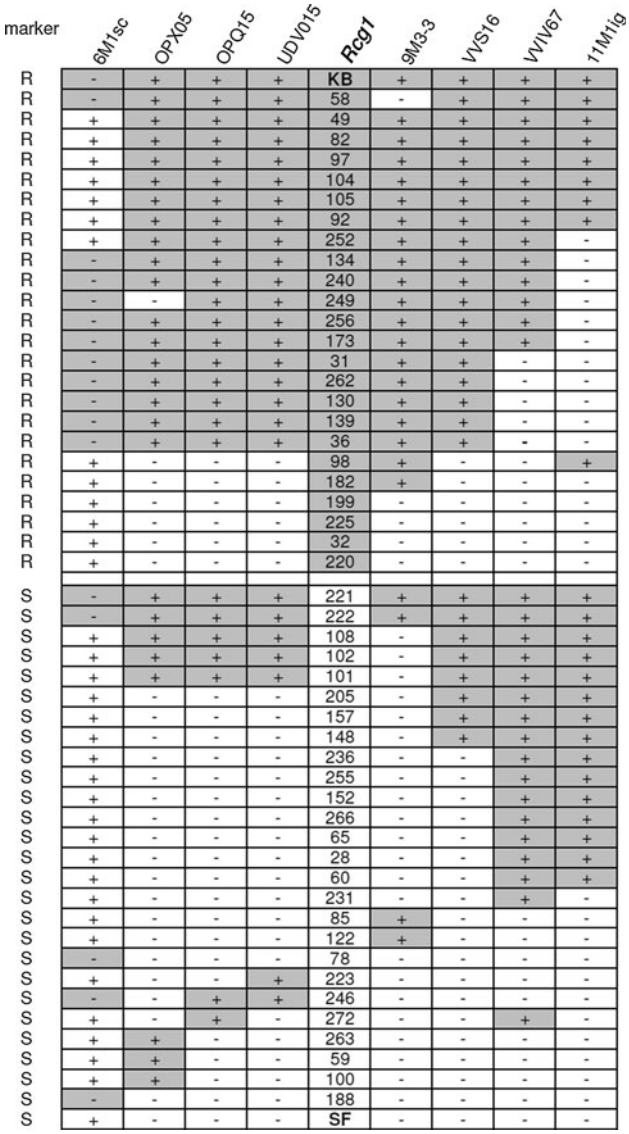

Fig. 3 Recombinant chromosomes of the mapping population. Markers specific for crown gall resistant (Kunbarát, KB) or for the susceptible (Sárfehér, SF) parents are shown in *gray* and *white*, respectively. All rows represent the genotype (genetic markers) of an individual recombinant LG15 of the mapping population. R, resistant progeny; S, susceptible progeny; +, the resistance-coupled SSR or SCAR marker was detected; −, the susceptible parent-specific SSR marker was detected or no SCAR marker was present. 6M1sc SCAR marker is linked to susceptible phenotype (see Tables 1 and 2)

Discussion

In this study, we identified several molecular markers linked to the *Rcg1* crown gall resistance locus in *V. vinifera*

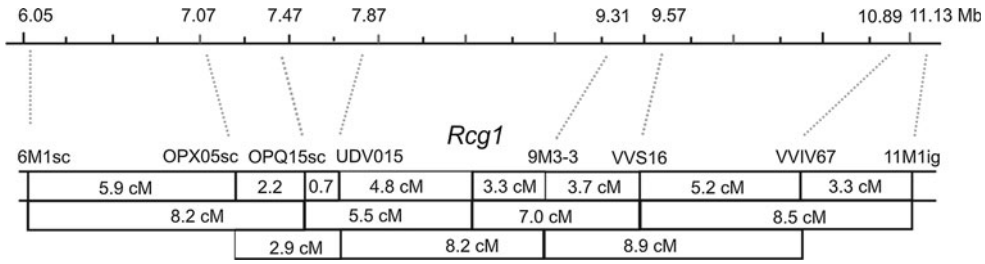

Fig. 4 Genetic map of the *Rcg1* region. The *upper line* represents the physical map (sequence) of the corresponding section of LG15. The *lower* part presents the order and genetic distances of the markers used in this work in the *Rcg1* region. The *dotted lines* show the positions of the DNA markers on the LG15 sequence. Map distances are indicated in centimorgans (cM)

that was previously introgressed from *V. amurensis* (Koleda 1974). We established the location of this resistance locus on LG15 and constructed a genetic map for the closely linked markers (Fig. 4).

The monogenic and dominant nature of crown gall resistance was previously determined in a variety of crosses (Szegedi and Kozma 1984; Szegedi et al. 1984). Szegedi et al. performed five resistant × susceptible and five susceptible × resistant crosses with different parents. Majority of the progeny populations show 1:1 segregation but three of them differed from the expected values ($\chi^2 = 3.40$, 9.38 and 10.86, respectively). In our mapping population, the segregation ratio also slightly differed from the expected 1:1 ratio resulting in more resistant progeny ($\chi^2 = 4.25$). This is just the opposite result which one can expect, if two (or more) genes determine the resistance. This segregation distortion can be the result of allelic differences between the homologous chromosomes, and therefore, may depend on the parents. Riaz et al. (2008) reported the first *Vitis*-segregation disorder region (*V-SDR1*) that distorts segregation and may carry male gametophytic factors.

Crown gall resistance-coupled molecular DNA markers were identified by three different approaches that were all useful in the final mapping. First, in different kinds of RAPD experiments, nine DNA bands were identified and sequenced that showed linkage to the *Rcg1* locus. Although these sequences showed homology mainly to repetitive elements, and therefore, did not carry enough information to localize them definitely in the grapevine genome sequence, we could develop five new SCAR markers of which two (OPQ15sc, OPX05sc) were involved in the final genetic map.

As a second approach, known microsatellite (SSR) markers were applied to localize the resistance gene. In these experiments, we showed that three SSR markers of the *V. vinifera* LG15 (VVIV67, VVS16, UDV015) are tightly linked to the *Rcg1* locus suggesting the location of crown gall resistance in this linkage group.

Finally, location of the resistance gene in LG15 was supported further when new SCAR markers were developed using the DNA sequence of LG15 for primer design. Through amplification of different intergenic or intron sequences with known chromosomal location, we found new markers linked tightly to the sensitivity (6M1sc) or resistance (9M3-3, and 11M1ig). The closest marker (9M3-3) is located 3.3 cM from the *Rcg1* locus which was calculated approximately to 576 kb.

The localization of strain-specific crown gall resistance in a wild apple (*Malus sieboldii*) genome has been reported recently (Moriya et al. 2008). To the best of our knowledge, our work is the second report on the genetic mapping of a natural crown gall resistance and the first one on grapevine. Although linked markers developed in this work are not close enough for map-based cloning of the resistance locus, they may be useful for marker-assisted selection (MAS) in breeding of crown gall resistant new grape cultivars.

Based on the molecular markers, the resistant parent (Kunbarát, a BC1 hybrid) carries the chromosome 15 of *V. amurensis.* An initial reference linkage map of *V. amurensis* was published recently (Blasi et al. 2011). This study provided some information on the degree of synteny with the *V. vinifera* genome. The number of linkage groups and arrangement of the markers on the map were consistent with the *V. vinifera* reference maps and genome sequence presenting a high colinearity between the two genomes. Our finding that crossing overs occur relatively random between a *V. amurensis* and a *V. vinifera* chromosome and that the genetic map of the *Rcg1* region is colinear with the genetic map and sequence of *V. vinifera* also support the above conclusion. It is important to note that there are some local differences in the marker order between the two genomes (Blasi et al. 2011). Accordingly, we have also observed fluctuation in the recombination frequency left of the *Rcg1* locus, suggesting an increased heterogeneity between homologous chromosomes in this region (Fig. 3). Even in Pinot Noir, sister chromosomes may differ by more than 10 % in DNA sequence (Velasco et al. 2007), which must influence the frequency of recombination.

Because of the dominant nature of the crown gall resistance, the *Rcg1* locus may carry a gain of function mutation or one (or more) extra gene that is present in *V. amurensis* but missing from or disrupted in the

V. vinifera genome. It provides resistance against wide range of agrobacteria carrying octopine, nopaline or vitopine-type Ti plasmids (Szegedi et al. 1984 and this study). The molecular nature of the resistance is still unknown. Limited propagation of bacteria or induction of *vir* genes within the plant, inefficient transfer or integration of the T-DNA may result in crow gall resistance as well as the block of the expression of oncogenes in the transformed cells (for review see Gelvin 2010). Our main goal in the future is to clarify the molecular events that result in crown gall resistance in *Rcg1* genetic background. This will include the isolation and sequence determination of the genetic locus itself as well as plant physiology and molecular biology studies to understand when the crown gall formation is halted.

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