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High-resolution mapping and chromosome landing at the root-knot nematode resistance locus *Ma* from Myrobalan plum using a large-insert BAC DNA library

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Abstract The *Ma* gene for root-knot nematode (RKN) resistance from Myrobalan plum (*Prunus cerasifera* L.) confers a complete-spectrum and a heat-stable resistance to *Meloidogyne* spp., conversely to *Mi-1* from tomato, which has a more restricted spectrum and a reduced efficiency at high temperature. This gene was identified from a perennial self-incompatible near-wild rootstock species and lies in cosegregation with the SCAR marker *SCAFLP2* on the *Prunus* linkage group 7 in a 2.3 cM interval between the SCAR *SCAL19* and SSR *pchgms6* markers. We initiated a map-based cloning of *Ma* and report here the strategy that rapidly led to fine mapping and direct chromosome landing at the locus. Three pairs of bulks, totaling 90 individuals from half-sibling progenies derived from the *Ma*-heterozygous resistant accession P.2175, were constructed using mapping data, and saturation of the *Ma* region was performed by bulked segregant analysis (BSA) of 320 AFLP primer pair combinations. The closest three AFLP markers were transformed into codominant SCARs or CAPS designated *SCAFLP3*, *SCAFLP4* and *SCAFLP5*. By completing the mapping population up to 1,332 offspring from P.2175, *Ma* and *SCAFLP2* were mapped in a 0.8 cM interval between *SCAFLP3* and *SCAFLP4*. A large-insert bacterial artificial chromosome (BAC) DNA library of P.2175, totaling 30,720 clones with a mean insert size of 145 kb and a 14–15× *Prunus* haploid genome coverage was constructed and used to land on the *Ma* spanning interval with few BAC clones. As P.2175 is heterozygous for the gene, we constructed the resistant and susceptible physical contigs by PCR screening of the library with codominant markers. Additional microsatellite markers were then designed from BAC subcloning or BAC end sequencing. In the resistant contig, a single 280 kb BAC clone was shown to carry the *Ma* gene; this BAC contains two flanking markers on each side of the gene as well as two cosegregating markers. These results should allow future cloning of the *Ma* gene in this perennial species.

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Introduction

Root-knot nematodes (RKN) *Meloidogyne* ssp. are important pests of fruit tree crops and numerous other perennial or annual plants. Three major RKN species *M. incognita*, *M. javanica* and *M. arenaria* are present all over the world in tropical and Mediterranean climates (Sasser 1977; Lamberti 1979). As nematicide chemicals are progressively removed from the market, genetic control of *Meloidogyne* species is gaining interest (Cook and Evans 1987; Roberts 1992). Many sources of RKN resistance have been identified and major resistance loci have been evidenced and mapped in diverse plant crops such as tomato (*Mi-1*, Kaloshian et al. 1998; *Mi-3*, Yaghoobi et al. 1995; *Mi-9*, Ammiraju et al. 2003), pepper (*Me3*, Djian-Caporalino et al. 2001), peanut (*Mae* and *Mag*, Garcia et al. 1996), carrot (*Mj-1*, Boiteux et al. 2000), peach (*Mi* and *Mij*, Lu et al. 1999, 2000; *Mia*, Yamamoto and Hayashi 2002; R_{Mia}, Claverie et al. 2004) and Myrobalan plum (*Ma*, Esmenjaud et al. 1996b; Lecouls et al. 1997, 1999). The *Mi-1* gene from tomato introduced into tomato cultivars from *Lycopersicon peruvianum* is currently the sole cloned RKN gene (Milligan et al. 1998). This gene for resistance to *M. incognita*, *M. arenaria*, *M. javanica*, together with the aphid *Macrosiphum euphorbiae* (Rossi et al. 1998) and the

whitefly *Bemisia tabaci* (Nombela et al. 2003), encodes a NBS-LRR protein which has been shown to function in interaction with the tomato *Rme1* locus (Martinez de Llarduya et al. 2001). *Mi-1* is also being studied with regard to its structure and function in regulating the resistance response (Hwang et al. 2000; Hwang and Williamson 2003) and has been shown to bind and hydrolyse ATP (Tameling et al. 2002). Unlike *Mi-1* (Holtzmann 1965; Dropkin 1969; Roberts et al. 1990; Kaloshian et al. 1996; Williamson 1998), the expression of resistance by the *Ma* gene from Myrobalan plum (*Prunus cerasifera* L.) is not affected by high temperatures (Esmenjaud et al. 1996a) and no virulent population overcoming this resistance gene is yet known (Esmenjaud et al. 1994, 1997).

In *Prunus* species, different ranges of resistance to RKN have been observed and corresponding genes used for rootstock breeding (Esmenjaud et al. 1997). In the Prunophora subgenus (grouping plums and apricots), the Myrobalan plum wild accessions P.2175, P.1079 and P.2980 carry several alleles of the *Ma* gene (*Ma1*, *Ma2* and *Ma3*, respectively) that confers high resistance to all tested *Meloidogyne* species including *M. arenaria*, *M. incognita*, *M. javanica* and *M. mayaguensis*. It is likely that many *Ma* resistance alleles also exist in other wild or recently domesticated *P. cerasifera* accessions. *Ma1*, *Ma2* and *Ma3* also confer resistance to a population of the recently described RKN species *M. floridensis* (the peach root-knot nematode; Handoo et al. 2004) previously known as the Florida (Fl) isolate that overcomes the resistance of the commonly used rootstocks from the subgenus Amygdalus (grouping peach and almond) such as 'Nemaguard', 'Nemared' or 'GF.557' (Table 1) (Esmenjaud et al. 1997; Lecouls et al. 1997; Rubio-Cabetas et al. 1998, 1999). Nematode juveniles were shown to penetrate the roots and emigrate from the roots in equivalent numbers in *Ma*-resistant and *Ma*-susceptible accessions, but they did not establish a feeding site nor develop in resistant accessions (Voisin et al. 1999). Molecular characterization of the *Ma* locus has been recently conducted. Two reliable SCAR (sequence characterized amplified region) markers, *SCAL19* and *SCAFLP2*, tightly linked to *Ma*, have been identified by bulked segregant analysis (BSA) and are currently used for marker-assisted selection (MAS) (Lecouls et al. 2004). *Ma* was then mapped on the linkage group 7 of the reference European *Prunus* map (Joobeur et al. 1998; Aranzana et al. 2002) in a 2.7 cM interval between the SSR (simple sequence repeats) marker *pchgms6* and *SCAL19* (Claverie et al. 2004).

We recently initiated a map-based cloning strategy of this gene, which conversely to most cloned resistance genes, segregates in a perennial, self-incompatible near-wild species with probably diverse *Ma* resistance alleles. Obtaining large segregating populations and evaluating them for RKN resistance is not easy. Nevertheless the cloning of this gene should be facilitated considering that Myrobalan plum is a diploid species ($2n=2x=16$) with a small genome estimated to be equivalent to the botanically closely related apricot species (*P. armeniaca*) (300 Mb/1C; Arumuganathan and Earle 1977) and an average physical/genetic correspondence of about 375 kb/cM. We report here the successful chromosome landing at the *Ma* locus based on the saturation of the *Ma* region with AFLP (amplified fragment length polymorphism) markers and conversion into SCARs and CAPSs (cleaved amplified polymorphic sequence), the construction of a Myrobalan plum large-insert bacterial artificial chromosome (BAC) DNA library, its screening with the *Ma* tightly linked markers, the elaboration of resistant (R) and susceptible (S) BAC contigs and the identification of a single BAC clone of 280 kb carrying the gene. This should allow

Table 1 Host suitability to *Meloidogyne* spp. of parental material used for *Ma* fine mapping. *R* Resistant, *S* susceptible, *R/S* variable behaviour in function of *M. javanica* isolates

Species	Accession	Resistance status to				Genotype
		M. arenaria	*M. incognita*	*M. javanica*	*M.* sp. Florida	
Myrobalan plum (*P. cerasifera*)	P.2175	**R**	**R**	**R**	**R**	(*Ma1 ma*)
	P.2032	S	S	S	S	(*ma ma*)
	P.16.5	S	S	S	S	Idem
	P.2646	S	S	S	S	Idem
	P.2794	S	S	S	S	Idem
	P.2069	S	S	S	S	Idem
Almond (*P. dulcis*)	Garfi (G)	S	S	S	S	(*ma ma*)
	Alnem1	**R**	S	**R**	S	Idem
	Alnem88	**R**	S	**R**	S	Idem
Peach (*P. persica*)	Shalil	**R**	**R**	S	S	(*ma ma*)
	Nemared (N)	**R**	**R**	**R**/S	S	Idem
Wild peach (*P. davidiana*)	P.1906	**R**	**R**	S	S	(*ma ma*)
Almond × peach	GF.557	**R**	**R**	S	S	(*ma ma*)
	$(G \times N)_9$	**R**	**R**	**R**/S	S	Idem
	$(G \times N)_{15}$	**R**	**R**	**R**/S	S	Idem
	$(G \times N)_{22}$	**R**	**R**	**R**/S	S	Idem

future cloning of *Ma* by identification of candidate DNA sequences and complementation experiments.

Materials and methods

Mapping population and rating of resistance to RKN

The diverse segregating progenies used in this study were created by INRA-UREFV Bordeaux, France, and propagated by softwood cuttings as previously described (Esmenjaud et al. 1995). Because some Amygdalus (peach and/or almond) parental accessions have a partial RKN host range (Table 1) due to resistance genes or factors other than *Ma*, the isolate *Meloidogyne* sp. Florida, controlled by this latter gene only, was used to discriminate *Ma* resistant individuals in all segregating progenies. RKN resistance was evaluated for 307 offspring as described in Esmenjaud et al. (1992).

AFLP screening and transformation into SCARs

Genomic DNA of *Prunus* material was extracted from frozen leaves according to the procedure of Saghai-Maroof et al. (1984) with some modifications. Among the available segregating material, one pair of resistant and susceptible bulks (15 individuals per bulk) was constructed from each of the intraspecific crosses P.2175 × P.2646, P.2175 × P.16.5, and P.2175 × P.2032 and these three pairs of bulks were used for BSA (Michelmore et al. 1991) of AFLP markers (Vos et al. 1995). The methylation sensitive *Pst*I endonuclease was chosen because the *Eco*RI/*Mse*I AFLP markers linked to *Ma* already obtained (Bergougnoux, personal communication) tended to accumulate at only one side of the gene and thus the *Pst*I/*Mse*I AFLP markers were supposed to have a better distribution, as described in Young et al. (1999). Furthermore, as *Ma* had been mapped on *Prunus* LG7 between the SSR marker *pchgms6* and the SCAR marker *SCAL19* (Claverie et al. 2004), the bulks were constructed from mapping data and all the individuals recombining just outside (but not inside) this interval were included into the R and S bulks. For AFLP screening, 100 ng of bulked or parental DNA was digested with the *Mse*I and *Pst*I restriction endonucleases.

Adaptor ligated DNA was then pre-amplified using the *Pst*I+1/*Mse*I+1 primers, and selectively amplified using the *Pst*I γ-[^{33}P] ATP labeled primer in 64 *Pst*I+3/*Mse*I+3 and 256 *Pst*I+2/*Mse*I+3 primer pair combinations. PCR products were separated on a 5% denaturing sequencing gel in 0.5× TBE buffer. The gel was dried on Whatman 3M paper and the AFLP bands visualized following autoradiography (Kodak Biomax MR, N.Y., USA). The *Ma* linked AFLP markers were firstly mapped in 50 individuals including all the available individuals recombining in LG7. For the three AFLP markers closest to *Ma*, the polymorphic bands were excised from the dried polyacrylamide gel, re-amplified using the appropriate primer combination plus one selective *Pst*I base pair and cloned into the pGEM-T Easy Vector (Promega, Madison, Wis., USA). At least ten clones per AFLP band were amplified using the selective primers and three clones per marker were sequenced (Genomex, Paris, France). Primers pairs were then designed using the Primer3 software (Rosen and Skaletsky 1998) in order to obtain the largest PCR products as possible. The markers *AFLP4* and *AFLP5* could be transformed respectively into the SCAR markers *SCAFLP4* and *SCAFLP5* which are both codominant as revealed by polyacrylamide denaturing gels. To recover the polymorphism of the *AFLP3* marker, 100 ng of P.2175 genomic DNA was digested with the *Mse*I restriction endonuclease, ligated with the corresponding adaptor and amplified using the *Mse*I primer as the forward primer and one specific reverse primer. A second amplification of the first PCR product (diluted 1:100) using the *Mse*I primer and another internal specific reverse primer allowed the amplification and sequencing of 200 bp upstream of the *Pst*I site of the AFLP fragment. A CAPS marker could be derived from the whole sequence using the *Pst*I restriction endonuclease.

PCR experiments

For the new SCAR, CAPS and SSR markers, the general PCR amplification protocol was as follows: amplifications were performed in a 15 µl final volume containing 40–60 ng genomic DNA, 0.7 U *Taq* polymerase (Invitrogen, Cergy-Pontoise, France), 0.2 µM of each primer, 200 µM of each dNTP (Promega), 1.5 mM $MgCl_2$ and 1× reaction buffer provided with the enzyme. The SSR *pchgms6* from peach was amplified using the primers pchgms6 F1 (5′-CATTGTTCATGGGAGGAATT-3′) and pchgms6 R2 (5′-CTAAAGGAGCACCAATTTTTG-3′), more specific to the P.2175 alleles (data not shown). PCR conditions were as follows: 94°C for 4 min, then 35 cycles of (94°C for 45 s, 55°C for 45 s, 72°C for 1 min 30 s), finishing with 72°C for 4 min. For *SCAFLP5*, *SCAFLP4* and the SSR markers (except *plgms17*), 0.3 pmol of the forward primer was γ-[^{33}P] ATP end labeled with polynucleotide kinase and PCR products were separated on a 5% denaturing sequencing gel in 0.5× TBE buffer and visualized following autoradiography. For *plgms17*, the PCR products were run on 2% agarose gels. For the CAPS *SCAFLP3*, 10 µl of the PCR product was digested with 10 U of *Pst*I restriction endonuclease (Roche, Meylan, France) for 5 h in a 30 µl final reaction volume with the appropriate reaction buffer. The digested products were then separated on a 2% agarose gel and stained with ethidium bromide. The *SCAL19* and *SCAFLP2* markers were amplified as described in Lecouls et al. (2004).

Additional recombinant scoring

One thousand and twenty-five additional offspring of P.2175 ranging into 800 intra- and interspecific G_1 (first

generation) and 225 intraspecific G_2 (second generation) individuals were used to complete the mapping population to a total of 1,332 individuals segregating for the gene (Table 3). These individuals were screened for two markers flanking *Ma*, i.e. the SCAR marker *SCAL19* and the SSR marker *pchgms6* (Claverie et al. 2004). Recombinant individuals between both markers were propagated either in vitro (Esmenjaud et al. 1993) or as softwood cuttings (Esmenjaud et al. 1995), and evaluated for RKN resistance.

BAC library construction

The Myroban plum P.2175 BAC DNA library was constructed as described in Chalhoub et al. (2004) with some modifications. Nuclei were isolated from 10 g of young leaves frozen in liquid nitrogen. Because the sucrose-based buffer added to the ground tissue became too viscous for the first step filtration, the buffer volume used was increased to 60 ml for 1 g of tissue and the concentration of sucrose had to be divided three times, resulting in a final concentration of about 160 mM sucrose. The next steps of high nuclear weight DNA isolation and BAC library construction followed the author's instructions. Restriction fragments were subjected to a triple size selection in a CHEF-DRIII apparatus (Bio-Rad) by pulse field gel-electrophoresis (PFGE) as this was shown to result in an increased mean insert size compared to other types of size selection. At the end of the gel-sizing process, slices of agarose containing DNA fragments of approximately 100–150 kb, 150–200 kb, 200–250 kb and 250–350 kb were removed separately. The DNA from the agarose slices was electroeluted. The pIndigo BAC vector was prepared for *Hin*dIII cloning according to the 'single tube vector preparation' method with no precipitation or gel electrophoresis steps, and used for ligation reactions. Ligation reactions were carried out using the four different size-selected partially digested DNA samples, resulting in four BAC sub-libraries. Competent *E. coli* DH10B cells (Invitrogen) were transformed by electroporation and transformants were selected on LB-Xgal-IPTG plates containing 12.5 μg/ml chloramphenicol. White colonies were picked using a Genetix Q-Bot and stored in 384-well microtiter plates (Genetix) at −80°C.

BAC library characterization

Pooling and screening by PCR

Because of the small genome size estimated for the Myrobalan plum genome, the BAC library was composed of only 80 plates corresponding to 30,720 total BAC clones. BAC clones from each plate were mixed into pools of 384 clones (designated 'plate pools'). The BAC clones from each plate pool were centrifuged, resuspended and boiled for 30 min into TE 10:0.1 buffer, and the supernatants were used directly in PCR reactions. The 24 clones of each of the 16 rows of the 384-well plates were pooled together. This resulted in 16 pools (for each 384-well plate), designated 'row pools' (one pool per row). Clones and row pools of clones were amplified directly in PCR wells. The strategy of BAC library screening with each of the PCR markers and using the different pools was done in three sequential steps. In the first step, positive plates were identified by screening the plate pools. The second step consisted of identifying the positive row in each of the positive plates identified in step 1. The third step consisted of identifying the positive BAC clone by screening the 24 clones of each of the positive row pools identified in step 2. PCR amplifications were carried with the parental DNA and empty plasmid vector as controls and run on 1.5% agarose gels. *SCAFLP4* and *SCAFLP5* resistant and susceptible alleles carried by the positive clones were determined after BAC clone radio-labeled PCR amplification and electrophoresis on poly-acrylamide denaturing sequencing gels.

Determination of the insert size of BAC clones and BAC subcloning

BAC clones were miniprepped, digested with *Not*I and the digestion products were subjected to pulsed-field gel electrophoresis as described in Chalhoub et al. (2004): 15 μl DNA from each clone was *Not*I digested in a 30 μl reaction mix and loaded on PFGE. Insert size was estimated using the PFGE lambda ladder (BioLabs, Frankfurt, Germany).

For BAC subcloning, 500 ng BAC plasmid DNA obtained from the minipreps was *Hin*dIII digested. Digested products were separated on a 1% agarose gel and products of more than 500 bp were removed from the gel using the Qiagen (Courtaboeuf, France) gel extraction mini-elution kit and cloned into the *Hin*dIII digested and dephosphorylated pBlueScript vector.

Results

Identification of AFLP markers by BSA and development of SCAR and CAPS markers

Three pairs of bulks, comprising a total of 90 individuals from half-sibling progenies derived from the *Ma*-heterozygous resistant accession P.2175, were constructed using mapping data. Additional markers in the *Ma* region were obtained by BSA of 320 AFLP primer pair combinations. Using the original segregating population of 307 individuals, 5 AFLP markers tightly linked to the *Ma1* allele from P.2175 were obtained and mapped to the 2.3 cM interval spanning the gene, between the previously obtained markers *SCAL19* and *pchgms6* (Fig. 1). Three of these markers were sequenced and transformed into SCAR or CAPS markers designated *SCAFLP3*, *SCAFLP4* and *SCAFLP5* (Table 2). The marker *SCAFLP4* is codominant on polyacrylamide denaturing gels (Fig. 2)

Table 2 Primer sequences and product sizes of *Ma*-linked SCAR and CAPS markers obtained from BSA of AFLP markers. *P Pst*I, *M Mse*I

Marker name	AFLP marker	Primer sequences	Restriction endonuclease	Product size
SCAFLP3	P-AA/M-ACC	5′-CCACCTTTAGATTACGTGCTGA-3′ 5′-GCAAAACCAGCCTCTGTCTC-3′	*Pst*I	508 bp (336 bp/172 bp)
SCAFLP4	P-AA/M-ACA	5′-TTCTCATATGGGCCATCTCCA-3′ 5′-TATTTCCAGGAGAGGAGGCGTA-3′	–	316 bp
SCAFLP5	P-CT/M-CAT	5′-CCCATTTACAGGAGCTTTTTG-3′ 5′-GCAGCTTCAACATGGAAGAG-3′	–	210 bp

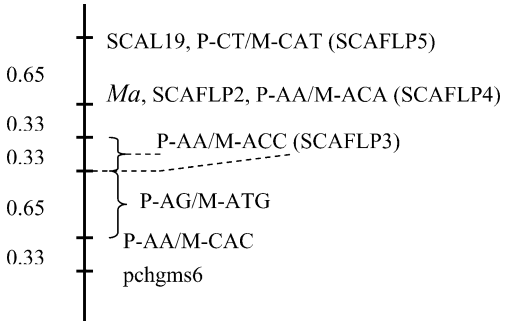

Fig. 1 Local map around *Ma* showing the position of AFLP and corresponding SCAR markers (*between parentheses*) obtained from BSA. Distances expressed in recombination percentages have been evaluated from a population of 307 individuals. The AFLP dominant markers P-AA/M-ACC and P-AG/M-ATG in coupling with resistance in P.2175 are also present in the susceptible accession P.2032 and therefore cannot be mapped precisely. Nevertheless, the CAPS *SCAFLP3* is codominant and can be mapped more precisely than its corresponding P-AA/M-ACC AFLP marker. *PPst*I, *MMse*I

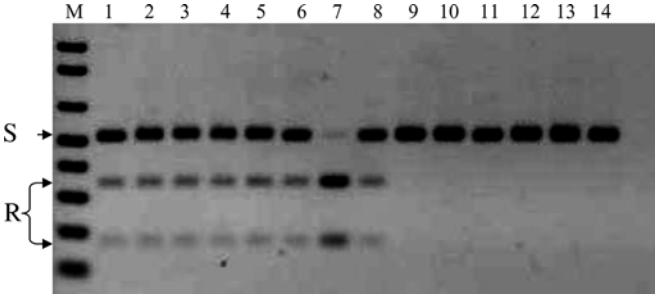

Fig. 3 Segregation of the *Pst*I digested CAPS marker *SCAFLP3* in intra and interspecific crosses involving P.2175. *Lanes 1–6 and 8 Ma* resistant offspring, heterozygous for the marker. *Lane 7 Ma* resistant individual from the cross P.2175 × P.2032, homozygous for the *Pst*I digested marker allele. *Lanes 9–14 Ma* susceptible offspring homozygous for the *Pst*I undigested marker allele. *S Pst*I undigested allele in coupling with susceptibility. *R Pst*I digested allele in coupling with resistance. *Lane M* 1 kb plus DNA marker

due to an insertion/deletion event of nine base pairs and cosegregates with both *Ma* and marker *SCAFLP2*. The marker *SCAFLP5* is also codominant, probably due to the presence of a short SSR $[(AG)_{11}]$ in its sequence and it cosegregates with *SCAL19* (data not shown). The CAPS *SCAFLP3* shows a *Pst*I restriction site polymorphism in coupling with resistance in P.2175 (Fig. 3).

High resolution mapping of the *Ma* gene

The genetic distances between the new and previous markers around the *Ma* gene were refined from new recombinant data. A total of 1,332 individuals, ranging into 21 different intra- or interspecific crosses segregating for *Ma*, were genotyped for the flanking markers *SCAL19* and *pchgms6* (Table 3). Thirty-one individuals recombining between these latter markers, separated by a genetic interval of 2.3 cM encompassing the gene, were detected. The recombinant individuals were genotyped with the markers *SCAFLP2*, *SCAFLP3* and *SCAFLP4* and RKN resistance tests allowed a finer location of the gene (Fig. 4a); *Ma* cosegregated with the *SCAFLP2* marker and was separated from *SCAFLP4* by a single recombination event.

The allele of the marker *SCAFLP4* in coupling with resistance in P.2175 (about 0.08 cM from *Ma*) is absent in the susceptible parental Myrobalan accessions whereas surprisingly, it is present at the homozygous state in the Amygdalus parental accessions. Consequently, the P.2175 allele in coupling with susceptibility segregates in the P.2175 × Amygdalus crosses. In contrast, the *SCAFLP2* allele in coupling with resistance in P.2175 is absent from any of the susceptible Myrobalan or Amygdalus parental accessions.

BAC library construction

We constructed a BAC library from P.2175. A total of 30,720 BAC clones distributed into four size classes (sub-

Fig. 2 Segregation of the SCAR marker *SCAFLP4* in 19 individuals from different intraspecific progenies segregating for *Ma*. *Lanes 1–10 Ma* resistant individuals, heterozygous for the marker. *Lanes 11–19 Ma* susceptible individuals, homozygous for the marker. The *arrows* indicate the alleles in coupling with susceptibility (*S*) and resistance (*R*) in P.2175

Table 3 Total number of *Ma* segregating offspring genotyped for recombination events between the *Ma* flanking markers *SCAL19* and *pchms6* and their distribution into 21 different crosses (direct and reciprocal crosses are pooled together). G_1 first generation, G_2 second generation

Crosses	Number of individuals
Intraspecific G_1	
P.2175 × P.16.5	33
P.2175 × P.18	20
P.2175 × P.2032	88
P.2175 × P.2069	15
P.2175 × P.2646	246
P.2175 × P.2794	2
Intraspecific G_2	
$(P.2175 \times P.16.5)_{18} \times P.16.5$	31
$(P.2175 \times P.16.5)_{20} \times P.16.5$	37
$(P.2175 \times P.2646)_{21} \times P.16.5$	72
$(P.2175 \times P.2646)_{23} \times P.16.5$	40
$(P.2175 \times P.2646)_{23} \times P.2646$	28
$(P.2175 \times P.2646)_{24} \times P.2646$	17
Interspecific G_1	
P.2175 × 'Alnem1'	72
P.2175 × 'Alnem88'	20
P.2175 × 'Garfi'	12
P.2175 × *P. davidiana* P.1908	16
P.2175 × 'Nemared'	60
P.2175 × ('Garfi' × 'Nemared')$_9$	62
P.2175 × ('Garfi' × 'Nemared')$_{15}$	64
P.2175 × ('Garfi' × 'Nemared')$_{22}$	233
P.2175 × GF.557	164
Total	1,332

libraries) were organized into 384-well plates. Insert sizes were estimated from 10 randomly chosen clones in each of the 4 size-selected sub-libraries. Sub-library 1 consists of 9,513 clones with insert size ranging from 50 to 150 kb and an average of 120 kb, sub-libraries 2 and 3 grouped 19,200 clones with insert sizes ranging from 80 to 200 kb and an average of 150 kb. Sub-library 4 grouped 2,007 clones with insert size ranging from 110 to 350 kb and an average of 210 kb. Thus the average insert size of the whole library is estimated to be approximately 145 kb with and insert distribution ranging from 50 to 350 kb and a 14–15× coverage of the Myrobalan plum haploid genome.

Construction of physical contigs spanning the *Ma* region and chromosome landing

As the accession P.2175 is heterozygous and carries both resistant and susceptible alleles of the *Ma* gene, we undertook construction of susceptible and resistant physical contigs by screening the BAC library with the codominant cosegregating or tightly linked markers, *SCAFLP2*, *SCAFLP3*, *SCAFLP4*, *SCAFLP5* and *SCAL19* (Fig. 4a). The positive BAC clones identified were considered as belonging to either the resistant or the susceptible contigs based on their detection with either the resistant or the susceptible alleles of the codominant markers. A total of 49 positive BAC clones were clearly identified with at least one of the markers and allowed the construction of resistant and susceptible BAC contigs. Overall there were between 12 and 18 (a mean of 14) positive BAC clones per marker (Figs. 4 and 5). Twelve positive BAC clones were detected for the *Ma* cosegregating marker *SCAFLP2* of which 4 carried the

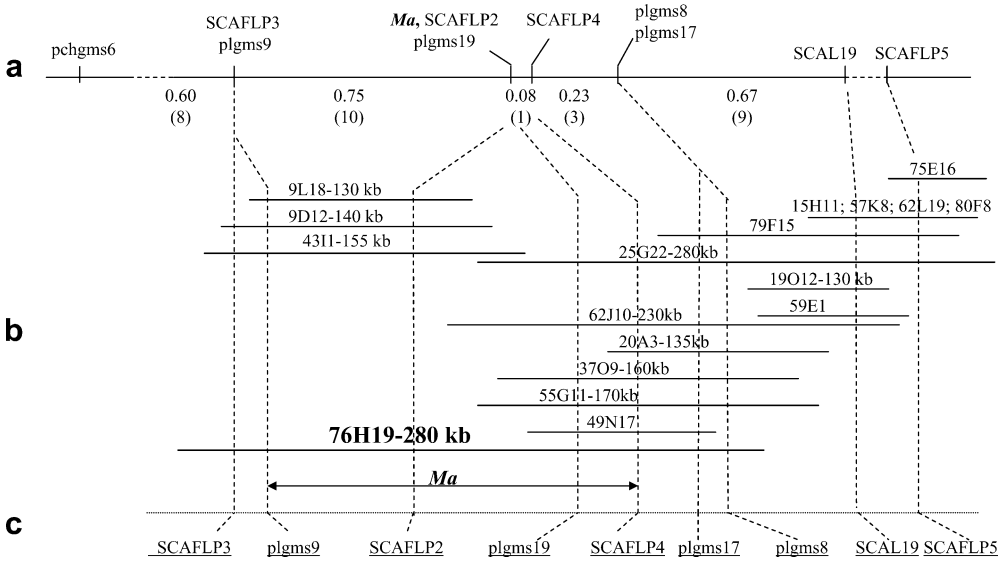

Fig. 4a–c Fine genetic mapping of *Ma* linked SSR (*plgms*) and SCAR (*SCAFLP*) markers (**a**) and physical mapping of positive BAC clones from the **resistant contig** (**b**). In (**a**) values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1,332 total individuals (*between parenthesis*, *lower row*). Amplification of the expected resistance allele of a marker from a BAC is represented by a cross between this BAC and the *dotted vertical line* joining the marker name (**c**). For some BAC clones insert sizes are indicated after the BAC designation. To alleviate the figure, several equivalent BAC clones are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (resistance allele)

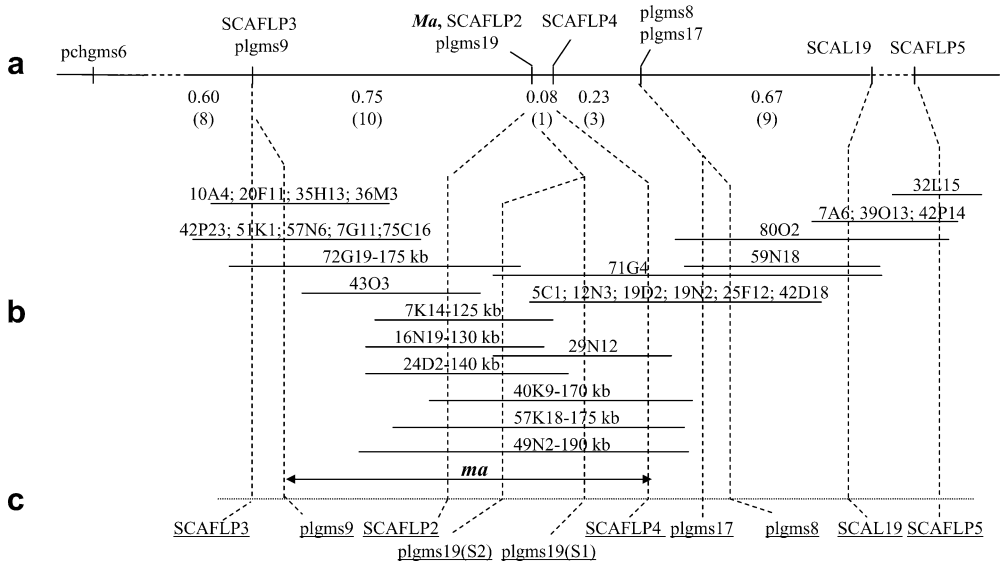

Fig. 5a–c Fine genetic mapping of *ma* linked SSR (*plgms*) and SCAR (*SCAFLP*) markers (**a**) and physical mapping of positive BAC clones from the **susceptible contig** (**b**). In (**a**) values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1,332 total individuals (*between parentheses*, *lower row*). Amplification of the expected susceptibility allele of a marker from a BAC is represented by a cross between this BAC and the *dotted vertical line* joining the marker name (**c**). For the SSR marker *plgms19* two amplification products (differing in length by 4 bp) in coupling with susceptibility and genetically cosegregating were physically separated and designated as *plgms19*(S1) and *plgms19*(S2). For some BAC clones insert sizes are indicated after the BAC designation. To alleviate the figure, several equivalent BAC clones are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (susceptibility *ma* allele)

resistant allele. Eighteen clones carried the next closest marker *SCAFLP4* of which 7 contained the resistant allele. Three clones from the resistant contig and a single clone from the susceptible contig carried both *SCAFLP3* and *SCAFLP2* (Fig. 4a). Surprisingly the markers *SCAFLP2* and *SCAFLP4*, separated by only 0.08 cM, were detected together in only a single clone of the R contig and 3 clones of the S contig. Finally a single BAC clone ('BAC76H19') carried all the resistant alleles of *SCAFLP2*, the flanking *SCAFLP3* and *SCAFLP4* markers and subsequently the *Ma* gene.

Thirteen random DNA sequences, from 224 to 827 bp long, were obtained from the 76H19 BAC subcloning and the sequencing of other BAC ends that anchor to the gene region. Four of these sequences were shown to contain microsatellite repeats and served to generate four polymorphic SSR markers tightly linked to *Ma* (Table 4). Amplifications were performed on parental material, recombinant individuals and BAC clones isolated from the *Ma*-resistant and *Ma*-susceptible contigs (Figs. 4 and 5). Genetically, the SSR *plgms9* from the 9L18 T7 BAC end cosegregates with *SCAFLP3*, the SSR *plgms19* cosegregates with *Ma* (and *SCAFLP2*) and the two others (*plgms8* and *plgms17*) cosegregate and fall between *SCAFLP4* and *SCAL19*. These newly developed markers were placed on the resistant and susceptible maps in parallel with the fine genetic mapping of the region surrounding *Ma* (Figs. 4 and 5). Two slightly different copies of the SSR marker *plgms19* could be separated on the susceptible contig since they were physically mapped between the *SCAFLP2* and *SCAFLP4* markers at two different loci.

Discussion

The precise mapping of the *Ma* gene on a well characterized segregating population was made possible thanks to the SSRs markers from the reference T × E (Texas almond × Earlygold peach) map (Joobeur et al.

Table 4 Primer sequences, repeat motifs and product sizes of SSR markers obtained from the BAC 76H19 subcloning (*plgms8*, *plgms17* and *plgms19*) or from T7 BAC-end sequencing of the included BAC 9L18 (*plgms9*)

Marker name	Primer sequences	Repeat motif	Product size
Plgms8	5′-AAACAGCCAGATCCGGAGTA-3′ 5′-TATAAGTCCGCCATCGCTTG-3′	$(AT)_{15}$	316
Plgms17	5′-CCAGTGATCCACGTGTTGAG-3′ 5′-AGCATCTCTCGTTCCCCTTT-3′	$(TA)_5(GA)(TA)_4(CA)_4$	759
Plgms19	5′-CGGGGTTCAAACTCAACAAG-3′ 5′-AGACGTGCTGCTTTGTTCAC-3′	$(CT)_{15}(CA)_{15}$	327
Plgms9	5′-GGTGGGAGAATTCGACTATCA-3′ 5′-CAACCCAATACCACGTACCC-3′	$(GA)_{22}$	156

1998; Aranzana et al. 2002) and this was the starting point of an efficient BSA and thus of the positional cloning strategy. By constructing three pairs of bulks, 90 individuals were screened by BSA at the same time and only markers located close to or less than 1 cM from the gene were obtained. Furthermore, by including in the bulks individuals recombining just outside the 2.3 cM interval between the closest flanking markers *SCAL19* and *pchgms6*, we could increase this marker selectivity and avoid the necessity for AFLP genotyping of superfluous markers. Three new AFLP markers tightly linked to *Ma* were transformed into specific codominant markers by valorizing three different kinds of polymorphism i.e. repeat numbers (for *SCAFLP 5*), insertion/deletions (for *SCAFL4*) and sequence differences (for *SCAFLP3*). This illustrates the particularly high potential of the AFLP technique for generating markers in the Myrobalan plum genome. The *Ma* gene was then more precisely mapped within a 0.8 cM interval.

The fine mapping of the SCAR markers using P.2175 as the resistance source involved six susceptible Myrobalan plum accessions (Tables 1 and 3). Since Myrobalan plum is a near-wild *Prunus* species which is fully self-incompatible (Salesses et al. 1993, 1994), we should not have observed any linkage between marker alleles and phenotypes for RKN resistance in these accessions. Nevertheless, for each of both markers *SCAFLP4* and *SCAFLP2* a single allele was observed in the susceptible Myrobalan parents (except for the P.16.5 accession) which was the same as the susceptibility allele from P.2175. The P.16.5 accession carries a third allele for the *SCAFLP2* marker. Those data suggest that a linkage disequilibrium remains in Myrobalan plum in the *Ma* region that will be interesting to investigate.

Thanks to the fine mapping strategy, the *Ma* gene was mapped in a 0.8 cM interval. Considering that the Myrobalan plum haploid genome covers 800 cM ($2n=2x=16$) and is presumably equivalent to that of the apricot (300 Mb/1C), 1 cM would correspond to a theoretical physical distance of 375 kb. Consequently, the 0.8 cM interval containing the locus is expected to cover approximately 300 kb. Thus the construction of a BAC library with inserts up to this size was supposed to facilitate further physical mapping and to maximize the chances of landing on this interval. This was relatively easily achieved using an improved BAC library construction protocol that has been developed recently (Chalhoub et al. 2004).

The BAC pooling technique for PCR screening of BAC clones applied to a heterozygous wild accession such as the Myrobalan plum P.2175 allowed the detection of both R and S markers alleles and their successful use for the construction of the resistant and susceptible contigs. Thanks to the construction of a large-insert BAC library with a high coverage of the Myrobalan plum genome, a single 280-kb insert size BAC clone encompassing *Ma* was directly isolated without the need for any chromosome walking steps. The physical mapping of the gene was also facilitated by the small size of the diploid *Prunus* genomes (Arumuganathan and Earle 1991), considering that reliable codominant markers tightly linked to and flanking *Ma* were available.

We observed that the markers *SCAFLP2* (cosegregating with *Ma*) and *SCAFLP4*, which are genetically separated by only one recombination event (which should correspond to a theoretical physical distance of less than 30 kb), share only one common BAC clone on the resistant contig and three common BAC clones on the susceptible contig. Although this might be due to a high number of *Hin*dIII restriction sites or to DNA hypomethylation between those later markers, this also suggests a lack of recombination. SSR development from physical and sequencing data in the region of a target gene may greatly improve the isolation of candidate genes. Nevertheless the SSRs directly developed from the BAC clones in our study cosegregate genetically with markers already obtained (e.g. *plgms19* and *plgms9* with *SCAFLP2* and *SCAFLP3*, respectively) or together (e.g. *plgms8* and *plgms17*) and do not resolve the distribution of the ten recombination events between *SCAFLP2* and *SCAFLP3* (Figs. 4 and 5). This result supports the idea of local variations in the recombination rate in the *Ma* region that could be linked to variations in polymorphism between the 'resistant' and the 'susceptible' chromosomes. Sequence data for the 76H19 BAC clone and additional markers information will allow us to precisely determine the distribution of recombination events around the *Ma* locus and the physical distances between the markers flanking this gene. Complete sequencing of the 280-kb insert will not only provide new markers and candidate genes for *Ma* identification but also data about *Prunus* genome organization (genes, microsatellites, structure and distribution of repeated sequences) in this particular region, which may be compared to the susceptible *Ma* region and extended via microsynteny to other *Prunus* or *Rosaceae* species.

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