4 Plum

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4.1 Introduction

4.1.1 History, Diversity, Domestication, Ploidy Level

According to Rehder (1947), *Prunus* are divided into three major subgenera: Prunophora (plums and apricots), Amygdalus (peaches and almonds) and Cerasus (sweet and sour cherries). The subgenus Prunophora is divided into two main sections: Euprunus which groups the plum species and Armeniaca which contains the apricot species. Plums have been domesticated independently in Europe, Asia and America (Weinberger 1975; Shaw and Small 2004). In Europe, *P. domestica* L. is the most important source of fruit cultivars and has been grown for over 2,000 years. Nevertheless, seeds of another European plum, *P. insititia* L., have been recovered in antiquity ruins and might be of a more ancient origin. The Myrobalan plum *P. cerasifera* Ehrh. probably originated in the Caucasus and Crimea regions (Eremin 1978). In Asia, the Japanese plum *P. salicina* Lindl. originated from China where it has been cultivated since very ancient times. Two to four centuries ago, it has been brought to Japan from where it has been spread all around the world as Japanese plum (Hedrick 1911). In North America, the third plum domestication source, a wide range of native species such as *P. americana* Marsh., *P. hortulana* Bailey, *P. munsoniana* Wight & Hedr., *P. angustifolia* Marsh. and *P. maritima* Marsh. (Okie 1987) are present.

Within the *Prunus* genus, plums are the most taxonomically diverse and are adapted to a broad range of climatic and edaphic conditions (Ramming and Cociu 1991; Salesses et al. 1993). Morphological taxonomy has long been difficult because species boundaries are blurred by interspecific similarities and hybridizations and intraspecific variations. Some plum species are used for their fruits but a majority is being used as rootstocks for plum and other stone fruits. As all the *Prunus* species, plums have a basic chromosomic number of 8 and range from diploid ($2n = 2x = 16$) to hexaploid ($2n = 6x = 48$). Most commercial varieties of plums belong to the European plums *P. domestica* and *P. insititia*, which both are hexaploid, and to the Japanese plum *P. salicina* which is diploid. The diploid Myrobalan plum *P. cerasifera* is widely used as a rootstock (Salesses et al. 1994) and is supposed to have been one of the genomic components of *P. domestica* ($6x$) in association with *P. spinosa* (blackthorn or sloe; $4x$) and might also be one of the components of this latter species (Salesses 1975; Reynders-Aloisi and Grellet 1994). As the peach genome size (diploid) is estimated of 280 Mbp/1C, diploid plums are expected to have equivalent genome sizes (what corresponds to twice the value of the *Arabidopsis* genome) while *P. domestica* genome size is estimated of 883 Mbp/1C (Arumuganathan and Earle 1991).

4.1.2 Economic Importance

In 2004, approx. ten billion tons of plums have been produced in the world of which ca. 3 and 5 billion tons are grown in Europe and Asia, respectively. After peaches and nectarines, this represents the second production among *Prunus* crops at the world, American and European scales. In Europe, the first producer is Germany with 450 millions tons (FAO 2005).

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4.1.3 Breeding Objectives

Breeding purposes in plum concern both cultivar and rootstock. In cultivars, besides the selection for large and good-flavored fruits and for wide ranges of production time, the main objective relates to resistance to *Plum pox virus* (PPV), the causal agent of the sharka disease. PPV, a quarantine pathogen naturally transmitted by aphids, is among the most important *Prunus* diseases and is widely disseminated in European plum orchards, causing significant economic losses. For rootstock breeding, the remarkable variability of wild plum species is starting to be exploited to enlarge the narrow genetic bases of most cultivated plum species (Rom and Carlson 1987; Ramming and Cociu 1991; Dosba et al. 1994). Rootstock programs are being conducted that use Myrobalan plum (alone or crossed with another *Prunus* species) (Eremin 1978; Salesses et al. 1993, 1994) for its positive traits such as good vegetative propagation and adaptation to waterlogged soils (Okie 1987). Some accessions of this species also exhibit a high and wide-spectrum resistance to root-knot nematodes (RKN) *Meloidogyne* spp. (Esmenjaud et al. 1994, 1997) or a graft compatibility with most peach varieties (Salesses et al. 1994).

4.2 Selection for Resistance to PPV

In reaction to the spread of PPV across European borders, control programs have included the development of plum cultivars tolerant or resistant to PPV infection, and programs of strict eradication. Localization (Hoffman et al. 1997), concentration (Polak 1998) and systemic spread of the virus in the plant (Ferry et al. 2002) as well as spatial spread at the orchard scale (Dallot et al. 2003, 2004) has been investigated in plum and other stone fruit species.

4.2.1 Classical Breeding Approach

Development of resistance to PPV in plum has followed the classical approach of searching for natural resistance and incorporating this resistance into new varieties (Kegler et al. 1998). Quantitative resistance has been estimated in a high number of cultivated plums (Paprstein and Karesova 1998) and a qualitative factor such as a hypersensitive character (Hartmann 1998; Hartmann and Petruschke 2000) has also been detected in the European plum cv. Jojo.

Strategies aiming at combining both types of resistances in the hexaploid genome of *P. domestica* are being deployed. As an example, three European plum cultivars, 'Cacanska najbolja', 'Cacanska rana' and 'Cacanska lepotica' (also called 'Cacak Best', 'Cacak Early' and 'Cacak Beauty' respectively) are being used extensively for the introduction of tolerance and partial resistance to PPV (Hartmann 1998). A study by Decroocq et al. (2004) using 10 nuclear microsatellite markers (simple sequence repeat = SSRs) designed for apricot and four chloroplastic SSR markers from dicotyledonous angiosperms (Weising and Gardner 1999) has established that these Cacak accessions were full siblings and were also half siblings of Jojo. These results based on a total of 15 European plum cultivars also showed the cross transportability of the nuclear markers between two *Prunus* species belonging to the same *Prunus* subgenus (Prunophora) and established from both nuclear and chloroplastic markers the pedigree of all four cvs, which had always been previously a matter of discussion (Paunovic et al. 1978).

For a successful identification of the QTLs in the *Prunus* resistance sources, genetic studies need to be associated to the detection of candidate genes. Analogues of virus resistance genes were identified (Decroocq et al. 2005) in *P. davidiana*, a wild relative of peach, that co-localize with genomic regions linked to PPV in this source.

4.2.2 Genetically Engineered Plums

To control PPV spread in plants, attempts to develop genetic engineering technology can be regarded as an alternative approach to the conventional breeding techniques. For this purpose, Sanford and Johnston (1985) have proposed the pathogen-derived resistance as a new strategy to combat viral diseases. Subsequently many research teams have focussed their research program in the creation of transgenic plants resistant to virus infection. Scorza et al. (1994) have successfully engineered the full-length PPV CP gene in *Prunus domestica*. Results about the preliminary greenhouse testing showed that a transgenic clone designated as clone C-5 has been identified as resistant (Ravelonandro et al. 1997; Jacquet et al. 1998). The molecular mechanisms involved have been re-

ported as the post-transcriptional gene silencing or PTGS (Scorza et al. 2001). To verify the stability of PPV resistance in plums, transgenic clones were released in field conditions. For over five years under high inoculum pressure, and regardless of the PPV strains, D or M, the transgenic plum C5 remained healthy when compared to control clones expected to show clear PPV symptoms (Ravelonandro and Scorza 2004). Interestingly, cross hybridization of the transgenic clone C-5 with other plum species permitted to show that the virus transgene can be inherited in the progeny as a single gene trait (Ravelonandro et al. 2001).

4.3 Breeding Efforts for Rootstocks

In plum, no mapping results have yet been used in the specific objective of breeding varieties and available data mainly relate to the Myrobalan plum as a central species in rootstock programs. Breeding efforts have been devoted to the introgression of resistance to root-knot nematodes (RKN) *Meloidogyne* spp. from this latter species into rootstocks (Dirlewanger et al. 2004c; Esmenjaud 2004). Genome mapping and molecular breeding concern in priority interspecific crosses also involving, besides Myrobalan plum, the peach resistance sources Nemared (Ramming and Tanner 1983) and Shalil (Layne 1987). Major results have been obtained on the cross Myrobalan plum 'P.2175' x almond-peach Garfi × Nemared (= 'GN'). The objectives of this *Prunus* rootstock breeding program are to provide an efficient alternative to the use of highly toxic nematicides by developing a new generation of *Prunus* rootstocks bearing high resistance to RKN, using marker-assisted selection (MAS) for pyramiding *Prunus* resistance genes, and several additional characters such as adaptation to chlorosis and drought (from almond), tolerance to water logging (from plum) together with graft compatibility with peach (from peach) and good rooting ability (from plum) (Dirlewanger et al. 2004c; Esmenjaud 2004).

The complete characterization of one major resistance gene to RKN (*Ma*) from Myrobalan plum has been achieved and the molecular cloning of this gene is in progress. Recent advances in this work through the steps of high-resolution mapping, construction of a BAC library for chromosome landing, isolation of one BAC clone carrying the gene, detection of candidate genes, will be reported in this chapter. As an introduction to the molecular aspects of these breeding efforts detailed further for RKN resistance, we develop hereafter the basic knowledge on genetics of resistance in *Prunus* sources.

4.3.1 Genetics of RKN Resistance in *Prunus* Sources

Genetics of resistance to RKN has been studied in the Myrobalan plums P.2175 and P.2980 and in the peach sources Nemared, Shalil, Juseitou and Okinawa (Table 1).

Accessions P.2175 and P.2980 have been shown to carry one dominant allele (heterozygous) of a single resistance gene, designated *Ma1* and *Ma3*, respectively (Esmenjaud et al. 1996b; Rubio-Cabetas et al. 1998). Each of these *Ma* alleles confers a high and wide-spectrum resistance to *M. arenaria, M. incognita*, *M. javanica* and *M. floridensis* (Esmenjaud et al. 1997; Lecouls et al. 1997; Rubio-Cabetas et al. 1999; Handoo et al. 2004) and to the minor species *M. mayaguensis* (Rubio-Cabetas et al. 1999) which overcomes the resistance of the *Mi* tomato gene (Fargette et al. 1996). This *Ma* resistance was not overcome by any of the over-30 RKN species and isolates tested (Esmenjaud et al. 1994, 1997; Fernandez et al. 1994) and was not modified under conditions usually known as affecting plant defences to RKN such as high temperature and high inoculum pressure (Esmenjaud et al. 1996a). Within perennials, where the genetics of RKN resistance is poorly documented, the *Ma* gene from Myrobalan plum is the first genetic system fully characterized for resistance to a plant pest (Lecouls et al. 1997, 1999; Lecouls 2000; Claverie et al. 2004a, b; Lecouls et al. 2004).

Resistance in Nemared peach has been firstly studied in an F_2 population derived from self-pollination of an F_1 peach hybrid Lovell × Nemared by Lu et al. (2000) who proposed the *Mi* and *Mij* genes for resistance to *M. incognita* and both *M. incognita* and *M. javanica*, respectively. Resistance in Nemared has also been studied from interspecific crosses [P. 2175 × (Garfi × Nemared)] segregating both for *Ma* and Nemared resistance (Claverie et al. 2004a). Resistance from Shalil (the peach parent for the almond-peach GF.557) was established from the cross P. 2175 × GF.557 segregating both for *Ma1* and Shalil resistance (Claverie et al. 2004a). As those interspecific crosses involving Nemared and Shalil segregated

Table 1. Spectrum and genetics of resistance of main sources to root-knot nematodes used in *Prunus* rootstock breeding

Subgenus	Species	Resistance status to *M. arenaria* (MA)	*M. incognita* (MI)	*M. javanica* (MJ)	*M. floridensis* (MF)	Resistance gene and genotype	References
Prunophora							
	Myrobalan plum (*P. cerasifera*)					*Ma* gene controlling MA, MI, MJ and MF	
	P.2175	R[1]	R	R	R	(*Ma1 ma*)	Esmenjaud et al. 1994, 1996, 1997
	P.2980	R	R	R	R	(*Ma3 ma*)	Lecouls et al.1997
	P.2032[2]	S[1]	S	S	S	(*ma ma*)	Rubio-Cabetas et al. 1999
	P.2646[2]	S	S	S	S	(*ma ma*)	
	P.16.5[2]	S	S	S	S	(*ma ma*)	
Amygdalus							
	Peach (*P. persica*)						
	Nemared		R			*Mi* gene controlling MI and (or) *Mij* controlling MI and MJ	Lu et al. 2000
	Shalil						
	GF.557 = almond × Shalil peach					R_{Mia557} gene controlling MA and MI	Esmenjaud et al. 1994, 1997
	GF.557	R	R	S	S	($R_{Mia557}r_{Mia557}$)	Claverie et al. 2004a
	Nemared					R_{MiaNem} gene controlling MA and MI	Esmenjaud et al. 1997
	Nemaguard	R	R	R/S [3]	S	($R_{MiaNem}R_{MiaNem}$)	Claverie et al. 2004a
	Nemared	R	R	R/S	S	($R_{MiaNem}R_{MiaNem}$)	Dirlewanger et al. 2004a
	Juseitou		R	R		*Mia* gene controlling MI and *Mja* controlling MJ	Yamamoto et al. 2001 Yamamoto and Hayashi 2002
	Okinawa		R			*Mi* gene controlling MI race 1	Sharpe et al. 1969 Gillen and Bliss 2005

[1] R = resistant; S = susceptible

[2] Susceptible control accessions

[3] R/S: variable behavior in function of *M. javanica* isolates

identically for resistance to *M. incognita* and *M. arenaria*, a single gene controlling both RKN species was hypothesized and designated, respectively, R_{MiaNem} and R_{Mia557} in Nemared and GF.557 (Claverie et al. 2004a). Resistances in 'Juseitou' to *M. incognita* (gene *Mia*) and *M. javanica* (gene *Mja*) and in 'Okinawa' to *M. incognita* race 1 (gene *Mi*) have been studied in the F_2 populations Akame × Juseitou (Yamamoto et al. 2001; Yamamoto and Hayashi 2002) and Harrow Blood × Okinawa (Gillen and Bliss 2005).

4.3.2 Mapping of the RKN *Ma* Gene in Plum – Comparison with Peach RKN Genes

Molecular studies concerning *Ma* have been conducted to develop a local map of the gene and to locate it on the plum and reference *Prunus* maps. The data on comparative locations of RKN resistance genes from plum and peach have been firstly reported in Claverie et al. (2004a) and then confirmed in Dirlewanger et al. (2004a).

4.3.2.1 Local Map and Marker-Assisted Selection (MAS) for *Ma*

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers have been identified by bulked segregant analysis (BSA) (Michelmore et al. 1991) using intraspecific progenies involving P.2175 (*Ma1 ma*) and several susceptible parents (*ma ma*). Two reliable SCAR (sequence characterized amplified region) markers, $SCAL19_{690}$ (derived from a RAPD marker) and $SCAFLP2_{202}$ (derived from an AFLP marker), were shown to be linked in coupling phase to the dominant resistance alleles *Ma1* and *Ma3* (Lecouls et al. 1999, 2004). SCAL19 is located less than 1 cM from *Ma* and SCAFLP2 is cosegregating with *Ma*, as shown by the analysis of over 1,300 individuals belonging to diverse intra- and interspecific progenies (Claverie et al. 2004b).

4.3.2.2 Location of RKN Genes in the Prunophora Subgenus (Myrobalan and Japanese Plums)

In Myrobalan plum, three RFLP markers among 46 probes distributed all over the *Prunus* genome, revealed polymorphic fragments between the resistant and the susceptible bulks. All three RFLP markers lie on the linkage group G7 of the reference map (Joobeur et al. 1998) and cover 32 cM. This preliminary position of *Ma* on G7 was confirmed by the detection of a polymorphism or difference in amplification signal intensity between bulks for three SSR markers located on this group, pchgms6, UDP98-405, and CPPCT033. Genotyping the individuals of the couples of bulks completed by all other individuals previously characterized for *Ma* allowed to locate these markers on the same side of the gene at 2.3, 9.5 and 21.3 cM, respectively. These SSR markers are placed on the other side of the gene relative to the SCAR markers SCAL19 and SCAN12 (Claverie et al. 2004a) (Fig. 1).

Additionally, in the Japanese plum, a single dominant gene designated R_{jap} was hypothesized from a segregating progeny of 26 individuals between the RKN resistant accession J.222 (heterozygous) and the RKN susceptible accession J.13 (homozygous). The SCAR markers linked to *Ma* and all the SSRs available in the reference map for this G7 region were evaluated for their polymorphism in parents and all individuals of the progeny. On this small-sized cross, the markers pchgms6, CPPCT022 and SCAL19 cosegregated with the R_{jap} gene (Fig. 1), which shows that this gene lies on the G7 probably in the same position as *Ma* (Claverie et al. 2004a). In Prunophora, differences in allelism and polymorphism of genetic markers linked to resistance associated with co-location of the *Ma* and R_{jap} genes suggest the conservation of a resistance locus acquired before separation of the species Myrobalan and Japanese plums. It is likely that this location is conserved in cultivated and wild plum species including diploid to hexaploid species.

4.3.2.3 Comparative Location of Plum and Peach Genes – Consequences for MAS

All studies concerning peach mapping located the RKN resistance genes from this species in the linkage group G2 of the reference *Prunus* map T × E (Joobeur et al. 1998, 2000; Aranzana et al. 2003). The peach genes R_{Mia557} and R_{MiaNem}, carried by two *a priori* unrelated resistance sources, Shalil and Nemared respectively (Table 1), were colocalized in a subtelomeric position on the G2 (Claverie et al. 2004a; Arús et al. 2004). This location was different from the more centromeric position previously

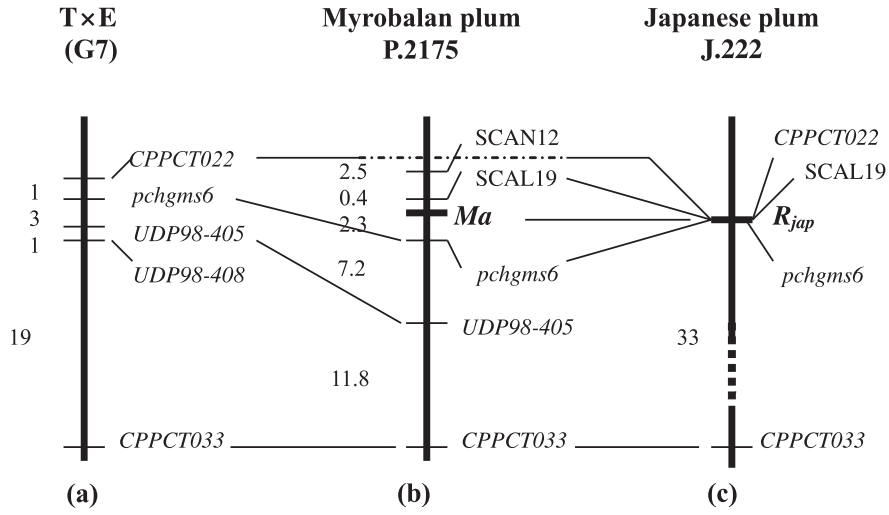

Fig. 1. Local maps of SSR (*in italics*) and SCAR (*normal letters*) markers linked to the *Ma* gene in the Myrobalan plum P.2175 (**b**) and to the R_{jap} gene in the Japanese plum J.222 (**c**) in comparison with SSR markers located on the linkage group G7 of the almond × peach reference *Prunus* map Texas × Earlygold (T×E) (**a**) (Aranzana et al. 2003). For the *Ma* gene, distances are expressed in cM using the Kosambi distance given by the MAPMAKER software version 3 (Lander et al. 1987) with a minimum LOD score of 3.0. For the *Rjap* gene, distances are expressed in recombination percentages

proposed by Lu et al. (1999) for the resistance gene *Mij* to *M. incognita* and *M. javanica* in Nemared near the SSR pchgms1 and the STS EAA/MCAT10. By contrast, R_{Mia557} and R_{MiaNem} were flanked by STS markers obtained by Yamamoto and Hayashi (2002) for the resistance gene *Mia* to *M. incognita* in the Japanese peach source Juseitou. Concordant results for the three independent sources, Shalil, Nemared and Juseitou, suggest that these peach RKN sources share at least one major gene for resistance to *M. incognita* located in this subtelomeric position.

The most beneficial and applied result is that *Ma* on the one hand and peach genes on the other hand are independent and can be pyramided into interspecific rootstock material. Construction of rootstock genotypes carrying *Ma* and peach genes by interspecific hybridization (e.g. Myrobalan plum × Amygdalus) is underway (Dirlewanger et al. 2004c; Esmenjaud 2004). These hybrids can thus cumulate favorable agronomic traits from both origins together with the complete-spectrum resistance controlled by the Myrobalan *Ma* gene and the more-restricted spectrum of Amygdalus genes. Indeed, the pyramiding of several genes in the same genotype may limit the risk of resistance breaking (Johnson 1983; Cook and Evans 1987; Roberts 1995) and thus extend the useful life of new rootstocks.

4.4 Construction of Maps for the 3-Way Interspecific Cross Myrobalan Plum × (Garfi × Nemared)

The mapping results reported here have been developed in Dirlewanger et al. (2004a). Inheritance and linkage studies were carried out with SSR markers in an F_1 progeny including 101 individuals of the cross between Myrobalan plum clone P.2175 and the almond-peach hybrid clone $(\text{Garfi} \times \text{Nemared})_{22}$ (= GN22). The *Ma* gene from P.2175 and the R_{MiaNem} gene from Nemared, are each heterozygous in the parents P.2175 and GN22, respectively. Two hundred and seventy seven *Prunus* SSRs were tested for their polymorphism. A genetic map was constructed for each parent according to the 'double pseudo-testcross' model of analysis (Fig. 2).

4.4.1 SCAR Analysis

$SCAL19_{690}$ and $SCAFLP2_{202}$, the two SCARs tightly linked to the *Ma* gene (Lecouls et al. 2004), were analyzed on the progeny. The five STS markers obtained

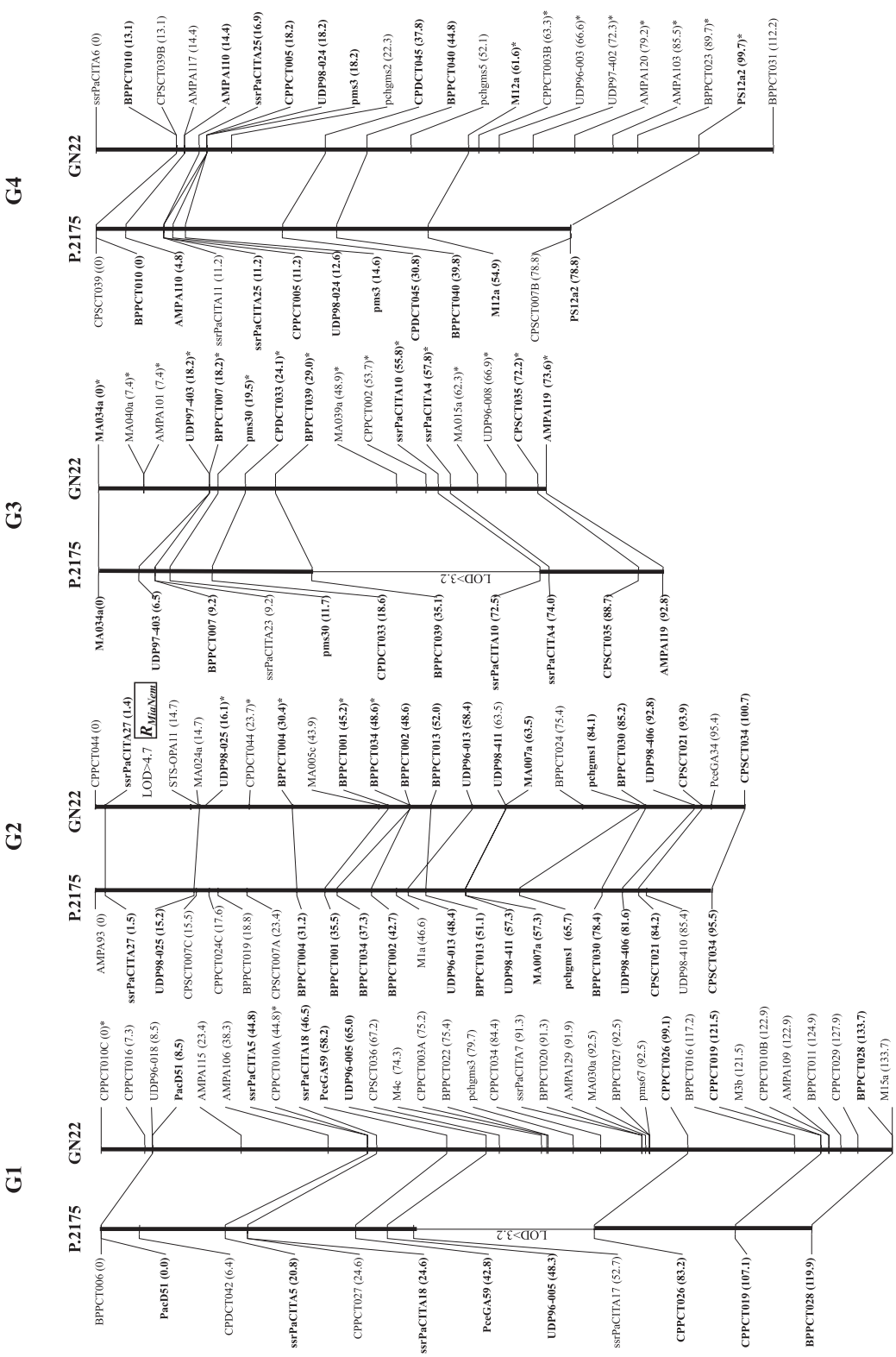

Fig. 2. Genetic maps obtained with the interspecific Myrobalan plum (P.2175) × almond-peach (GN22) F_1 progeny. Anchor-loci between the P.2175 and the GN22 maps (*in bold*) are connected by *lines*. Distorted loci ($P < 0.01$) are indicated by a *star* after the name

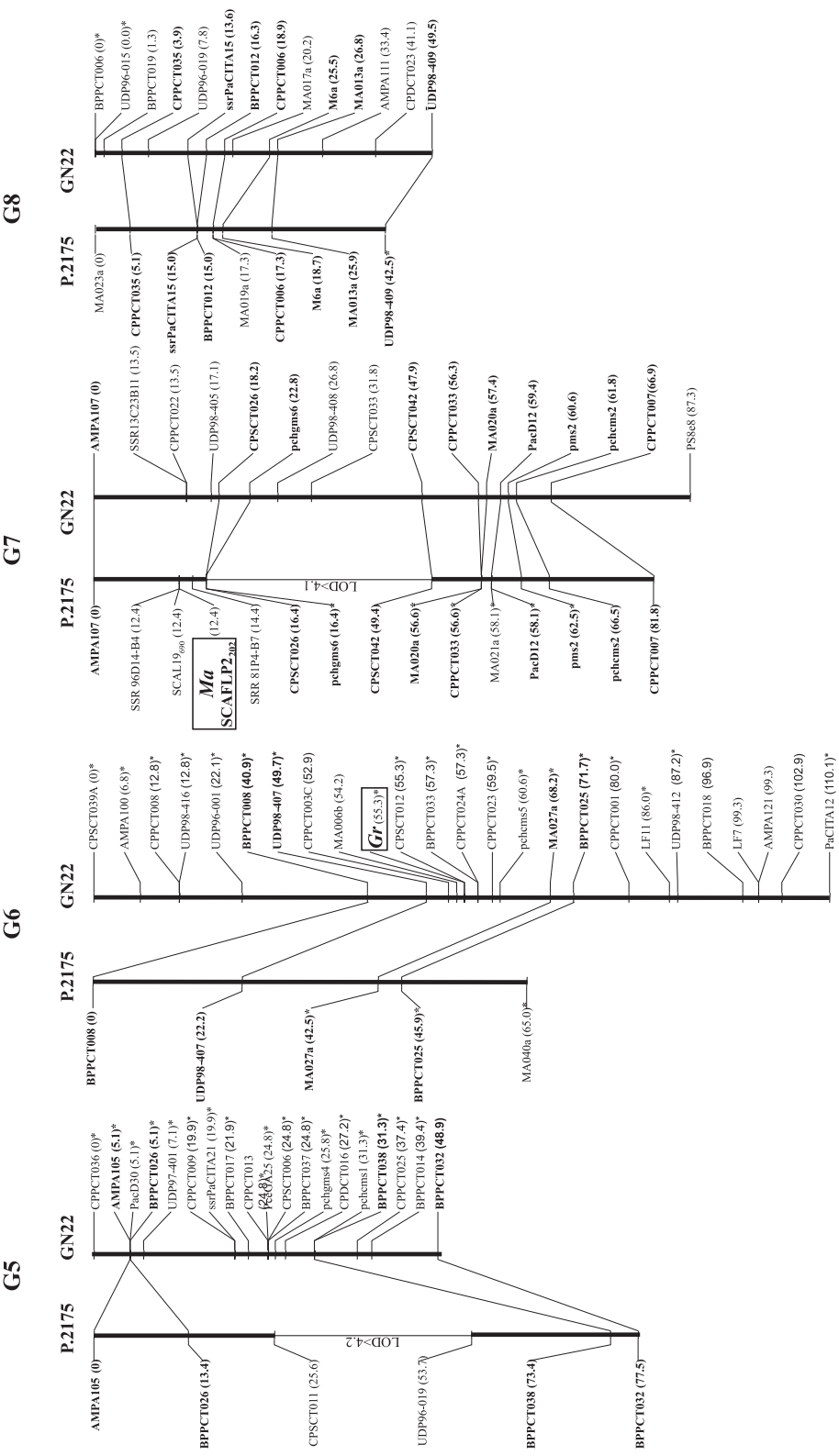

Fig. 2. (continued)

by Yamamoto and Hayashi (2002) linked to the resistance loci of the peach Juseitou, were also tested but only STS-OPA11 had a readable profile with a fragment of 481 bp segregating in the P.2175 × GN22 F_1 progeny.

4.4.2 SSR Analysis

Among the 277 SSRs originated from several *Prunus* species, 46 (16.6%) had complex profiles on acrylamide gels, 14 (5%) revealed no polymorphism, 104 (37.5%) revealed polymorphism in P.2175, 184 (66.4%) revealed polymorphism in GN22 and 84 (30.3%) were polymorphic in both parents. Thus the polymorphism detected in GN22 (66.4%) was much higher than in P.2175 (37.5%). The high degree of heterozygosity in GN22 results from its interspecific hybrid status. Most heterozygous SSRs in P.2175 were also heterozygous in GN22 (80.8%). These data confirm the high degree of microsatellite portability among *Prunus* previously reported by Cipriani et al. (1999) and Dirlewanger et al. (2002, 2004b). This high polymorphism between the Myrobalan plum P.2175 and GN22 has also been observed by Mnejja et al. (2004) between Japanese plum and peach (85%) or almond (78%), using 27 single-locus microsatellites. More SSRs deviated significantly from the expected ratio in GN22 (41.5%) than in P.2175 (10.6%); the interspecific status of GN22 may explain these results. In most cases, distorted segregations are more frequent in interspecific crosses than in intraspecific ones (Guo et al. 1991; Kianian and Quiros 1992) considering that mistakes between the coupling of homologous chromosomes during the metaphase 1 may occur in interspecific crosses. Among the 166 SSRs heterozygous in GN22, all those located on G3 and nearly all those located on G5 and G6, had a distorted segregation. In P.2175, distortions are located mainly in G6 and G7. Only the middle part of the G6 contained distorted segregating markers in both maps.

Many SSR markers (92) were already located on the T×E map (Aranzana et al. 2003), others were mapped on the peach P×F map (Dettori et al. 2001), on the apricot Stark Early Orange and Polonais maps (Lambert et al. 2004) or in the almond Ferragnès and Tuono maps (Joobeur et al. 2000). Here, 75 SSR markers were mapped for the first time.

4.4.3 Inheritance and Map Construction

4.4.3.1 Segregation of the *Ma*, R_{MiaNem} and *Gr* Genes

The 101 individuals from 2175 × GN22, tested to *M. floridensis* (Handoo et al. 2004) to evidence the *Ma* gene (Table 1), were shown to segregate into 40 resistant: 61 susceptible. This segregation deviated from the expected 1:1 ratio (P = 0.036). The 61 susceptible individuals were then processed for evaluation to *M. incognita*, in order to evidence the R_{MiaNem} resistance gene. Nevertheless, only a subset of both mapping populations could be evaluated, due to unsuccessful rooting of the cuttings. Within the 27 P.2175 × GN22 hybrids evaluated to *M. incognita*, 13 were resistant and 14 were susceptible thus fitting the expected 1.1 ratio. A high distorted segregation ratio (P = 0.00059) was observed for the color of the leaf, with 32 red-leaf and 66 green-leaf individuals.

4.4.3.2 *P.2175* Myrobalan Linkage Map

The P.2175 Myrobalan linkage map was constructed by analyzing the segregation of the *Ma* gene and 94 markers (92 SSRs, 2 SCARs) (Fig. 2). The P.2175 linkage map covered 524.8 cM with a LOD > 5.0 and 653.8 cM with a LOD > 3.2.

The *Ma* gene, already reported to cosegregate with the SCAR marker SCAFLP2 (Lecouls et al. 2004), cosegregated also with the SCAR $SCAL19_{690}$ and the SSR 96D14-B4. This SSR was identified within a BAC clone from the Nemared library (Georgi et al. 2002), containing $SCAFLP2_{202}$ (Lecouls, Personal Communication). The *Ma* gene and SSR 96D14-B4 segregated with the expected Mendelian 1:1 ratio; the two SCARs had distorted segregation (P = 0.037 each). They were located on P.2175 G7 at 12.4 cM from the top of the linkage group.

4.4.3.3 *GN22* Linkage Map and Evidence of Translocation

The map of the interspecific almond-peach GN22 parent from the P2175 × GN22 progeny was constructed by analyzing the segregation of the R_{MiaNem} gene, the *Gr* gene, and 166 markers (165 SSRs and 1 STS) (Fig. 2). With a LOD > 5.0, all markers were grouped into 7 linkage groups instead of the 8 expected. The

27 plants evaluated for resistance to *M. incognita* allowed the R_{MiaNem} gene to be mapped to linkage group 2 (G2) with a LOD>4.7. Six of the linkage groups, G1, G2, G3, G4, G5, G7, were homologous to those found in T×E with identical locus order and similar distances.

Thirty-eight markers formed a single group in the GN22 map and among them, 16 were already mapped in linkage group 6 and 11 in linkage group 8 in other maps [T×E (Aranzana et al. 2003), P×F (Dettori et al. 2001), J×F (Dirlewanger et al. 2006)]; 11 markers were not previously mapped. Ordering of this group (G6-G8) was difficult and linear order containing all loci could not be established. The map with the most markers included only 29 loci: 11 were already mapped in other maps on G6 and 11 on G8 (Fig. 2). The top of G6-G8 contained markers already mapped in the G6 but in the inverse order comparing to the T×E map, the bottom of G6-G8 contained markers already mapped in the G8 in exactly the same order as in T×E map. This pseudolinkage between G6 and G8 groups is a consequence of a reciprocal translocation between the chromosomes corresponding to G6 and G8. This was already reported in a 'Garfi' × 'Nemared' F_2 population (Jauregui et al. 2001). Reciprocal translocations are one of the most common structural chromosome rearrangements and have been detected in many species through the study of pollen viability and chromosome pairing during meiosis (Garber 1972).

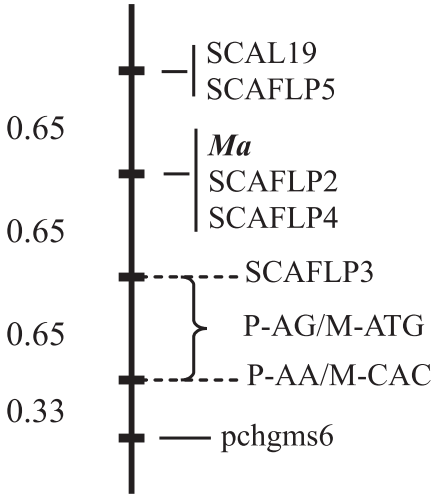

Fig. 3. Local map around *Ma* showing the position of AFLP and SCAR markers obtained from BSA. Distances expressed in recombination percentages have been evaluated from a population of approx. 300 individuals. P = *Pst*I; M = *Mse*I

With a LOD > 5.0, the GN22 map covered 716.0 cM, including the *Gr* gene located on G6 and 166 markers (165 SSRs and 1 STS). With a LOD > 4.7, the R_{MiaNem} gene controlling the nematode resistance from 'Nemared' was placed, as expected from location previously established by Claverie et al. (2004a), on G2 near the top of the group, between ssrPaCITA27 (13 cM) and the SCAR STSOPA11 that cosegregates with the SSR MAO24a (7.4 cM). The mean density of the map was 4.3 cM between markers.

4.4.4 Comparison of the *P.2175* and *GN22* Maps

Among the 73 SSRs markers polymophic in both parents and tested in the progeny, 68 were placed on both maps on homologous linkage groups. This shows a high level of colinearity between Myrobalan plum and the peach and almond genomes. This was already observed between apricot Stark Early Orange, Polonais and T×E (Lambert et al. 2004). These results reveal a strong homology of the genomes belonging to the *Prunophora* and *Amygdalus* sub-genera. By comparing all the *Prunus* maps sharing common markers, it is now possible to identify a set of single SSR loci covering all the genomes as it was proposed by Aranzana et al. (2003). Translocation events are now easily detected by using already mapped markers.

4.5 Strategy for Map-Based Cloning of the *Ma* Gene

Developing a positional cloning strategy for the *Ma* locus for resistance to RKN from the rootstock species *P. cerasifera* is a challenging task in particular because of the time and space required for producing and characterizing adequate populations. This project has been undergone because of the remarkable properties of the *Ma* gene which confers to this allogamous diploid plum, a complete-spectrum and a heat stable resistance to *Meloidogyne* spp. Conversely, the *Mi* gene from tomato, which is the RKN reference resistance (R) gene and the sole RKN R

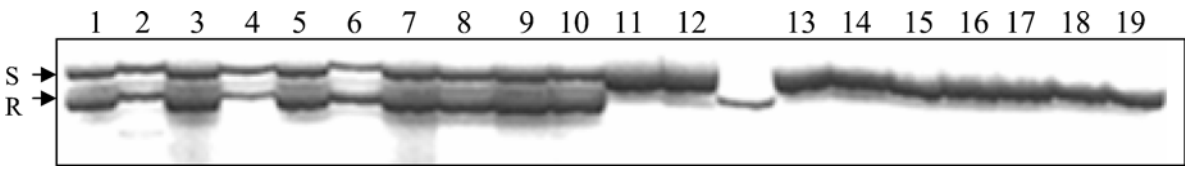

Fig. 4. Segregation of the SCAR marker SCAFLP4 in 19 individuals from different intraspecific progenies segregating for *Ma*. *Lanes 1–10*: *Ma* resistant individuals, heterozygous for the marker. *Lanes 11–19*: *Ma* susceptible individuals, homozygous for the marker. The *arrows* indicate the alleles in coupling with susceptibility (*S*) and resistance (*R*) in P.2175

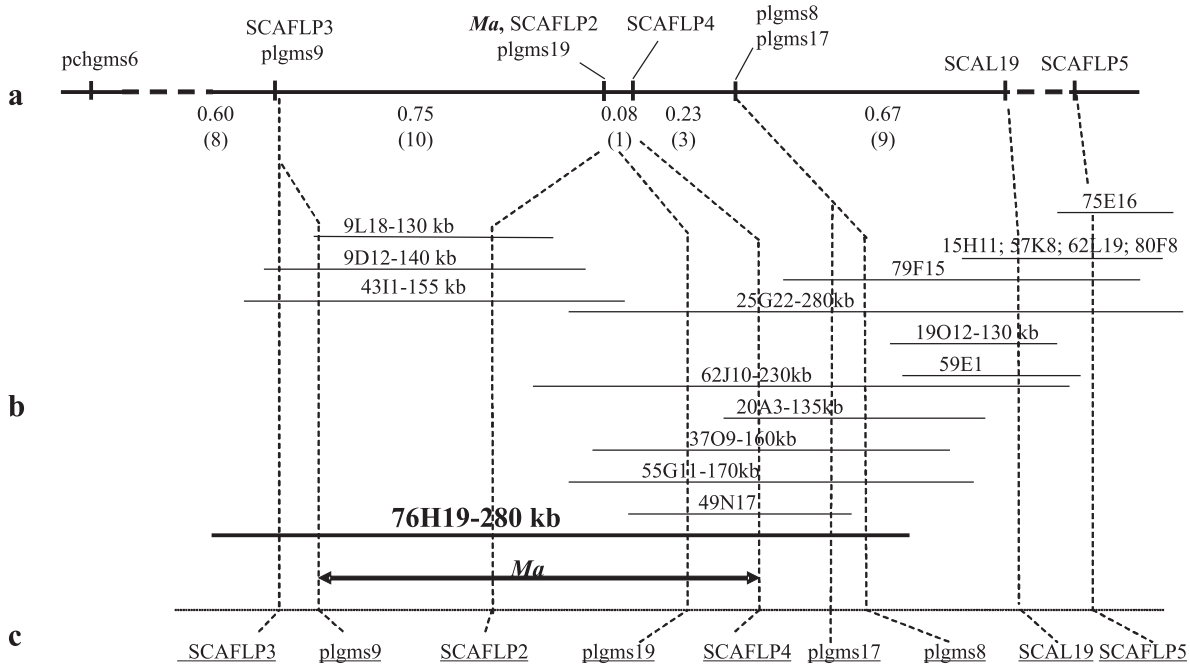

Fig. 5. Fine genetic mapping of *Ma* linked SSR (plgms) and SCAR (SCAFLP) markers (**a**) and physical mapping of positive BAC clones from the resistant contig (**b**). In (**a**), values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1332 total individuals (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. BAC clones experimentally characterized by the same markers are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (resistance allele)

gene cloned up-to-now, has a more restricted spectrum and a reduced efficiency at high temperature. Another favorable argument is that Myrobalan plum is a diploid species with a small and compact genome ($2n = 2x = 16$) estimated to be equivalent to the botanically closely related apricot species (*P. armeniaca*) (300 Mbp/1C; Arumuganathan and Earle 1991) i.e. with an average physical distance of about 300–400 kb per cM. The different steps of this strategy reported hereafter have been developed in Claverie et al. (2004b).

4.5.1 Detection of AFLP Markers by BSA, Development of PCR Markers and High-Resolution Mapping of the *Ma* Gene

Additional markers in the *Ma* region were obtained by BSA of 320 AFLP primer pairs combinations. Using a segregating population of 307 individuals, five AFLP markers tightly linked to the *Ma1* allele from P.2175 were obtained and mapped in the 2.3 cM interval spanning the gene, between the previously obtained

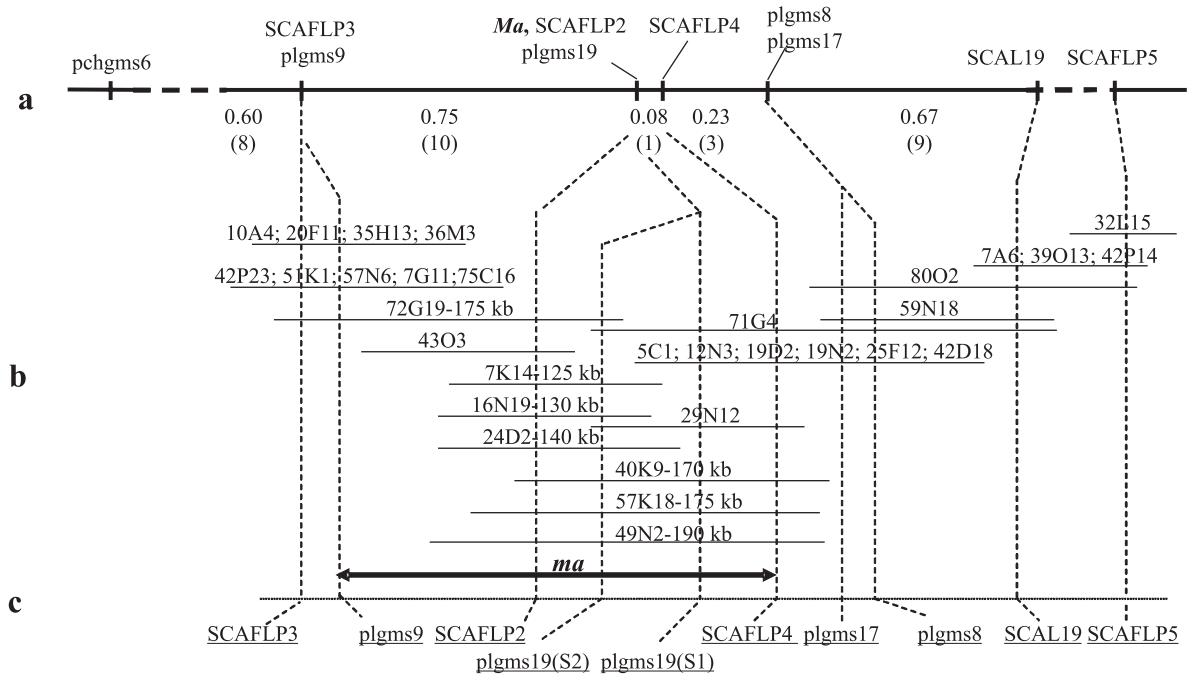

Fig. 6. Fine genetic mapping of *ma* linked SSR (plgms) and SCAR (SCAFLP) markers (**a**) and physical mapping of positive BAC clones from the susceptible contig (**b**). In (**a**), values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1,332 total individuals (between parenthesis, *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 base pairs) 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. BAC clones experimentally characterized by the same markers are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (*ma* susceptibility allele)

markers SCAL19 and pchgms6 (Fig. 3). Three of these markers were sequenced and transformed into SCAR or CAPS (cleaved amplified polymorphism sequence) markers designated SCAFLP3, SCAFLP4 (Fig. 4) and SCAFLP5.

A total number of 1,332 individuals, from 21 crosses segregating for *Ma*, revealed 31 individuals recombining between the flanking markers SCAL19 and pchgms6 in the genetic interval of 2.3 cM encompassing the gene. These recombinant individuals were then genotyped with the markers SCAFLP2, SCAFLP3 and SCAFLP4 and RKN resistance tests allowed a finer location of the gene (Figs. 5a and 6a): *Ma* co-segregated with the SCAFLP2 marker and was separated from SCAFLP4 by a single recombination event.

4.5.2 BAC Library Construction

A total of 30,720 BAC clones distributed into four-size classes (sub-libraries) were organized into 384-well plates. 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 size 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 145 kb with insert distribution ranging from 50 to 350 kb and the library has a 14–15× coverage of Myrobalan plum haploid genome. Considering that Myrobalan plum is highly heterozygous, this coverage must be expressed as a 7–8× coverage of the diploid genome.

4.5.3 Construction of Physical Contigs Spanning the *Ma* Region and Chromosome Landing

As the accession P.2175 is heterozygote and carries both R and S alleles of the *Ma* gene, R and S physical contigs were constructed by screening the BAC library

with the codominant co-segregating or tightly linked markers, SCAFLP2, SCAFLP3, SCAFLP4, SCAFLP5 and SCAL19 (Figs. 5a and 6a). The identified positive BAC clones 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. Surprisingly the markers SCAFLP2 and SCAFLP4 only separated by 0.08 cM were detected together only in a single clone of the R contig and three clones of the S contig. Finally a single BAC clone ('BAC76H19') carried all together 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*. SSR amplifications were performed on recombinant individuals (and on parental material as controls) for a refined genetic mapping of the region surrounding *Ma*; the SSR plgms9 from the 9L18 T7 BAC end cosegregated with SCAFLP3, the SSR plgms19 cosegregated with *Ma* (and SCAFLP2) and the two others (plgms8 and plgms17) cosegregated and fell between SCAFLP4 and SCAL19. From amplification data in the BAC clones from the *Ma*-resistant and *Ma*-susceptible contigs, these newly developed markers were then placed on the resistant and susceptible physical maps (Figs. 5b and 6b).

4.6 Conclusion and Future Scope of Works

4.6.1 Resistance to Plum Pox Virus

Several genes are involved in the resistance to Plum pox virus in peach and apricot and identification of QTLs is in progress in both species (Guillet and Audergon 2001; Villanova et al. 2003; Decroocq et al. 2005). Because of the high synteny within *Prunus* and the close genetic relationships between them and in particular between apricot and plum, it is assumed that most genetic and mapping information acquired in peach or apricot about resistance to PPV will be easily transferable and thus exploitable in plum. In *P. davidiana* for example, identification of distinct genomic regions involved in resistance and their co-localization with virus resistance gene analogues (Decroocq et al. 2005) are the first steps towards marker-assisted selection of PPV resistance for peach from this wild peach species and might be useful later in plum species. The numerous SSR markers now characterized in plum (Decroocq et al. 2004; Dirlewanger et al. 2004a) are powerful tools in this way.

As a complement to conventional breeding, transgenic clone C5 is a promising source of high level PPV resistance transferable to progeny through cross-hybridization experiments (Ravelonandro and Scorza 2004). To alleviate the concern of consumers about genetically modified organisms (GMOs), research work is still necessary to evaluate the safe use of genetically modified fruits and the ability of such GMOs to contribute to a sustainable agriculture.

4.6.2 Genome Mapping

The *Ma* gene and 93 markers (2 SCARs, 91 SSRs) were placed on the P.2175 Myrobalan map covering 524.8 cM. In peach, the R_{MiaNem} gene, the *Gr* gene controlling the color of leaves, and 166 markers (1 SCAR, 165 SSRs) were mapped to seven linkage groups instead of the expected eight in *Prunus*. Markers belonging to groups 6 and 8 in previous maps formed a single group in the GN22 map and evidenced a reciprocal translocation, already reported in a Garfi × Nemared F_2, near the *Gr* gene. By separating markers from linkage groups 6 and 8 from the GN22 map, it was possible to compare the eight homologous linkage groups between the two maps using the 68 SSR markers heterozygous in both parents (anchor loci). All but one of these 68 anchor markers are in the same order in the Myrobalan plum map and in the almond-peach map, as expected from the high level of synteny within *Prunus*. The *Ma* and R_{MiaNem} genes confirmed their previous location in the Myrobalan linkage group 7 and in the GN22 linkage group 2, respectively. The SCAR markers ($SCAL19_{690}$, $SCAFLP2_{202}$) (Lecouls et al. 2004) cosegregated with *Ma* and 4 SSRs (SSR 96D14-B4, SSR 81P4-B7, SSR6, SSR12) were located in the same region.

All these data will be used in the *Prunus* rootstock breeding program aiming at developing a new generation of *Prunus* rootstocks bearing high resistance to RKN using MAS and several additional characters such as adaptation to chlorosis and drought (from almond), tolerance to waterlogging (from plum) together with graft compatibility with peach (from peach) and good rooting ability (from plum) (Esmenjaud 2004; Dirlewanger et al. 2004c). These data will also be available for the other rootstock programs relative to *Prunus* crops and particularly peach (Reighard 2002). The genetic linkage maps constructed from the interspecific F_1population issued from the cross P.2175 × GN22 will be used for the detection of QTLs involved in drought, waterlogging and chlorosis resistance. A subset of the progeny has already been evaluated for different ecophysiological parameters (predawn leaf water potential, conductance, transpiration, photosynthesis and growth parameters). A high variability of response was observed, especially for the water use efficiency, an essential condition for a breeding program (Kleinhentz et al. 2005), confirming that this material is promising for the selection of a new generation of *Prunus* rootstock associating the favorable characters of each species.

4.6.3 Towards Map-Based Cloning of *Ma*

The *Ma1* allele from the heterozygous parent P.2175 was accurately located using SSR markers available from *Prunus* maps. Applying an adapted BSA strategy resulted in three extra AFLP markers tightly flanking *Ma1* which were transformed into codominant SCAR markers. These markers, as well as the two closely linked markers obtained in previous studies, were used to build a high-resolution map, based on recombination events at the *Ma1* locus from segregating intra- and inter-specific crosses including more than 1,300 individuals. A BAC library of the parent P.2175 characterized by a large mean insert size (145 kb) and a 14–15 × haploid genome coverage was constructed. The markers tightly linked to the gene allowed the elaboration of the R and S contigs at the *Ma* locus. One 287 kb insert BAC carrying *Ma1* was detected in the R contig.

Because of the characteristics of *Ma* (i.e., complete RKN spectrum, heat stability, and no virulent isolate known), it may be of an outstanding interest to identify the gene and to study its structure, function and evolution. In this objective, a complete sequencing of the 280-kb insert has been performed. Bioinformatic sequence analysis revealed a cluster of three TIR-NBS-LRR (TNL) open reading frames (ORFs) lying between candidate ORFs from other multigenic families. New SSR markers directly derived from the BAC sequence reduced the physical interval encompassing *Ma* to a 70 kb region including a putative lectin/kinase receptor (LecRK) and the TNLs. Additional fine mapping, using 1,700 young Myrobalan plum segregating seedlings, still reduced this interval to 54 kb only containing the three TNLs as candidate resistance genes (Claverie 2004). The analysis of these sequences, in combination with a linkage disequilibrium study among Myrobalan plum accessions should allow to identify the best candidate to encode the *Ma* gene. Sequence analysis will also generate data about *Prunus* genome organization (genes, microsatellites, structure and distribution of repeated sequences) in this particular region that will be compared to the susceptible *Ma* region and extended via microsynteny to other *Prunus* or *Rosaceae* species. This study may also provide new information about the dynamics of the natural evolution of a resistance locus from a perennial, near-wild and self-incompatible plant (Salesses et al. 1993, 1994).

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