ORIGINAL PAPER

Cloning and characterization of *XiR1*, a locus responsible for dagger nematode resistance in grape

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Abstract The dagger nematode, *Xiphinema index*, feeds aggressively on grape roots and in the process, vectors grapevine fanleaf virus (GFLV) leading to the severe viral disease known as fanleaf degeneration. Resistance to *X. index* and GFLV has been the key objective of grape rootstock breeding programs. A previous study found that resistance to *X. index* derived from *Vitis arizonica* was largely controlled by a major quantitative trait locus, *XiR1* (*X. index Resistance 1*), located on chromosome 19. The study presented here develops high-resolution genetic and physical maps in an effort to identify the *XiR1* gene(s). The mapping was carried out with 1,375 genotypes in three populations derived from D8909-15, a resistant selection from a cross of *V. rupestris* A. de Serres (susceptible) × *V. arizonica* b42-26 (resistant). Resistance to *X. index* was evaluated on 99 informative recombinants that were identified by screening the three populations with two markers flanking the *XiR1* locus. The high-resolution genetic map of *XiR1* was primarily constructed with seven DNA markers developed in this study. Physical mapping of *XiR1* was accomplished by screening three bacterial artificial chromosome (BAC) libraries constructed from D8909-15, *V. vinifera* Cabernet Sauvignon and *V. arizonica* b42-26. A total of 32 BAC clones were identified and the *XiR1* locus was delineated within a 115 kb region. Sequence analysis of three BAC clones identified putative nucleotide binding/leucine-rich repeat (NB-LRR) genes. This is the first report of a closely linked major gene locus responsible for ectoparasitic nematode resistance. The markers developed from this study are being used to expedite the breeding of resistant grape rootstocks.

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Introduction

Plant parasitic nematodes are among the most damaging agricultural pests. Ectoparasitic nematodes live and reproduce in soil and feed by puncturing the root surface with a stylet; in contrast, endoparasitic nematodes physically enter the roots to feed and reproduce. Economically, the most important are endoparasitic sedentary nematodes of the genera *Meloidogyne*, *Heterodera* and *Globodera*. Examples of plant genes that confer resistance to these three genera have been reported. For example, *Hs1^{pro-1}* from sugar beet confers resistance to the beet cyst nematode *H. schachtii* (Cai et al. 1997). The tomato *Mi-1.2* gene mediates resistance to three different root-knot nematode (*Meloidogyne*)

species and to the potato aphid *Macrosiphum euphorbiae* (Milligan et al. 1998; Rossi et al. 1998). The *Hero A* gene from tomato confers resistance to various pathotypes of potato cyst nematodes *G. rostochiensis* and *G. pallida* (Ernst et al. 2002). Both *Gpa2* (van der Vossen et al. 2000) and *Gro1-4* (Paal et al. 2004) from potato confer resistance to *G. pallida* and *G. rostochiensis*, respectively. These genes have been cloned and characterized and are being used by breeders in their efforts to produce resistant plant material (Williamson and Kumar 2006). However, no plant genes that confer resistance to ectoparasitic nematodes have yet been physically mapped or identified due to the transient association between a mobile nematode and the site of feeding, which makes phenotypes more difficult to evaluate.

The grapevine fanleaf degeneration disease complex can reduce crop yields by 80% (Martelli and Savino 1990) and is a major worldwide viticultural problem (Andret-Link et al. 2004). Disease symptoms consist of disrupted fruit set caused by grapevine fanleaf virus (GFLV) and depressed plant growth caused by severe root damage from GFLV's vector, the ectoparasitic nematode *Xiphinema index* (Thorne and Allen 1950). *Xiphinema index* feeds primarily on actively growing root tips and causes the root tip to swell and gall followed by decay and proliferation of lateral roots behind the root tip (Martelli and Savino 1990). GFLV is a nepovirus of the family *Comoviridae* (Wellink et al., 2000), and possesses a bipartite genome. The gene responsible for encoding the coat protein, $2C^{CP}$, resides on RNA 2 and has been found to dictate the specific transmission of GFLV by *X. index* (Andret-Link et al. 2004). The virus resides in the anterior region of the nematode stylet at the odontostyle–odontophore junction (Raski et al. 1973), and can be successfully transmitted after approximately 5 min of feeding (Alfaro and Goheen 1974).

Because of the very high value of vineyard land, fallow and crop rotation are not practiced as a means of controlling soil-borne pests and diseases. In addition, grape roots are known to survive and support nematodes for at least 7 years (Villate et al. 2008). The use of fumigants to control *X. index* in vineyards is no longer recommended because of their limited soil penetration, their high cost, and their detrimental environmental effects (Raski and Goheen 1988). Thus, the development of resistant rootstocks using *Vitis* sources of resistance has been a breeding goal for many years. Resistance to *X. index* exists in several *Vitis* species including *V. arizonica*, *V. candicans*, *V. rufotomentosa*, *V. smalliana* and *V. solonis* (Kunde et al. 1968). Genetic studies leading to the isolation and characterization of genes conferring resistance to *X. index* would further our understanding of resistance and assist molecular and classical breeding efforts to control *X. index*.

Grape ($2n = 2x = 38$) has a relatively small genome ($C = 483$ Mbp for *V. vinifera*) and an average genetic mapping unit (cM) corresponding to about 288 kb, given a grape genetic map of 1,676 cM (Di Gaspero et al. 2007), and is comparable to that of rice (~280 kb/cM) (International Rice Genome Sequencing Project, 2005). In addition, whole-genome sequences of *V. vinifera* are now publicly available in GenBank (http://www.ncbi.nlm.nih.gov/). These qualities and genome resources make grape a suitable woody fruit crop for molecular isolation of genes using map-based cloning strategies. In a previous report, a fast and reliable *X. index* resistance screening system based on gall numbers was developed and used to demonstrate that host resistance to *X. index* derived from *V. arizonica* is primarily controlled by a major quantitative trait locus (QTL) designated *XiR1* on chromosome 19 (Xu et al. 2008). Combining these results with the recently available resources in grape genomics suggested that map-based cloning could lead to the identification and characterization of *X. index* resistance at the *XiR1* locus. In an effort to further this goal, the research presented here details the development of a high-resolution genetic map around *XiR1*, construction of BAC contigs encompassing the *XiR1* locus and sequence analysis of three BAC clones from the *XiR1* region containing nucleotide binding/leucine-rich repeat (NB-LRR) genes *XiR1.1* and *XiR1.2*, which are strong candidates for *XiR1*.

Materials and methods

Plant materials and screening for resistance to *X. index*

Three mapping populations with a total of 1,375 F_1 genotypes were used to construct a high-resolution genetic map of the *XiR1* region. The first population, 9621, was derived from a cross of D8909-15 × F8909-17, in which the *XiR1* locus was initially identified (Xu et al. 2008). The 9621 population was expanded to 943 F_1 individuals for this study. The second was the 0023 population consisting of 178 F_1 individuals from a cross of D8909-15 × *V. vinifera* B90-116. The third population, 05384, consisted of 253 F_1 individuals and derived from a cross of D8909-15 × *V. vinifera* Aíren. The common female parent D8909-15 was the source of resistance to *X. index*, while all three male parents F8909-17, *V. vinifera* B90-116 and *V. vinifera* Aíren were susceptible. D8909-15 is a selection derived from a cross of *V. rupestris* A. de Serres × *V. arizonica* b42-26. The maternal grandparent *V. rupestris* A. de Serres is susceptible to *X. index* while the paternal grandparent *V. arizonica* b42-26 is highly resistant. To distinguish the two haploid genomes as well as the genome origin for a specific homologous chromosome of D8909-15, the genomes and

individual homologous chromosomes of b42-26 origin and those of A. de Serres origin were designated D8909-15-R and D8909-15-S genome/chromosome, or simply "R" and "S" genome/chromosome, respectively.

To minimize the number of plants required for the screening of *X. index* resistance, the three populations were analyzed with two previously identified markers M4F3F and VMCNg3a10 (Xu et al. 2008) that flank the *XiR1* locus so that informative recombinant genotypes could be identified. Four plants of each of the resulting 99 recombinants along with the parents and the highly susceptible control *V. rupestris* St. George were propagated from herbaceous two-bud cuttings. The rooted plants were then evaluated for resistance to *X. index* based on the number of galls formed in 4–8 weeks after inoculation with 100 nematodes, as described previously (Xu et al. 2008).

BAC libraries

Three BAC libraries were used to identify BAC contigs encompassing the *XiR1* locus. The first BAC library was constructed by Yimin Jin (M. A. Walker, unpublished data) from D8909-15 using a vector/enzyme combination of pECSBAC4/*Eco*RI. The D8909-15 BAC library has 33,792 clones with an average insert size of approximately 50 kb. The coverage of this library was estimated at $3.4\times$ genome equivalents. A total of 88 DNA pools with one for each of the 384-well plates were prepared for the D8909-15 library, enabling PCR-based screening for clones of interest. The second BAC library was constructed from *V. vinifera* Cabernet Sauvignon by the E&J Gallo Company (Modesto, CA, USA) using two vector/enzyme combinations: pECBAC1/*BamH* I and pECBAC1/*EcoR* I and is now available from the laboratory of M. A. Walker at the University of California, Davis. This BAC library consists of 56,448 clones with a combined coverage of $13.9\times$ genome equivalents. The number of clones, average insert size and genome equivalents are 27,648, 151 kb and $7.5\times$ for the *Bam*HI sub-library, and 28,800, 125 kb and $6.4\times$ for the *Eco*RI sub-library, respectively. The third BAC library was constructed from *V. arizonica* b42-26 using a vector/enzyme combination of pCC1/*Hin*dIII (Amplicon Express, Pullman, WA, USA). The *V. arizonica* b42-26 library consists of 36,864 clones with a coverage of 6.1X genome equivalent with an average insert size of 140 kb. The Cabernet Sauvignon library and the *V. arizonica* b42-26 library were gridded onto a set of three and two 22.5×22.5 cm^2 Hybond N+ filters, respectively. These filters were screened by hybridization using ECL Direct Nucleic Acid Labeling and Detection System (Amersham, GE Healthcare, Buckinghamshire, UK).

BAC sequencing and marker development

Markers were developed based on combined information from BAC end sequences and the polymorphisms identified between the two homologous chromosomes of D8909-15 as well as between the parents from the three populations. BAC DNA was prepared from 500 ml LB overnight culture using a Qiagen Large Construct Isolation Kit (Valencia, CA, USA) and BAC end sequences were obtained by direct sequencing using BigDye terminator V3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Primers were designed for each sequenced BAC end using Vector NTI program (Invitrogen, Carlsbad, CA, USA), and then used to: (1) directly screen the D8909-15 BAC library; (2) isolate the corresponding BAC ends for hybridization-based screening of the Cabernet Sauvignon BAC library; and (3) amplify, clone and sequence the corresponding genomic fragments from all the parents for marker development by identifying polymorphisms between the parental genomes. To estimate the size of inserts, BAC DNA was digested with restriction enzyme *Not*I (New England Biolabs, Ipswich, MA, USA) and analyzed with a Chef gel DRIII system (Bio-Rad Laboratories, Hercules, CA, USA) run for 16 h at 14°C with 6 V/cm and pulse 5–40 s. BAC clones S29J16 and M10J21 were sequenced using a shotgun strategy by Macrogen (Seoul, Korea) and Amplicon Express (Pullman, WA, USA), respectively. VA31P21 was sequenced by Macrogen (Seoul, Korea) using 454 technology. The DNA and putative protein sequences of *XiR1.1* and *XiR1.2* are available as GeneBank accession numbers GU471231 and GU471232, respectively.

Nested RT-PCR

Total RNA was isolated from 5 g of D8909-15 leaf and 10 g of root tissue using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described previously (Iandolino et al. 2004), with minor modifications. Poly (A)$^+$ RNA was isolated using the Dynabeads mRNA purification kit (Dynal A.S, Oslo, Norway). First-strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was performed as described by the manual. A 2-μl aliquot of leaf and root first-strand cDNAs was used for the first-round PCR reactions, and 2 μl of the first-round PCR products were used as templates for the nested PCR reactions. The amplification of both PCR reactions were performed for 30 cycles (1 min at 94°C, 2 min at 55°C, 3 min at 72°C) followed by 7 min at 72°C. PCR products were separated on 1.2% agarose gels and visualized with ethidium bromide. Primers for nested RT-PCR had the following sequences: 1: 5′-GGCT GACCAAATTCCATTCAGTGTTGTGGAG-3′; 2: 5′-GC TGGATGACTATGCAACCCATTATCT-3′; 3: 5′-AGGAT

TTTCGTTCCAAACATCATCAAG-3′; 4: 5′-CAACTAT
GGATAGCACAAGGTTATATTCAA-3′; 5: 5′-ATTTCT
CCCCTGGATACCAGTTCAACATCCCTCACGTT-3′;
6: 5′-AGTAAGTCCATCCTCCACAATTCCTTCAACTT
TGGCAT-3′.

Annotation of BACs and sequence analyses

The editing and compiling of DNA sequence data as well as
comparisons and analysis of DNA and deduced amino acid
sequences were made using Vector NTI program (Invitrogen,
Carlsbad, CA, USA). DNA and protein sequences of
predicted genes were analyzed by NCBI databases Blast
(http://www.ncbi.nlm.nih.gov/) and GeneMark.hmm (http://
exon.biology.gatech.edu/), the latter using both *Arabidopsis
thaliana* and *Medicago truncatula* settings. The CurLRR
program at http://www.bioinf.manchester.ac.uk/curlrr/index.
html, the COILS server at http://www.ch.embnet.org/software/
COILS_form.html and the Motif Scan program at http://
hits.isb-sib.ch/cgi-bin/motif_scan were used for the prediction
of protein functional motifs.

Results

Marker development and high-resolution mapping

Xu et al. (2008) found that the SSR marker VMC5a10 was
tightly linked to the *XiR1* locus on chromosome 19
(Fig. 1a) in the 9621 population, which then consisted of
188 progeny. To identify additional markers linked to the
XiR1 locus, the D8909-15 BAC library was screened by
PCR with VMC5a10. Markers developed from the ends of
the identified BAC clones were then used to screen the
D8909-15 and *V. vinifera* Cabernet Sauvignon BAC
libraries. As a result, a total of seven markers (Table 1;
Fig. 1b) were developed from the end sequences of 21 BAC
clones (Fig. 1c, d) identified from these two BAC libraries.
Because grape is a highly heterozygous crop and the two
BAC libraries were constructed from two different sources
of genomic DNA, it was expected that the D8909-15 BAC
clones could be from either the D8909-15-R genome (one
of the *V. arizonica* b42-26 genomes) or the D8909-15-S
genome (one of the *V. rupestris* A. de Serres genomes). The
Cabernet Sauvignon BACs are similarly from its two differ-
ent haploid *V. vinifera* genomes. Detailed analysis based on
specific alleles of markers suggested that seven D8909-15
BAC clones were derived from the D8909-15-R genome
carrying the resistant allele of *XiR1* (Fig. 1c). These seven
BACs covered about 500 kb with three gaps in the *XiR1*
region of the "R" genome. Five BACs were derived from
the D8909-15-S genome (Fig. 1c), and one M10J21 cov-
ered an estimated 50 kb gap between BAC clones S8O16

and S29J16. Among the nine BACs from the Cabernet
Sauvignon library, four clones (23P24, 9I17, 1N2 and
34E12) were considered to originate from the first haploid
genome origin of Cabernet Sauvignon, whereas the other
five (5K4, 22H13, 2L17, 8N8 and 37P14) were from the
second genome (Fig. 1d).

Screening for informative recombinants and evaluation of resistance to *X. index*

XiR1 was previously mapped within a 5.6 cM interval
between two markers, VMCNg3a10 and M4F3F, on chro-
mosome 19 (Fig. 1a; Xu et al. 2008). To fine-scale map the
XiR1 region, these two flanking markers were used to iden-
tify informative recombinant genotypes from the three
mapping populations 9621, 0023 and 05384. Genotypic
data showed that the two markers segregated normally with
an expected 1:1 ratio ($\chi^2 = 1.34$, $P = 0.10–0.25$ for
VNG3a10, and $\chi^2 = 0.16$, $P = 0.50–0.75$ for M4F3F) in the
three combined populations. There were 99 recombinants
identified within this interval from the 1,375 individuals
analyzed (Table 2). The overall percentage of recombined
D8909-15 gametes in the three mapping population was 7.2%,
and ranged from 5.6 to 7.9%. The two types of recombined
D8909-15 gametes, type A (where a D8909-15-S allele at
marker Vng3a10 recombined with a D8909-15-R allele
at marker M4F3F) and type B (where a D8909-15-R allele at
marker Vng3a10 recombined with a D8909-15-S allele
at marker M4F3F), were significantly distorted ($\chi^2 = 8.49$,
$P < 0.005$) from the expected ratio of 1:1 (Table 2). How-
ever, the overall distorted 1:1 ratio was due primarily to the
9621 population ($\chi^2 = 11.09$, $P < 0.005$) as the expected 1:1
ratio was observed in the 0023 ($\chi^2 = 1.60$, $P = 0.10–0.25$)
and 05384 ($\chi^2 = 0.00$, $P > 0.95$) populations. These results
suggested that male parents had significant effects on the
ratio of the two recombinant types in their F_1 progeny
since the female parent D8909-15 was the same in the three
populations.

These 99 recombinants were further evaluated for
X. index resistance based on the number of galls observed on
their root systems, resulting in 61 resistant and 38 suscepti-
ble genotypes. In addition, all 99 recombinant plants were
screened with BAC-derived genetic markers (Table 1),
which allowed the identification of ten key recombinants
(Fig. 2). These data permitted the positioning of the *XiR1*
locus within an interval of 0.51 cM (7 recombinants) between
the two closest flanking markers 1N2R3b and M4F3R in
the D8909-15-R genome (Figs. 1b, 2).

Cloning and analysis of candidate *XiR1* sequences

Sequence analysis of two overlapping BAC clones, one
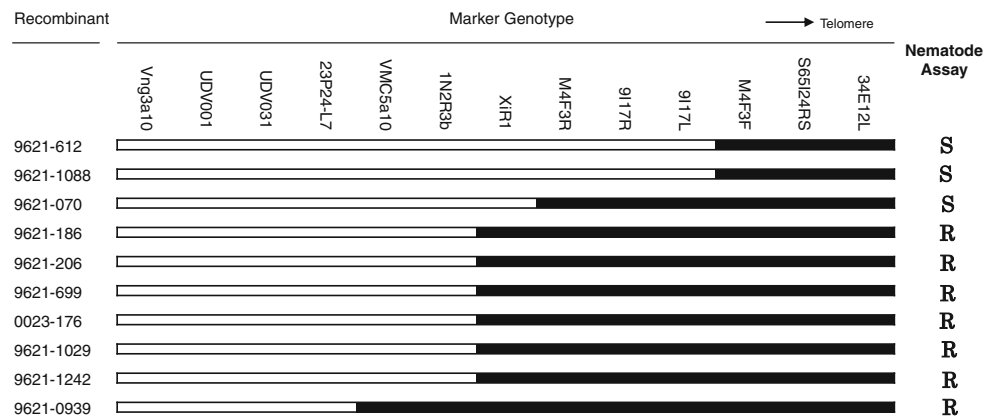from the resistant source (S29J16, ~52 kb) and one from
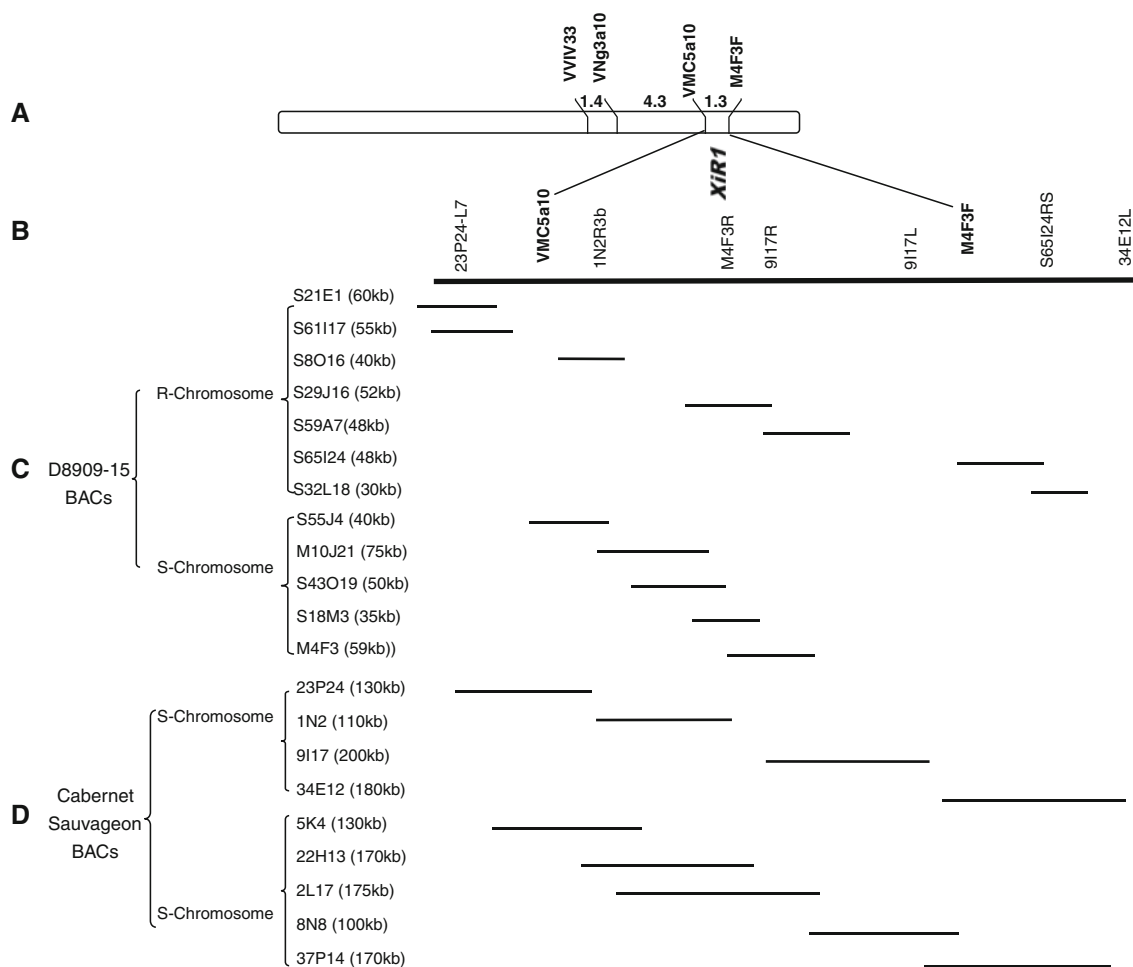
Fig. 1 A high-resolution genetic and physical map of the *XiR1* region on chromosome 19. **a** Genetic linkage map showing the relative position of *XiR1* with SSR markers on chromosome 19. The *numbers* along the chromosome are genetic distance in centimorgans. **b** The *horizontal bar* represents a segment of the D8909-15-R genome, with BAC clones spanning the *XiR1* region and positions of newly developed markers from this study. **c** The names, positions and sizes of twelve BAC clones derived from the D-8909 library. Seven of them are from the resistant background with three gaps, and five are from the susceptible genome. M10J21, which is from the susceptible genome that covers one of the gaps, is also shown. **d** The names, positions and sizes of nine BAC clones derived from the Cabernet Sauvignon library in two different genomes

Table 1 Primer information and means of detection for DNA markers developed in the *XiR1* region	Marker name	Primer sequences (5′–3′)	Detection
	34E12L	GAATCCTCAATTCATTTTTAATGATTTGTCTG CCTATCTGCATTGCCTCTGATATTAGTATG	PAGE
	S65I24RS	TTGCAGTTTAGCAGCTTTTGTTTGA AGAGCAGTGTTCAATTTGAGTTTGGA	Agarose
	9I17L	TTTATGACCTTAACAGCCAATTTGGAGT GTAATACAAGTTATACAACTGTTATCATGTGGCA	PAGE
	9I17R	AGAACCCATTCTAGATTCAAATGTAGTAGGA TCACTATCATGCACAAATTCCATATTTACT	Agarose
	M4F3R	CCAATTTCACAAAAAAAAAATGTAGAATCC TTACCAAAAATTCTTCATGAAACCATCC	Agarose
	1N2R3b	CATTGTTGATTTTCCAACTTCATTAGTATTGCCATGAT AACCTGTGATCGTTCAAACTGGAG	Agarose
PAGE polyacrylamide gel electrophoresis	23P24-L7	CCTTCAATCGGATCTTGACCAGACC CCCTTTTTTTCGGTTGAGGTCCC	PAGE

Recombinant | Marker Genotype | → Telomere | Nematode Assay

Vng3a10 · UDV001 · UDV031 · 23P24-L7 · VMC5a10 · 1N2R3b · XiR1 · M4F3R · 9I17R · 9I17L · M4F3F · S65I24RS · 34E12L

9621-612 — S
9621-1088 — S
9621-070 — S
9621-186 — R
9621-206 — R
9621-699 — R
0023-176 — R
9621-1029 — R
9621-1242 — R
9621-0939 — R

Fig. 2 Schematic representation of ten key recombinant genotypes surrounding the *XiR1* locus with nematode assay data. The *solid bars* represent chromosomal segments from D8909-15 that originated from the *X. index* resistant *V. arizonica* b42-26 ("R" genome) and the *open* *bars* represent segments from the susceptible *V. rupestris* A. de Serres ("S" genome). The crossover break-points are shown by the junctions between the *solid* and *open bars*

Table 2 Screening for informative recombinants in the *XiR1* region

	Markers		Population			
	Vng3a10	M4F3F	9621	0023	05384	Total
Recombinants (Type A)*	S	R	47	7	10	64
Recombinants (Type B)**	R	S	22	3	10	35
Non-recombinants (Type 1)	S	S	431	85	129	645
Non-recombinants (Type 2)	R	R	443	83	104	631
Total			943	178	253	1375
Recombinants (%)			7.3	5.6	7.9	

* Type A recombinants: A D8909-15-S allele at marker Vng3a10 recombined with a D8909-15-R allele at marker M4F3F

** Type B recombinants: A D8909-15-R allele at marker Vng3a10 recombined with a D8909-15-S allele at marker M4F3F

the susceptible parent (M10J21, ~75 kb) was completed. Sequencing data from BACs S29J16 and M10J21 revealed that both had a putative LRR region at the overlapping region, and an additional NB-LRR gene was identified in M10J21 only. There are four additional predicted genes in BAC S29J16 including a putative D-galactoside/L-rhamnose binding lectin, a reverse transcriptase and two hypothetical proteins which are unlikely to be the *XiR1* candidates. These results suggest that the *XiR1* locus in the D8909-15-S genome has been sequenced; however, a BAC clone spanning the corresponding region of the D8909-15-R was not identified in the D8909-15 BAC library, and the putative *XiR1* genes most likely reside in the 50-kb gap between clones of S8O16 and S29J16.

Primers corresponding to the overlapping LRR regions in BAC clones of S29J16 and M10J21 were designed and used to re-screen the D8909-15 BAC library. Three new BAC clones were obtained; however, none of them covered the gap after detailed marker analysis (data not shown). PCR amplification using primers generated from the BAC ends of S29J16 and S8O16 was unsuccessful at "filling-in" the missing sequence data. These results prompted the construction of a new BAC library from *V. arizonica* b42-26, the homozygous *X. index* resistance source (Y. Jin and M. A. Walker, unpublished data). Eleven clones spanning the gap were isolated by screening this library with the end sequence from BAC clone S29J16. Database homology searches using sequence data from one of the shortest clones, VA31P21, which completely overlapped the BAC clone M10J21, revealed two tandem repeats of NB-LRR genes (*XiR1.1* and *XiR1.2*). The deduced open reading frame was 1,268 amino acids for *XiR1.1*, 1,272 amino acids for *XiR1.2* and 1,250 amino acids for the *XiR1* susceptible homologue (*XiR1sh*) found in M10J21 (Fig. 3). The predicted polypeptides have 62.1% amino acid sequence identity. The major differences between these three predicted polypeptides are in the LRR domains. There are 12 LRRs in *XiR1.1*, 14 LRRs in *XiR1.2* and 14 LRRs in *XiR1sh* (predicted by the CurLRR program). No apparent N-terminal signal sequence was found, indicating that the encoded proteins may be located in the cytoplasm. Because no Toll interleukin receptor (TIR) domain was found, the *XiR1* genes are considered to belong to the non-TIR subfamily of R genes. *XiR1.1*, *XiR1.2* and *XiR1sh* each contain a leucine zipper motif and an NB domain including the kinase-1a (P-loop), kinase-2 and kinase-3a motifs predicted by Motif Scan program (Fig. 3). Additionally, both VA31P21 and M10J21 contain two retrotransposon genes (GenBank accession #: ABA94354 and ABA98656), a putative intregase (GenBank accession #: ABH08430) and an ATP-dependent transporter (GenBank accession #: XP_001779695). Because plant NB-LRR genes are known for conferring resistance to diseases and pests, *XiR1* and

Fig. 3 Alignment of the deduced amino acid sequences of *XiR1.1*, *XiR1.2* and *XiR1sh*. *Solid background* indicates identity and conservative substitutions. Putative leucine zipper, kinase-1a, kinase-2 and kinase-3a motifs are *underlined*. The boundaries of LRR domains are indicated. Sequence gaps inserted to maintain alignment are shown as *dashes*. The GeneDoc program (http://www.psc.edu/biomed/genedoc/) was used for formatting the alignment

XiR1.2 are likely candidates for the *XiR1* locus conferring resistance to *X. index*.

XiR1.1, *XiR1.2* and *XiR1sh* are transcribed

The GeneMark.hmm program predicted that *XiR1.1* contains five exons and four introns, and that *XiR1sh* contains three exons and two introns; however, *XiR1.2* was predicted to contain a single exon with no introns (Fig. 4a). To determine whether the *XiR1* candidate genes were expressed, poly(A)$^+$ RNA was isolated from both leaf and root tissues of D8909-15. Genomic DNA contamination was tested by using these RNA samples as templates with primers 1/3 (Fig. 4a) for PCR amplification and no bands were observed (data not shown). The poly(A)$^+$ RNA samples were then subjected to nested RT-PCR analysis. The amplified fragments were designed to include both N-terminus (NT) and LRR regions (Fig. 4a). PCR using primer sets (1/3 and 4/6) yielded an NT-840 bp band and an LRR-1,150 bp band, respectively. These PCR products were further used as templates for PCR using primer sets (2/3 and 4/5) to generate a 600-bp band from the NT region and an 800 bp from the LRR region (Fig. 4b). The populations of NT-nested PCR products (600 bp) derived from both leaf and root tissue mRNAs were subcloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and transformed into *E. coli*. The nucleotide sequence analysis of 15 colonies from each of the resulting transformations confirmed the expression of cDNA sequences corresponding to *XiR1.1*, *XiR1.2* and *XiR1sh* from both leaf and root tissues of D8909-15.
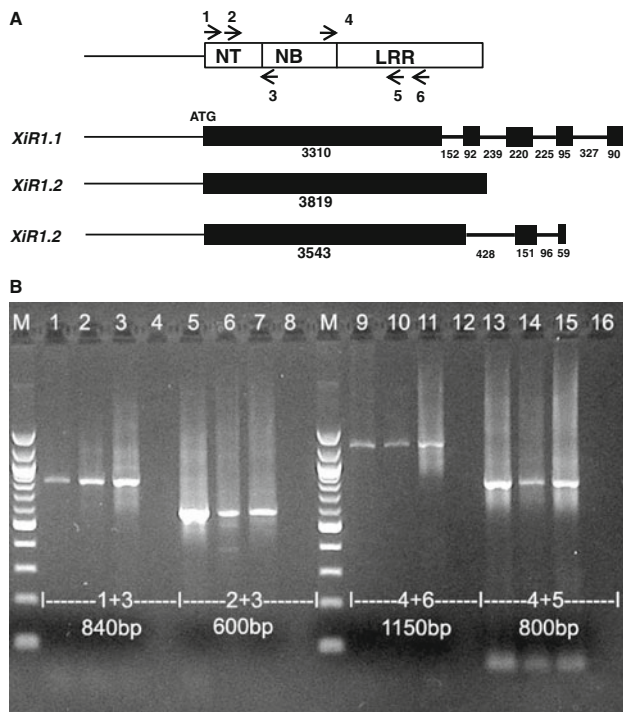
Fig. 4 Prediction of intron–exon organization and expression of *XiR1* homologues in both root and leaf tissues of D8909-15. **a** Schematic overview of the putative *XiR1* gene. The positions of PCR primers (*1–6*) are indicated by *arrows*. Exons containing coding regions are indicated as *black boxes*. Introns are shown as *black lines*. *Numbers* given below indicate the sizes of exons and introns in base pairs. *Thin lines* before ATG are the 5′ untranslated regions. **b** Agarose gel electrophoretic analysis of PCR products, obtained using primer pairs (1 + 3 and 4 + 6) and (2 + 3 and 4 + 5). The cDNA samples used in lanes of *1*, *5*, *9* and *13* were from leaf mRNA of D8909-15; cDNA samples used in lanes of *2*, *6*, *10* and *14* were from root mRNA of D8909-15. Lanes *3*, *7*, *11* and *15* are positive controls that amplified PCR fragments from BAC clone VA31P21. Lanes *4*, *8*, *12* and *16* are negative controls that contain PCR reagents only without adding cDNAs. Lane *M* indicates DNA size markers

Discussion

XiR1 fine-scale map

The ability to produce novel grapevine cultivars by conventional breeding is frequently hampered by a high degree of heterozygosity, the polygenic inheritance of desired characters and the long juvenile period before grape seedlings produce fruit. Molecular markers tightly linked to agriculturally important traits expedite the grape breeding process by allowing very early screening for single and preferably multiple traits, and therefore the earlier selection for additional traits and for next generation parents. Using 188 F_1 progeny derived from a cross between D8909-15 and F8909-17, the *XiR1* locus was mapped near marker VMC5a10 on chromosome 19 (Xu et al. 2008). Then, the

positional cloning strategy was employed to isolate the putative *XiR1* QTL using the tightly linked marker VMC5a10 to identify BAC clones covering the *XiR1* region. The BAC end sequences of these clones were used to develop PCR-based markers to re-screen BAC libraries and to analyze recombinant plants in order to fine-scale map the *XiR1* locus. The positions of the new markers were determined to refine the *XiR1* vicinity to an interval size of 115 kb between M4F3R and 1N2R3b at seven recombination events using 1,375 F_1 plants in three populations. This data further led to the isolation of the *XiR1* locus, which contains members of NB-LRR type plant resistance genes.

Three mapping populations were used to develop a high-resolution map of the *XiR1* region. Since the D8909-15 resistance source was used as the female parent in the three populations, it was expected that similar recombination frequencies would be seen in the interval between markers VNG3a10 and M4F3F. The recombination frequency ranged from 5.6 to 7.9% across the three populations, confirming the expected limited variation (Table 2). However, the number and frequency of recombinants identified in the *XiR1* region suggested that there were large differences among the three populations. Nine recombinants were found within this region in the 9621 population of 943 genotypes; a frequency of 0.95% in a cross of two half-siblings. There were a total of 431 genotypes in the 0023 and 05384 populations, which shared *V. rupestris* × *V. arizonica* parent D8909-15 as the female parent, but had different *V. vinifera* male parents. The number and frequency of recombinants in the *XiR1* region in these populations were 1 and 0.23%, respectively, and much lower than that observed in the 9621 population, suggesting that the male parents had a large impact on the frequency of *XiR1* region recombinants. The cause for this observation is unknown, but these data suggest that the male parent F8909-17 of the 9621 population was genetically superior for producing informative recombinants in the *XiR1* region.

The accuracy of positional cloning of the *XiR1* locus

Many plant QTLs that have been mapped, including those with large and small effects, and have subsequently been verified using tagged or cloned genes. Examples of verified QTLs with large effect include *fw2.2* for fruit size in tomato (Frary et al. 2000), *Gpc-B1* for wheat grain protein, zinc, and iron content (Uauy et al. 2006), *FT1* for flowering time in soybean (Yamanaka et al. 2005), and *Sub1* for submergence tolerance in rice (Xu et al. 2006). Examples of verified QTLs with small effects include *tb1* for shoot morphology in maize × teosinte (Doebley et al. 1997), *Pup1* for P uptake in rice (Wissuwa et al. 2002) and *inv/GE* for sugar content in potato (Li et al. 2005). The *XiR1* QTL was previously mapped near marker VMC5a10 at the

telomeric end of chromosome 19 (Xu et al. 2008). In this study, *XiR1* was physically located within 115 kb between markers 1N2R3b and M4F3R, which are within 1 LOD from the peak of the QTL, confirming that the position of *XiR1* was very accurate. Although the *XiR1* region physically spans 115 kb in the D8909-15-S genome, the corresponding region in the D8909-15-R genome was 40–70 kb with BACs S8O16 and S29J16 of the "R" genome origin, thus there is a gap estimated at 50 kb between the two BACs. This initial speculation was later confirmed using sequence data from BAC clone V31P21. The inability to completely cover the *XiR1* region in the "R" genome may have been caused by the limited coverage of the D8909-15 library, which has 3.4× genome equivalents.

The major differences in the genetic and physical location of marker VMC5a10 were observed between the D8909-15 "S" and "R" genomes. This marker mapped with marker 1N2R3b on one side of *XiR1* in the "R" genome, while it was physically mapped to the other side of *XiR1* in the "S" genome. When the two *V. vinifera* Cabernet Sauvignon genomes were examined, a large insertion that carried a partially duplicated *XiR1* region was inserted near marker 9I17R in one genome, resulting in a much larger *XiR1* region than that in the other *V. vinifera* genome (data not shown). However, the 115 kb *XiR1* region defined by the two markers 1N2R3b and M4F3R was largely conserved across genomes from the three *Vitis* species: the D8909-15-R (*V. arizonica*) genome, the D8909-15-S (*V. rupestris*) genome and the *V. vinifera* Cabernet Sauvignon genome without the large insertion. Thus, the *XiR1* region provides a unique opportunity to examine evolutionary impacts on the microstructure of a given region specific to each of the genomes.

Structure and expression of genes in the *XiR1* locus

Sequencing analysis of two BAC clones, one resistant (VA31P21) and one susceptible (M10J21), revealed several similar genes in both clones including two retrotransposon genes. Among the predicted candidate genes, the NB-LRR encoding gene is of particular interest because four out of the five characterized endoparasitic nematode resistance (*R*) genes are also NB-LRR genes (Williamson and Kumar 2006). It is intriguing to find that a NB-LRR gene may also confer resistance to an ectoparasitic nematode; perhaps, employing a similar strategy as tomato *Mi-1.2* gene-based resistance to potato aphid. In both cases, the pest uses a stylet to penetrate and obtain nutrients while feeding from outside the host. There are two *R* gene homologues, *Mi-1.1* and *Mi-1.2*, at the *Mi-1* locus, only *Mi-1.2* confers resistance to both nematodes and aphids (Milligan et al. 1998; Rossi et al. 1998). The predicted proteins from XiR1.1 and XiR1.2 have 70.3% identical amino acids. However, the

alignment of the deduced amino acid sequences of XiR1.1, XiR1.2 and Mi-1.2 revealed 26% identity with structural similarity only to the LZ and NB domains. This is primarily due to the lack of a long N-terminus in XiR1.1 and XiR1.2, which is present in Mi-1.2 and only exists in Solanaceous plants. A search in GenBank found that these predicted proteins had highest similarity with the NB-LRR protein RGH1 (GenBank accession # AAO37645.1) from cassava (*Manihot esculenta*), an accession that currently has no known function (López et al. 2003). The finding that *XiR1.1*, *XiR1.2* and *XiR1sh* transcripts were expressed in both root and leaf tissues of D8909-15 suggests that biological functions other than nematode resistance may be encoded by these three genes.

Plant *R* genes are usually found in tandem arrays of paralogues (Hulbert et al. 2001) clustered together with retroelememts (Michelmore 2000). It has been proposed that intragenic recombination is important in *R* gene evolution by combining parts of different alleles to create new specificities (Van der Hoorn et al. 2001). Sequence comparisons between our data and the French/Italian Pinot noir genome sequencing database (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) indicate that the *XiR1* region at the telomeric end of chromosome 19 contains both NB-LRR-encoding and retrotransposon genes. The majority of plant *R* genes cloned to date belong to the NB-LRR family. These genes can be subdivided into two broad classes based on whether the N-terminal region contains a TIR domain or a predicted coiled coil (CC) that contains the leucine zipper domain. XiR1.1, XiR1.2 and XiR1sh each contain a possible CC structure with the predicted leucine zipper motif based on the COILS program and thus fit well into the CC–NB–LRR subclass. In addition, these three genes contain the kinase-1a (P-loop), kinase-2 and kinase-3a motifs in their conserved NB domains. The variable C-terminal LRR domain is involved in pathogen recognition specificity and in regulating the signalling activity of the R protein and mutations or single amino acid changes in this domain can lead to constitutively active proteins (Hwang et al. 2000; Bendahmane et al. 2002; Hwang and Williamson 2003). XiR1.1, XiR1.2 and XiR1sh are predicted to have 12, 14 and 14 LRRs, respectively.

To test whether *XiR1.1* or *XiR1.2* is responsible for conferring *X. index* resistance, several nematode-susceptible grape lines will be transformed with constructs containing each of these genes using *Agrobacterium*-mediated (*A. tumefaciens* and *A. rhizogenes*) transformation. Although the efficiency of *Agrobacterium*-mediated genetic transformation for grape has improved, the confirmation of *XiR1* function will take at least 1 year. Nevertheless, the map-based cloning approach for grape resistance genes has directly aided the grape rootstock breeding program by providing DNA markers that have accelerated screening for resistance

saving time and cost in the greenhouse and field. This study has identified the first locus responsible for ectoparasitic nematode resistance and serves as a model for additional molecular genetic studies on the mapping, cloning and characterization of key grape traits.

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