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EST contig-based SSR linkage maps for *Malus × domestica* cv Royal Gala and an apple scab resistant accession of *M. sieversii*, the progenitor species of domestic apple

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Abstract *Malus sieversii* is a progenitor species of domestic apple *M. × domestica*. Using population “GMAL 4595” of 188 individuals derived from a cross of Royal Gala × PI 613988 (apple scab resistant, *M. sieversii*), 287 SSR (simple sequence repeats) loci were mapped. Of these SSRs, 80 are published anchors and 207 are newly developed EST (expressed sequence tag) contig-based SSRs, representing 1,630 *Malus* EST accessions in GenBank. Putative gene functions of these EST contigs are diverse, including

regulating plant growth, development and response to environmental stresses. Among the 80 published SSRs, 18 are PI 613988 specific, 38 are common and 24 are Royal Gala specific. Out of the 207 newly developed EST contig-based SSRs, 79 are PI 613988 specific, 45 are common and 83 are Royal Gala specific. These results led to the construction of a *M. sieversii* map (1,387.0 cM) of 180 SSR markers and a Royal Gala map (1,283.4 cM) of 190 SSR markers. Mapping of scab resistance was independently conducted in two subsets of population “GMAL 4595” that were inoculated with *Venturia inaequalis* races (1) and (2), respectively. In combination with the two major resistance reactions Chl (chlorotic lesions) and SN (stellate necrosis) to each race, four subsets of resistance data, i.e., Chl/race (1), SN/race (1), Chl/race (2) and SN/race (2), were constituted and analyzed, leading to four resistance loci mapped to the linkage group 2 of PI 613988; *SNR1* (stellate necrosis resistance to race (1)) and *SNR2* are tightly linked in a region of known scab resistance genes, and *ChlR1* (Chlorotic lesion resistance to race (1)) and *ChlR2* are also linked tightly but in a region without known scab resistance genes. The utility of the two linkage maps, the new EST contig-based markers and *M. sieversii* as sources of apple scab resistance are discussed.

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Introduction

The genus *Malus* belongs to the *Rosaceae* family, subfamily *Pomoideae* (the pome fruits). There are at least 25 *Malus* species (Robinson et al. 2001), among which domestic apple (*Malus* × *domestica* Borkh.) is considered as an interspecific hybrid. To facilitate genetic improvement of apple with marker-assisted selection (MAS), development of transferable molecular markers, construction of genetic maps and association of molecular markers with QTL (quantitative trait loci) and major genes of economic and horticultural importance are essential.

A number of genetic maps using various marker systems have been constructed in apple. Early maps were mostly constructed with RAPD (rapid amplified polymorphic DNA), RFLPs (restricted fragment length polymorphisms) and isozymes (Conner et al. 1997; Hemmat et al. 1994) or in combination with AFLP (amplified fragment length polymorphism) markers and a limited number of SSR (simple sequence repeats) markers (Maliepaard et al. 1998). AFLPs have played an important role in the construction of several maps (Igarashi et al. 2008; Liebhard et al. 2003; N'Diaye et al. 2008). SSR markers have become increasingly important in recent maps (Celton et al. 2009b; Fernandez-Fernandez et al. 2008; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) because of their relative abundance, rich polymorphism, high reliability and ease of use. These characteristics of SSR markers make them indispensable in the construction of framework maps. SNP (single nucleotide polymorphism) markers have become the most abundant and important marker system in genetic and genomic studies, and efforts to develop *Malus* SNP markers have been reported in apple (Chagne et al. 2008; Han et al. 2009). More recently, a high-quality sequence draft of the apple genome has been released, marking a major breakthrough in apple genetics and genomics (Velasco et al. 2010).

As of November 2010, there are 335,682 accessions of *Malus* ESTs (expressed sequence tags) deposited in GenBank, corresponding to at least 23,777 unigenes in GenBank. These EST sequences are mostly generated by several functional genomics studies of apple in recent years (Gasic et al. 2009a; Naik et al. 2006; Newcomb et al. 2006; Wisniewski et al. 2008). To take advantage of the EST sequence

information, the *Malus* SSR Microsatellite Analysis Project (Jung et al. 2008) analyzed 260,581 *Malus* EST accessions, which were ultimately aligned to 23,284 contigs and 53,200 singlets. A total of 56,356 SSRs were identified from these contigs and singlets. Primer sequences for most of these SSRs have also been designed and maintained at the Genome Database for *Rosaceae* (GDR) (<http://www.rosaceae.org/>), which is accessible to the public. These EST-based SSRs and their corresponding primer sequence information represent invaluable genomic resources in *Malus*. However, these resources remain largely unexploited to date. Approximately 400 SSR markers have been mapped in *Malus* (Celton et al. 2009b), a lower number compared with other crops. If a fraction of these EST-based SSRs were mapped, it would significantly increase the number of *Malus* SSR markers. Moreover, EST-based SSRs are frequently more transferable in related species compared with genomic SSRs, making them a relevant tool for comparative genomic studies in *Rosaceae* (Celton et al. 2009a; Gasic et al. 2009b; Sargent et al. 2009).

Malus sieversii from Central Asia is widely regarded as the major progenitor species of domestic apple, based on previous morphological and molecular studies (Harris et al. 2002; Juniper 2007; Juniper et al. 1999; Robinson et al. 2001) and the latest comprehensive study in sequencing the apple genome (Velasco et al. 2010). US scientists conducted collection expeditions to Central Asia, including Kazakhstan, Tajikistan and Uzbekistan, to collect *M. sieversii* germplasm (Forsline et al. 2003; Forsline and Hummer 2007; Luby et al. 2001) in order to overcome one of the bottlenecks in apple improvement, lack of genetic diversity in breeding materials. Approximately 130,000 seeds from nearly 900 trees were collected. Vegetative clonal materials were also collected from 44 trees with desirable horticultural traits, designated “elite”. These collections, along with other collections of *Malus* species, have been maintained primarily by a USDA plant germplasm repository at Geneva, NY, USA. To better use and manage these materials, studies have focused on development of core collections that capture the majority of the diversity using a minimal number of accessions (Richards et al. 2009a, b; Volk et al. 2005, 2009).

Evaluation of these *M. sieversii* collections has concentrated on disease and pest resistance,

environmental stress tolerance, plant growth habit and genetic diversity (Luby et al. 2001). Apple scab disease resistance has been identified in eight out of the 39 elite *M. sieversii* accessions (Luby et al. 2006). Investigations of GMAL 3631 (PI 600520), also a *M. sieversii* accession, led to identification of *Rvi8* (*Vh8*) (Bus et al. 2005a), a major apple scab resistance gene with resistance to *V. inaequalis* races (1) to (7) (Bus et al. 2009). This new scab resistance gene may become significant as *Rvi6* (*Vf*), the major apple scab resistance gene used in breeding of modern apple cultivars, has been overcome by the pathogen in Europe and New Zealand (Guerin and Le Cam 2004), and recently in North America (Beckerman et al. 2009). Other studies using these collections have investigated mechanisms of fruit abscission (Sun et al. 2009), genome size (Korban et al. 2009; Tatum et al. 2005) and evolution of *Malus* species (Gharghani et al. 2009). However, basic genomic information for this important *Malus* species remains scarce, although progress in construction of a different *M. sieversii* genetic map has been reported (Lalli et al. 2010). The objectives of this study are (1) to construct genetic maps for Royal Gala and PI 613988 (*M. sieversii*) to better understand the *M. sieversii* genome in contrast to that of *M. × domestica*, (2) to explore the potential utility of the existing EST-based SSR resources to increase the number of SSR markers for *Malus*, and (3) to map the apple scab resistance gene(s) from *M. sieversii* accession PI 613988.

Materials and methods

Plant materials and DNA isolation

The mapping population “GMAL 4595” of 188 F₁ individuals was made in 2002 from a cross Royal Gala (*M. × domestica* Borkh.) × PI 613988 (plant introduction number 613988, an apple scab resistant accession of *M. sieversii*). After seeds were germinated in winter 2003, the seedlings were first evaluated for apple scab (*Venturia inaequalis*) resistance (see details below), and then planted on their own roots in 2003 in a field nursery in Geneva, NY. In the following year (2004) the seedlings were planted in a high-density orchard in Geneva, where they remain at present. The maternal parent, Royal

Gala, is a widely grown commercial variety, whereas the paternal parent PI 613988 was one of the elite *M. sieversii* clones collected from Site 4 in Kazakhstan (Forsline et al. 2003). PI 613988 bears fruits of size and quality close to commercial apples (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1531529>), and has resistance to apple scab based on evaluations (Aldwinckle and Forsline, unpublished data) of six replications with an equally mixed frozen inoculum (2.7×10^5 conidia/ml) of *V. inaequalis* isolates 1805-2, 1777-8, 1771-2, 1778-6 and 1810-1 that represent the five races (1–5) present in North America (Williams and Kuc 1969; Yepes and Aldwinckle 1993). Resistance reactions of PI 613988 were complex, including three replicates of stellate necrosis (SN), two replicates of chlorotic lesions (Chl) and non-sporulating, and one replicate of hypersensitive response (Aldwinckle and Forsline, unpublished data). Genomic DNA was isolated from young leaves of population “GMAL 4595” and its parents using a CTAB (cetyl trimethylammonium bromide)-based DNA isolation protocol (Cullings 1992; Doyle and Doyle 1987).

Scab resistance evaluation

The seedlings of the mapping population “GMAL 4595” at the stage of two or more true leaves were divided into two subpopulations of 81 and 107 plants, and were inoculated with individual *V. inaequalis* race (1) (1805-2) and (2) (1777-8), respectively. Preparation of inoculum, inoculation and resistance evaluation were conducted as described previously (Malnoy et al. 2008; Yepes and Aldwinckle 1993). Briefly, frozen suspensions of the *V. inaequalis* spores were thawed and diluted to a concentration of 2.7×10^5 conidia/ml to prepare the inocula. The plants were sprayed with the inoculum of either race (1) or (2) using an atomizer connected to a compressed air supply, and were then incubated in a mist chamber for 48 h under the following conditions: 16-h photoperiod of white fluorescent light ($40 \mu\text{E}/\text{m}^2/\text{s}$), $18 \pm 1^\circ\text{C}$, and 100% relative humidity. At the end of the incubation period, the plants were moved to a growth chamber at $24 \pm 1^\circ\text{C}$. Plant reactions were evaluated two weeks after inoculation, and were scored with ‘S’ (susceptible) for extensive sporulation, and a range of resistance reaction scores, including ‘Chl’ (chlorotic lesions), ‘SN’ (stellate

necrosis), ‘0’ (no symptoms), ‘HR’ (hypersensitive response, i.e., pit type) and ‘N’ (necrotic lesions).

SSR development

A total of 533 SSR primer pairs were screened, including 111 previously published, and 422 EST contig-based SSRs selected from the Genome Database for Rosaceae (<http://www.rosaceae.org/>). For the 111 published SSRs, 82 were chosen from the core set of 88 SSRs reported to be suitable for apple linkage group anchoring and framework map construction (Patocchi et al. 2009a; Silfverberg-Dilworth et al. 2006). The remaining 29 were chosen from publications (Celton et al. 2009b; Fernandez-Fernandez et al. 2008; Gessler et al. 2006; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) for gap filling and for mapping the apple scab resistance loci *ChlR1*, *ChlR2*, *SNR1* and *SNR2* more precisely. Of the 422 EST contig-based SSRs, 384 contained 11 or more dinucleotide repeats, and 38 had 9 or more trinucleotide repeats. All these EST contig-based SSR markers were named using abbreviations of their original corresponding contig numbers, e.g., an SSR derived from “malus_v4_contig10052” in the GDR database was named “C10052” (Electronic Supplementary Material Table S1). All the SSR markers were initially screened for polymorphisms between the two haploid genomes within a parent and between the two parents. Polymorphic SSRs were then applied to the entire “GMAL 4595” progeny of 188 trees for segregation analysis.

The PCR amplification was set up in 10 μ l reactions, containing 3–7 ng genomic DNA, 0.5 units of AmpliTaq 360 DNA Polymerase (Applied Biosystems), 1 \times AmpliTaq 360 Buffer supplemented with 2.0 mM of magnesium chloride, 200 μ M of each dNTP and 0.5 μ M of each primer. The reactions were carried out with a Mastercycler (Eppendorf) using the following conditions: an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final 5-min extension at 72°C. PCR products of the entire reaction of 10 μ l, along with 20 bp Molecular Ruler (BioRad #170-8201), were similarly separated using a low-cost, high-throughput PAGE (polyacrylamide gel electrophoresis) system as described previously (Wang et al. 2003). Briefly, 6% polyacrylamide gels (Fisher BP1408-1) in 0.5 \times TBE buffer were used in

combination with a Mega-gel Dual High-Throughput Vertical Electrophoresis Unit (C-DASE-400-50, CBS Scientific, CA, USA). For each run, a pre-run of 60 min at 350 V and a run of 120 min at 300 V were conducted with ethidium bromide at a concentration of 0.15 ng/ μ l in the lower buffer chamber using an FT-1000 power supply (Fisher FB1000Q). The gel image was taken after each run using the UVP DigiDoc-It Imaging System (Fisher UVP97010501) and bands segregating in the population of 188 individuals were scored manually for linkage analysis.

Annotation of the mapped ESTs

The putative function of the mapped EST contigs was deduced by comparison with proteins in the database. Briefly, the GenBank non-redundant protein sequences database was searched with the BLASTX program for each of the mapped EST contig sequences. A cutoff expected value of 10^{-9} was applied in the BLASTX search process. Putative functions of the EST contigs were then annotated with the GenBank accession numbers of the highest similarities and associated functions if known.

Genetic mapping

SSR marker genotypic data were organized into two independent sets with one for each parent according to the two-way pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). Linkage analyses were performed using JoinMap 3.0 (Van Ooijen and Voorrips 2001) with grouping threshold LOD = 5.0. The Kosambi mapping function was used to convert recombination frequency into genetic map distances (Kosambi 1944). In the map construction process, a few markers were removed from the final maps as their presence in a linkage group made it difficult to determine the linkage phase using JoinMap. Graphic presentation of the linkage maps was generated using a drawing program Mapchart 2.1 (Voorrips 2002). Linkage group (LG) numbers were assigned in accordance with published SSR anchors (Maliepaard et al. 1998; Patocchi et al. 2009a). Mapping of apple scab resistance was conducted in four combinations of datasets, based on two *V. inaequalis* races (1,2) and two major resistance reactions—Chl (chlorotic lesions) and SN (stellate necrosis), i.e., Chl/race (1), SN/race (1), Chl/race (2) and SN/race (2). The first

two (Chl/race (1) and SN/race (1)) datasets were observed in one subset of population “GMAL 4595” of 81 plants, and the second two (Chl/race (2) and SN/race (2)) in the other subset of 107 plants. Susceptible data corresponding to each of the two races were included in the analyses accordingly. Therefore, dataset Chl/race (1) was composed of 38 S and 26 Chl progeny plants and SN/race (1) of 38 S and 17 SN plants. Similarly, dataset Chl/race (2) was composed of 43 S and 23 Chl plants, and SN/race (2) of 43 S and 34 SN. The map positions of the apple scab resistance calculated from the four datasets were represented by *ChlR1*, *SNR1*, *ChlR2* and *SNR2*, respectively.

Results

Development and analyses of the *Malus* EST contig-based SSRs

To expand the pool of SSR markers in *Malus*, a total of 422 *Malus* EST contig-based SSRs primer pairs were screened using the two parents. Of the 422 EST SSRs, 392 (92.9%) amplified a PCR product(s). However, 203 EST SSRs (48.1%) yielded polymorphic bands and mapped to a sum of 207 loci, including 79 specific to PI 613988, 83 specific to Royal Gala and 45 in common to both parents (Fig. 1; Supplementary Table S2). Marker C306 amplified two loci of PI 613988, one on LG 1 and the other on LG 7. Marker C6799 generated two loci of Royal Gala located on LG 3 and LG 11, respectively. C11819 and C3824 are common markers to the parents, but the two markers were mapped to four linkage groups, i.e., C11819 was on LG 1 of PI 613988 and LG 3 of Royal Gala, and C3824 was on LG 3 of PI 613988 and LG 9 of Royal Gala (Fig. 1).

Details of the EST contig-based SSR markers, including forward and reverse primer sequences, targeted SSR motifs, linkage group locations, and marker sizes, are presented in Table 1. To associate the 203 mapped EST contigs with GenBank, the individual GenBank accessions encompassed in each of the contigs are listed in Supplementary Table S1. There are 1,630 individual EST accessions that are covered by the 203 contigs mapped.

Construction of the Royal Gala and *M. sieversii* PI 613988 maps

The Royal Gala map was constructed with 190 markers, including 62 published SSR anchors, and 128 EST contig-based SSRs in the population of 188 progeny (Fig. 1; Supplementary Table S2). Of these 190 markers, 83 were commonly shared with the *M. sieversii* PI 613988 map (see immediately below). With a cumulative length of 1,283.4 cM, the Royal Gala map covers all 17 linkage groups. The mean length of linkage groups was 75.5 ± 16.9 cM with LG 14 the shortest (42.6 cM) and LG 15 the longest (108.8 cM) (Fig. 1; Supplementary Table S2). The average marker density was 6.8 cM per marker with LG 5 the most dense (3.3 cM/marker) and LG 13 the least (12.1 cM/marker). LG 13 has the least number of markers (6) and LG 5 and LG 15 have the most (19). There are two gaps larger than 25 cM in the map, with LG 10 having the largest gap (33.0 cM).

The *M. sieversii* PI 613988 map was constructed with 180 markers (56 published SSR anchors and 124 EST contig-based SSRs), in the same population (Fig. 1; Supplementary Table S2). Of the 180 markers, 83 were shared with the Royal Gala map (described above). The *M. sieversii* map, with a cumulative length of 1,387.0 cM, covers 17 linkage groups. However, the total map length of *M. sieversii* was longer than that of the Royal Gala map by 103.6 cM (8.1%). The mean length of linkage groups was calculated to be 81.6 ± 28.3 cM with LG 3 the shortest (19.5 cM) and LG 15 the longest (154.8 cM) (Fig. 1; Supplementary Table S2). The average marker density was 7.7 cM per marker with LG 3 the most dense (3.9 cM/marker) and LG 6 the least (15.3 cM/marker). LG 3 also has the lowest number of markers (5) while LG 10 has the most (19). There are six gaps larger than 25 cM in the map, with LG 14 and LG 17 having the largest gap (35.7 cM).

On average, there were 4.9 ± 2.2 common markers bridging the 17 homologous linkage groups, with LG 7 having the least (one) and LG 17 the most (10) common markers (Fig. 1; Supplementary Table S2). Comparison of the linear orders of the 83 common markers suggested that the marker orders are largely conserved between the *M. sieversii* (PI 613988) and *M. × domestica* (Royal Gala) maps. However, non-collinear orders were observed not only in the homologous linkage groups, including linkage groups

4, 5, 8, 9, 10, 12, 15, 16 and 17, but also in non-homologous linkage groups, i.e., marker C3824 was mapped to LG 5 of *M. sieversii* and LG 9 of Royal Gala (Fig. 1).

Thirty-eight (21.1%) and 40 (21.1%) markers with segregation distortion ($P = 0.05$ – 0.00005) were found in the *M. sieversii* and Royal Gala maps, respectively. Distribution of these markers did not appear to be random. In *M. sieversii*, six linkage groups (1, 3, 8, 10, 11 and 17) had three or more (up to eight) markers with segregation distortion, accounting for 34 out of the 38 markers. In Royal Gala, 28 out of 40 markers with segregation distortion were similarly clustered in five linkage groups (3, 7, 10, 12 and 17), each of which had also three or more (up to nine) distorted markers. There was no segregation distortion evident in eight linkage groups (2, 4, 5, 6, 7, 12, 15 and 16) of *M. sieversii* and four linkage groups (2, 4, 5 and 14) of Royal Gala (Fig. 1; Supplementary Table S2).

Annotation of the mapped ESTs

The putative functions of these mapped EST contigs are diverse, including regulating plant growth, development and response to environmental stresses (Supplementary Table S3). A few examples are given below: C4576 (LG 7 of PI 613988)—a putative auxin response factor; C7524 (LG 9 of PI 613988)—a WRKY transcription factor; C13449 (LG 6 of PI 613988)—a putative F-box family protein; C17597 (LG 17 of Royal Gala)—a putative serine/threonine-protein kinase; C14133 (LG 9 of Royal Gala)—a putative MYB transcription factor; and C3656 (LG 16 of Royal Gala)—a stress response suppressor. However, there are 21 (10.4%) mapped EST contigs returned with no significant similarities with the cutoff expected value of 10^{-9} , suggesting that genes represented with these contigs are likely unique to *Malus* (Supplementary Table S3).

Mapping of apple scab resistance

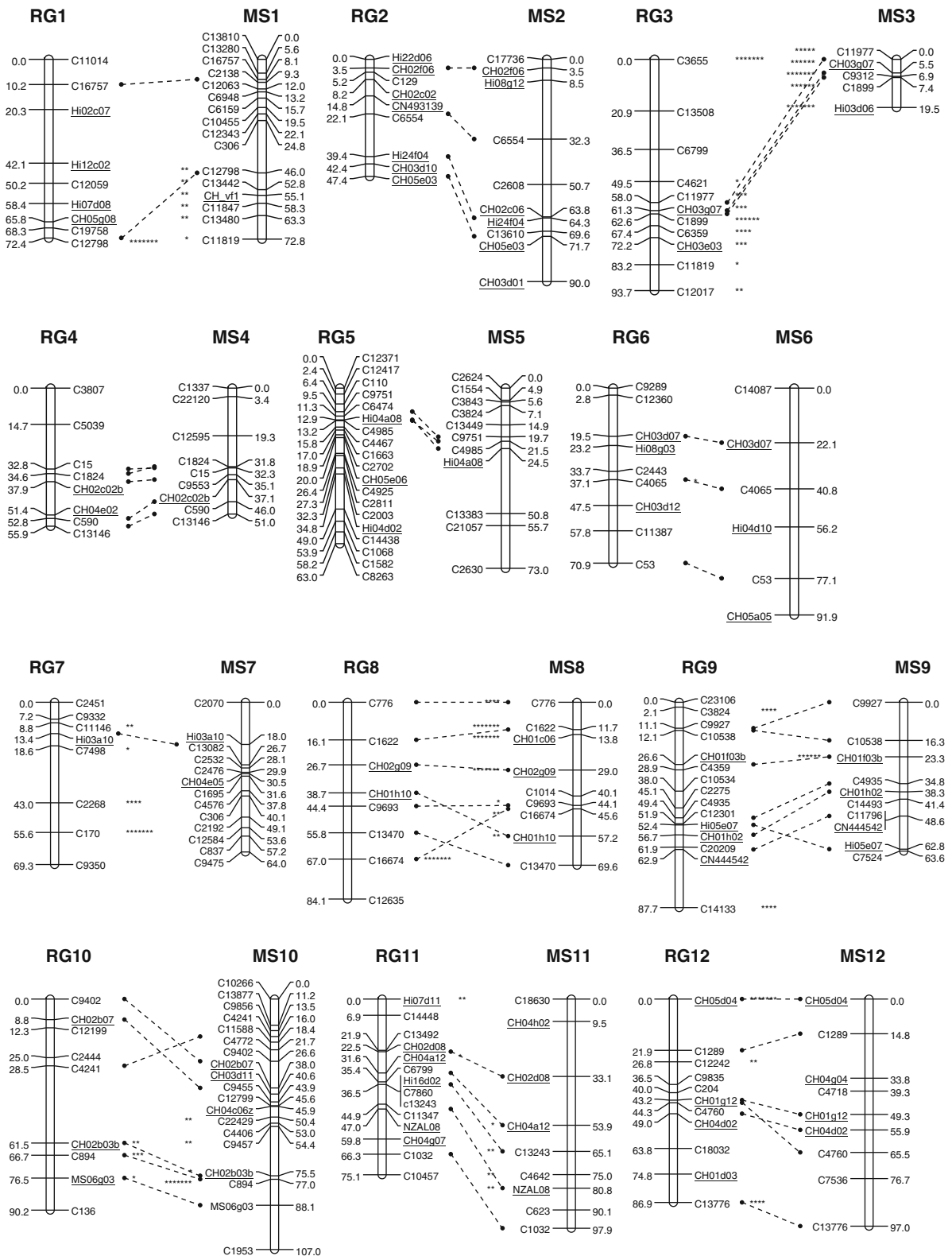
The two subsets of population “GMAL 4595” showed different responses to *V. inaequalis* races (1) and (2) (Table 2). In the first subset of 81 seedlings inoculated with race (1), 38 (46.9%) susceptible and 43 (53.1%) resistant plants were observed, suggesting a pattern of 1:1 ($P_{(\chi^2 > 0.309)} = 0.58$) segregation for a single

Fig. 1 Genetic maps of Royal Gala (RG) and *Malus sieversii* PI 613988 (MS). The linkage groups are numbered following previous publications (Maliepaard et al. 1998; Patocchi et al. 2009a), but by prefixing the numbers with RG for Royal Gala and MS for PI 613988. The names for the published SSR anchors are *underlined*. The newly developed *Malus* EST contig-based SSRs from the Genome Database for Rosaceae (GDR) are named with their corresponding contig numbers in an abbreviated form; e.g., the SSR developed from contig ‘malus_v4_Contig10052’ in GDR is named as “C10052”. The *dotted lines* are used to collect identical loci between homologous linkage groups. *Asterisks* indicate significant levels of segregation distorted markers based on chi-squared tests: * $P = 0.05$, ** $P = 0.01$, *** $P = 0.005$, **** $P = 0.001$, ***** $P = 0.0005$, ***** $P = 0.0001$, ***** $P = 0.00005$

dominant resistance gene. However, within the 43 resistant plants, two distinct types of resistance responses, chlorotic lesions (Chl) and stellate necrosis (SN), were noted with 26 and 17 seedlings, respectively (Table 2), suggesting a possible involvement of two major resistance genes. In the second subset of 107 seedlings challenged with race (2), 43 (40.2%) were susceptible, whereas 64 (59.8%) were resistant (Table 2). A majority of the resistant seedlings showed Chl (23/64) and SN (34/64) types, and a minor fraction (7/64) were contributed by necrosis (N), hypersensitive response (HR) and no symptoms (0) combined (Table 2), indicating once again a possible involvement of two major resistance genes.

To address the distinction in resistance reactions of two major types (Chl and SN) as well as the variation of *V. inaequalis* races (1) and (2) in inocula, genetic mapping of apple scab resistance was independently conducted with four subsets of data, i.e., Chl/race (1), SN/race (1), Chl/race (2) and SN/race (2). Mapping with the subsets of Chl/race (1) (38 S and 26 Chl plants) and Chl/race (2) (43 S and 23 Chl plants) mapped the Chl resistance to 4.4 cM, designated *ChlR1* (*Chlorotic lesion resistance to race (1)*), and 1.0 cM, designated *ChlR2* (*Chlorotic lesion resistance to race (2)*), respectively, downwards from marker C2608 on LG 2 of PI 613988 (Fig. 2), indicating that *ChlR1* and *ChlR2* are tightly linked with a genetic distance of 3.4 cM.

For the datasets SN/race (1) (38 S and 17 SN plants) and SN/race (2) (43 S and 34 SN plants), the SN resistance was also mapped to LG 2 of PI 613988, but downwards from marker CH05e03 by 2.1 cM, designated *SNR1* (*stellate necrosis resistance to race (1)*), and 5.1 cM, designated *SNR2* (*stellate necrosis resistance to race (2)*), respectively



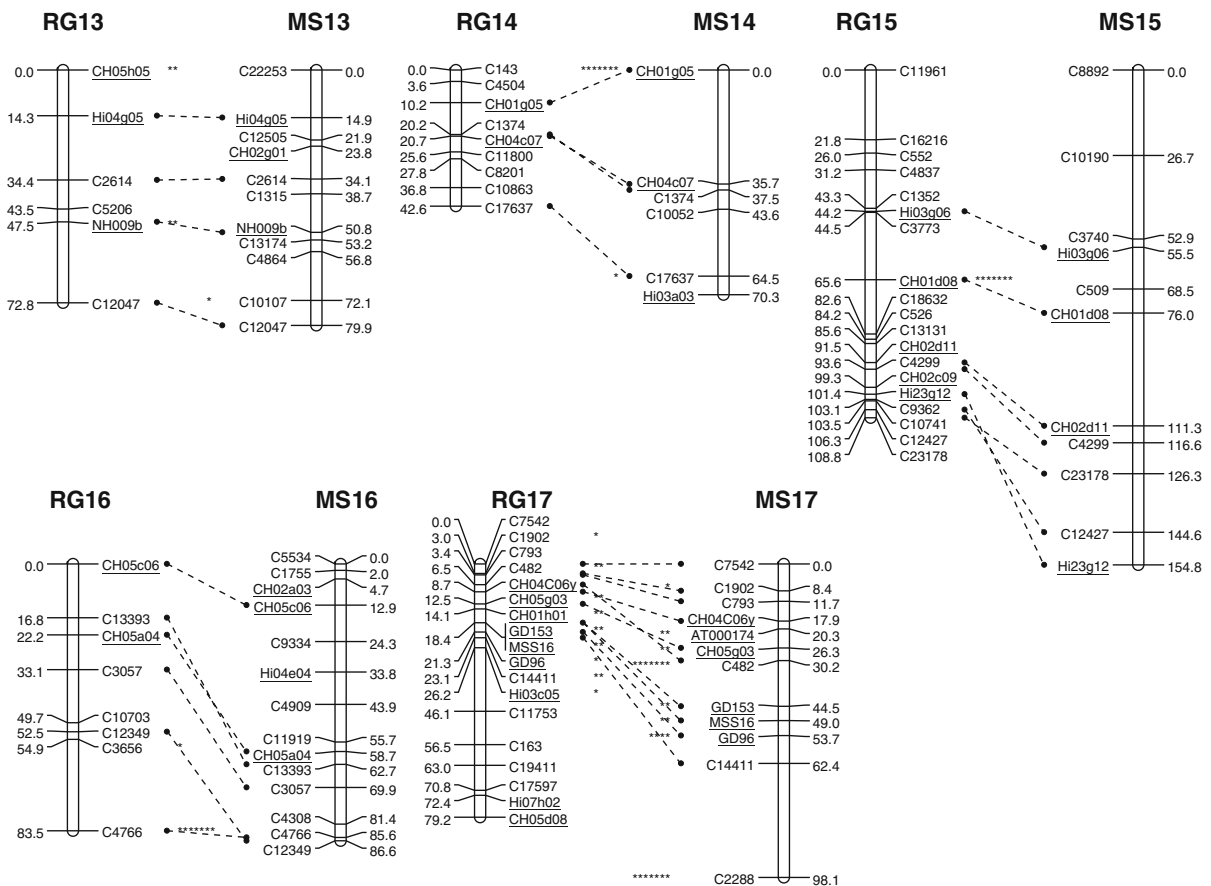


Fig. 1 continued

(Fig 2). *SNR1* and *SNR2* appear to have genetic positions comparable to known genes, such as *Rvi8* (*Vh8*), an apple scab resistance gene also previously identified from *M. sieversii* (Bus et al. 2005a). To examine the map relationship between *Rvi8* (*Vh8*) and *SNR1* and *SNR2*, the *Rvi8* (*Vh8*) closely linked markers OPL19SCAR and OPL18SCAR were tested, and marker OPL19SCAR was successfully mapped within the interval of 3.0 cM between *SNR1* and *SNR2* (Fig. 2), suggesting that *SNR1* and/or *SNR2* may be allelic to *Rvi8* (*Vh8*).

Discussion

Analyses of the published SSR anchors

One hundred and eleven published SSRs were screened and 79 (80 loci) were used to anchor the linkage groups. This was completed in two steps:

first, a set of 82 SSRs was chosen from a core set of 88 SSRs often polymorphic across domestic apple varieties (Patocchi et al. 2009a). Of this set, 77 (93.9%) amplified bands and 62 (75.6%) were mapped successfully. Second, a set of 29 SSRs were selected from other maps (Celton et al. 2009b; Fernandez-Fernandez et al. 2008; Gessler et al. 2006; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) to fill the gaps in linkage groups 1, 6 and 17, and to examine the relationship between *ChlR1*, *ChlR2*, *SNR1* and *SNR2*, and the six scab resistance genes on LG 2 (Gessler et al. 2006). Of the 29 SSRs, 24 (82.7%) produced amplicons and 17 (58.6%) were mapped, resulting in a much lower rate than the first set. These results suggested that the core set of 88 SSRs (Patocchi et al. 2009a) is an effective starting set of markers for apple linkage group anchoring and framework map construction, including *M. sieversii*. Marker Hi03a03 from the second set was mapped to LG 14 of PI 613988, different from LG 6 as reported

Table 1 Names, primer sequences and other details for the 203 *Malus* EST contig-based SSRs developed

SSR name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Motif	Linkage group ^a	Type of marker ^b	Expected size (bp) ^c	Estimated size (bp) ^d
C10052	TTCAGGAATGTCAATTTGCG	TGCAGCCATGGATGAAGTAA	(ag)14	MS14	SL	105	100–140
C10107	AGCACCATGGTAAGCCAAAG	GGATTCGGTCCTCTAGCC	(at)22	MS13	SL	275	240–250
C1014	AAACCCCATGGCTTTTTCTC	TCTCATGGAGTTGTGCAAGC	(at)21	MS8	SL	238	180–240
C10190	CGGAAGTGGAAAGCTGAAAG	GACTGAGTTTCGCAAGAGGG	(tc)20	MS15	SL	169	150–185
C10226	CCCTAACCCCTAACCCCTAACCA	ACGGCTTTCCATTGTCATAC	(ct)22	MS1	PML	263	210–270
C1032	GAGGGTAAAGCTCCCTCAAC	GGGTTATTGCTCTGACGAGG	(tc)13	MS11/RG11	SL	286	400–440
C10455	TTCACACCGAAAGCTCTCT	AAGGCAATACAAGACGGACG	(ct)12	MS1	SL	221	200–220
C10457	CCGCCTTACTCTCTCTCAA	GAGAATGAACGGAGGATGGA	(ag)23	RG11	SL	270	105–140
C10534	CATGCTGCATTCAAGGAAGA	CAATCCATTACACATGCGGT	(ag)11	RG9	SL	251	330–375
C10538	CATGCTGCATTCAAGGAAGA	AAAGGACAGGAAGAAACCCA	(ag)18	MS9/RG9	SL	229	220–235
C1068	GGCCATCATCCATCTTTTTTC	TATGTAAGGCCACCCCATTTG	(ag)21	RG5	PML	270	265–300
C10703	GAATCCAAACAACCCCATTTG	ATCCATCTGCGGTTTTGAAG	(cag)12	RG16	SL	287	270–320
C10741	TCTCTGTTTTTGCTCGTTCCG	ACTTCTCTTGCTCCGCATA	(ga)21	RG15	SL	145	140–160
C10863	TTGGCTCCCTCTAAACCGTA	AGCTTGAACCTGTGCTAGATG	(ta)16	RG14	SL	187	180–210
C110	TACTGCTGGCACAAACTTCC	TCACCTCTCTCCCTCTGCAT	(ct)11	RG5	SL	117	100–110
C11014	TGAAAATTTTGTTCGAGGGC	CTCCCACCTTTCTCTTGAC	(ct)11	RG1	SL	212	300–330
C11146	GTCCCAACTGCAAGGAGTA	AAGGAGAGAGAAGGAAGCGG	(ct)12	RG7	SL	155	150–170
C11347	GCTCTGATTCCCTCAATCACT	TTTCAGGAAACGGCAATAC	(ct)15	RG11	SL	199	180–210
C11387	TTGCTCCTCTAAACACTCGGA	GGTTGTGGCTCATCCACTTT	(ct)12	RG6	SL	268	900–980
C11588	CAACCTTATATTTCCCTCCC	GCGTTTCTCACTCGAAAAGG	(ct)16	MS1	SL	208	200–235
C11753	GGCAAAACGAAGGTTGCTAT	CGCCGTTATCTGTGCTGTA	(ct)16	RG17	SL	142	140–170
C11796	CCAGTTGCAGTGGATTGATG	GAATTGAATAAGCCAGGCCA	(ct)25	MS9	PML	151	120–160
C11800	GGACAAGTCACAAAAACCAGA	GTCGGAGAAGCTTCCATTTG	(tc)12	RG14	SL	107	80–105
C11819	GTGGCATAAGCAATGCTTGAA	AGCACAAAAGGAGGTTGCACT	(ca)13	MS1/RG3	ML	218	210–240
C11847	AAGATCGCAAATTTACCCCC	TTCAACGAGAGAGCAGAGCA	(tc)14	MS1	SL	142	140–170
C11919	GCTGCATCTCTGCACTTGT	TGCACGTCTCTACGAACTG	(ga)13	MS16	PML	223	200–260
C11961	TCCCCAGTACCCAAACTCTG	AGAATCGCAGCTAAAACCGA	(ag)26	RG15	SL	279	250–300
C11977	ACTCCCTCCCTTCTTTTCA	GGGAGATTTGTTGGGGTTTT	(ct)16	MS3/RG3	PML	257	250–330
C12017	CCTTCACTCTCTCATCCCC	AGAAGATGGCGAGCTGGTTA	(ct)13	RG3	SL	236	400–460
C12047	GCCAAGACTCTTTCATCCAG	TTTTTCGATCTGGGTGGTCTC	(ct)11	MS13/RG13	SL	298	300–350
C12059	TTCTCACAGACAGTGACCACC	TGGTTTGGGTTGAAAATGGT	(ag)11	RG1	PML	129	125–140
C12063	CAAACCTCTCATCGCAACCT	CTTGGAGCTGTGAGAGTCCC	(ct)15	MS1	SL	172	600–670
C12199	GCCCACTTCCACCTTATCTC	GGAACAATGAAGTTGCCGTT	(ct)18	RG1	SL	212	200–220
C12242	TGAAATCACCTCAACCCCTC	GCCAATTAATAGGTGGCGA	(cac)10	RG12	PML	144	300–350
C12301	TGGAGAAGTGCAAAGTGCAA	AACTGGTTTTCCCACTCCC	(tc)15	RG9	SL	277	1000–1200
C12343	GCCAACACTCACTACTTCTC	TTCGTCTGGCCTTTCAACT	(ag)15	MS1	SL	115	110–140
C12349	TTTCGGAATTCGGACCT	TCTTTCTGTGGGTTTTGG	(ct)16	MS16/RG16	SL	120	90–125
C12360	ACCCTGCTGCTCTGGAAGTA	GAATGAGACCCCAATCTCA	(ct)17	RG6	SL	130	280–305
C12371	TTGTTGTGCTTAATGCTCCC	CCCACAAAGCTCACGAATTT	(ct)14	RG5	SL	161	155–190
C12417	GCTTCGTATTCGAGGGGG	CAAGGAAAGATGGGGTCTGA	(ga)11	RG5	SL	164	160–200
C12427	GAGAGAGACCACCAGAAACA	ACTTCTCTTGCTCCGCATA	(ag)17	MS15/RG15	SL	167	150–175
C12505	TATTTGGCAAACCATCTCCC	ATGCGCTTGTTAATGAGGCT	(ct)18	MS13	SL	268	270–280
C12584	AATCGGACCGTTGTTTTCAAG	TGTCCTCTTGAATCCCTG	(ct)11	MS7	SL	102	145–175
C12595	AAACCATACACAACGCCACA	ATGAAAACCCACAAAACCCA	(ct)11	MS4	SL	274	230–280
C12635	CAAATCACAACAGCCAGAGC	CCATGGGAGCAGCTGATAAT	(tc)14	RG8	SL	186	180–240
C12798	TCTACCCTGTGTTTTGGG	GGAAGTGGGAGGGGAGATAG	(tc)13	MS1/RG1	PML	185	205–260
C12799	CCCACCATATACCTCCATCG	CATCAGGCCTTTCTCTTTTCG	(ga)18	MS1	PML	180	160–180

Table 1 continued

SSR name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Motif	Linkage group ^a	Type of marker ^b	Expected size (bp) ^c	Estimated size (bp) ^d
C1289	TGCCGCATCTGAAGTGAATA	ATCTTCGGCTCCATTTTCT	(ac)14	MS12/RG12	SL	222	200–230
C129	CCAAGGATTAGAGACGCAGC	CGCTCTGTGACAAGAATTGA	(ga)12	RG2	SL	224	210–225
C13082	TCAACCCGATACCAATTTCC	TACCCAATAAAAACGCCAGAG	(at)15	MS7	SL	234	220–270
C13131	GCAGCAGAGCACAGACGAT	GAGGAGGGAGAGGGAGAGAA	(tc)11	RG15	SL	201	185–220
C13146	GCTTTCCTTTCTTCTTCAA	CTGGGAAAAATGGGGAAAAAT	(ct)20	MS4/RG4	SL	182	175–200
C1315	CCTCCTTGAATTCTTCTCTCC	CAATCAAGGAAAGCTGCACA	(ct)11	MS13	SL	137	130–150
C13174	TTACCTTCTCTTCCCTCCG	GGGATCAATGAGCAAGCATT	(ct)11	MS13	SL	182	180–220
C13243	ACCCCTTCTTTCTTCTTCAA	TTCTTTGGCTTGGTCTTGCT	(ct)13	MS11/RG11	PML	112	90–120
C13280	CCTTCACTCACCTTTCTCGC	CTCCTCTCCCTCAGTACCC	(ct)14	MS1	SL	294	250–300
C1337	AGAGAGATGAACCGCGACAT	TGAACGAGACAAACTGTGGC	(ct)13	MS4	SL	164	170–200
C13383	GCGTGGCATTTCGTATTT	CAAAGTCGCGGTGGTTTTAT	(tc)14	MS5	SL	156	140–185
C13393	CACACTCCATCTCTCATTTC	ATTGATAGGCTTGTACGGCG	(ct)15	MS16/RG16	SL	191	180–220
C13442	TGTGAGACCTCCCTCCCTC	ATCAGTTGGAGGTCAATGCC	(ct)17	MS1	PML	199	170–200
C13449	GACCATGGCCATAACAATC	GGGATACGCATGCCTTAAAA	(cac)10	MS5	PML	163	150–165
C13470	TCGATTCTCAATCTCTCTCA	ATCGGAGAAAACCCAAATCC	(ct)16	MS8/RG8	PML	239	230–280
C13480	ACGAATCTCTCTCAGCGCA	GATTTCGGAGGGGAGAGAAAAG	(ga)12	MS1	SL	236	235–285
C13492	AATGAAGTGTCCCATCGAC	GTCCAGCTCCCCAAATTGTA	(ga)13	RG11	SL	147	140–170
C13508	TTCTTCTCTTTCCCTCTCC	TTGGAATTTGGATTGGTGGT	(ct)14	RG3	SL	272	290–320
C1352	TCCTCAGAAAGCCGTTTCGTT	GAGAGCCTCTAGAGCAGCA	(ag)15	RG15	PML	192	190–220
C136	GCACTTGCAGGCCAATAACT	TTGTTGCTGCGAAACAAAGT	(cat)10	RG1	PML	119	100–120
C13610	TCCCCTTCTCCTTTCGATT	GAAAGCATCAGGCGTTTCAT	(ga)13	MS2	SL	180	160–210
C1374	CGGATCACAGACGCCAT	GCGTCATTCAACAGCTTCA	(tc)13	MS14/RG14	SL	197	170–230
C13776	ACCCCACTTCTCAAATTTCC	GGCACAGCTAGGATCTGCTC	(tc)17	MS12/RG12	SL	298	140–160
C13810	CAGGACTCTAAGGACTGCCG	CGTCCTAGATAGATGCCCCA	(ta)13	MS1	SL	203	220–250
C13877	TTTCTTTGTGAGATTCCGGC	TGAGGAGTTTTATGGGCCAG	(ct)14	MS1	SL	204	205–255
C14087	CACCGCGTCAAAAATACCTT	CTTGTGTTTCCCTCCCAAA	(tc)12	MS6	PML	232	210–280
C14133	CTCTCTGATGAGGGCGTTTC	TATTACAGCCGACACCACCA	(ct)21	RG9	SL	161	350–360
C143	TTAATTGGGTCTGAAAGCCG	GAAGAATGTCCGAAAGTTCGC	(ct)12	RG14	SL	292	260–320
C14411	GCCTATGGCTGTTTGAGAGG	TTGCCATCCATGTTTTCTCA	(ct)15	MS17/RG17	PML	248	230–255
C14438	CCTCACTCAGAGTTGGCAGA	GTGAAGACGAGATGCTGGGT	(tc)19	RG5	SL	205	190–210
C14448	CTCTAACCTACGCTGCTGGG	TGTGGACATCAAGCTTCTGC	(ga)17	RG11	SL	258	220–300
C14493	ACTGCAACCACACCACACAC	ACAAGGGTGGAGGAAGGTCT	(tc)20	MS9	SL	185	180–190
C15	CAGACTCTGCAACCCCTCTC	TTGCGAGAAAGCTAAAAACCA	(tc)14	MS4/RG4	SL	180	170–190
C1554	GCTTCAATCACTTCGCAAAT	TTTCCAGCCAATTCAAAAC	(ct)13	MS5	SL	276	380–420
C1582	GAACCCAGACCAGACCAT	TTTCTTCCCACCCATCTCAG	(tc)17	RG5	SL	159	150–170
C16216	GCATTAACCCTGTCCCAGAA	TGTTTGATTCAAGCTGGCTG	(aag)17	RG15	SL	189	360–380
C1622	TCTGACACGGGATAAACGAA	ACTTCAATCCCCCGAAGTCT	(ag)16	MS8/RG8	SL	274	270–290
C163	GCAAAAATTTCTGGAGAGAGG	TGCAAGATCAGGAACACCAG	(tc)16	RG17	SL	254	260–280
C1663	GGTGACTCCTTCTCCACCAA	GCTGAAACTGGCATGGTTTT	(ct)15	RG5	SL	117	95–115
C16674	AAACGGGTGCACAAAGAAAAG	GAGCAAGATGGCCGAGTTTA	(tc)23	MS8/RG8	SL	289	280–300
C16757	AATGGGACCCAACCTGGTACA	TCGACCATACAAATTGCTGC	(gta)13	MS1/RG1	PML	279	280–300
C1695	GTATTCAAGCGGATCATTCCC	TCGACTCTGGCCCTTCTCTA	(ct)12	MS7	SL	181	320–360
C170	TCAAGTGCAGATTCAGACCG	TTGCGAAGCTCGCTGTATAA	(tc)11	RG7	PML	276	270–310
C1755	TCCCTCCCTACTCTCAAACG	AGAAGACGGGAGGGGTAATA	(ct)20	MS16	SL	196	180–210
C17597	TCCTTTCGCTGGTGTCTCTT	GGGGTGTCTGTCAAGTGTGTG	(tc)18	RG17	SL	145	125–150
C17637	TAGATCGTAGGCTGGGATGG	CCAGCAGAAAAGCAAAAGACC	(ag)30	MS14/RG14	SL	258	225–285
C17736	TTGTGTGTGTGCGTGTTT	GGGGTTGGAATTTGATGATG	(ta)19	MS2	SL	232	200–240

Table 1 continued

SSR name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Motif	Linkage group ^a	Type of marker ^b	Expected size (bp) ^c	Estimated size (bp) ^d
C18032	CAACACTTCCAGGGCCAC	TTGGGAAATTGGGTGTGTGT	(tc)19	RG12	SL	251	240–260
C1824	CAGACTCTGCAACCCCTCTC	TTGCGAGAAAGCTAAAAACCA	(tc)17	MS4/RG4	SL	186	160–200
C18630	AATGCCTAACGAAGACACCG	GCTCTAGCCAAAGTGCCATC	(tc)20	MS11	SL	296	180–300
C18632	GCTTCTCAGCAGCTCAGTT	AGGAAAGGGAGGAGGTGTGT	(ct)19	RG15	PML	209	200–210
C1899	TATCCGCCCTTACTCCCT	ACACCACCTCCCAGAACTTG	(tc)12	MS3/RG3	PML	215	185–250
C1902	CCTCTGCCTCTCCAAAATA	ATCGTCGTGACCAGAAGGAC	(ga)19	MS17/RG17	SL	203	185–205
C19411	CTCCCTTTTCTACCGAGTCC	AAGTTCGCAACCGATCAAGT	(ta)18	RG17	SL	206	175–205
C1953	AGAACAGAGGGAAGCAGACG	TCACATTCTCCATGGCGTTA	(aga)10	MS10	SL	248	240–260
C19758	CCAACCGCCAAAAGTAGAAA	GGCGGCCATACAGTAAAAAGA	(tc)20	RG1	SL	276	265–290
C2003	GAGACCGTGGACAAGCAAGT	AAGTGAACCTCAGACACCGCC	(tc)20	RG5	PML	276	260–280
C20209	AATTTGTTCCGAGAAGTCGC	ATGGATCAGTGAGGCAGAGG	(ga)65	RG9	SL	242	280–300
C204	GCTCTCTCAGACAACGCACA	GAATTAGGCGGCAAATTTCA	(ct)16	RG12	SL	287	270–320
C2070	TAACCAAAACAAAAGCCACCC	GGGAGTAGATGTGACCGAA	(cca)10	MS7	SL	274	260–280
C21057	AAGCCATGGATATTTGCAGC	GGCCATATATTGTTGCCTTTT	(at)21	MS5	SL	243	210–250
C2138	GCTCCAGGACAAACCACCTA	CAACCGGAGGGACAAAGATA	(ct)11	MS1	SL	265	260–290
C2192	CAAACAATCCGAACACAACG	TTCAGGAAGCTGGTTTGCTT	(tc)11	MS7	PML	122	700–750
C22120	TCATCAAGTATCCGCGACCT	GGAAGGGGGAGAGCATAAAG	(ct)18	MS4	SL	127	120–150
C22253	CTGCTCACTCGGATTTGTGA	TATGTTGGCCATGCTTTGAA	(atc)10	MS13	SL	281	350–370
C22429	GTGGCTGGTGGATTTCTGTT	TCATGTTCTTCTCCCTTG	(tct)12	MS1	SL	246	220–240
C2268	ATCTTCGTCCAAAGCAGCAT	TTGAAGAGTCTTGGGCGAGT	(ag)23	RG7	SL	195	170–200
C2275	AATTGTCTCTGGGTCGGTG	GATGGTGAGGAGAGTCCAGG	(tc)22	RG9	SL	238	220–255
C2288	GCCGTGGTACGTTTGCTATT	AGGGCACCCACCATTTTTAC	(tg)11	MS17	SL	259	260–340
C23106	CCAACCAAACCCCTTCTCTCA	CGGAGCTTGATAGTGCTTC	(tc)19	RG9	SL	160	140–160
C23178	CTTTGGGCCAACAAATCAAT	GCAACATTACAAGGACGCAG	(ag)22	MS15/RG15	PML	224	220–240
C2443	TCCTCACCTCTCGTACCACC	GCTTTGCCCTAACGATGAAC	(ct)18	RG6	SL	128	120–130
C2444	GCATGAACCTCTCCACCTC	GGTTTGCAGAAAAGGACTGC	(cca)11	RG1	SL	136	130–165
C2451	TATCCGGAAGTGAAGTGGTG	AGTTTTTCGCTTTTCCGACA	(ct)17	RG7	SL	267	480–500
C2476	AGTCACTGCCCTCTCACTCG	TAGAGAACAGGGCGTCGTTT	(tc)13	MS7	SL	156	140–160
C2532	ACATGTCTAAGGGTCTCAG	CTCCATACACTGTTGGGGCT	(ct)21	MS7	SL	147	130–145
C2608	GATTGGTGTGGTTCTTGCT	AAAGCTTTTGCAGTTGCCAT	(tc)13	MS2	SL	283	260–280
C2614	CGTCAGGCTTTTCTCTGCTT	GGATAAAGGGCTCGGAAAAG	(tc)17	MS13/RG13	PML	171	150–175
C2624	CTCCGAATTC AACCCCTCAA	CATCGTCATCGTCTTCTT	(ct)18	MS5	SL	161	160–180
C2630	CCTCCATTTACAACCAAAGGG	CAGCTTTTCTGTCCGAAGG	(tct)9	MS5	SL	220	200–230
C2702	CTTGGGAGGGTAAAATGGCT	TGCGGCCCTAATATTGTCTT	(ct)19	RG5	SL	215	220–230
C2811	CAGAATCCCCTCTGTCAAA	TGACCGAAAAGGCAGAAACT	(at)18	RG5	SL	216	195–240
C3057	TTCCAATACACTGCTCATCCA	TGAAATGGAGAGGAACCCAG	(tc)12	MS16/RG16	SL	183	170–185
C306	CTTTCTCTCCCTCAATCCCC	CGTCTGTTTGCAAGTGAGA	(ct)13	MS1/MS7	ML	110	80–120
C3655	CCTTTCTGCTGCCTTTTAC	ACTGAGGCTGCAACATACC	(ct)17	RG3	SL	292	270–340
C3656	AATGGTTCGCAAACCTCAAAG	TGGCTCTGATTATTTTCGG	(aag)9	RG16	SL	289	230–320
C3740	AAGCAAAGAGAAAGCAAGCTG	CGTTCCTTGTAAAGAGCCG	(ag)13	MS15	SL	233	205–240
C3773	CGAGGGCACGAGGAATTA	GAGAGGCAGAGAGGCAGAGA	(ag)11	RG15	SL	279	260–280
C3807	TTCAGAAAACCCACACTCA	TTTGCCTCGATGCTGAAAC	(ag)12	RG4	SL	269	480–500
C3824	CCAACCAAACCCCTTCTCTCA	AGTGAGGGAGTGAAGGAGCA	(tc)13	MS5/RG9	ML	275	240–270
C3843	GCTTTGGCCTATGTCCTCAC	GGATACCAACAGCAGGCATT	(tc)16	MS5	SL	164	120–170
C4065	CTCCGTGAACCTGTGAAAT	AGGAGGAGGAGGAAGCAGAG	(ct)18	MS6/RG6	PML	187	175–205
C4241	GATGGGAAATTTGGGGTTTT	ATCAATTGGAATCCCAACCA	(ag)15	MS1/RG1	PML	251	350–390
C4299	ACCACAGCGCCACAAAAAGT	GACGGTTCTGGTTCGACATT	(tc)16	MS15/RG15	PML	144	130–150

Table 1 continued

SSR name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Motif	Linkage group ^a	Type of marker ^b	Expected size (bp) ^c	Estimated size (bp) ^d
C4308	TAACACCTCCCCTCCTTCCT	TGATGAGACCCAGAACGACC	(tc)11	MS16	SL	188	180–210
C4359	GCCTCTCTCGTTAAACCT	CCGAGGCGGTGTATAACCTA	(tc)17	RG9	SL	290	260–300
C4406	ATATTCTCAGCCACCAGCCA	GTACGGGGAGGGAGAGAGAG	(ct)20	MS1	PML	123	100–140
C4467	CCTCACTAAACGCATTGCAC	ATTTACAGCAGCCAAATGACC	(ag)15	RG5	SL	144	120–145
C4504	GAAATAACATTTTGACCGCCA	CAGAGCTCAATTCGTGACA	(tc)18	RG14	SL	272	290–300
C4576	TTCTTGTTTCGTAATGGGGC	GGAGGAGCAGAGAGCAGAGA	(ttc)14	MS7	SL	158	155–205
C4621	CCAACCTCCTCTCCCGTTT	CCAGTACTCTGCTGGGCTTC	(ct)12	RG3	PML	125	100–135
C4642	GAGCAGTTGCAACAAGTCCA	GTGGAAATGGCTAAGCAAGC	(ag)13	MS11	SL	247	230–265
C4718	CGTGGACTCCCAGACAAAGT	GAGCCAAAGAAAGTAGGGGG	(ct)13	MS12	SL	126	120–140
C4760	AGCTCTTCACATCACCACA	CAAAAGGGTGCCAATGAACT	(tc)11	MS12/RG12	SL	221	200–250
C4766	TCACTCCCTCCAAGTTTTGC	GACCGAGTGCAGAGAAAAGG	(ct)17	MS16/RG16	SL	129	110–140
C4772	CATATCGCAGTCTCAGTGGC	CTCTCCCTGAGCCAAACAG	(tc)11	MS1	SL	137	125–160
C482	CCTCTACACCTACCCCTCC	CTTGAATTGGAAACATGGGG	(tc)19	MS17/RG17	PML	212	200–250
C4837	CATCCTTGCAACTTTCACCA	GCTTTGGGTGCTGAGTTTTTC	(tc)15	RG15	PML	151	140–160
C4864	AAGCCCTGAAAATCCAAACC	ATCGGACTGTGACCCTTCTG	(ga)15	MS13	PML	228	205–220
C4909	AGCTCTGGTTTTTCTGGGGT	TGACCGATGAGCTGTCTCAG	(ag)15	MS16	SL	231	220–250
C4925	ACTCCCACAGACACTCAGG	CCAGGTATAAGCGTCGGTGT	(ct)17	RG5	SL	268	400–450
C4935	TTTCCAGCTGAAAACCTCG	GCAGAGAAATCCGCAGAAAC	(ct)14	MS9/RG9	SL	252	250–265
C4985	GGGGGCACAGAAACCTCAT	CATTGTGAAACTGAAGCCA	(tc)12	MS5/RG5	SL	148	100–160
C5039	TAATTCGCTCCCTCTCTAT	GCCAATGCCTGTAGAGAGC	(tc)16	RG4	SL	136	120–140
C509	TCTTCACACCTTCAATCCC	GGAGAGCTGAAGAGCCAAGA	(cat)10	MS15	SL	143	130–145
C5206	TAATGGCGGCTCTCAGTCT	GCGAGCAGAGGTAGCAAAC	(ct)14	RG13	SL	294	270–300
C526	CGATACGAGTGGGTTCGATT	CTGGCGAAGAACGGAECTTA	(tta)10	RG15	SL	158	130–160
C53	GCCACTGTGGGTGCTTTAT	AAAACATGCTGCTGTTGGAA	(ct)14	MS6/RG6	SL	192	170–220
C552	GGGGATGATGCTTCAACAC	CCGACAGAGTTGCAGAACAA	(tc)11	RG15	SL	300	500–540
C5534	GAAGAGTACGCTTGATGGGG	TTGGGTTTGTGGGACAAAAT	(ga)15	MS16	SL	125	125–140
C590	TCACTTCAGAGCCGCATTAG	CCATGAGAAGGCTTGGTGT	(ag)11	MS4/RG4	PML	281	240–270
C6159	CCATCTCATTTTCACTCCC	GGCCAAGACGAAATCGAATA	(tc)11	MS1	SL	188	180–200
C623	GGGTCTAGTGAGGGAAAGG	TTCTGCGGGGAAGATTACTG	(tc)13	MS11	SL	124	120–150
C6359	TGGGACGGACACACACAC	CGGAAATGGTCACTGGAAC	(tc)11	RG3	SL	238	230–270
C6474	CCAGGCAAAAATAGAAAAGGG	CTGATTTCTCTGACTCTGCC	(ct)12	RG5	PML	287	280–350
C6554	TCAGAGCAATGGAATGTGGA	CGAGAGAAGAGGAACATCGAG	(tc)15	MS2/RG2	SL	282	290–310
C6799	GAGGGACGTGAGCAACTAC	GCCAATCTTTCGTTTTTGGT	(taa)10	RG3/RG11	ML	292	240–260
C6948	CAAACCTCTCATCGCAACCT	CTTGGAGCTGTGAGAGTCCC	(ct)15	MS1	SL	157	600–660
C7498	AATGCCAAAATTACAAGCG	CAGACTCGACTTGCCTTCC	(cac)11	RG7	SL	267	250–320
C7524	TACTACCACCGCCTTGTTTC	AGCTCTAATGGGAGGATCTCA	(at)13	MS9	SL	220	200–215
C7536	AACGCCAAGAGAAAGTGGA	GGAAGGAGGGAGGAGAGAGA	(ag)12	MS12	SL	206	200–240
C7542	CCCTCTCTCCTCTGCCTCTT	ATCTGCGTCTTATGAACCG	(tc)15	MS17/RG17	SL	221	205–235
C776	GAGGCACCATCTTGCTCTG	ATCTGGGAAATCTTGGGGAG	(ag)13	MS8/RG8	SL	103	80–110
C7860	TTCTTTTGCCCAAGCATCA	GGCTATCGGATAATGGGGTT	(ga)13	RG11	SL	149	145–160
C793	ACGAGGCCCTCCTCCAC	GAGCTTGGTGGGTTTGTAGA	(ct)18	MS17/RG17	SL	192	190–220
C8201	CATCAAGCGTGTGGTTATGG	CAAAAGCAAGCAAAGCATCA	(ta)12	RG14	SL	176	150–170
C8263	TGAGGATCGGGAGTTGTACC	CCCCATTCCTTCTTCCCTTC	(ag)11	RG5	SL	289	280–320
C837	GGTCGACACTTCCCAATTCT	TAGCATGCCTGGTCTCTCCT	(ct)17	MS7	SL	163	230–260
C8892	AGACAAGGGCCTGACTAGGG	AGCTTCATCAACGATTGGCT	(ag)16	MS15	SL	170	280–310
C894	GGCTGGTTTTAGAGCGACAC	ATCCCATGACTCACCAGCTC	(ga)20	RG1	PML	193	280–400
C9289	AACATCCAACAACCACACG	GAGCCTTTTTATTTGCAGCG	(ag)15	RG6	SL	131	110–130

Table 1 continued

SSR name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Motif	Linkage group ^a	Type of marker ^b	Expected size (bp) ^c	Estimated size (bp) ^d
C9312	AGGATTCAATCAGCTACGCC	TCCACCAGTGACAAGAGCTG	(ag)18	MS3	SL	153	140–170
C9332	CAGAGCTTTCAACTCGCACA	ATTAGGACCTCCCTCGCATT	(ag)11	RG7	SL	153	150–165
C9334	GGACACTGGTATTTTCGGCA	ACTAGGTGGTCGCTCATTGC	(tc)11	MS16	SL	153	290–310
C9350	ATCTTCGTCCAAAGCAGCAT	AAGAAGCGGAAGAGGAGGAG	(ga)20	RG7	SL	291	270–295
C9362	TCTCTGTTTTTGCTCGTTCG	ACTTCTCTTGCTCCGCATA	(ga)21	RG15	PML	145	140–155
C9402	CCCGACTCAGAAACCCAGTA	CGAAATCGATATCTCGGGAA	(ct)14	MS1/RG1	SL	156	160–175
C9455	CTTCCCGTACATAGGGACCA	CAGTTAGCATTACCCGCATC	(ct)13	MS1	SL	261	280–300
C9457	GTGTTTTCCCTTCAAGCAGC	TGAGGAACCGAGACCAAACCT	(ct)13	MS1	SL	131	140–170
C9475	AGCCATGAAAAGCAATCGAG	GGATCCGAACGTGGTGTATG	(ag)22	MS7	SL	298	300–310
C9553	ACCCAAGCACAAATCATT	GAATGCAAGAATCTGACGCA	(ct)16	MS4	SL	264	270–300
C9693	GGCTCAAAATTCAAAACCCA	GCCATCTACCCACAAACCTC	(ag)16	MS8/RG8	PML	256	250–275
C9751	TGCGAATGAAATCACCGTAA	GCCGGTATAGTATACGCATGG	(at)13	MS5/RG5	SL	254	270–290
C9835	TGATTTTTCCGGCTTGGTTA	CCAGAATAAAATTGGTTCGTCC	(at)16	RG12	SL	278	295–310
C9856	AACCGACAAGGCAACAGAAG	TTGGTCCGACTGCCTAATCT	(ct)15	MS1	SL	300	330–380
C9927	AGGGCCTTGGGCTAGTTTA	ATACACACCCACACGTGCAT	(tg)17	MS9/RG9	PML	264	270–320

^a RG, Royal Gala; MS, *M. sieversii*

^b SL, single locus; ML, multi-locus; PML, presumed multi-locus because of multiple bands

^c Calculated based on EST sequences

^d The allele ranges of Gala and PI 613988 estimated on polyacrylamide gels (6%) by comparison with 20 bp Molecular Ruler (BioRad #170-8201)

Table 2 Evaluation of apple scab resistance in the two subsets of population “GMAL 4595”

Host reactions	Race (1)		Race (2)		Total	
	No. of seedlings	%	No. of seedlings	%	No. of seedlings	%
Susceptible (S)	38	46.9	43	40.2	81	43.1
Necrotic lesions (N)	0	0.0	2	1.9	2	1.1
Chlorotic lesions (Chl)	26	32.1	23	21.5	49	26.1
Stellate necrosis (SN)	17	21.0	34	31.8	51	27.1
Hypersensitive response (HR)	0	0.0	2	1.9	2	1.1
No symptoms (0)	0	0.0	3	2.8	3	1.6
Subtotal of resistance (R) ^a	(43)	(53.1)	(64)	(59.8)	(107)	(56.9)
Total ^a	81	100	107	100	188	100

^a The numbers in parentheses are excluded in the total

(Silfverberg-Dilworth et al. 2006). This discrepancy was likely caused by the fact that the Hi03a03 primers amplified several bands in this study, and thus multiple loci. Indeed, marker Hi03a03 has already been “presumed multi-locus” by the High-quality Disease Resistant Apples for a Sustainable Agriculture (HYDRAS) project of Europe (<http://www.hidras.unimi.it/>). In addition, marker CN493139 could not be confirmed with two closely linked loci (CN493139x and CN493139y) as shown elsewhere (Pacocchi et al. 2009a), but with one locus on LG 2 of

Royal Gala (Fig. 1), which appeared to be consistent with other reports (Celton et al. 2009b; Fernandez-Fernandez et al. 2008; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006).

Development and analyses of the *Malus* EST contig-based SSRs

Advances in genome sequencing technology have resulted in an exponential increase in DNA sequences deposited in GenBank. As of November 2010, there

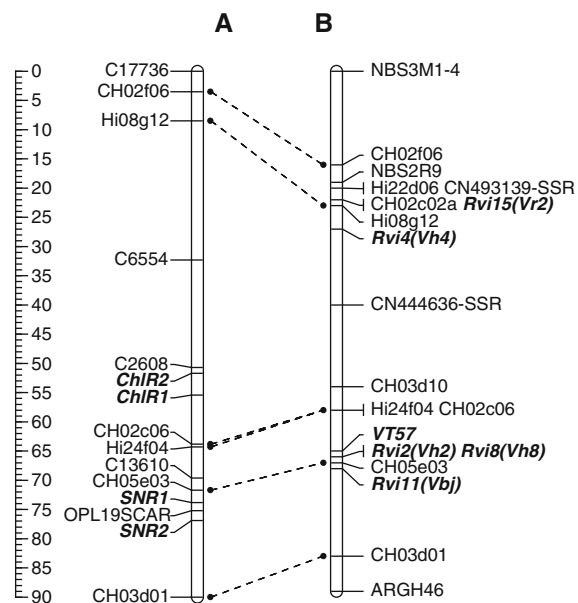


Fig. 2 Genetic mapping of *ChlR1*, *ChlR2*, *SNR1* and *SNR2* and comparison with the six scab resistance genes known on LG 2. **a** Genetic map of LG 2 of PI 613988. OPL19SCAR is an *Rvi8* (Vh8) tightly linked marker (Bus et al. 2005a). **b** Map of LG 2 (Gessler et al. 2006) (adapted with removal of markers for concise and better comparison). The ruler measures map genetic distance in cM

are 335,682 accessions of *Malus* EST in the databases. The *Malus* SSR Microsatellite Analysis Project (Jung et al. 2008) was completed in 2008, and analyzed 260,581 *Malus* EST accessions (>77% of the 335,682 ESTs). This had led to identification of 56,356 SSRs from unique *Malus* EST contigs and singlets, representing a remarkable genomic resource for *Malus*. A test trial of 422 SSRs designed from these unique EST contigs (presumably equivalent to unigenes) resulted in successful mapping of 203 of them in the two parental genomes, yielding a success rate of 48.1%. The 422 EST contig-based SSRs contained an SSR region of at least eleven dinucleotide repeats or nine trinucleotide repeats. The success rate in mapping SSRs with fewer repeats or with complex SSR motifs needs to be determined. Further efforts are needed as well to take advantage of EST-based SSRs available in the GDR database to develop a high density EST-based SSR genetic map in *Malus*. These potential EST SSR markers together with the upcoming sequence-anchored integrated genetic linkage map of apple (Troggio et al. 2010)

would further facilitate understanding of the apple genome.

Malus EST-derived markers have been used in various studies, including SSRs (Celton et al. 2009b; Gasic et al. 2009b; Silfverberg-Dilworth et al. 2006; Yao et al. 2010), SNPs (Chagne et al. 2008); CAPS (cleaved amplified polymorphic sequence) markers (Igarashi et al. 2008), “universal” gene-specific markers (Sargent et al. 2009) and others. A search was performed to investigate if any ESTs reported in the literature were present in the 1630 ESTs mapped in this study. Six ESTs, two unmapped and four mapped, were already reported. SSRs CN849428 and CN857442 were the two unmapped (Gasic et al. 2009b) and were found in contigs C9332 (LG 7 of Royal Gala) and C9927 (LG 9 of both parents), respectively. SNP CN917681 (Chagne et al. 2008) and SSRs CN444542 (Silfverberg-Dilworth et al. 2006), CN898349 and CN943067 (Celton et al. 2009b) were the four mapped and were probably allelic with the four EST contig-based SSRs C2630 (LG 5 of PI 613988), C11796 (LG 9 of PI 613988), C14438 (LG 5 of Royal Gala) and C4576 (LG 7 of PI 613988), respectively (Fig. 1; Supplementary Table S1). CN444542 and C11769 are mapped to the same position on LG 9 of PI 913988, implying that they are allelic markers. Together, this suggests that 203 of the 207 loci defined by these EST contig-based SSRs are reported for the first time.

Several EST markers had a non-allelic locus on other non-homologous linkage groups, including C306 (LG 1 and LG 7), C6799 (LG 3 and LG 11), C3824 (LG 5 and LG 9) and C11819 (LG 1 and LG 3). An SSR marker CH03g12y near marker C11819 on LG 3 was also reported to have a non-allelic locus CH03g12z on LG 1 (Liebhard et al. 2002). Such non-allelic markers were likely caused by extensive chromosomal duplications in the *Malus* genome that had arisen from an apple ancestor genome by autopolyploidization (Velasco et al. 2010).

Construction of the Royal Gala and *M. sieversii* PI 613988 maps

The *M. sieversii* PI 613988 map has been constructed with 180 SSR markers, including 56 published SSR anchors and 124 new EST SSRs developed from *Malus* unigenes. The map covers 17 linkage groups

with a total length of 1,387.0 cM, i.e., 103.6 cM (8.1%) longer than the Royal Gala map, suggesting that the *M. sieversii* accession had a relatively higher recombination rate during meiosis in this population. Compared with existing *Malus* maps and the Royal Gala map constructed here, the majority of the linkage groups were covered. But more markers are needed to improve the coverage of LGs 3 (by extending the length) and 15 (by filling the large gaps). Chromosomal rearrangements do not appear to be extensive between *M. sieversii* and Royal Gala genomes since the map orders of the 83 common markers are largely conserved. Non-collinear orders were seen between homologous LGs 4, 5, 8, 9, 10, 12, 15, 16 and 17 in the two parental genomes. But a similar degree of non-collinear orders was also reported between cultivars of domestic apple (Igarashi et al. 2008; Silfverberg-Dilworth et al. 2006). The *M. sieversii* linkage map of PI 613988, along with that of another *M. sieversii* elite to be published (Lalli et al. 2010), represents the first efforts towards improving the understanding of the genome of the major progenitor species of domestic apple. The availability of this map is a first step towards facilitating the usage of *M. sieversii* in breeding programs, in studies aimed at discovering traits and genes of great economic and horticultural importance.

The mapped ESTs contigs provide resources for identifying candidate genes underlying known QTL and/or major gene loci. For instance, C9751 encodes a putative glutamate receptor (GLR)-like gene and is mapped to LG 5 of Royal Gala. The map position of C9751 is close to *Dwarfing* (*Dw1*), a major gene locus controlling the dwarfing ability of the apple rootstock M.9 (Pilcher et al. 2008). There are 20 GLR-like genes in the *Arabidopsis* genome, and expression of the 20 genes was detected in *Arabidopsis* roots, with five root-specific (Chiu et al. 2002). GLR-like genes may be critical for organization and functioning of the rice primary root apices (Li et al. 2006). At least two GLR-like genes from *Arabidopsis* have functional Na⁺-, K⁺-, and Ca²⁺-permeable ion pore domains (Tapken and Hollmann 2008). This is consistent with observations that overexpression of a GLR-like gene in transgenic *Arabidopsis* led to impaired calcium utilization and sensitivity to ionic stress, and to stunted and bushy stature with large numbers of short secondary inflorescences (Kim et al. 2001). Thus the putative GLR-like gene C9751 may

be a potential *Dw1* candidate gene worth more detailed study.

In addition, C4576 and C17597 appear to be candidate genes for several tree architecture QTL (Segura et al. 2009) and the *Rvi5* (*Vm*) locus for apple scab resistance (Patocchi et al. 2005), respectively. C4576, encoding a putative auxin response factor (ARF), was located 7.2 cM south from marker CH04e05 on LG 7 of PI 613988. The map position of C4576 is likely within the interval of approximately 16 cM between markers CH04e05 and MS06c09 on LG 7 of the Starkrimson × Granny Smith map, where seven QTL conferring primary and secondary growth of apple trees were identified (Segura et al. 2009). C17597, encoding a member of the putative serine/threonine-specific protein kinase family, some of which are plant resistance genes, was mapped 1.6 cM upstream of marker Hi07h02 on LG 17 of Royal Gala, which co-segregates with *Rvi5* (*Vm*), a major apple scab resistance gene (Patocchi et al. 2005).

Apple scab resistance and *M. sieversii*

Eighteen apple scab resistance genes have been reported previously (Bus et al. 2009, 2010; Erdin et al. 2006; Galli et al. 2010a, b; Patocchi et al. 2009b; Soriano et al. 2009) with six located on LG 2 (Gessler et al. 2006), including *Rvi2* (*Vh2*) (Bus et al. 2005b), *Rvi4* (*Vh4*) (Bus et al. 2005b), *Rvi8* (*Vh8*) (Bus et al. 2005a), *Rvi11* (*Vbj*) (Gygax et al. 2004), *Rvi15* (*Vr2*) (Patocchi et al. 2004) and *VT57* (Bus et al. 2005b). In this study, four apple scab resistance loci were mapped when resistance data were grouped by races (1) and (2) and resistance reactions Chl (chlorotic lesions) and SN (stellate necrosis): *ChlR1*, *ChlR2*, *SNR1* and *SNR2*. But the first two were tightly linked to each other (by 3.4 cM) and the second two were also similarly linked (by 3.0 cM). This suggests that there are probably only two scab resistance genes from the genome of PI 613988: one represented by *ChlR1* and/or *ChlR2*, the other by *SNR1* and/or *SNR2*. In comparison with the six known scab resistance genes, the genomic region of *ChlR1* and *ChlR2* is unique, an indication of new apple scab resistance gene(s) identified from *M. sieversii* in this study. However, the *SNR1* and *SNR2* region appears to be comparable to *Rvi8* (*Vh8*) (Bus et al. 2005a). It is likely that *SNR1* and *SNR2* may be allelic to *Rvi8* (*Vh8*), but a test with more races (1) to (8) is required to ascertain this.

It is important to note that *Rvi8* (*Vh8*) is not only mapped to LG 2, but also identified from a *M. sieversii* (GMAL 3631, PI 600520) selection (W193B) (Bus et al. 2005a; Forsline and Hummer 2007; Luby et al. 2001). One of the major objectives for collecting and maintaining the *M. sieversii* germplasm is to utilize their disease resistance genes for apple genetic improvement. A scab resistance inheritance study has found diverse patterns in apple scab resistance in *M. sieversii*, since, among the seven resistant *M. sieversii* elite accessions crossed with Royal Gala, the ratio of resistance progeny varied from 9% to 67% when screened with *V. inaequalis* races (1) and (2) (Luby et al. 2006). Together with these complex inheritance patterns, the identification of *Rvi8* (*Vh8*), and *ChlR1*, *ChlR2*, *SNR1* and *SNR2* suggests that, as the major progenitor species of domestic apple, *M. sieversii* is indeed a rich resource for improving scab resistance in apple.

Conclusions

The maps constructed provide the first insight into the genome of *M. sieversii*, the major progenitor species of domestic apple. The new EST contig-based SSR markers will be useful in a range of genetic and genomic studies. Our test of 422 SSRs suggests that the EST-based SSRs and their corresponding primer sequence information maintained in GDR are invaluable and worthy of future efforts for the development of high-density EST-based SSR genetic maps in *Malus*. Identification of *ChlR1*, *ChlR2*, *SNR1* and *SNR2* enhances the view that as the major progenitor species of domestic apple, *M. sieversii* is a rich resource for improving scab resistance in apple. The Royal Gala × PI 613988 progeny that are resistant to *V. inaequalis* would be desirable breeding materials for pyramiding apple scab resistance in breeding programs.

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