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ORIGINAL ARTICLE

Fine mapping of the rosy apple aphid resistance locus *Dp-fl* on linkage group 8 of the apple cultivar 'Florina'

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Abstract Rosy apple aphid (*Dysaphis plantaginea*), is one of the major insect pests of apple, causing serious physical and economic damage to fruit production. A dominant resistance gene *Dp-fl* was previously mapped at the bottom of linkage group LG8 from the cultivar 'Florina', linked to the SSR CH01h10. The development of additional genetic markers mapping closer to *Dp-fl* was needed to position the gene accurately and to improve the effectiveness of marker-assisted breeding (MAB). The aims of this study were to identify single nucleotide polymorphisms (SNPs) in the region of *Dp-fl* and to position these SNPs relative to *Dp-fl*. To generate a fine map of the *Dp-fl* interval, a total of 191 plants segregating for resistance and derived from four different populations were tested with temperature-switch PCR (TSP) markers developed for SNPs located in the region of CH01h10. All the plants were phenotypically evaluated for aphid resistance and those data compared with the genetic data. These efforts resulted in positioning the *Dp-fl* resistance locus in a genetic interval corresponding to a physical distance of about 330 kb on the 'Golden Delicious' genome. The new markers were tested on several apple founder cultivars in order to test the specificity of the SNPs and, thus, the best markers for the MAB were identified. Finally, the 330-kb interval was analyzed for the identification of coding sequences and putative candidate genes for *D. plantaginea* resistance were identified.

Keywords *Dysaphis plantaginea* · Graphical genotyping · *Malus* × *domestica* · SNP · TSP markers

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Introduction

Rosy apple aphid (RAA), *Dysaphis plantaginea* (Passerini) (Hemiptera: Aphididae), one of the aphid species regularly infesting apple trees (*Malus* × *domestica*), is considered one of the major insect pests of apple orchards in Europe and North America (Brown and Mathews 2007; Miñarro and Dapena 2008; Brown and Myers 2010; Parisi et al. 2013). RAA causes shoot distortion and leaf rolling, and in the case of severe infestation, the fruit remains smaller and become deformed, hence yields and its commercial value are reduced (De Berardinis et al. 1994). Control of RAA relies mainly on pesticide sprays since naturally occurring antagonists are not effective enough to control aphid populations (Miñarro et al. 2005; Brown and Mathews 2007). However, new alternative strategies to control RAA should be adopted for a more sustainable apple production. Cultivar resistance may be a good alternative strategy to manage RAA, considering that significant differences among cultivars in aphid abundance and damage level have already been well documented (Angeli and

Simoni 2006; Arnaoudov and Kutinkova 2006; Miñarro and Dapena 2007, 2008; Razmjou et al. 2014). The presence of single dominant resistance (R) genes controlling aphid resistances in fruits and other crops, such as cereals and vegetables, has been reported. This process may involve the gene-for-gene recognition of aphid-derived elicitors by plant resistance genes (Smith and Boyko 2007). The cloning and identification of some aphid R genes in different plant species support this hypothesis. For instance, Rossi et al. (1998) found that the nematode resistance gene *Mi* of tomato provides resistance to the potato aphid, *Macrosiphum euphorbiae* (Thomas). *Mi* is a member of the nucleotide-binding site and leucine-rich region (NBS-LRR) class II family, and a model for *Mi* interaction with elicitors from the aphid was proposed by Kaloshian (2004). Several NBS-LRR sequences putatively involved in aphid resistance have been cloned and mapped in barley, wheat, sorghum, melon, and *Medicago truncatula*, too (Smith and Boyko 2007). Sequences from *M.* × *domestica* with a significant degree of similarity to NBS-LRR genes were also retrieved during the development of the BAC contig spanning the region of *Dysaphis devecta* (Walker) resistance locus *Sd-1* (Cevik and King 2002).

Different parameters describe the way a plant resists to insects, such as antixenosis, antibiosis, and tolerance. Antixenosis deters or reduces colonization by insects, whereas antibiosis causes adverse effects on insect developmental traits. Tolerance is the ability of the plant to grow with little or no damage despite infestation (Hesler and Tharp 2005). Apple accessions may differ in their characteristics associated with RAA resistance, as has been described for woolly aphid resistance (Sandanayaka et al. 2003, 2005). 'Florina' is one of the best known cultivars resistant to RAA. It is considered tolerant to the aphid because it does not show the typical foliar or shoot deformations caused by RAA, despite possible small aphid development. 'Florina' RAA resistance has also been classified as antibiosis since RAA is less fecund and has a high mortality after feeding on 'Florina' leaves (Rat-Morris 1993). In addition, Marchetti et al. (2009) demonstrated that aphids on 'Florina' need a longer period before the first probe and do not show signs of complete phloem sap ingestion, proving that surface features and phloem factors are involved in the cultivar resistance, which may in turn indicate antixenosis mechanisms (Hesler and Tharp 2005).

In a preliminary study, 'Florina' RAA resistance was shown to be under the control of a single resistance gene, named *Dp-fl*, located at the distal end of linkage group (LG) 8, at about 6 cM below the SSR marker CH01h10 (Charles-Eric Durel, unpublished data). The high heritability of RAA resistance (Rat-Morris and Lespinasse 1995; Miñarro and Dapena 2004, 2008) based on its simple dominant genetic determinism was confirmed by phenotypic and genotypic evaluations on several 'Florina' progenies, in which the genetic linkage between *Dp-fl* and the SSR marker CH01h10 was re-confirmed, too (Dapena et al. 2009). Thus, 'Florina' is considered a good parent for the transmission of this resistance to *D. plantaginea*, which was inherited from its ancestor, the wild apple genotype *Malus floribunda* 821. Some scab-resistant cultivars derived from this accession and its derivative F2-26829-2-2, such as 'Galarina', 'Golden Orange', 'GoldRush', and 'Liberty', showed resistance to RAA, whereas others, for example, 'Ariane', 'Redfree', and 'Topaz', do not. Many other cultivars have been classified as susceptible, such as 'Golden Delicious' and 'Melrose' as well as various local cultivars, such as 'De la Riega', 'Perico', and 'Raxao' (Angeli and Simoni 2006; Arnaoudov and Kutinkova 2006; Miñarro and Dapena 2007, 2008). Further sources of RAA resistance have also been described in other genetic backgrounds and a *Malus robusta* derivative was shown to carry a single dominant gene for hypersensitivity to RAA, the gene *Smh* (Alston and Briggs 1970), but to date, this gene has not been mapped. Furthermore, a QTL for RAA resistance was reported on LG17 of 'Fiesta' (Stoeckli et al. 2008). Two major genes for woolly aphid resistance on LG8 have also been reported, *Er1* and *Er3*, but were mapped closer to the opposite end of the chromosome (Bus et al. 2008).

The phenotypic evaluation for RAA resistance is time-consuming and expensive in terms of money and labor costs. Other ways of selecting resistant genetic material are therefore needed, and the use of molecular markers is a good option, as already proposed by Bus et al. (2008) with markers for three major resistance genes to woolly apple aphid. To date, CH01h10 has been a useful tool for the screening of *Dp-fl* resistance (Dapena et al. 2009), but the identification of new markers more closely linked to the gene will be crucial for more efficient marker-assisted breeding (MAB) for resistance. SNPs are good candidates for marker development, as they constitute the most common source of DNA sequence variations found in genomes of most organisms, including apple. Moreover, they are found in both non-coding and coding regions, thus rendering them effective markers for mapping genes (Newcomb et al. 2006; Chagné et al. 2008; Jänsch et al. 2015). Finally, the availability of three high-density SNP arrays greatly facilitates genome analyses in *M.* × *domestica* (Chagné et al. 2012; Bianco et al. 2014, 2016).

Thus, the overall goals of our work were to construct a fine map of the *Dp-fl* region on 'Florina' LG8 through the development of specific and reliable SNP markers tightly linked to the resistance locus and to select the most effective markers for the implementation of MAB screening for *D. plantaginea* resistance. Finally, a search for candidate genes in the *Dp-fl* region was performed on the apple genome sequence (Velasco et al. 2010).

Materials and methods

Plant material and genomic DNA extraction

A panel of 191 plants derived from four progenies of 'Florina' was included in this work for the fine mapping of the region of interest. The resistant cultivar 'Florina' was either used as the female parent with the American cultivar 'Melrose', or as the male parent with the three local cider cultivars 'Perico', 'Raxao', and 'De la Riega' (Rat-Morris and Lespinasse 1995; Miñarro and Dapena 2004), resulting in 28, 79, 41, and 43 seedlings, respectively.

A panel of seven apple "founder" cultivars was analyzed to evaluate the specificity of the newly identified SNP markers: 'Braeburn', 'Cox's Orange Pippin', 'Delicious', 'Golden Delicious', 'Granny Smith', 'Jonathan', and 'McIntosh' (Durel et al. 1998; Salvi et al. 2014). Furthermore, they were tested on the progenitor *M. floribunda* 821, on its derivative F2-26829-2-2 from which 'Florina' has been bred, and on 'GoldRush', another known resistant cultivar. Another 25 apple accessions were also analyzed: four resistant to RAA, four susceptible, and 17 of unknown reaction. Among these, 21 directly derive from *M. floribunda* 821 and were selected for the *Rvi6* (*Vf*) gene for resistance to apple scab (*Venturia inaequalis*) (Online Resource 1).

For all the samples, total genomic DNA was isolated from young leaf tissues, using the Qiagen DNeasy Plant mini kit (Qiagen) following the manufacturer's protocol. DNA quantity and quality were measured spectrophotometrically with a Nanodrop ND-8000® (Thermo Scientific, USA).

Phenotypic evaluation

The response to RAA was evaluated on apple plants after infestation with aphids in two different locations: the three crossing populations 'Raxao' × 'Florina', 'Perico' × 'Florina', and 'De la Riega' × 'Florina' were evaluated in a greenhouse at SERIDA, Villaviciosa, Asturias, Spain, and the population 'Florina' × 'Melrose' was evaluated both in a greenhouse (until their shoot was 50–80 cm high) and in an unsprayed orchard at INRA, Angers, France.

At SERIDA, the number of replicates per individual seedling varied from three to eight depending on the cross. The same numbers of plants of 'Florina' and 'Golden Delicious' were used as resistant and susceptible controls, respectively. Plants were grafted on M.7 rootstocks and kept outdoors in 4-L pots. Plants were fertilized with 8 g of Osmocote plus. In mid-June, when new shoots were about 20 cm, plants were introduced into the greenhouse and randomly distributed. They were irrigated three times a week and maintained at a temperature below 22 °C using an automatically regulated cooling system. Plants were infested when new shoots were about 30–60 cm, and aphid movement from one plant to another was prevented by putting the pots in dishes filled with water. Secondary shoots were periodically pruned to keep the aphids on the principal stem. There were no overlapping branches.

Methodology at INRA was similar with same greenhouse conditions, but the number of replicates was two or three. Ten replicates were used in the case of the resistant ('Florina') and susceptible ('Melrose') controls. The plants were infested for two consecutive years, twice each year. Plants were then transferred into an experimental orchard, and field assessments were carried out for two consecutive years following natural infestation.

Aphids

Infestation was performed by placing young adult apterous virginiparous females on the upper side of the first leaf beneath the terminal cluster with a paint brush. At SERIDA, aphids were collected in the field from different apple cultivars to capture some of the natural variability. Individuals from each cultivar were reared separately on susceptible apple plants. Thus, several distinct populations of RAA were maintained in the laboratory. Four adults, each from a different population, were placed on each plant. Re-infestation was performed when necessary during the first 4 days to ensure aphid settlement. At INRA, aphids derived from a clonal aphid line of RAA obtained from one founder collected in the field and raised on grafted 'Golden Delicious' plants were used. Five adults were placed on each plant. Plants were considered as infested and the experiment as successful when a second generation was produced, i.e., more than five aphids were observed on the plants.

Damage assessment

The plants were inspected regularly for damage and scored 21 days after the placement of the aphids for tolerance (level of leaf distortion) and antibiosis (number of aphids on leaves). A clear relationship between antibiosis and tolerance was observed in the progenies although some tolerant plants developed aphid populations (75 % of tolerant plants were infested by fewer than five aphids while 79 % of the susceptible individuals were infested by more than five aphids or a colony, data not shown). Therefore, the leaf roll reaction was chosen as the criterion for damage assessment. The following scoring scale was used for tolerance: 0 = no leaf distortion; 1 = leaf very slightly curled; 2 = leaf slightly curled; and 3 = typical leaf roll (i.e., susceptible). The most distorted leaf was used for classifying the plant, and any plant showing just one distorted leaf was classified as susceptible. Each genotype was classified with the highest damage level recorded for any of the replicates (e.g., for a given genotype, if only one of the replicates showed typical leaf roll symptoms, the

genotype was scored as 3 and considered susceptible). Because the scoring was performed independently by different people and at different times at SERIDA and INRA, class 2 was not scored exactly in the same way at both places. Consequently, at SERIDA, plants exhibiting shoot damage classes of 0, 1, or 2 were considered tolerant and class 3 susceptible, whereas at INRA, plants scored as classes 0 and 1 were considered tolerant and plants scored as classes 2 and 3 were considered susceptible both in the greenhouse and in the orchard. Regarding antibiosis, the scale used at SERIDA was described by six indices: 0 = no aphids; 1 = 1 to 5 aphids; 2 = 6 to 25 aphids; 3 = 26 to 125 aphids; 4 = 126 to 625 aphids; and 5 = more than 625 aphids. At INRA, the scale was described by four indices: 0 = no aphids; 1 = 1 to 5 aphids; 2 = more than 5 aphids, no reproduction; and 3 = aphid colony.

SSR genotyping and *Dp-fl* mapping

Four apple SSRs spanning LG8 already available from the literature and heterozygous in the resistant parent 'Florina' were chosen to create the backbone of the genetic map of the chromosome: CH01h10, CH01c06, Ch01f09, and C13470 (http://www.hidras.unimi.it; Liebhard et al. 2002; Wang et al. 2012) in the largest progeny, 'Perico' × 'Florina'. The SSR primers were synthesized with a generic non-complementary nucleotide sequence at their 5′-end, with the forward and reverse primer for each marker synthesized with the nucleotide sequence 5′-ACGACGTTGTAAAA-3′ and 5′-CATTAAGTTCCCATTA-3′, respectively (Hayden et al. 2008). Two generic tag primers, tagF and tagR with the sequences 5′-ACGACGTTGTAAAA-3′ and 5′-CATTAAGTTCCCATTA-3′, respectively, were also synthesized. The tagF primer was labelled at its 5′-end with either the VIC or the FAM fluorescent dye (Applied Biosystems, Vienna, Austria). The amplification of SSR markers was performed using the PCR protocol as described by Hayden et al. (2008), and the thermal cycling conditions were the same as those reported by Liang et al. (2015).

Single amplification products (1 μL) of each SSR were multiplexed post-PCR with 0.25 μL of internal lane standard, Gene Scan™–500 LIZ® Size Standard and 9.75 μL of Hi-Di formamide (Applied Biosystems). Separation was performed using a 3730 DNA Analyzer (Applied Biosystems). Data analysis was carried out using the Peak Scanner™ Software v1 (Applied Biosystems).

For the resistance gene mapping, the RAA susceptible plants were classified as recessive (*aa*), while the resistant plants were coded as heterozygous for the presence of the resistance gene (*Aa*). The genetic map of LG8 was calculated using the JoinMap 4.1 software (Van Ooijen 2006).

SNPs identification in the *Dp-fl* region and TSP marker development

First, the physical position of SSR CH01h10 was determined through the blast tool on 'Golden Delicious' genome (http://www.rosaceae.org/tools/ncbi_blast; Velasco et al. 2010). Then, in order to identify SNPs linked to the *Dp-fl* gene, the 8K and 20K apple Infinium® SNP chips (Chagné et al. 2012; Bianco et al. 2014) were exploited. The parents 'Florina' and 'Melrose', as well as *M. floribunda* 821 and its derivative F2-26829-2-2, were genotyped with the two Illumina arrays following the standard Illumina protocol (www.illumina.com). The SNP data were analyzed using GenomeStudio Data Analysis software (Illumina Inc.) with a GenCall threshold of 0.15. All the SNPs with GenTrain score >0.6 in the region of the CH01h10 physical position were checked and the SNPs heterozygous in 'Florina', *M. floribunda* 821 and F2-26829-2-2, and homozygous in 'Melrose' were selected. The homozygous condition in 'Melrose' was preferred for the SNP to be more informatory.

For each SNP marker selected as described above, the temperature-switch PCR (TSP) assay was designed to perform the genotyping in the parents and segregating progenies, as described by Hayden et al. (2009) and Tabone et al. (2009). All the primers were designed using Primer3 Software (www.bioinformatics.nl/primer3plus). Primer sequences and PCR product sizes are provided in Online Resource 2. Some technical details were modified from the protocol of Hayden et al. (2009) and Tabone et al. (2009), including standard PCR reagents and the addition of 50 ng diluted DNA instead of 20 ng dessicated DNA. PCR assays were performed using 1 U Taq DNA Polymerase (Thermo Fisher Scientific), 5 ng of bovine serum albumin Fraction V (Thermo Fisher Scientific), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Sigma), 0.1 μM each of forward and reverse LS primer, 0.5 μM of forward/reverse AS primer, and 50 ng genomic DNA in a total reaction volume of 10 μl. PCRs were performed in an Applied Biosystems 2720 thermal cycler. After the test with standard TSP thermal cycling program, specific optimization of PCR conditions was needed for each SNP assay in order to ensure accurate and clear SNP genotyping. In particular, the number of cycles and the Tm were specifically adjusted (Online Resource 3). The PCR products were visualized on an Image Station 440 CF (Kodak, Rochester, NY, USA) after electrophoresis on 1.5 % (*w*/*v*) agarose gels and GelRed™ (Biotium) in precast.

Once the best TSP amplification system was set up for each assay, the parents and the four crossing populations were tested in order to detect: (i) which SNPs were informative ('*Aa*x*aa*' or '*Aa*x*Aa*') and (ii) whether the seedlings did or did not have the allele of 'Florina' at the analyzed locus in each population. The graphical genotyping approach was used in order to visualize the genotype of individuals to detect the

recombinant plants easily (Young and Tanksley 1989). This approach allowed the identification of the markers delimiting the *Dp-fl* gene region.

Identification of candidate genes in the *Dp-fl* interval region

As long as the *Dp-fl* interval region was well defined, the putative CDSs included in it were traced back from the 'Golden Delicious' genome sequence, version 1.0 (Velasco et al. 2010). The nucleotide sequences of these CDS, their physical position, and their biological function descriptions were retrieved from *M.* × *domestica* Gbrowse (http://www.rosaceae.org/) and NCBI database (http://www.ncbi.nlm.nih.gov/).

Results and discussion

Segregation for resistance and *Dp-fl* mapping

The results observed on 'Florina' (0 for tolerance to RAA, no damage, and 2 for antibiosis), 'Melrose' (classified 3 for tolerance and 3 for antibiosis, i.e., typical leaf roll and thriving colonies), and their progeny showed a clear relationship between antibiosis and tolerance although a little proportion of tolerant plants developed aphid populations like susceptible plants (i.e., they do not show antibiosis). These plants did not show damages, and therefore, it is possible to conclude that the damage in tolerant plants is independent from the number of aphids. Tolerance, our main criterion for resistance, is considered the most important from an agronomic and economic point of view. Moreover, the field tests conducted in the population 'Florina' × 'Melrose' at INRA showed a good correlation between results of greenhouse and field tests (data not shown).

The observed RAA resistance segregations fit the 1:1 model in all the progenies except the 'Perico' × 'Florina' one (Table 1). The skewing of the segregation towards susceptibility of this progeny had already been partially reported by Miñarro and Dapena (2004). In order to create the backbone of the genetic map of LG8 of 'Florina', four SSRs spanning the whole LG8 were tested on the 'Perico' × 'Florina' progeny (Table 2 and Fig. 1). The resulting map confirmed the previously reported position of the *Dp-fl* gene at the bottom of LG8 (Charles-Eric Durel, unpublished data). It mapped between the SSR markers CH01h010 and C13470, at 4.3 and 1.2 cM, respectively. The two SSRs located at the top of the linkage group (CH01c06 and CH01f09) showed a 1:1 segregation, while the *Dp-fl* flanking SSRs (Ch01h10 and C13470) were distorted. Therefore, we can conclude that the distortion in the 'Perico' × 'Florina' progeny is more likely related to a general skewness of the whole *Dp-fl* region instead of a different inheritance model. Segregation distortions are common in apple and, i.e., they were reported also for the two major genes for woolly aphid resistance, *Er1* and *Er3*, on LG8 (Bus et al. 2008).

Fine mapping of the *Dp-fl* locus

The genotyping of the parents 'Florina' and 'Melrose' with the 8K and 20K Illumina SNP chip resulted in the identification of 16 SNPs in the region of SSR CH01h10, according to their physical position on the 'Golden Delicious' genome. These 16 SNPs were distributed in a region of about 6 million bp at the bottom of LG8. They were chosen because of their heterozygous state in 'Florina' to fit with its R/S heterozygous state for *Dp-fl*. Thus, depending on the genotype of the susceptible parent at each locus, two types of segregation were expected: '*Aa*x*aa*' and '*Aa*x*Aa*'. Initially, TSP assays were developed for the 16 SNPs in order to obtain an efficient and specific method of SNP genotyping. TSP provides several advances, including simplified assay design, being cheap and easy-to-use because they are based on endpoint PCR and the detection of polymorphisms on agarose gel (Hayden et al. 2009). Then, the TSP assays were tested on the parents and the protocol was specifically optimized for each SNP (Online Resources 2 and 3). Among the 16 SNPs, 10 were further selected because of their homozygous state in at least one of

Table 1 Chi-square test for segregation calculated with the phenotypic data of the four apple progenies derived from crosses of 'Florina' (resistant) with susceptible cultivars

Progeny	No. of plants	RAA phenotype		Chi-square	*p*
		Resistant	Susceptible		
Florina × Melrose	28	16	12	0.57	n.s.
Perico × Florina	79	29	50	5.58	*
Raxao × Florina	41	16	25	1.98	n.s.
De la Riega × Florina	43	19	24	0.58	n.s.
Total	191	80	111		

n.s. not significant

*$p \leq 0.05$

Table 2 Chi-square test on genotypic (SSRs) and phenotypic (RAA resistance) data in the 'Perico' × 'Florina' progeny

Locus	Position (cM)	Number of seedlings		Chi-square	*p*
		Aa	*aa*		
CH01c06	0	40	39	0.01	n.s
CH01f09	12.4	41	38	0.11	n.s
CH01h10	40.6	26	53	9.23	**
Dp-fl	45.2	29	50	5.58	*
C13470	46.4	29	50	5.58	*

n.s. not significant

*$p \leq 0.05$, **$p \leq 0.01$

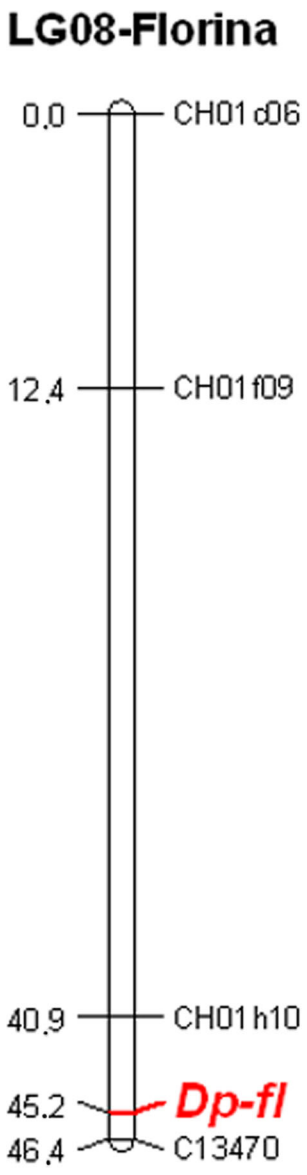

Fig. 1 Genetic linkage map of four SSRs and the locus for the resistance to RAA located on the LG8 of the apple cultivar 'Florina', based on 79 seedlings the 'Perico' × 'Florina' progeny. The resistance locus *Dp-fl* is indicated in *bold*

the susceptible parents, giving fully informative segregation ('*Aaxaa*', Table 3). The remaining six TSP markers were discarded because they did not perform well and were not accurately scored on agarose gel. Finally, the 10 selected TSPs were tested on the four populations. The mapping of these TSP markers confirmed their positions in the region of SSR CH01h10 with all of them linked with each other and showed that the genetic position of the SNPs was in full agreement with the physical order in the 'Golden Delicious' genome sequence (Table 3 and Fig. 2).

The *Dp-fl* resistance locus could be easily integrated into the SSR/SNPs linkage map of the distal part of 'Florina' LG8 thanks to the comparison of molecular and phenotypic data for RAA resistance in the four segregating populations. A total of 14 plants showing recombination in the *Dp-fl* region were identified, and therefore, the *Dp-fl* gene was fine mapped in a small genetic window (Fig. 2). The graphical genotypes of two recombinants (FM_F145 from 'Florina' × 'Melrose' and RF_X-9104-8 from 'Raxao' × 'Florina') made it possible to identify the borders of the *Dp-fl* window between SNP_104 and SNP_585, considerably narrowing down the physical interval to about 330 Kb.

As reported in Fig. 2, three SNPs (SNP_205, SNP_783, and SNP_585) exhibited a fully informative segregation pattern ('*Aaxaa*') in all the populations. For the other SNPs, an '*AaxAa*' segregation prevented assignment of the exact genotype to all seedlings because only the '*aa*' genotypes could be unambiguously assigned. For almost all of these plants, the genotype could be imputed according to the scoring of the surrounding markers. Only a few genotypes remained uncertain because they were directly flanking a recombination event, as was the case for PF_X-9504-07 with markers SNP_494 and SNP_104, and RF_89-13 and DF_X-9401-4 with marker SNP_494.

Molecular markers for MAB

To evaluate the applicability of the newly identified SNP markers in marker-assisted breeding (MAB) for the RAA resistance in apple, they were tested on seven apple breeding founder varieties and on the 'Florina' progenitor *M. floribunda* 821 and its direct derivative F2-26829-2-2 (Table 4).

Besides 'Florina', the whole *Dp-fl* haplotype was also present in *M. floribunda* 821 and F2-26829-2-2, which is consistent with the expected strong linkage disequilibrium in a genomic region introgressed from a wild progenitor into a cultivated species. The alleles linked to resistance of three SNPs (SNP_585, SNP_205, and SNP_398) appeared highly specific to the *M. floribunda* 821 genome (Table 4). Of course, the specificity has to be evaluated case by case in each cross because a lack of informativeness of these SNPs in other genetic backgrounds cannot be excluded. Besides SNP_585, the other flanking marker, SNP_104, has a good level of specificity as

Table 3 Information on SNPs selected and converted into TSP markers and used for the fine mapping of the *Dp-fl* region in apple. The genotypes of the parents of the segregating populations are reported; the homozygous state is indicated in italics

SNP name (Bianco et al. 2014)	SNP short name	SNP type	Segregating base	Florina	Melrose	Perico	Raxao	De la Riega	Contig[f]	Position of the SNP[f]
SNP_TC_28804875[a,b,c]	SNP_875	A/G	G	AG	*AA*	*AA*	AG	AG	MDC009462.72	23449468
SNP_AC_30391205[a,d]	SNP_205	T/G	G	TG	*TT*	*TT*	*TT*	*TT*	MDC005666.521	25034145
SNP_GA_31139398[a,e]	SNP_398	T/C	T	TC	*CC*	*CC*	TC	TC	MDC018465.71	25583790
SNP_FB_0764494[b]	SNP_494	T/C	C	TC	*TT*	TC	TC	TC	MDC015692.160	26453335
SNP_FB_0765104[b]	SNP_104	A/G	G	AG	*AA*	AG	*AA*	*AA*	MDC022778.432	26733535
SNP_FB_0765585[b]	SNP_585	A/G	A	AG	*GG*	*GG*	*GG*	*GG*	MDC015891.57	27068075
SNP_FB_0766783[b]	SNP_783	T/C	C	TC	*TT*	*TT*	*TT*	*TT*	MDC017260.184	28110544
SNP_FB_0767171[b]	SNP_171	T/C	C	TC	*TT*	*TT*	TC	TC	MDC015301.273	28475549
SNP_FB_0767496[b]	SNP_496	T/C	C	TC	*TT*	*TT*	TC	TC	MDC011723.416	28646203
SNP_FB_0769013[b]	SNP_013	A/C	C	AC	*AA*	*AA*	AC	AC	MDC009876.228	29401434

[a] SNP included in the 8K apple Infinium® SNP chip

[b] SNP included in the 20K apple Infinium® SNP chip

[c] Full name from Bianco et al. (2014): RosBREEDSNP_SNP_TC_28804875_Lg8_MDP0000319749_MAF40_MDP0000319749_exon1

[d] Full name from Bianco et al. (2014): RosBREEDSNP_SNP_AC_30391205_Lg8_00342_MAF10_MDP0000898538_exon1

[e] Full name from Bianco et al. (2014): RosBREEDSNP_SNP_GA_31139398_Lg8_01576_MAF10_1636849_exon2

[f] Data derived from the 'Golden Delicious' Apple genome v1.0, contigs

well, but its resistance-linked allele is also present in 'Braeburn' and 'Cox's O.P.', which are susceptible to RAA; therefore, this SNP is not fully informative in crosses with these cultivars and their derivatives. Interestingly, 'Braeburn' is reported to carry the *Sd1* gene for resistance to *D. devecta* (Alston and Briggs 1970).

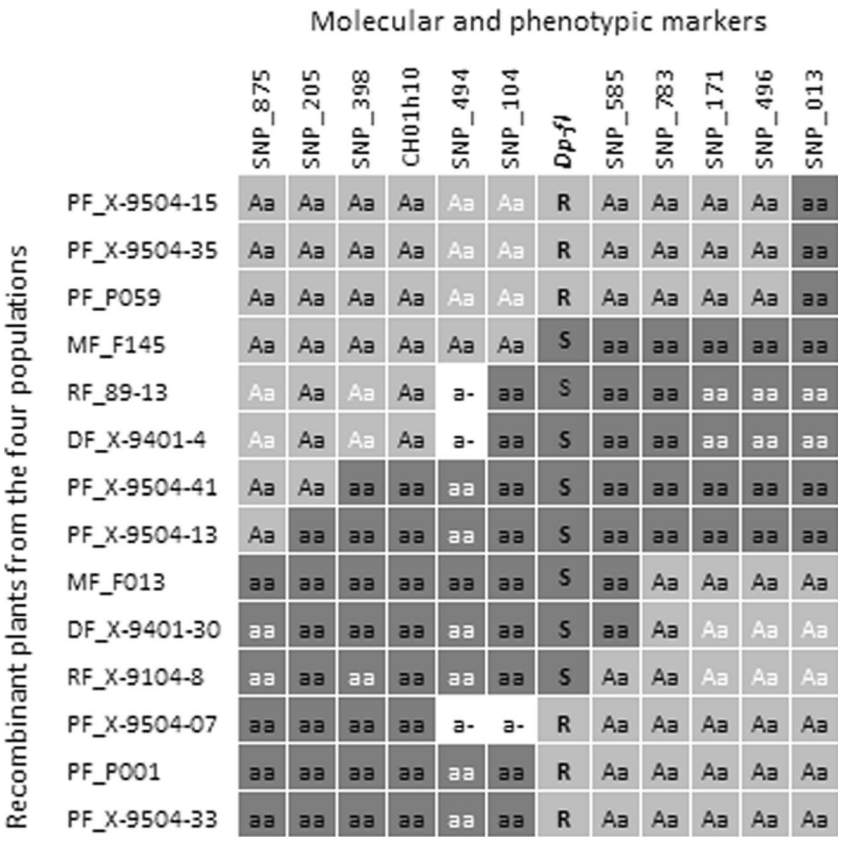

Fig. 2 Graphical genotyping of the *Dp-fl* region in the apple cultivar 'Florina'. Molecular and phenotypic data are reported for the recombinant plants of the four populations: *PF* 'Perico' × 'Florina', *FM* 'Florina' × 'Melrose', *RF* 'Raxao' × 'Florina', and *DR* 'De la Riega' × 'Florina'. The *white letters* are used for the SNPs segregating in an '*AaxAa*' pattern in some of the progenies (not fully informative meiosis). This indicates that the genotype was inferred from the surrounding samples; the "*a*-" is used when it is not possible to deduce the full genotype from the scoring. *R* resistant, *S* susceptible

Table 4 Informativeness of SNPs of the *Dp-fl* region in apple genotypes: seven apple cultivars ('Braeburn', 'Cox's Orange Pippin', 'Delicious', 'Golden Delicious', 'Granny Smith', 'Jonathan' and 'McIntosh') were included because they are important founders of breeding programs worldwide, 'Florina' because it is the donor of resistance in the analyzed progenies, *Malus floribunda* 821 and its derivative F2-26829-2-2 because they are the original source of the resistance and 'GoldRush' because it is another known resistant cultivar. For each SNP, "*A*" and "*a*" are the alleles in coupling and repulsion with *Dp-fl* gene, respectively

Founders genotypes	SNP_875	SNP_205	SNP_398	SNP_494	SNP_104	SNP_585	SNP_783	SNP_171	SNP_496	SNP_013	Phenotype	
M. floribunda 821	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	R	Rat-Morris and Lespinasse (1995)
F2-26829-2-2	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	R	Rat-Morris and Lespinasse (1995)
Florina	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	R	Rat-Morris (1993); Miñarro and Dapena (2008)
GoldRush	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	*Aa*	R	Miñarro and Dapena (2008)
Braeburn	*Aa*	*aa*	*aa*	*Aa*	*Aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	S	Miñarro and Dapena (pers. communication); E. Rat-Morris and F. Laurens (pers. communication)
Cox's O.P.	*aa*	*aa*	*aa*	*Aa*	*Aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	S	Briggs and Alston (1969)
Delicious	*Aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	S	Angeli and Simoni (2006)
Golden Delicious	*aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	*Aa*	*Aa*	*Aa*	*Aa*	S	Angeli and Simoni (2006); Miñarro and Dapena (2008)
Granny Smith	*aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	S	Arnaoudov and Kutinkova (2006); E. Rat-Morris and F. Laurens (pers. communication)
Jonathan	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	S	E. Rat-Morris and F. Laurens (pers. communication)
McIntosh	*Aa*	*aa*	*aa*	*Aa*	*aa*	*aa*	*aa*	*Aa*	*Aa*	*Aa*	S	Briggs and Alston (1969)

Unfortunately, for the other SNPs (SNP_875, SNP_494, SNP_783, SNP_171, SNP_496, and SNP_013), the allele coupled with resistance is also present in some known susceptible cultivars. Therefore, these SNPs are not fully suitable for MAB. Conversely, using a set of these SNPs and also considering the haplotype should be an efficient way to perform MAB for RAA in apple.

The efficiency of the two closest *Dp-fl* markers (SNP_104 and SNP_585) was also demonstrated by scoring them in another set of 25 apple cultivars (Table 5), mostly of unknown reaction. The molecular and phenotypic data were in

Table 5 Results of a parental marker-assisted selection by using the two flanking SNPs (TSP_104 and TSP_585) closely linked to the *Dp-fl* gene. In 25 apple cultivars, the *Dp-fl*-resistant accessions are indicated with R and the susceptible ones with S. 'Florina' is also included in this list as the resistant control and 'Melrose' and 'Golden Delicious' as susceptible controls

Genotype	Phenotype	SNP_104	SNP_585	Deduced phenotype
Florina	R	*Aa*	*Aa*	R
Galarina	R	*Aa*	*Aa*	R
Golden Orange	R	*Aa*	*Aa*	R
GoldRush	R	*Aa*	*Aa*	R
Liberty	R	*Aa*	*Aa*	R
Brina	–	*Aa*	*Aa*	R
PRI-Coop 15	–	*Aa*	*Aa*	R
PRI-Coop 17	–	*Aa*	*Aa*	R
PRI-Coop 39	–	*Aa*	*Aa*	R
Delorina	–	*Aa*	*Aa*	R
Modì	–	*Aa*	*Aa*	R
Primiera	–	*Aa*	*Aa*	R
Ariane	S	*aa*	*aa*	S
Gala[a]	S	*aa*	*aa*	S
Golden Delicious[a]	S	*aa*	*aa*	S
Melrose[a]	S	*aa*	*aa*	S
Pink Lady[a]	S	*aa*	*aa*	S
Redfree	S	*aa*	*aa*	S
Topaz	S	*aa*	*aa*	S
Amasya[a]	–	*aa*	*aa*	S
Ariwa	–	*aa*	*aa*	S
Dalinette	–	*aa*	*aa*	S
Doriane	–	*aa*	*aa*	S
Enterprise	–	*aa*	*aa*	S
Initial	–	*aa*	*aa*	S
Macfree	–	*aa*	*aa*	S
Prime Red	–	*aa*	*aa*	S
Renetta Grigia di Torriana[a]	–	*aa*	*aa*	S

[a] Cultivars susceptible to scab (*Venturia inaequalis)*. All the others derive from *M. floribunda* 821 and are carrying the *Vf* gene

agreement for the eight genotypes of known resistance/susceptibility to RAA ('Ariane', 'Gala', 'Galarina', 'GoldRush', 'Golden Orange', 'Liberty', 'Redfree', and 'Topaz'). Of the 17 cultivars of unknown reaction, seven cultivars ('Primiera', 'Modì', 'Delorina', 'Brina', PRI-Coop 17, PRI-Coop 39, and PRI-Coop 15), all deriving from *M. floribunda* 821, are postulated to carry the *Dp-fl* gene on the basis of the molecular analysis, making them putatively interesting genotypes for breeding. Conversely, several other cultivars deriving from *M. floribunda* 821 are not carrying the *Dp-fl* gene ('Doriane', 'Enterprise', 'Initial', and 'MacFree'). The results on the efficiency of these two *Dp-fl* flanking markers in MAB are promising, and a survey for the presence of the *Dp-fl* gene in scab-resistant accessions can thus be performed since the donor of the *Dp-fl* gene (*M. floribunda* 821) is well known also as the source of the *Rvi6* (*Vf*) scab resistance gene and has been widely used in breeding worldwide (Gessler and Pertot 2012). RAA resistance-associated markers are particularly desirable because resistant cultivars are an efficient and sustainable method to control RAA and reduce chemical inputs in apple orchards. In fact, apple genotype strongly affects RAA population and damage levels (Miñarro and Dapena 2007, 2008; Razmjou et al. 2014).

Identification of the genomic region spanning the *Dp-fl* locus in the apple genome sequence

Our efforts to fine map the *Dp-fl* locus greatly facilitated the molecular identification of putative functional candidate R genes. The sequences of the SNP_104 and SNP_585 markers were aligned to the 'Golden Delicious' genome sequence version 1.0 (Velasco et al. 2010). The survey of the 330 Kb region between the two flanking markers resulted in 35 principal contigs, organized mainly in two clusters because of the presence of three gaps (Fig. 3).

A total of 54 CDSs were retrieved from the 35 contigs (Online Resource 4). The annotation of these predicted genes showed variability in their biological functions, spanning from transporter activity, cell division activity to DNA binding function. Among these CDSs, some were identified as putative candidate genes thanks to the gene ontology (GO) annotation. Indeed, for 19 CDSs putative involvement in the RAA was postulated (indicated in bold in Online Resource 4). Out of these 19, twelve genes might be directly related to *M.* × *domestica-D. plantaginea* specific interaction by acting as R genes. More precisely, MDP0000399704 is homologous to a Defensin Ec-AMP-D2-like gene and, according to the GO annotation, it encodes for a protein involved in reactions triggered in response to the presence of a foreign body (biological process: defense response). Two CDSs, MDP0000129202 and MDP0000255502, showed a leucine-rich repeats (LRRs) domain. NBS-LRR genes are one of the largest plant R gene classes and have already been identified in members of the Rosaceae family (Zhong et al. 2015). Most interestingly, sequences from *M.* × *domestica* with a significant degree of similarity to NBS-LRR genes have already been found in the region of locus *Sd-1*, the resistance gene for another aphid, *D. devecta* (Cevik and King 2002). Eight CDSs showed homology with a TMV resistance protein (MDP0000283149, MDP0000285698, MDP0000273990, MDP0000234409, MDP0000210537, MDP0000292484, MDP0000642458, and MDP0000122099), and their GO annotation imputes a role for them in defense response, transmembrane signaling receptor activity, innate immune response, and apoptotic process. Moreover, these predicted genes contain an LRR conserved domain, putatively important in plant response as explained above. It is known that R gene families possess a higher proportion of duplicate genes than other gene families (Meyers et al. 2005), and the rapid gene expansion or contraction of these families might be a survival strategy to combat rapidly-evolving, species-specific pathogens (Chen et al. 2010). Thus, the cluster organization of these last eight CDSs supports

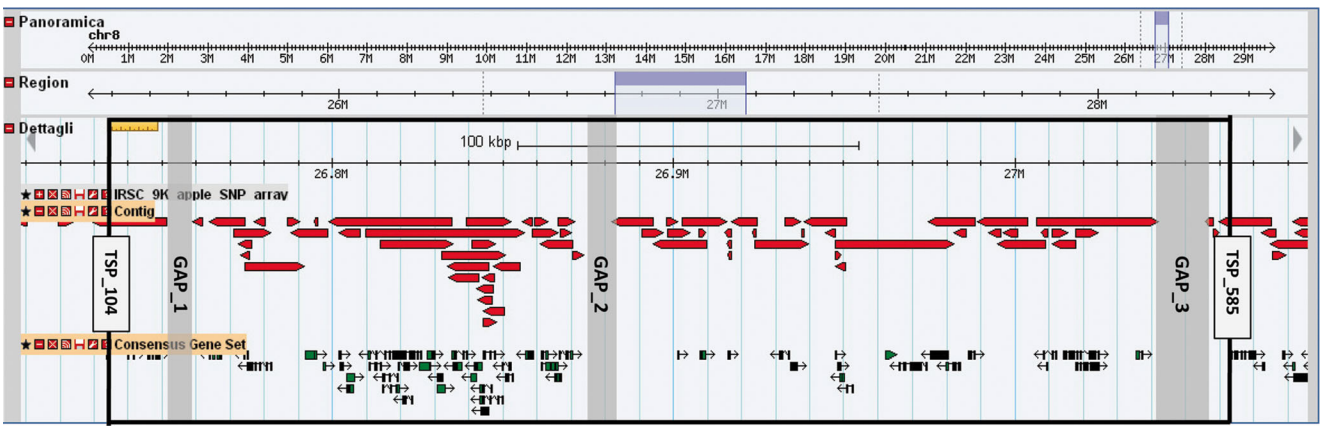

Fig. 3 Image of the *Dp-fl* window on LG8 of 'Golden Delicious' from GDR Gbrowse, *Malus* × *domestica* v1.0 (http://www.rosaceae.org). The flanking TSP markers and the gaps in the sequence are reported with *black* and *gray boxes*, respectively

the hypothesis of their involvement in RAA resistance. Finally, MDP0000756536 is a protein belonging to the pectin acetylesterase family which is responsible for catalyzing the deacetylation of pectin. It might be important for the defense mechanism since it has been found to be up-regulated in 'Florina' after RAA infestation and not in the susceptible cultivar 'Topaz' (Qubbaj et al. 2005). By employing the cDNA-AFLP method, Qubbaj et al. (2005) showed that several defense-related genes are specifically up- or down-regulated after RAA infestation, such as the pectin acetylesterase and also a vacuolar H(+)-ATPase subunit-like protein and an ADP-ribosylating enzyme. Indeed, this comparison of differentially expressed genes in resistant and susceptible apple cultivars after RAA infection suggested that the resistance might be regulated by signal transduction mechanisms similar to other biotic and abiotic stresses.

Specific aphid recognition by R genes is not the only possible resistance mechanism of apple, and other processes could be involved in the elicitation of a general stress response. Aphid saliva secreted into host leaves while feeding contains peroxidases, β-glucosidases, and other potential signal-generating enzymes able to alter the expression of inducible plant physiological factors and to trigger a general stress response similar to those involved in defense against pathogens, for instance, PR proteins (Smith and Boyko 2007). In fact, besides the 12 candidate R genes, the other seven genes are homologous to genes coding for serine/threonine kinase proteins and they may be involved in non-specific plant biotic/abiotic stress responses by acting in the signaling pathways (see Online Resource 4). Moreover, the Pto-like serine/theronine kinase gene was classified as a good candidate for the resistance gene in wheat for another aphid, *Diuraphis noxia* (Boyko et al. 2006).

With all the information acquired in this work about candidate genes for RAA resistance, the finding and validation of the R gene will be greatly facilitated.

Conclusions

A fine map of the region containing the locus responsible for the resistance of apple to the aphid *D. plantaginea* was developed and several easy-to-use SNP markers tightly linked to the *Dp-fl* locus were identified. The mapping efforts were greatly accelerated by the availability of two high-density SNP arrays that were a good starting point for the identification of SNPs in the *Dp-fl* gene region segregating in the four populations involved in the study. Each SNP was then tested on different genotypes with a cheap but efficient approach, the TSP method. This panel of SNPs, tightly linked among them and with the resistant allele of the *Dp-fl* gene, will be useful to introgress the resistance to the aphid *D. plantaginea*.

The characterization of founders and cultivars with the new markers developed in the region of the *Dp-fl* gene was useful to identify the best markers for MAB, indeed the most specific markers, in coupling with the resistance gene and present only in resistant genotypes. This will help breeders to identify the best parents for breeding programs, and also to pyramid *Dp-fl* with other RAA resistance genes such as *Smh* from *Malus robusta* (Alston and Briggs 1970) when markers are available.

Moreover, our high-resolution genetic map of the *Dp-fl* locus allowed the identification of interesting candidate genes in the small genomic region enclosed by the two most closely linked SNP markers, thus paving the way for the cloning of *Dp-fl*. The full sequencing of the *Dp-fl* region is essential for the identification of the R gene. It should be noted that the candidate genes identified in this work are from 'Golden Delicious', which is susceptible. Thus, we cannot exclude a different set of genes in this region in a resistant cultivar such as 'Florina'. The identification of the R gene will contribute to a better understanding of the functional mechanism involved in apple aphid resistance.

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Conflict of interest The authors declare that they have no competing interests.

Data archiving statement The SSR and TSP data for this research are available (tfGDR1027) at the Genome Database for Rosaceae (www.rosaceae.org).

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