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High-resolution genetic map of the *Rvi15* (*Vr2*) apple scab resistance locus

Paolo Galli · Giovanni Antonio Lodovico Broggini · Markus Kellerhals · Cesare Gessler · Andrea Patocchi

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Abstract The *Rvi15* (*Vr2*) apple scab resistance locus found in the GMAL 2473 accession has been previously mapped to the top of the Linkage Group 2 (LG2) by analyzing 89 progeny plants of a cross between 'Idared' and GMAL 2473. A new population of 989 progeny plants, derived from a cross between 'Golden Delicious' and GMAL 2473, has been analyzed with the two SSR markers CH02c02a and CH02f06, previously found to be associated with *Rvi15* (*Vr2*), and with two published markers derived from NBS sequences (ARGH17 and ARGH37) estimated to map close to the *Rvi15* (*Vr2*) locus. ARGH17 and ARGH37, were found to be the closest markers to the resistance locus, bracketing it within an interval of 1.5 cM. The SSRs mapped one on each side of *Rvi15* (*Vr2*). CH02f06 mapped at 2.9 cM from ARGH37 while CH02a02a mapped at 1.7 from ARGH17. The position of *Rvi15* (*Vr2*) respect to CH02a02a indicates that *Rvi15* (*Vr2*) and *Rvi4* (*Vh4*), a second apple scab gene mapped on the top of LG2, are two different resistance genes. In order to develop even more tightly linked markers to *Rvi15* (*Vr2*), ARGH17 was used as the starting point for chromosome walking through the *Rvi15* (*Vr2*) homolog region of the cv. 'Florina'. A single 'Florina' BAC clone, 36I17, was sufficient to span the homologous locus in the new population's recombinant progeny. Sequencing of the 36I17 BAC clone allowed identifying seven putative ORFs, including two showing a TIR-NBS-LRR structure. Ten additional markers could be developed mapping within a 1.8 cM interval around the *Rvi15* (*Vr2*) resistance gene. ARGH17 and GmTNL1 markers, the latter also derived from NBS-LRR resistance gene homolog sequence, are the closest markers to *Rvi15* (*Vr2*) bracketing it within a 0.5 cM interval. The availability of 12 markers within the *Rvi15* (*Vr2*) region, all within a small physical distance (kbp) in 'Florina', suggests that cloning of the *Rvi15* (*Vr2*) apple scab resistance gene from GMAL 2473 will be possible.

Keywords Resistance gene · *Venturia inaequalis* · *Malus* × *domestica* Borkh · Positional cloning

P. Galli · G. A. L. Broggini · C. Gessler · A. Patocchi
Plant Pathology, Institute of Integrative Biology, ETH-Z, Universitaetstrasse 2, 8092 Zurich, Switzerland

M. Kellerhals · A. Patocchi (✉)
Agroscope Changins-Wädenswil (ACW), Schloss, 8820 Wädenswil, Switzerland
e-mail: andrea.patocchi@acw.admin.ch

Introduction

Apple breeding programs founded in the last century were developed to increase the profitability of the fruit. These programs focussed, mainly, on traits such as high productivity, fruit quality, attractive

appearance and long storage life (Kellerhals et al. 2000). Breeding for pest and disease resistant cultivars was the second major objective (Laurens 1999). Most apple production regions around the world have climates that are favourable to *Venturia inaequalis* (Cooke) Aderhold, the casual agent of apple scab. This disease is considered the most damaging fungal disease in economic terms (MacHardy 1996) and the selection for resistant cultivars is now a central theme in most breeding programs. To date, the most successful breeding programs for apple scab resistant cultivars have used the major resistance (*R*) gene *Rvi6* (*Vf*) found in the crab apple *Malus floribunda* 821 (Janick et al. 1996). (Please note that throughout all the manuscript we will indicate both names of the apple scab resistances: the new name following the nomenclature system proposed by Bus et al. (2009), and the historical name between parentheses). *Rvi6* (*Vf*) resistance was thought to be durable until 1993, when two new races of the pathogen (races 6 and 7), able to produce scab lesions on some apple cultivars carrying *Rvi6* (*Vf*), were found (Parisi et al. 1993; Roberts and Crute 1994; Bénaouf and Parisi 2000). This finding indicated that new resistance breeding strategies should be set up carefully, in order to increase the durability of major *R* genes in commercial cultivars. A promising strategy to achieve durable resistance is the incorporation of two or more functionally different *R* genes (pyramiding) in the same cultivar (MacHardy et al. 2001). This is thought to delay, or even prevent the breakdown of the resistance.

In apple, 16 major *R* genes against apple scab have been identified and mapped (Gessler et al. 2006; Bus et al. 2009; Patocchi et al. 2009; Soriano et al. 2009). The availability of tightly linked markers to these resistances is an important prerequisite for pyramiding resistance genes. Five single scab-resistance loci (*Rvi2* (*Vh2*) and *Rvi4* (*Vh4*), Bus et al. 2005a; *Rvi15* (*Vr2*), Patocchi et al. 2004; *Rvi8* (*Vh8*), Bus et al. 2005b; *Rvi11* (*Vbj*), Gygax et al. 2004) have been mapped on linkage group (LG) 2 together with Quantitative Trait Loci (QTLs) (Calenge et al. 2004). The close proximity of resistances on the top of LG 2 has important consequences for breeding of scab-resistant cultivars, as it would be difficult to combine tightly linked genes on the same LG by traditional breeding strategies. Development of tightly linked markers for each gene would help to identify particular (rare) recombination events that couple beneficial alleles or uncouple a beneficial and an unfavorable allele combination.

The availability of tightly linked markers to a particular R gene is also a crucial prerequisite for successful map-based cloning, as they provide the starting point for chromosome walking. Additionally, the accurate mapping of the target gene based on a large number of individuals allows identification of the recombinant plants, which enable orientation of Bacterial Artificial Chromosome (BAC) clones during chromosome walking. Tightly linked markers associated with apple scab *R* gene *Rvi6* (*Vf*) have been used for its positional cloning. Patocchi et al. (1999a) analyzed 2,071 plants derived from seven crosses segregating for *Rvi6* (*Vf*). Using this large population, they located the *R* gene on LG 1 between two markers (M18 and AM19) in an interval of 0.9 cM. The availability of these markers together with the BAC library of 'Florina' (*Rvi6, i.e.Vf*) (Vinatzer et al. 1998), allowed first the construction of a 550 kb BAC contig spanning the *Rvi6* (*Vf*) genomic region (Patocchi et al. 1999b), then the identification of four candidate genes for resistance (Vinatzer et al. 2001) and, finally, the identification of the *Rvi6* (*Vf*) gene (*HcrVf2*), the only *R* gene cloned in apple (Belfanti et al. 2004; Malnoy et al. 2008).

Rvi15 (*Vr2*) was mapped on the top of LG 2 using 89 individuals of an 'Idared' x GMAL 2473 population (Patocchi et al. 2004). The resistance, expressed as slow hypersensitive response (sHR, Galli et al. 2010), was found to co-segregate with a Single Sequence Repeat (SSR) marker, CH02c02a, and two Amplified Fragment Length Polymorphism (AFLP) markers, EA37MA39 and EA35MA41 (Fig. 1a). Two additional markers were also found to be linked to *Rvi15* (*Vr2*). The AFLP EA42MA39 was mapped at 6 cM on one side of the resistance locus, while the SSR CH02f06 mapped at 6.9 cM on the other side of *Rvi15* (*Vr2*).

In this paper we report the saturation of the *Rvi15* (*Vr2*) region with molecular markers using a new mapping population. First, published markers mapping in the *Rvi15* (*Vr2*) region such as ARGH17 and ARGH37 (Apple Resistance Genes Homologues) reported by Baldi et al. (2004) were tested. In a second approach, further markers were developed from the homologous region of 'Florina' performing chromosome walk with the available 'Florina' BAC

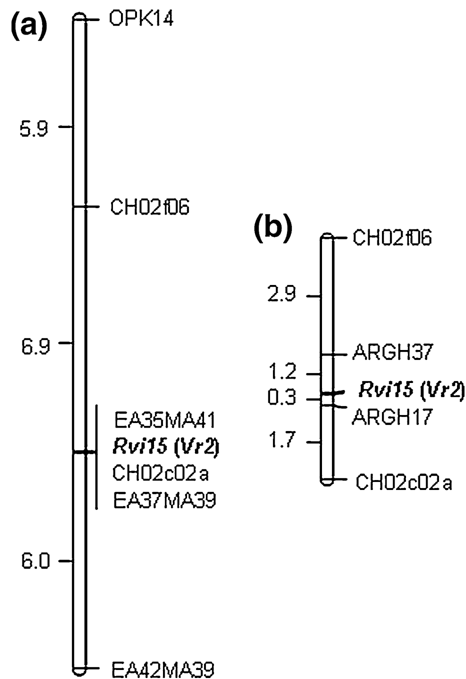

Fig. 1 Genetic maps of the *Rvi15* (*Vr2*) resistance locus. **a** Genetic map based on 89 progenies from the cross 'Idared' × GMAL 2473 (modified from Patocchi et al. 2004). **b** Genetic map based on data from 989 progeny plants of the cross 'Golden Delicious' × GMAL 2473 using polymorphic markers from the literature. Distances are indicated in cM

library (Vinatzer et al. 1998). A 'Florina' BAC clone spanning the whole homologous region was identified and sequenced. Subsequently, new markers were derived from this clone and were mapped in the cross 'Golden Delicious' × GMAL 2473 to *Rvi15* (*Vr2*).

Materials and methods

Plant material and scab inoculation

A cross between the apple scab susceptible 'Golden Delicious' and the resistant selection GMAL 2473 was performed at the Swiss Federal Station of Wädenswil (ACW) in spring 2004. A total of 999 progeny plants was inoculated with a local field-derived conidial suspension ("mixed" strains) of *V. inaequalis* (10^5 conidia/ml), as described by Gianfranceschi et al. (1996). Symptoms were evaluated on young leaves 14–21 days after inoculation according to Chevalier et al. (1991): class 0, no symptoms; class 1, pin point pits; class 2, chlorotic lesions, possibly small necrotic spots; class 3a, chlorotic and necrotic spots; class 3b, chlorotic and necrotic spots, presence of sporulation; class 4, sporulation. All the progeny were re-inoculated about 3–4 weeks after the first inoculation in order to verify their classification. Only the progeny plants classified in the same class in both scoring assessments were considered for further analysis. Chi-square test was used to perform pair-wise comparisons of the progeny distributions within classes.

Mapping of published *Rvi15* (*Vr2*) markers in 'Golden Delicious' × GMAL 2473

After DNA extraction (Patocchi et al. 2004), progenies of the cross 'Golden Delicious' × GMAL 2473 were analyzed with the two SSR markers, CH02c02a and CH02f06, associated with *Rvi15* (*Vr2*) (Patocchi et al. 2004) according to Liebhard et al. (2002). The two Sequence Characterized Amplified Region (SCAR) markers ARGH17 and the ARGH37, mapping to the top of LG 2 (Baldi et al. 2004) were searched for Single Nucleotide Polymorphisms (SNPs) allowing their mapping. Amplicons of both markers were amplified in a Perkin Elmer Cetus Gene Amp PCR System 9600 from the parents and a set of 10 resistant and 10 susceptible seedlings, using 25 ng of genomic DNA (Baldi et al. (2004). The amplicons were then purified with the NucleoFast kit (Macherey–Nagel, Germany) following the manufacturer's instructions and re-suspended in 50 µl ddH$_2$O. Five micro liter were used as template in a sequencing reaction using 1 µl BigDye Terminator kit 3.1 (Applied Biosystems, USA) together with 20 pmol primer in a total reaction volume of 10 µl. Sequencing reactions consisted of 99 cycles of 30 s at 96°C, 20 s at 50°C and 4 min at 60°C. Reaction products were separated on an ABI 3100 DNA sequencing system (Applied Biosystems, USA). The obtained sequences were then aligned with the software Sequencer (Genecodes) and searched for Single Nucleotide Polymorphisms (SNPs). The ARGH17 SCAR marker was transformed in a Cleaved Amplified Polymorphic Sequence (CAPS) marker: amplicons obtained from all progeny of the cross were *Alu*I digested following manufacturer's instructions (New England Biolabs, USA) and subsequently run on a 1% agarose gel in order to distinguish the alleles. The

alleles of the ARGH37 presented a single SNP and the marker was mapped by sequencing the amplicon of the 56 recombinant plants detected between the CH02c02a and CH02f06 markers (Note: these two SSRs have been found to bracket the *Rvi15* (*Vr2*) gene, see results). Genetic maps were calculated using a LOD score of 10 with the software JoinMap version 3.0 (Van Ooijen and Voorrips 2002).

'Florina' BAC library screening and BAC clone analysis

^{32}P radioactive labelled ARGH17 probe (NEBlot kit, New England Biolabs, USA) was used to screen the apple BAC library of 'Florina' (Vinatzer et al. 1998) as described by Patocchi et al. (1999b). The 'Florina' BAC library has an average insert size of 120 kb, is cloned in the pECBAC1 vector and represents approximately 5× apple haploid-genome equivalents (769Mbp/1C *Malus* × *domestica* Borkh; Arumanagathan and Earle 1991); however, it does not contain the functional *Rvi15* (*Vr2*) *R* gene.

Single colonies of ARGH17 positive BAC clones were inoculated into 10 ml LB medium culture (chloramphenicol 12.5 µg/ml) and allowed to grow overnight at 37°C in an orbital shaker (250 rpm). Plasmid DNA was recovered following standard alkaline lysis procedure (Birnboim and Doly 1979). After DNA quantification, BAC extremities were sequenced: 4.5 µl DNA solution (approximately 1 µg DNA) was mixed with 20 pmol (1.5 µl) of T7 (5′-TA ATACGACTCACTATAGGG-3′) or SP6 (5′-ATTTA GGTGACACTATAG-3′) universal primers and 4 µl BigDye Terminator kit 3.1 (Applied Biosystems, USA). Reactions consisted in 99 cycles of 30 s at 96°C, 20 s at 50°C and 4 min at 60°C. Specific primers were then designed for the sequences obtained using Primer3 software (Rozen and Skaletsky 2000).

To assess BAC-insert size, 10 µl of extracted BAC plasmid DNA was restriction-digested with *Not*I according to the manufacturer's instructions (New England Biolabs, USA). Fragments were separated by Pulsed Field Gel Electrophoresis (PFGE) under the following conditions: 1xTBE, 1% agarose, 14°C, 5–20 s switch time, 13 V/cm on a CHEF-DR II apparatus (Bio-Rad, USA). BAC-insert size was estimated comparing the position of the obtained bands with the size standard MidRage PFG MarkerI (New England Biolabs, USA).

BAC contig construction and orientation

Overlapping BAC-ends were checked with PCR based assays using specific primers designed from the BAC-end sequences to verify the presence or absence of the fragment in the other BAC clones. Whenever the PCR-based assay gave contradictory results, the PCR product of the BAC-end was used to hybridize the BAC fingerprint as described by Patocchi et al. (1999b). The blotted fingerprints were prepared by digesting 15 µl of the extracted BAC DNA solution with *Eco*RI following the manufacturer's instructions (New England Biolabs, USA). Digested DNA was then run over-night in a 1% agarose gel in 1xTBE. Gels were stained with an ethidium bromide solution, photographed and blotted on Hybond-N + membranes (Amersham, USA). BAC clones showing similar *Eco*RI digestion and hybridization patterns were assigned to the same contig.

In order to develop new polymorphic markers and identifying the direction of the chromosome walking, specific BAC-end primers were used to amplify the fragment from 25 ng genomic DNA from the two parents and a set of 10 resistant and 10 susceptible progenies. If no length polymorphism was readily detected, and if the PCR reaction showed a unique band, the amplicons were sequenced as previously described. The obtained sequences were aligned with the software Sequencher (Genecodes) to search for SNPs. Newly developed polymorphic markers were then mapped analyzing the 56 recombinant plants detected between the CH02c02a and the CH02f06 markers.

Shotgun sequencing of BAC clone 36I17

A shotgun library of 'Florina' BAC 36I17 clone was developed as described by Broggini et al. (2009). 480 single colonies of the library were randomly picked and grown overnight in 1.4 ml LB medium (50 µg/ml kanamycin) at 37°C in 96 plates. Plasmid DNA was recovered following the standard alkaline lysis procedure (Birnboim and Doly 1979). Plasmid inserts were sequenced using (-49)M13Reverse (5′-GAGCG GATAACAATTTCACACAGG-3′) and (-43)M13-Forward (5′-AGGGTTTTCCCAGTCACGACGTT-3′) as previously described. The sequences obtained were analyzed with the software Sequencer

(Genecodes). The vector was trimmed using a specific software function and assembly was performed at high stringency (99% match, 20 bp minimum overlap). Specific primers were designed to fill the gaps in the BAC sequence and used to sequence the corresponding sub-clone. Finally, the assembled sequence was blasted against all nucleotide sequences deposited at NCBI. Open reading frame (ORF) predictions were performed with the FGENESH 2.6 software (Salamov and Solovyev 2000). Obtained ORFs were then compared by BLASTX to all protein sequences deposited at NCBI database. Identity at nucleotide level between sequences was calculated with ClustalW2 (Larkin et al. 2007) software of EMBL-EBI (www.ebi.ac.ukl).

Development and mapping of markers associated with *Rvi15* (*Vr2*) from the complete sequence of the 36I17 BAC clone

SSRs were identified from the BAC 36I17 clone sequence and flanking primers were designed using Primer3 software (Rozen and Skaletsky 2000). PCR amplifications were performed from 25 ng of genomic DNA with 35 cycles of 45 s denaturation at 96°C, 45 s annealing at 55°C and 1 min extension at 72°C. Microsatellite alleles were analyzed as described by Gianfranceschi et al. (1998).

Primers were designed for Resistance Gene Homologs (RGHs) found in the sequence (FloTNLfor: 5′-TTCGGCAACACAACACAAC-3′ and FloTNLrev: 5′-AATCCAAAATTTATTGGGAATG AAC-3′). Long PCR amplification was carried out in a total volume of 25 µl containing 2.5 µl of 10× long PCR buffer with $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 1 U of long PCR enzyme mix (containing a *Taq* DNA Polymerase as well as a second thermostable polymerase that exhibits 3′–5′ exonuclease activity; Fermentas, Switzerland) and 5 µl (25 ng) of genomic DNA solution from the two parents. The thermal cycling conditions were: initial denaturation at 94°C for 2 min followed by 10 cycles of 15 s denaturation at 94°C, 30 s annealing at 60°C, 4 min extension at 68°C and 20 cycles with the same profile as above, but with 15 s more in the extension step. Finally, a 10 min PCR extension was carried out at 68°C. PCR products were purified and cloned with the TOPO® TA Cloning® Kit (Invitrogen, USA) following the manufacturer's instructions. Sequencing of 10 clones for each parent was then performed as described above. The obtained sequences were aligned with the software Sequencer (Genecodes) to search for SNP. Specific primers designed flanking the SNP (TNL1for: 5′-CGATACCTTCATCAACACA GC-3′and TNL1rev: 5′-AGAAAGAAAATTGTACA TGTACTG-3′) were used to amplify the fragment from a set of 10 resistant and 10 susceptible progeny and the 56 recombinants. PCR conditions for GmTNL1 were the same as for the amplification of SSRs described above, except for the annealing temperature which was 61°C. All the genotypic data was used to calculate a genetic map using a LOD score of 10 and the software JoinMap version 3.0 (Van Ooijen and Voorrips 2002).

Results

Scab evaluation of the progeny derived from the cross ‘Golden Delicious’ × GMAL 2473

989 progeny of the 999 plants derived from the cross ‘Golden Delicious’ × GMAL 2473 were either classified as susceptible (class 4, strong sporulation) or as resistant (class 1, pinpoint pits) in both phenotypic assessments. The remaining ten plants were not used for further analysis: nine plants presented differences in disease symptoms between the two inoculations, while one plant did not show any symptoms in both inoculation attempts. Considering the 989 progeny presenting no ambiguous phenotype classification, the segregation in this population was 51.8% resistant and 48.2% susceptible individuals. This result does not significantly differ from a 1:1 segregation ratio ($\chi^2 = 0.130$) (Table 1).

Initial mapping of *Rvi15* (*Vr2*) in the cross ‘Golden Delicious’ × GMAL 2473 with markers from the literature

The 989 progeny of the ‘Golden Delicious’ × GMAL 2473 population were analyzed with the SSR markers CH02f06 and CH02c02a previously used by Patocchi et al. (2004). CH02f06 mapped at 4.1 cM from *Rvi15* (*Vr2*), while CH02c02a mapped at 2 cM on the opposite side of the resistance locus (Fig. 1b). These two SSR markers allowed identifying the 56 recombinant plants (37 between CH02f06 and

Table 1 Scab resistance scoring of the 999 progeny plants of the cross 'Golden Delicious' × GMAL 2473

	Resistance classes					
	0	1	2	3a	3b	4
First scoring						
Number of progeny plants per class	1[a]	517	0	0	5[b]	476
Percentage of progeny plants per class	0.1	51.8	0	0	0.5	47.6
Second scoring						
Number of progeny plants per class	1[a]	515	0	2[b]	2[b]	479
Percentage of progeny plants per class	0.1	51.6	0	0.2	0.2	47.9
Used for mapping						
Number of progeny plants per class	0	512	0	0	0	477
Percentage of progeny plants per class	0	51.8	0	0	0	48.2

[a] The progeny plant showing no symptoms in both phenotype rounds was excluded from further analysis

[b] Nine plants were scored in different classes during the two phenotype assessments and were not considered for genetic map construction

Rvi15 (*Vr2*) and 19 between CH02c02a and *Rvi15* (*Vr2*)) which have been used to map all other markers developed to fine map *Rvi15* (*Vr2*).

The two SCAR markers ARGH17 and ARGH37 had been previously mapped in the proximity of the *Rvi15* (*Vr2*) locus (Baldi et al. 2004) and were therefore chosen to determine their linkage to the resistance. The ARGH17 sequence (378 bp) revealed two nucleotide differences between the two alleles of GMAL 2473, generating two additional *Alu*I restriction sites on the allele not associated with the resistant phenotype. Digestion of the ARGH17 amplicon with *Alu*I resulted in three fragments of size 321, 49, and 8 bp for the allele coupled with the resistance. The susceptible allele, with its two additional restriction sites, was digested into five bands of size 227, 56, 49, 38, and 8 bp. Three recombinants were detected between the *Rvi15* (*Vr2*) and the ARGH17 marker, placing ARGH17 between CH02c02a and *Rvi15* (*Vr2*) at 0.3 cM from the resistance locus (Fig. 1b). The ARGH37 amplicon sequenced from GMAL 2473 (419 bp) revealed a SNP at the position 289 from the reverse primer (T in coupling with the resistance and C in repulsion with the resistance) which allowed the mapping of this marker. ARGH37 mapped between the resistance locus and the CH02f06 marker at a distance of 1.2 cM (10 recombinants) from *Rvi15* (*Vr2*) (Fig. 1b).

Isolation of 'Florina' BAC clones from the homologous *Rvi15* (*Vr2*) region and contig construction

In order to develop new markers tightly linked to the *Rvi15* (*Vr2*) locus, the ARGH17 marker was used as a probe to screen the 'Florina' BAC library (Vinatzer et al. 1998). Five BAC clones hybridized to ARGH17: 6M11, 9C10, 36I17, 51C11 and 96H21. *Not*I digestions revealed an insert size of approximately 100 kb for the 51C11 and 36I17 and 120 kb for the other BACs. After *Eco*RI digestion, four BACs (9C10, 36I17, 51C11 and 96H21) could be ordered in a single contig (Fig. 2). The BAC 36I17 clone was considered to be a sub-clone of clone BAC 96H21, because its entire fingerprint pattern was present in the BAC 96H21 clone fingerprint. Several bands were found to be unique to the BAC 96H21 clone pattern. Additionally, it was the only clone with a *Not*I digestion in the insert (data not shown), indicating that this BAC clone is located on an extremity of the contig. The BAC clone on the other side of the contig was determined to be 9C10, because of a unique 6.5 kb band not present in the other fingerprints. The 6M11 BAC clone exhibited a different fingerprint pattern from the other clones and was not used for further analysis.

After sequencing of the BAC extremities, an SSR (named 36I17T7) was identified on the T7 extremity of the 36I17 BAC clone. The SSR mapped between ARGH37 and *Rvi15* (*Vr2*) at a distance of 0.9 cM from the resistance locus (Fig. 3). This indicated that the 36I17 and 96H21 BAC clones each span the whole *Rvi15* (*Vr2*) region. When specific primers designed on all the other BAC-ends were used to search for polymorphism between the two parents, two SNP markers were identified on 9C10 T7 and 51C11 T7 extremities. The two markers mapped between ARGH17 and the microsatellite CH02c02a, remaining outside of the region between ARGH17 and *Rvi15* (*Vr2*). No polymorphic markers could be identified on the SP6 insert-ends of the 51C11 and

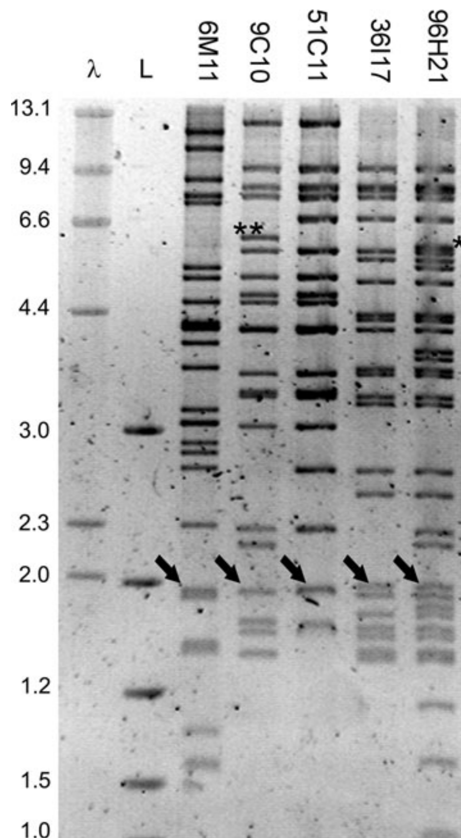

Fig. 2 *Eco*RI digestion of the five BAC clones hybridizing to ARGH17, the probe used to screen the 'Florina' BAC library. *Eco*RI fragment hybridizing to radio-labelled probe prepared from ARGH17 are indicated with *arrows*. Contig extremities are indicated with *asterisks*, single for 96H21 T7 BAC-end and double for 9C10T7. λ = λ DNA-*Hind*III Digest; L = 100 bp DNA size standard in kb

9C10 BAC clones, which would have been physically closer to *Rvi15* (*Vr2*) than the marker 36I17T7 (Fig. 3).

Analysis of the sequence of the BAC clone 36I17 and development of additional markers associated with *Rvi15* (*Vr2*)

The 36I17 'Florina' BAC clone is the smallest BAC clone containing the two closest *Rvi15* (*Vr2*) flanking markers (ARGH17 and 36I17T7). Therefore, it was decided to sequence this clone in order to identify even more tightly linked markers. 862 sequences were assembled in two contigs (accession number: GU295057); one with a length of 94,370 bp (containing the SP6 BAC-end) and a second with only 500 bp (containing the T7 BAC-end). The gap between the two contigs could not be sequenced because the presence of two different SSRs at the gap extremities. BLASTN and ORF prediction analysis of the two contig sequences revealed the presence of seven putative genes. Five ORFs were characterized by different functional motifs: namely ATP-binding cassette (ABC), Lis homology domain (LisH), single-stranded DNA binding protein (SSDP), WD40 domain, transmembrane domain, protein kinase and pentatricopeptide repeat (PPR) domains. The remaining two ORFs were characterized by the TIR-NBS-LRR (TNL-) structure common to the majority of known *R* genes (Dangl and Jones 2001). One of the RGHs showed 99% identity to the sequence of the ARGH17 marker and was named FloARGH17. The other RGH, named FloTNL, was amplified with long PCR from GMAL 2473 and 'Golden Delicious' using specific flanking primers. Two amplicons, 1,985 and 2,241 bp, were obtained and cloned from each parent, respectively. Ten clones of each amplicon were sequenced and aligned in order to detect SNPs between the alleles of the two parents. The sequence alignment revealed no polymorphism between the GMAL 2473 alleles in the bigger fragment. On the other hand, two polymorphic sites were identified in the smaller fragment. After constructing specific primers for the smaller fragment (Table 2), the new marker, named GmTNL1, was mapped at 0.2 cM from *Rvi15* (*Vr2*) (Fig. 4b).

In addition to the SNP marker GmTNL1, 15 SSRs were selected from the BAC 36I17 clone sequence. Six were polymorphic and could be mapped (Table 2). The 1-F1R SSR mapped at the same position as the marker 36I17 T7 (developed on the T7 BAC extremity). The CTRE, 3-B5F and 1-G12F SSRs mapped between 36I17T7 and GmTNL1 markers at 0.8 cM from *Rvi15* (*Vr2*) (Fig. 4b). The 1-G12F SSR could only be mapped in a small portion of the progeny, because the two parents had the same alleles (a/b). Therefore, only homozygous progeny (aa or bb) for one of the two alleles could be mapped. The 4-A2R marker was mapped between the CTRE group and the GmTNL1 at 0.7 cM (5 recombinants) from the *R* locus. Finally, the 3-B6R was mapped to 1.1 cM from the *Rvi15* (*Vr2*) between the ARGH37 and the 36I17 T7 extremity. When comparing the physical order of the markers in 'Florina' (Fig. 4a) and the genetic map of the *Rvi15* (*Vr2*) region

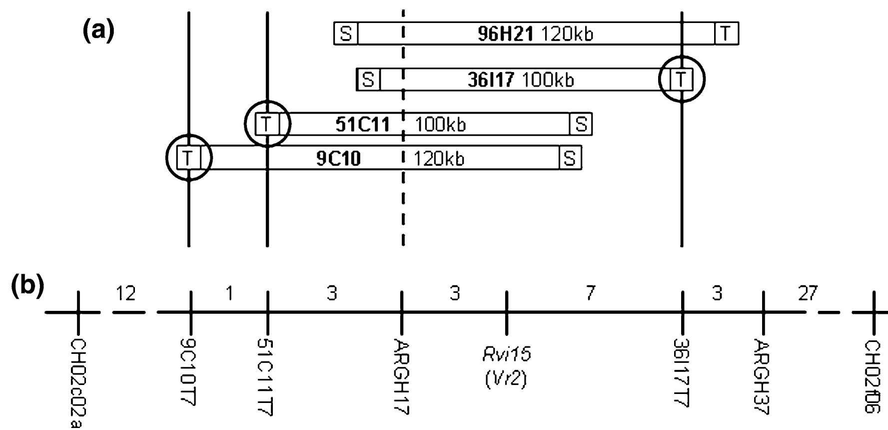

Fig. 3 Schematic representation of the BAC contig spanning the *Rvi15* (*Vr2*) homologous region in 'Florina'. **a** Representation of the BAC contig constructed with 'Florina' BAC clones. BAC extremities, on which a polymorphic marker was developed, are circled and reported on the genetic map of the *Rvi15* (*Vr2*) resistance locus. **b** *Rvi15* (*Vr2*) genetic map. *Values* indicate the number of recombinants found in 989 progenies of the cross 'Golden Delicious' × GMAL 2473. T: T7 BAC insert-end; S: SP6 BAC insert-end

(Fig. 4b) it is evident that there is some disagreement in the order of some markers.

Discussion

Phenotype and segregation ratio of the 'Golden Delicious' × GMAL 2473 mapping population

The large majority of the progeny (989 plants) of the cross between 'Golden Delicious' and GMAL 2473 14-21 days after inoculation, presented either pin-point pits (resistant) or lesions with heavy sporulation (susceptible). Only nine plants displayed inconsistent symptom development in both phenotyping rounds and were excluded from further analysis. This exclusion is justified by the fact that construction of accurate genetic maps relies heavily on the correct classification of susceptible versus resistant plants (Gygax et al. 2004). In addition, one progeny plant showing no symptoms in both phenotype assessments was excluded from further analysis because of the possibility of disease escape. The clear 1:1 segregation of resistant and susceptible phenotypes in the remaining 989 progeny plants, suggested that *Rvi15* (*Vr2*) mediated resistance is conferred by a single, dominant, major *R* gene or closely linked *R* genes. Patocchi et al. (2004) did not find such a clear 1:1 ratio when studying the segregation of *Rvi15* (*Vr2*) in the cross between 'Idared' and GMAL 2473. Moreover, Patocchi et al. (2004) reported a wider range of phenotypes in the resistant progenies. Variation in the symptom typology of progeny plants is well known in populations derived from crosses between *Rvi6* (*Vf*) carrying plants. This was ascribed to minor genes or modifiers of the *Rvi6* (*Vf*) gene that alter the *Rvi6* (*Vf*) resistance expression (Gessler 1989). Analogously, the *Rvi15* (*Vr2*) resistance might require other heritable factors than the *R* gene itself, to be completely expressed in the form of a slow hypersensitive response, leading to the formation of pinpoint pits. These heritable factors could influence, quantitatively, the *Rvi15* (*Vr2*) resistance expression, resulting in a range of phenotypes in a cross. Simply stated, 'Idared' might not carry all the alleles that would allow a complete expression of the *Rvi15* (*Vr2*) resistance. In comparison, the genetic background of 'Golden Delicious' might carry additional genes or the "right" alleles that result in a classical hypersensitive response. Inoculation tests of other populations segregating for *Rvi15* (*Vr2*) or in different genetic backgrounds will allow better understanding of the genetics of the *Rvi15* (*Vr2*) resistance.

High-resolution genetic map and development of new markers

All of the 989 progeny plants were analyzed with the two microsatellites CH02c02a and CH02f06 and the CAPS ARGH17 marker. The SNP of ARGH37 was

Table 2 Markers mapped in the *Rvi15* (*Vr2*) region

Marker	Primers[e]	Type	Annealing	Resistant[f]	Susceptible[f]
CH02f06[a]	F: 5′-CCCTCTTCAGACCTGCATATG-3′	SSR	60	146	158
	R: 5′-ACTGTTTCCAAGCGCTCAGG-3′				
CH02c02a[a]	F: 5′-CTTCAAGTTCAGCATCAAGACAA-3′	SSR	60	176	180
	R: 5′-TAGGGCACACTTGCTGGTC-3′				
ARGH17[b]	F: 5′-TTGCCGACGTTCGTGATGCT-3′	CAPS	55	321/8/49	56/38/227/8/49
	R: 5′-GATATCCTTTGTTTGGACAACC-3′				
ARGH37[b]	F: 5′-TGCACGACATTAGCAACACTG-3′	SNP	55	T (289)	C (289)
	R: 5′-GAAACAACTTCTTTTGAGAGTTC-3′				
9C10T7[c]	F: 5′-TTGAGTGTTCGGACTGATGG-3′	SNP	55	T (100)	C (100)
	R: 5′-CCCTTGCACTAAAGCTCCAC-3′				
51C11T7[c]	F: 5′-CAATTTTGCAGGTTGTTCCTC-3′	SNP	55	T (202)	G (202)
	R: 5′-CGGGCAACGTAATTTATTGG-3′				
36I17T7[c]	F: 5′-AGGATTTGAGGAGCATGGAG-3′	SSR	55	320	328
	R: 5′-AGAAACCAACACCCCCTCAC-3′				
1-F1R[d]	F: 5′-TATGCCATGTGGCTTCAGTG-3′	SSR	55	378	380
	R: 5′-AGTAGAGAATCGGCCGTGTG-3′				
3-B5F[d]	F: 5′-GGAGTTGATACGGTGGTTCG-3′	POLY-A	55	330	0
	R: 5′-CATGCACATTGGAGGAAGTG-3′				
4-A2R[d]	F: 5′-TGCAATTCAGAGCGTTCAAG-3′	SSR	55	310	316
	R: 5′-TGATAGCGCACGTGGTAGAG-3′				
3-B6R[d]	F: 5′-GCAAATCAGTGCAAATGTAGC-3′	SSR	55	254	256
	R: 5′-ACCAAAAAGACGATGATAGGG-3′				
CTRE[d]	F: 5′-CAAATACATGGATGGCATGG-3′	SSR	55	200	220
	R: 5′-CCGGAACATTCGTAAGTTGTC-3′				
GmTNL1[d]	F: 5′-CGATACCTTCATCAACACAGC-3′	SNP	61	T (206)	G (206)
	R: 5′-AGAAAGAAAATTGTACATGTACTG-3′				
1-G12F[d]	F: 5′-GTTTCCAGTCCCTGACAGC-3′	SSR	55	340	336
	R: 5′-CAGAACAAAACCCACCACAG-3′				

[a] From Liebhard et al. (2002)

[b] From Baldi et al. (2004)

[c] Developed during 'Florina' BAC contig construction

[d] Developed from the 'Florina' BAC 36I17 clone sequence

[e] Underlined primers are used to sequence the PCR product for SNPs detection (F = Forward; R = Reverse)

[f] Size or nucleotide difference of the alleles

analyzed on a set of resistant and susceptible seedlings and the 56 recombinants detected between the two microsatellites. The two AFLP markers that co-segregated with *Rvi15* (*Vr2*) in the study of Patocchi et al. (2004) were not analyzed in the progenies of 'Golden Delicious' × GMAL 2473 because no other markers could be developed from the sequencing of the two AFLPs (data not shown). The larger population, generated from the 'Golden Delicious' × GMAL 2473 cross, clearly resulted in a more detailed genetic map of the resistance locus. The CH02c02a SSR, co segregating with *Rvi15* (*Vr2*) in the cross 'Idared' × GMAL 2473 cross, could be separated from the *Rvi15* (*Vr2*) locus and found to map on the other side of the resistance locus opposite of CH02f06 (Fig. 1). Bus et al. (2005a) questioned whether *Rvi4* (*Vh4*) and *Rvi15* (*Vr2*) were the same *R* genes. However, the 'Golden Delicious' × GMAL 2473 genetic map indicates that *Rvi15* (*Vr2*) maps to 2.0 cM above the CH02c02a, while *Rvi4* (*Vh4*),

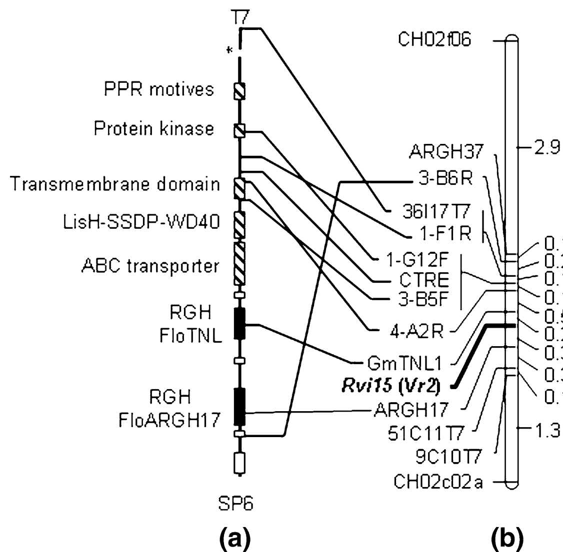

Fig. 4 ORF predictions on the 'Florina' BAC 36I17 clone sequence and comparison of the physical order of the markers in 'Florina' with the genetic order on the 'Golden Delicious' × GMAL2473 map. **a** Schematic representation of marker position and position of predicted proteins on the sequence of the 'Florina' BAC 36I17 clone. *Black boxes* indicate TIR-NBS-LRR putative genes, while *white boxes* represent putative transposable elements. Predictions of other types of proteins are indicated with *dashed boxes*. The asterisk indicates the position of the gap present in the BAC 36I17 clone sequence. **b** Saturated genetic map of the *Rvi15* (*Vr2*) region based on data from 989 progeny plants of the cross 'Golden Delicious' × GMAL 2473. Distances are indicated in cM

mapped by Bus et al. (2005a), sits 5 cM below CH02c02a. Therefore, the two resistance genes should be considered different, as they are located in two close but different region of LG2.

As ARGH17 is the closest marker to *Rvi15* (*Vr2*) identified from the literature among those available for the top of LG 2, we decided to use this marker to screen the 'Florina' BAC library. All BAC clones shown to contain this marker were then used to develop additional markers in the *Rvi15* (*Vr2*) region. The use of RGH derived marker, based on an NBS-LRR sequence, for screening of BAC libraries by hybridization could have been problematic because NBS-LRR sequences are frequent in plant genomes (e.g., 150 NBS–LRR in the *Arabidopsis* genome, Meyers et al. 2003). However, the screening of the 'Florina' BAC library (Vinatzer et al. 1998) identified only five positive BAC clones, indicating the high specificity and the suitability of ARGH17 as a probe. One BAC clone, 6M11, was excluded from further analysis because it showed different *Eco*RI fingerprints compared to the other BACs. One possible explanation for the identification of this clone by hybridization is that it comprises the same region but on the homologous chromosome.

A single BAC clone, 36I17 (100 kb), was found to span the whole *Rvi15* (*Vr2*) homolog region of 'Florina'. From the BAC-insert ends and from the BAC 36I17 clone sequences, ten new *Rvi15* (*Vr2*) tightly linked markers were developed and mapped within 1.8 cM of the resistance locus. This allowed saturating the "hot" region with additional 8 markers. A similar strategy was adopted by Cevik and King (2002) to saturate the genetic map of the *Sd-1* aphid *Dysaphis devecta* resistance gene of apple. In this latter study, the 'Florina' BAC library (Vinatzer et al. 1998) was employed to construct a BAC contig of approximately 800 kb spanning the *Sd-1* locus. Although 'Florina' does not carry the functional *Sd-1* resistance allele, five polymorphic markers were developed from the isolated BAC clones allowing saturation of the region *Sd-1* and restricting the region to 180 kb.

In this study, the order of the markers between the 'Florina' physical map and the *Rvi15* (*Vr2*) genetic map was not always consistent. The extreme case is the 3-B6R marker designed 5 kb downstream of the start codon of the FloARGH17 ORF and which maps 1.4 cM upstream of the ARGH17 marker in GMAL 2473. An in-depth analysis of the 3-B6R sequence revealed that the marker is designed on a long terminal repeat of a TRIM-like retrotransposon (Terminal-repeat retrotransposon in miniature). Therefore, the anomalous position of the marker in the genetic map could arise from the repetition in the genome of the retrotransposon that could be derived from the transposition of the retrotransposon, or from the duplication of the region where the retrotransposon was inserted. Depending on which SSR alleles (polymorphic or not) the retrotransposon contains, a different locus will be mapped, resulting in a different genetic position from the one used to design the primers. This degree of uncertainty is even greater if we consider that markers developed on a sequence from a genotype (in this case 'Florina') could be different from the genotype used for the mapping (GMAL 2473 in this case). Complete sequencing of the homologous GMAL 2473 sequence will be required to verify whether the marker sequence is repeated or not.

Conclusion

Two markers derived from RGH sequences (ARGH17 and GmTNL1) bracket the *Rvi15* (*Vr2*) resistance locus within an interval of 0.5 cM. Therefore, one could postulate that *Rvi15* (*Vr2*), located between these NBS-LRR derived markers, might also belong to the NBS-LRR type of *R* gene. Other studies showing a large cluster of NBS-LRR RGHs in the region of *Rvi15* (*Vr2*) have reinforced this hypothesis. Calenge et al. (2005) developed three markers using NBS profiling technology and mapped them at 1–2 cM from the CH02c02a microsatellite and 3–2 cM from the CH02f06 marker, placing them in the *Rvi15* (*Vr2*) region. In another study, a cDNA-derived marker designed on an LRR similar sequence (UIUC-27), which was not polymorphic in our cross (data not shown), was mapped in this region (Naik et al. 2006). However, the fact that no *Rvi15* (*Vr2*) co-segregating markers could be developed from the two NBS-LRR clustered sequences of 'Florina' suggests that molecular events, such as duplication or insertion, could have occurred at the *Rvi15* (*Vr2*) resistance locus. Therefore, we can not exclude a *priori* that *Rvi15* (*Vr2*) possesses another type of *R* gene structure. The structure of the resistance gene will be clarified upon completion of the positional cloning of the gene and the demonstration of its functionality. To accomplish this, a large-insert apple genomic-DNA library of GMAL 2473 needs to be constructed. Subsequently, the library can be probed with the highly specific ARGH17 marker, which mapped very close to the *Rvi15* (*Vr2*) gene (0.3 cM). In addition, the other markers developed in this study can be used to orient the BAC contig during its construction, simplifying the cloning procedure of the *Rvi15* (*Vr2*) apple scab resistance gene.

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