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ORIGINAL PAPER

Development of marker sets useful in the early selection of *Ren4* powdery mildew resistance and seedlessness for table and raisin grape breeding

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Abstract The single, dominant powdery mildew resistance locus *Ren4* from *Vitis romanetii* prevents hyphal growth by *Erysiphe necator*. Previously, we showed that when introgressed into *V. vinifera* in the modified BC_2 population 03-3004, *Ren4* was linked with the simple sequence repeat marker VMC7f2 on chromosome 18—a marker that is associated with multiple disease resistance and seedlessness. However, in the current study, this marker was monomorphic in related breeding populations 05-3010 and 07-3553. To enhance marker-assisted selection at this locus, we developed multiplexed SNP markers using three approaches: conversion of bulked segregant analysis AFLP markers, sequencing of candidate genes and regions flanking known *V. vinifera* SNPs, and hybridization to the Vitis9KSNP genotyping array. The Vitis9KSNP array was more cost-efficient than all other approaches tested for marker discovery and genotyping, enabling the genotyping of 1317 informative SNPs within the span of 1 week and at a cost of 11 cents per SNP. From a total of 1,446 high quality, informative markers segregating in 03-3004, we developed a haplotype signature of 15 multiplexed SNP markers linked with *Ren4* in 03-3004, 5 of which were linked in 05-3010, and 6 of which were linked in 07-3553. Two of these populations segregated for seedlessness, which was tightly linked with *Ren4* in 03-3004 (2 cM) but not in 05-3010 (22 cM). Chromosomal rearrangements were detected among these three populations and the reference genome PN40024. Since this is the first application of the Vitis9KSNP array in a breeding program, some suggestions are provided for application of genotyping arrays. Our results provide novel markers for tracking and pyramiding this unique resistance gene and for further functional characterization of this region on chromosome 18 encoding multiple disease resistance and seedlessness.

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Introduction

Grapevine powdery mildew caused by *Erysiphe necator* (syn. *Uncinula necator*) affects fruit yield and quality everywhere grapes are grown, necessitating fungicide application due to the nearly universal susceptibility of *Vitis vinifera* grapes. As a result, grape breeding programs around the world are introgressing resistance from wild grapes (Akkurt et al. 2007; Barker et al. 2005; Coleman et al. 2009; Dalbo et al. 2001; Eibach et al. 2007) into high quality *V. vinifera* grapes. Breeding seedless raisins and table grapes has the added complexity of requiring embryo rescue and tissue culture (Ramming 1990), a process that is labor-intensive and requires 2–3 years of growth and transplanting before progeny can be evaluated for fruit characteristics. Therefore, molecular markers linked with seedlessness and disease resistance are desired for raisin and table grape breeding, as they would enable early testing of progeny while in tissue culture and discarding of progeny that lack these two important traits.

Recently, a single dominant resistance phenotype (*Ren4*) from an Asian species *V. romanetti* was identified as conferring rapid non-race-specific resistance to powdery mildew when introgressed into *V. vinifera*, preventing hyphal growth (Ramming et al. 2011). Prevention of hyphal growth typically occurs due to penetration resistance and is associated with recessive resistance genes, such as *mlo* (Jorgensen 1992), or with non-host resistance genes (Zhang et al. 2007). Two dominant resistance genes have been described as preventing hyphal growth: the race-specific penetration resistance gene *Mlg* from barley (Gorg et al. 1993), and the broad-spectrum *Arabidopsis* gene *RPW8.2,* which allows formation of the powdery mildew haustorium (Wang et al. 2009). Thus, *Ren4* could be a novel resistance gene with promising potential for durable, broad-spectrum resistance and is being introgressed into *V. vinifera* raisin and table grape breeding lines (Ramming et al. 2011). However, given the few examples of durable powdery mildew resistance genes, experience suggests that *Ren4* should be pyramided with other resistance genes, which requires development and application of molecular markers linked with *Ren4*.

With the recent mapping of several disease resistance genes in grapevine, similarities with resistance genes in other crops have been uncovered. For instance, co-localization, or clustering, of functional disease resistance genes has been shown in a number of plant species including wheat, lettuce, soybean, and several solanaceous crops, and resistance genes often co-localize with resistance gene analogue (RGA) sequences (Hayes et al. 2004; Kuang et al. 2004; Marano et al. 2002; van der Voort et al. 1999). In grapevine, the *Run1* gene conferring resistance against powdery mildew is in the same RGA cluster on *V. rotundifolia* chromosome 12 as the downy mildew resistance gene *Rpv1* (Donald et al. 2002; Bellin et al. 2009). Also, the *Ren1* powdery mildew resistance gene corresponds to an area on *V. vinifera* chromosome 13 containing coiled-coil NBS-LRR sequence of unknown function and specificity plus several additional RGA pseudogenes (Coleman et al. 2009). Finally, *Rpv2* and *Rpv3*, both conferring resistance to downy mildew, have been mapped to chromosome 18, which also has an extensive cluster of RGAs (Bellin et al. 2009). Previously, we reported that *Ren4* was linked with the simple sequence repeat (SSR) marker VMC7f2 on chromosome 18 (Mahanil et al. 2011), and this result has been independently confirmed (Riaz et al. 2011).

Molecular marker technology for these studies has advanced from relatively anonymous sequence polymorphisms between uncharacterized areas of the genome (e.g. AFLP) to direct assays of sequenced genomes. The recent decline in re-sequencing costs enabled the development of the Vitis9KSNP array, a genotyping microarray that assays nearly 9,000 SNPs segregating among several *Vitis* spp. (Myles et al. 2010). SNPs queried by this array were discovered by Illumina GA sequencing of 10 *V. vinifera* and 6 wild *Vitis* species, but most of the SNPs segregate within *V. vinifera* while only a limited number of SNPs segregating among wild *Vitis* species were included.

Here, we present our cumulative results from genotyping AFLP, SSR, and SNP markers for the purpose of tagging the *Ren4* resistance gene, refining interval maps around *Ren4*, and tracking its segregation along with seedlessness. Our results provide a crucial step towards marker-assisted selection in raisin and table grape breeding programs.

Materials and methods

Plant populations

Vitis romanetii 'C166-026' was obtained from the USDA-ARS repository in Davis, CA. Two modified-BC_1 (mBC_1), resistant full siblings (C87-41 and C87-14) resulting from the cross Raisin de Palestine × (C166-026 × *V. vinifera*) were used as the maternal parent in the development of three mBC_2 populations. C87-41 is powdery mildew resistant and seedless, whereas C87-14 is powdery mildew resistant and seeded. The segregating mBC_2 population 03-3004 (C87-41 × B70-57) was generated with 57 progeny and was used to develop a linkage map described here and to identify AFLP markers by bulked segregant analysis, as described previously (Mahanil et al. 2011). This population is currently maintained as mature vines by the USDA San Joaquin Valley Agricultural Sciences Center in Parlier, CA, USA. Two additional populations were developed to

validate marker–trait associations: 07-3553 (C87-14 × B82-43; $n = 185$) and 05-3010 (C87-41 × A85-40; $n = 69$).

Phenotyping

Powdery mildew resistance of 57 progeny in the 03-3004 population was evaluated for three seasons (2007–2009) under no-spray vineyard conditions during which time natural epidemics occurred from diverse populations of *E. necator*. Vineyard data were confirmed using natural infection in a greenhouse in Parlier, CA and artificial inoculation of detached leaves in Geneva, NY. Up to eight leaves per genotype were collected for detached leaves assay: the fourth fully expanded leaf and a mature leaf, from two replicate shoots per vine and from two replicate vines per genotype, as previously described (Ramming et al. 2011; Cadle-Davidson et al. 2011). One hundred eighty-five progeny of 07-3553 and 69 progeny of 05-3010 were evaluated following natural epidemics in a greenhouse in Geneva, NY and Parlier, CA, respectively. Evaluations were performed when the susceptible checks 'Ruby Seedless' or 'Chardonnay' had more than 70% incidence (Ramming et al. 2011), to reduce the likelihood of escapes, i.e., susceptible progeny appearing resistant due to random lack of inoculum. Foliar disease incidence (% of leaves that exhibited powdery mildew symptoms) and disease severity, or coverage (% leaf area infected), were evaluated. However, due to the extreme phenotype of resistance (no signs of the pathogen), seedlings with any sporulating powdery mildew were rated as susceptible.

Seedlessness was characterized in arbitrary subsets of 31 progeny of 03-3004 and 66 progeny of 05-3010 by sampling the largest seed trace from each of 10 berries. The ten traces were weighed, and data were averaged and categorized as seedless if this average was smaller than 15 mg.

DNA isolation

DNA was extracted from young leaf material using DNeasy 96 plant kits (Qiagen, Valencia, CA, USA) according to manufacturer directions. Concentration and purity of DNA were determined by Picogreen dsDNA Quantitation kit (Invitrogen, Carlsbad, CA, USA) and electrophoresis on 0.8% w/v agarose gels. The DNA was normalized to 10 ng/µl and stored at −20°C.

SSR markers

A total of 227 SSRs were used to screen parental DNA of the 03-3004 mapping population. Markers that were monomorphic, multicopy or difficult to amplify were not included in further analyses. Those markers polymorphic between resistant and susceptible parents were used to amplify 45 progeny of the 03-3004 mapping population (Table 1). SSR primer sequences have been reported by Bowers et al. (1996, 1999) (VVMD); Sefc et al. (1999) (VrZAG); Di Gaspero et al. (2000) (VMC); Adam-Blondon et al. (2004) (VMC); Arroyo-Garcia and Martinez-Zapater (2004) (VMC); Merdinoglu et al. (2005) (Vvi); and Welter et al. (2007) (VMC). The remaining SSR markers are reported in NCBI databases dbSTS and uniSTS (http://www.ncbi.nlm.nih.gov). Three SSR primer combinations were used per multiplex, each forward primer being labeled with a different (either VIC, TAMRA, FAM, or NED) fluorophore. PCR conditions were: 30 s at 95°C followed by 35 cycles of 45 s 94°C, 45 s at specific primer T_m, 45 s at 72°C; followed by 1 h at 72°C. Detection by capillary electrophoresis was performed at the Core Laboratories Center (CLC) at Cornell University using an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, CA, USA) with standard setting of 1 bp resolution relative to GeneScan-500 Liz Size Standard (Life Technologies). Raw SSR data were analyzed using Genemapper 4.0 (Life Technologies).

SNPlex

Mapping population parents were screened for SNPs using 170 existing primer sets including: 140 SNP primer pairs developed by Troggio et al. (2007), 9 primer pairs from grape RGAs (rgVamu092, rgVcin 109, rgVcin 123, rgVcin 165, rgVhyb 121, rgVhyb 149, rgVrip 064, rgVcin 139 and rgVhyb 101; Mahanil et al. 2007), 16 primer pairs from multigene families of grape *VvMlo* and *VvPmr6*, and five primer sets develop from BSA-AFLP (Mahanil et al. 2011). Amplifications were carried out in a volume of 20 µl with 10 ng genomic DNA, 1× PCR GoTaq reaction buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 pM each primer and 1 U of GoTaq (Promega). PCR cycles were: 4 min at 94°C denaturation; 35 cycles of 1 min 94°C, 45 s at specific primer T_m, 45 s 72°C; followed by a final 10 min extension at 72°C. Products were evaluated by gel electrophoresis in 0.8% agarose gel and visualized by SYBR green staining (Promega).

Direct sequencing of PCR products was performed at CLC and SNP mutations discovered using Sequencher 4.7 (Genecodes, Ann Arbor, MI, USA). Criteria for selecting sequences for SNPlex assays were (1) mutations were homozygous in one parent and heterozygous in the other; (2) at least 20–30 monomorphic nucleotides were present on each side of the SNP; and (3) the GC content was greater than 40%. Sequences were then used to design a SNPlex kit that was subsequently manufactured by Life Technologies. Forty-seven SNPlex assays were performed at CLC on all three breeding populations, and the data were analyzed using Genemapper 4.0.

Table 1 Segregation types observed for markers used in mapping Ren4 in the population 03-3004

Linkage group	Marker		
	SSR	SNP/SNPlex	Segregation type[a]
1	VMC8a7, VMC3g		lmxll
	VMC8e8, VMC9d3		nnxnp
	VMC8d1		efxeg
	VMC9f2		hkxhk
2	*Vvib23f, VMC8c2*		*abxcd*
	Vvib01		*efxeg*
	VMC6b11f		*lmxll*
3	VMC8f10, VVMD36		abxcd
	VMC1g7f		lmxll
	Vvin54		nnxnp
4	*VMCNG2e1*		*nnxnp*
	VMCSsrVrZAG_21, VMC2b5f		*abxcd*
	VMCSsrVrZAG_83, VMC6e10, Vvip37	*1070M03F*	*lmxll*
		1081F18F	*hkxhk*
5	Vvip52f, Vvit68, VMC3c7, VMC9b5		lmxll
	VVMD14f		abxcd
	Vvin40f, VMC4c6f		efxeg
6	*VMC2h9f, VMC2g2f, GR0246*	*1077F12F*	*lmxll*
	VMC2f10f, Vvim43		*abxcd*
7	VVMD7, VMCSsrVrZAG_62f, VMC5h5f,VMC1a12		abxcd
	VMC1a2, Vviv36	1076M05F	hkxhk
	VMC8d11, VVMD6f	1082P02F, 1077H02R, 1094K03F	lmxll
		1031J11F, 1031N12R,1082P02F	nnxnp
	Vviv04		efxeg
8	*VMC2f12*		*nnxnp*
	VMC6g8, VMC7h2, Vvip04		*efxeg*
	Vvim07	*1076I02R*	*lmxll*
	VMC1b11		*abxcd*
9	Vviu37, UDV-132, VMC9f4f		nnxnp
	VMC6d12		efxeg
	Vviq52	1078B24F	lmxll
	VMC4h6, VMC1c10f		abxcd
10	*VMC3d7, Vvih01*		*lmxll*
	VMCSsrVrZAG_25, UDV-063		*abxcd*
	VMCSsrVrZAG_67, Vviv37		*efxeg*
11	VMCNG2h1, VVMD25		nnxnp
12	*VMC2h4f*	*1073N16R, 1037L03F, 1074F10R,1082I20R*	*lmxll*
	Vvim11		*efxeg*
13	Vvic51, VMC3b12	1070F10R, *VvMlo*A, *VvMlo*B	lmxll
	Vvin62	1071K14F, *VvPmr*A	nnxnp
	VMC3g11		abxcd
14	*VMCNG1e1, VMC1e12, VMC2h12,VMCNG1e1-1*		*efxeg*
	Vvin64	*1030B14F*	*hkxhk*
	VMC6c10, VMC2b11		*abxcd*
	Vvis70		*nnxnp*

Table 1 continued

Linkage group	Marker		
	SSR	SNP/SNPlex	Segregation type[a]
15	Vvib63	1037C12F	nnxnp
	Vvip33	1071N19R	lmxll
16	*Vvin52, UDV-104, VMC5a1*		*efxeg*
	UDV-013, UDV-052	*1089N12R*	*lmxll*
	VMC1e11		*nnxnp*
	VMC4b7.2		*abxcd*
17	Vvis63		abxcd
	Vvin75, Vvip22bf		lmxll
18	*VMC3e5f,Vvim93, VMC7f2*	*1082L02F*	*lmxll*
	Vvin16, VVMD17f, VMC2a3, GR0520, IN0954	*E4M1R1*	*nnxnp*
19	VMC5e9f, Vvi33f		nnxnp
	Vvivq31, VMCSsrVrZag_15f, VMC3b7.2f		lmxll
Unknown		*E3M4S1*	*nnxnp*

[a] Segregation types in JoinMap 4.0 (Van Ooijen 2006) were based on a cross-pollinated (CP) population, specifically <lmxll> and <nnxnp> when heterozygous in one parent and homozygous in other; <hkxhk> when both parents were heterozygous; and <efxeg> or <abxcd> when parents were heterozygous and three or four alleles were segregating, respectively. Expected ratios of each segregation type were: 1:1 for <lmxll> and <nnxnp>, 1:2:1 for <hkxhk> and 1:1:1:1 for <efxeg> and <abxcd>

Linkage map construction and QTL detection

Simple sequence repeat and SNPlex markers were scored as co-dominant markers to construct a linkage map for the 03-3004 mapping population, based on a cross-pollinated (CP) population in JoinMap 4.0 (Van Ooijen 2006). A chi-squared goodness-of-fit test was used to test individual loci for expected segregation. Linkage groups were determined based on LOD scores of at least 3. The parental maps were analyzed with MapQTL 6.0 (Van Ooijen 2009) using powdery mildew phenotypic data averaged over 3 years to identify QTL. The non-parametric Kruskal–Wallis (KW) test was used for determining marker linkage to powdery mildew resistance.

Vitis9KSNP array

DNA was isolated as described above, amplified with bacteriophage Phi29 DNA polymerase provided in the Genomiphi whole-genome amplification kit (GE Healthcare, Piscataway, NJ, USA), and processed as described elsewhere (Myles et al. 2010). SNPs with GenTrain scores <0.3, GenCall scores <0.2, or at least 20% missing data (Myles et al. 2011), or with more than one Mendelian inconsistency (not meeting the expectation of one allele from each parent) failed to meet quality thresholds and were not included in further analyses, unless otherwise noted.

Results

Populations and phenotyping

Powdery mildew resistance segregated as a qualitative, single dominant locus as follows for the three populations: 38 resistant (R):19 susceptible (S) in 03-3004, 37 R:32 S in 05-3010, and 91 R:94 S in 07-3553. Progeny were either highly susceptible or lacked any powdery mildew symptoms (Fig. 1); categorization into resistant and susceptible classes was consistent across environments, tissues, and plant age, as previously noted (Ramming et al. 2011). Seedlessness and powdery mildew resistance were tightly linked in 03-3004, with only one recombinant (03-3004-113), which was seedless and susceptible (Table 2). In 05-3010, the phenotypic variation between seeded and seedless based on seed trace weight was continuous (Table 2), and recombination between powdery mildew and seedlessness was more frequent than observed in 03-3004 (Table 2).

Marker development and mapping

Previously, BSA-AFLP was used to identify three low-copy sequences putatively associated with *Ren4* and meeting SNPlex design criteria (Mahanil et al. 2011). Of these, only 2 SNPlex-BSA-AFLP segregated in population 03-3004 (Fig. 2). To develop additional markers for mapping resistance, 25 candidate genes (e.g. RGAs, *Mlo*)

Fig. 1 Full-sibling progeny from population 03-3004 showing qualitative segregation for powdery mildew susceptibility (*left*) and resistance (*right*) at the *Ren4* locus. Reprinted from Ramming et al. (2011)

Table 2 Distribution of seed trace sizes and the association of seedlessness with foliar powdery mildew severity in 03-3004 and 05-3010 families

Population	Average largest trace (mg)[a]	Resistant genotypes[b]	Susceptible genotypes[b]
03-3004	0.0	17	1
	15.4–70.1	0	13
05-3010	0.0	10	0
	0.1–4.9	7	0
	5.0–9.9	1	2
	10.0–14.9	5	2
	15.0–81.4	13	26

[a] The largest trace from each berry was weighed and averaged across a 10 berry sample

[b] The number of resistant and susceptible progeny in each category is provided here. Progeny with any signs of powdery mildew were considered susceptible

for powdery mildew resistance were resequenced in the 03-3004 parents, but only three polymorphic amplicons were successfully converted to segregating SNPlex markers (Fig. 2). For 140 SNPlex regions previously identified in *Vitis* (Troggio et al. 2007), resequencing of the 03-3004 parents resulted in the development of 23 segregating SNPlex markers (Fig. 2).

In total, 101 SSRs and 28 SNPlex SNPs were polymorphic between the parents of 03-3004, segregated among the progeny (Table 1; Fig. 2), and were used to create a linkage map. The expected marker–trait associations on chromosome 18 were detected in 03-3004 in spite of the relatively small population size. The SSR marker VMC7f2 perfectly correlated with powdery mildew resistance [Kruskal–Wallis (KW) = 39.9, $p < 0.0001$] and significantly predicted seedlessness (KW = 18.0, $p < 0.0005$). An AFLP-derived SNP (E4M1R1) mapping near VMC7f2 on chromosome 18 was slightly less predictive of resistance (KW = 23.6, $p < 0.0001$) and seedlessness (KW = 8.2, $p < 0.005$). The parents of 05-3010 and 07-3553 were genotyped using VMC7f2 and E4M1R1 but were monomorphic.

Marker saturation and application

To identify additional markers on chromosome 18 near VMC7f2 and E4M1R1, 03-3004 parents and 18 progeny as well as 07-3553 parents were genotyped using the Vitis9KSNP array. DNA of one progeny (03-3004-77) failed quality controls due to low GenCall scores (Supplementary Fig. 1) and was not included in further analyses. By evaluating Mendelian inconsistencies among the polymorphic SNPs, we determined that a second progeny (03-3004-114) was likely a half sibling of the other progeny because of its high frequency of Mendelian inconsistencies (Supplementary Fig. 2). This was likely due to an accidental pollination or a seed contamination, and this sample was excluded from further analyses.

Most SNPs (6413) passed quality filters, 1910 (30%) of which were polymorphic between parents. Across the remaining 16 progeny in population 03-3004, 1317 SNPs (21%) were informative and 21 SNPs from chromosome 18 corresponded perfectly with resistance. However, one out of 21 markers (15533998) did not pass SNPlex design requirements. Twenty-four additional markers were selected based on polymorphism in 07-3553 (without regard to GenCall quality scores), and three were selected flanking the region giving a total of 47 new SNPlex assays. These were applied across all three populations.

For markers developed only based on 07-3553 parental polymorphism, eighteen failed GenCall and/or GenTrain quality thresholds and did not segregate in any population. The remaining three segregated in 07-3553, but none were predictive of resistance in any of the three populations (Supplementary Table 1).

Overall, six of the 47 SNPlex markers co-segregated perfectly with resistance in 03-3004 (KW = 41.9, $p < 0.0001$) (Fig. 3; Table 3). In population 05-3010, markers 13947255 and 14267463 co-segregated perfectly with resistance (KW = 23.0 and 26.0, respectively, $p < 0.0001$), though 13947255 had missing data for three progeny (Fig. 3). In 07-3553, 6 of 187 progeny had an apparent recombination between the predictive markers and the trait; for all other progeny, only the marker 16429479 perfectly predicted resistance (KW = 115.0, $p < 0.0001$; Table 3). Flanking markers were linked to *Ren4* (Fig. 3) and have potential for application in marker-assisted selection of resistant as well as seedless individuals.

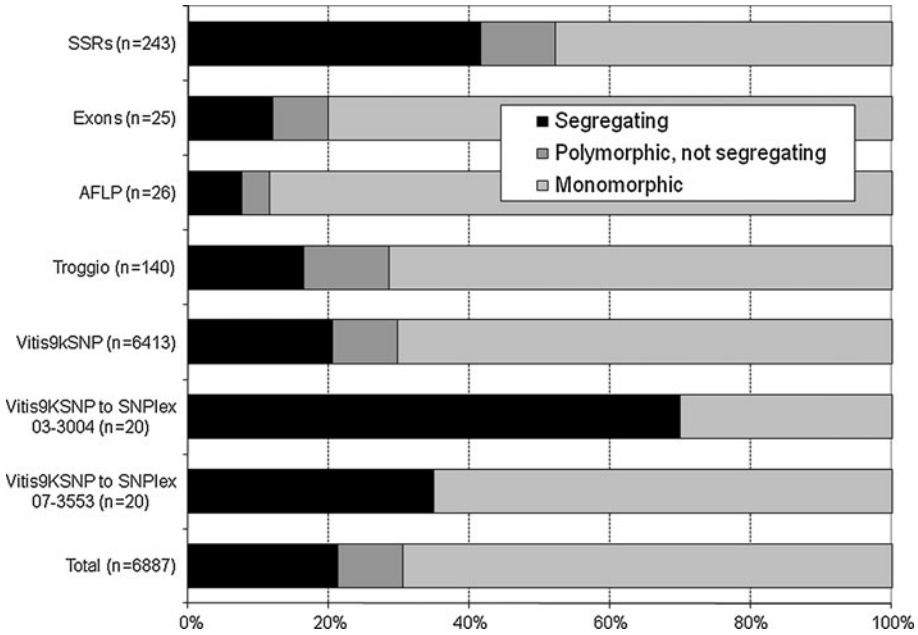

Fig. 2 Marker conversion rates for SSRs and SNPs in population 03-3004. Several sources of markers were tested for application in 03-3004, as follows: SSRs were selected from a collection of previously published markers to cover all chromosomes; exons were selected from RGA and other powdery mildew candidate genes (including 9 primer pairs from grape RGAs; rgVamu092, rgVcin 109, rgVcin 123, rgVcin 165, rgVhyb 121, rgVhyb 149, rgVrip 064, rgVcin 139 and rgVhyb 101; Mahanil et al. 2007, 16 primer pairs from multigene families of grape *VvMlo* and *VvPmr6*, Mahanil et al. 2009); AFLP polymorphisms from were sequenced, as reported previously (Mahanil et al. 2011); regions flanking SNPlex markers from Troggio et al. (2007) were sequenced; parents and 16 arbitrarily chosen progeny from 03-3004 were genotyped by Vitis9KSNP array; and *V. vinifera* PN40024 flanking reference sequence was used for SNPlex assay development. Vitis9KSNP to SNPlex 03-3004 shows those SNPs selected near the *Ren4* locus based on genotypes of parents and 16 progeny; Vitis9KSNP to SNPlex 07-3553 shows the same markers applied in the related population 07-3553. *n* Number of markers tested for polymorphism in parents. The proportion of markers that segregated among progeny is depicted in *black*. The proportion that provided high quality sequence or size polymorphisms as SSR or SNPlex markers but did not segregate is depicted in *dark grey*. For SSRs, some polymorphic markers did not segregate in progeny or did not give scoreable markers in multiplex PCR. For SNPlex, some polymorphic markers failed the assay design

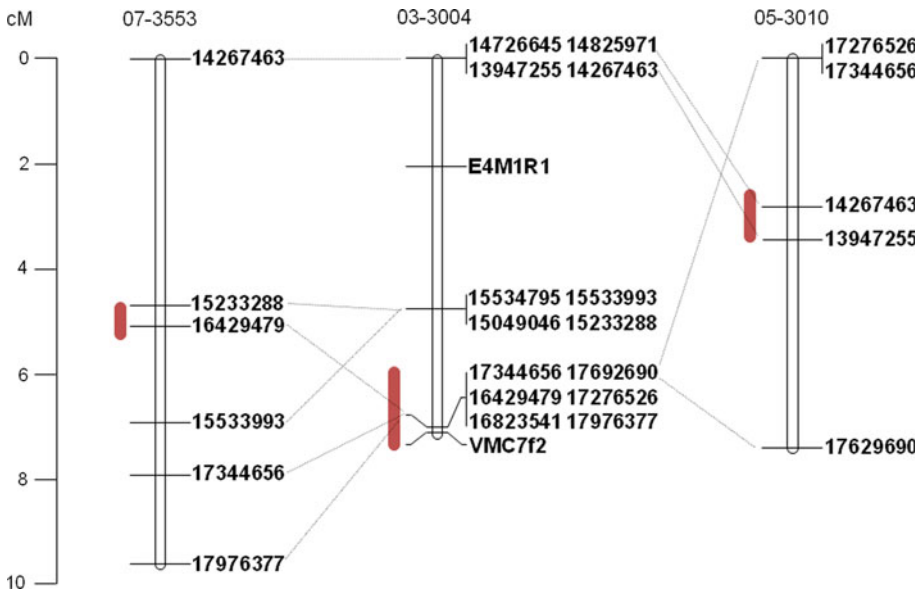

Fig. 3 Interval maps of the *Ren4* locus on chromosome 18 for three segregating populations. The *bars* alongside each map depict peaks in the Kruskal–Wallis test statistic, exceeding the following thresholds for each population: 113 for 07-3553, 40 for 03-3004, and 23 for 05-3010 (each significant at $p < 0.0001$)

Table 3 Marker and trait recombination rates near the *Ren4* locus in three breeding populations

Marker	03-3004		05-3010		07-3553
	P[a]	S	P	S	P
13947255	6.7[b]	4.4	0.0	19.1	–
14267463	6.7	4.4	0.0	21.7	4.9
14726645	6.7	4.4	–	–	–
14825971	6.7	4.4	–	–	–
E4M1R1	4.4	4.4	–	–	–
15049046	2.2	4.4	–	–	–
15233288	4.4	4.4	–	–	2.1
15533993	2.2	4.4	–	–	3.2
15534795	2.2	4.4	–	–	–
16429479	0.0	0.0	–	–	1.8
16823541	0.0	0.0	–	–	–
17276526	0.0	0.0	2.2	30.4	–
17344656	0.0	0.0	2.2	30.4	3.9
17629690	0.0	0.0	4.3	30.0	–
17976377	0.0	0.0	–	–	4.6
VMC7f2	0.0	2.2	–	–	–
P versus S:[c]	2.2		19.7		

[a] *P* powdery mildew resistance, *S* seedlessness. Seedlessness did not segregate in the 07-3553 seeded × seeded population

[b] For each marker listed here, the marker recombination rate (%) with P or with S is provided, calculated as the frequency of incongruity. – not determined due to lack of marker transferability among populations

[c] P versus S indicates recombination rates (%) between these two traits and is not associated with a specific marker

Discussion

Accessions of several Asian grape species including *V. amurensis, V. romanetii, V. piazekii, V. davidii, V. davidii* var. *cyanocarpa, V. liubanensis* and *V. bashanica* are known sources of resistance to powdery mildew (Wan et al. 2007). From one accession of *V. romanetti*, we mapped the broad-spectrum powdery mildew resistance locus, *Ren4*, to chromosome 18 and identified 15 SNPs and 1 SSR flanking the locus. Seedlessness, already known to involve a major QTL on chromosome 18 (Cabezas et al. 2006; Costantini et al. 2007; Mejia et al. 2007), also segregated in both seedless mapping populations and showed tight linkage with *Ren4* in one but not in the other population.

At least five different powdery mildew resistance loci are known (*Run1* on Chr 12, *Ren1* on Chr 13, *Ren2* QTL on Chr 14, *Ren3* QTL on Chr 15, and *Run2* on Chr 18) with sources in several North American *Vitis* species or eastern European *V. vinifera* (Coleman et al. 2009; Pauquet et al. 2001; Akkurt et al. 2007; Dalbo et al. 2001; Fischer et al. 2004; Welter et al. 2007; Riaz et al. 2011). These studies produced several SSR and SNP markers that were tested in population 03-3004, but only *Run2* markers were shown to be linked or even on the same chromosome as *Ren4*. Interestingly, *Run2* and *Ren4* are tightly linked with SSR marker VMC7f2, which is linked with *Rpv3* resistance to the oomycete *Plasmopara viticola*, causing grapevine downy mildew. *Rpv3* is in turn linked at an unknown distance to a second downy mildew resistance, *Rpv2*, and is in a cluster of RGAs with as yet unproven function. This locus is one of three clusters of putatively functional resistance genes in the midst of numerous RGA sequences found in grapevine (Bellin et al. 2009). Further, the *Run1*/*Rpv1* resistance locus introgressed from *V. rotundifolia* similarly colocalizes to RGAs that collectively confer resistance to powdery mildew and downy mildew (Barker et al. 2005; Moroldo et al. 2008).

One of the purposes of the populations used here was to generate markers and useful breeding lines for seedlessness. Thus, two of the initial crosses were resistant seedless × susceptible seedless (03-3004 and 05-3010). Seedlessness and *Ren4* loci were tightly linked in 03-3004 but not in 05-3010 possibly indicating suppressed recombination associated with these loci. Further, we observed quantitative variation in seed trace mass, supporting previous observations that minor genes on other chromosomes contribute to seedlessness (Mejia et al. 2007). The SSR marker VMC7f2 has been previously shown to map 4 cM away from the *Seed development Inhibitor* (*SdI*) locus (Cabezas et al. 2006); similarly, in populations 03-3004 this marker had a recombination frequency of 2.2% with seedlessness and was absolutely linked to *Ren4* (Table 3). While markers for seedlessness can be informative in seedless × seedless crosses with infrequent seeded progeny, as in the current study, they would be even more valuable in the early introgression of traits from wild grapes, which are seeded and frequently produce seeded progeny. Additional work is needed to determine the utility of seedless markers in crosses involving a seeded parent.

Here, we present our cumulative results of attempts to convert markers from several sources. We experienced varying success rates, providing useful guidance for marker development. Many of our AFLP sequences had low homology to the 8× *V. vinifera* PN40024 genome or matched repetitive DNA (Mahanil et al. 2011). Therefore, considering all 26 polymorphic bands that we sequenced, the success rate for segregating marker development was poor (8%). Resequencing candidate genes and regions previously used for SNPlex marker development resulted in similarly low success rates (12 and 16%, respectively), due to lack of relevant polymorphism between the parents (i.e., segregating biallelic SNPs flanked by 20–30 nt of homology

with >40% GC). In striking contrast, Vitis9KSNP array markers were frequently converted to SNPlex within population 03-3004 (80%). In addition, 1,317 SNP markers were identified in 1 week using the Vitis9KSNP array, in strong contrast to the months of effort required to identify dozens of SNPs by AFLP and resequencing. At a price of about US$150 per DNA sample, each informative SNP from the Vitis9KSNP array costs only 11 cents, for a process that both discovers and genotypes SNPs for a given population at a known chromosomal location. This approach was significantly cheaper and more time efficient than AFLPs, SSRs, and resequencing.

Previously, SNPlex assays were used to test heterozygous state SNP transferability, with 31.5% success rate across the unrelated cultivars of *V. vinifera*, 18.8% across the wild forms of *V. vinifera*, and 2.3% among non-*vinifera Vitis* species (Vezzulli et al. 2008). For the three BC_2 populations in the current study, SNPlex markers segregating in 03-3004 had higher levels of transferability to 05-3010 (50%) and 07-3553 (40%) than previously described.

Our data reveal an interesting artifact of bi-allelic SNP analysis in introgression populations in which the unique parental haplotype apparently segregates with the opposite phenotype in progeny. That is, we selected bi-allelic markers that were heterozygous in one parent (e.g. resistant = T^*G) and homozygous in the other (e.g. susceptible = TT) and found very tight linkage wherein the resistant progeny were T^*T and the susceptible TG, such that the unique haplotype was in repulsion with the trait. The resistance haplotype in this particular case is T^*, not G, and is therefore not equivalent to either T haplotype from the susceptible parent. The G, in this case, belongs to yet another susceptibility haplotype. There are several reasons why this may be the case, foremost among them being the fact that the populations at hand involve resistance introgressed from a species distantly related to *V. vinifera*, yet the majority of markers used here were discovered from *V. vinifera*.

Further, in this study, there were 10 different haplotypes (5 different parents) segregating across the 3 different populations. Thus, when applied blindly in a new population, single vinifera SNPs may not be informative for selection. Instead, phased haplotype signatures spanning the locus in coupling and/or repulsion should be more useful than single markers for SNP-based marker-assisted selection (Menzel et al 2010). While applying phased haplotype signatures may seem more complicated than SSR genotyping, we demonstrated a benefit of having a haplotype signature of 15 SNPs multiplexed into a single assay, a subset of which was informative each new population, rather than relying on a single SSR (VMC7f2), which was monomorphic in populations 05-3010 and 07-3553.

This study describes the first application of the Vitis9KSNP array in a breeding program and provides an opportunity for guiding future studies with this or similar genotyping arrays. Vitis9KSNPs that were polymorphic and segregating in 03-3004 were frequently converted to segregating markers in a SNPlex assay (80%). In contrast, Vitis9KSNPs that were polymorphic in 07-3553 but had low GenTrain and/or low GenCall scores did not convert into co-segregating SNPlex markers. For the six selected Vitis9KSNPs that passed both quality filters and were polymorphic between 07-3553 parents, only three were polymorphic as SNPlex markers, and these did not co-segregate with resistance, due to chromosomal rearrangements between the PN40024 reference and 07-3553. Therefore, success in using a genotyping array to discover SNPs useful in breeding populations will require quality filtering and may require genotyping of some progeny to confirm marker segregation. Based on our experiences with the Vitis9KSNP array in these and other populations, we recommend the following quality filters after genotyping the parents and at least ten progeny: (1) discard SNPs with GenTrain scores <0.3 or GenCall scores <0.2; (2) discard individuals or SNPs with 20% or more missing data; and (3) discard progeny with greater than 4% Mendelian inconsistency (indicative of pollen contamination). Even then, some high quality SNPs (20% here) may not convert successfully from the Vitis9KSNP assay to other SNP platforms due to technical differences.

While the novel phenotype of *Ren4* (Ramming et al. 2011) may suggest novel resistance gene structure, we have now mapped this gene to a region on chromosome 18 that contains the race-specific resistance gene *Rpv3* (Peressotti et al. 2010) and *Run2* (Riaz et al. 2011) as well as several RGA sequences (Bellin et al. 2009). Most commonly in other powdery mildew pathosystems, *R*-gene-mediated resistances associated with RGAs involve effector triggered immunity that is race-specific. Uncovering the genetic basis of *Ren4* has the potential to uncover novel biological insights into powdery mildew pathogenesis, particularly the mechanisms of penetration and haustorial formation. Our data provide markers tightly linked to *Ren4* potentially useful for both fundamental and applied research, including both SSRs and SNPs that will be useful for seedling selection of resistant as well as seedless individuals in fine mapping and breeding populations.

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