New Data Completing the Spectrum of the *Ma*, *RMia*, and *RMja* Genes for Resistance to Root-Knot Nematodes (*Meloidogyne* spp.) in *Prunus*

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ABSTRACT

Root-knot nematodes (RKN) (*Meloidogyne* spp.) are worldwide pests that affect a considerable number of plants, among which stone fruit (*Prunus* spp.) are severely attacked. Prevalent RKN species are *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* in stone fruit but the emergent *M. ethiopica* and *M. enterolobii* are also reported to challenge perennial crops. In *Prunus* spp., the complete-spectrum resistance (*R*) gene *Ma* from plum and the more restricted-spectrum *R* genes *RMia* from peach and *RMja* from almond completely inhibit nematode multiplication and gall formation of the RKN species that they control. This study aimed to update the resistance spectra of these three major genes by evaluating their activity toward one isolate of the yet-untested RKN species mentioned above. To state whether a given gene controls a particular species, the principle of our experiment was to genotype with appropriate markers a number of individuals

segregating for this gene and then to phenotype these individuals. A perfect matching of the genotype and the phenotype of individuals indicates that the gene of interest is active against and, thus, controls the corresponding isolate of this RKN species. Segregating materials used were an *Ma* F1 plum progeny, an *RMia* F2 peach progeny, and an *RMja* F2 almond progeny. In addition to previous data, our results establish a clear spectrum for each of the three genes toward isolates from both the three prevalent species and the two emerging species. Ultimately, our results reveal that (i) *Ma* controls all of them, (ii) *RMja* controls all species except *M. incognita* and *M. floridensis*, and (iii) *RMia* controls *M. arenaria*, *M. incognita*, and *M. ethiopica* but not *M. javanica* or *M. enterolobii*. Our data should have wide implications for RKN resistance management and breeding and for deciphering the molecular mechanisms of the spectrum of RKN *R* genes.

Root-knot nematodes (RKN) (*Meloidogyne* spp.) are major crop pests throughout the world (Jones et al. 2013; Lamberti 1979). *Meloidogyne arenaria*, *M. incognita*, and *M. javanica*, the predominant RKN species under Mediterranean and hot climates, are highly polyphagous and reproduce through mitotic parthenogenesis (Triantaphyllou 1985) on hundreds of cultivated and wild plant species (De Guiran and Netscher 1970). Several RKNs have been described and reported recently as emerging species with a putatively high impact on woody crops (Saucet et al. 2016). These species include the invasive *M. ethiopica*, prevalent in Chile (Carneiro et al. 2007a; Meza et al. 2016) and Brazil (Correa et al. 2014), where new intensive perennial crops such as stone fruit and kiwi are set up. These RKNs also include the polyphagous species of tropical origin, *M. enterolobii* (formerly *M. mayaguensis*), which is uncontrolled by the *Mi-1* gene from tomato and highly aggressive toward many plants (Castagnone-Sereno 2012; Rammah and Hirschmann 1988; Saucet et al. 2016; Yang and Eisenback 1983), including perennial guava crops (Carneiro et al. 2006; Carneiro et al. 2007b). Another species is *M. floridensis*, detected first in *Prunus* spp. in the southeastern United States and designated as the

peach RKN because it attacks peach resistance sources (Handoo et al. 2004; Saucet et al. 2016).

The genus *Prunus* is divided into five main subgenera and comprises more than 400 species distributed worldwide (Rehder 1947). Though the plum species range from diploid to hexaploid ($2n = 6x = 48$), most species are diploid ($2n = 2x = 16$) (Salesses et al. 1992). A majority of fruit-producing and rootstock species belong to the subgenera *Amygdalus* (almond and peach) and *Prunophora* (plums and apricot). In stone fruit crops, *Prunus* spp. rootstock material is predominantly susceptible to RKNs. Resistance sources have been sought from a wide range of *Prunus* crops with the objective of controlling these pests (Saucet et al. 2016) and they express different resistance spectra (Esmenjaud et al. 1997, 2009; Scotto La Massese et al. 1984).

Genes conferring RKN resistance have been identified especially in plum (*Ma*), peach (*RMia*), and almond (*RMja*). The major dominant gene *Ma* from Myrobalan plum (*Prunus cerasifera*) confers a high-level resistance to all tested RKN species (*M. incognita*, *M. arenaria*, *M. javanica*, *M. floridensis*, and *M. enterolobii*) (Lecouls et al. 1997; Rubio-Cabetas et al. 1999). *Ma*, located on the chromosome 7 of the peach (*P. persica*) genome, the reference sequence for *Prunus* spp. (Verde et al. 2013), belongs to the toll interleukin 1 receptor nucleotide-binding leucine-rich repeat (TNL) class of genes (Claverie et al. 2011). The *RMia* gene carried by the peach cultivars Nemaguard and Nemared (Ramming and Tanner 1983) confers resistance to *M. incognita* and *M. arenaria*. Additionally, several genetic hypotheses have been put forward regarding the control of *M. javanica* in these accessions: two independent genes (Sharpe et al. 1969) or a single gene (Lu et al. 1998; Yoshida and Seike 1981). Nevertheless, the genetic determinants of this resistance remain to be identified and studies have proposed that resistance (*R*) genes for resistance to *M. incognita* might have a partial effect on *M. javanica* (Lu et al. 1998, 1999). *RMia* is a putative TNL gene which is located on chromosome 2 (Claverie et al. 2004a; Duval et al. 2014). Finally, the

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RMja gene (Van Ghelder et al. 2010) in the Alnem series (Alnem1, Alnem88, and Alnem201) of bitter almond (*P. dulcis*) confers a specific resistance to *M. javanica* (Kochba and Spiegel-Roy 1975) (Table 1). *RMja* belongs to the same cluster as the *Ma* gene and is presumably its ortholog (Van Ghelder et al. 2018).

This article considers the five RKN species *M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii*, and *M. ethiopica*. It aims to supplement the data on the spectrum of resistance carried by the three *Prunus* *R* genes previously reported, by evaluating them toward an isolate of the RKN species yet untested (Table 1). Because each of the genes completely inhibits nematode multiplication and gall formation of the RKN species that they control, we used the gall index rating criterion to phenotype individuals as resistant (R) or susceptible (S). All three genes have been evaluated to *M. ethiopica*. The *RMja* almond gene has also been assessed to *M. arenaria* and *M. enterolobii*. In addition, the *RMia* peach gene has been evaluated toward the isolate of *M. javanica* to assess whether it might have a partial effect on this latter species using *M. incognita* as a reference controlled RKN species. To state whether a given gene controls a particular species, we genotyped a number of individuals segregating for the gene studied using appropriate markers, then phenotyped these individuals for resistance to this RKN species. From the genotype-phenotype matching, we finally established whether this latter gene controls the RKN species represented by its tested isolate.

MATERIALS AND METHODS

Plant material. The plant material belonged to plum (*Ma* gene), almond (*RMja* gene), and peach (*RMia* gene). In plum, an *Ma* segregating cross between the *Ma*-resistant accession P.2980 (heterozygous; *Ma/ma*) (Lecouls et al. 2004; Rubio-Cabetas et al. 1998) and the susceptible accession P.1090 (rootstock cultivar Myrocal; *ma/ma*) (D. Esmenjaud, unpublished) has been specifically produced for our study (Fig. 1). The F1 progeny segregated into the two classes *Ma/ma* (R) and *ma/ma* (S). Forty-one random individuals were genotyped at the seedling stage. All individuals were subsequently phenotyped for resistance to *M. ethiopica* (Table 2). The *Ma* heterozygous donor P.2175 (*Ma/ma*) (Claverie et al. 2004a,b, 2011) and three other *Ma*-susceptible accessions (homozygous recessive, *ma/ma*) P.2032, P.16.5, and P.1254 were also used as controls (Lecouls 2000; Lecouls et al. 1999).

In peach, a four-way cross involving two F1 hybrids obtained from four peach accessions was used (Fig. 1). The first F1 individual was (Pamir × Rubira)140 (= PR140) between the two peach accessions Pamir and Rubira, both susceptible to *M. arenaria*, *M. incognita*, and *M. javanica*, and homozygous recessive for *RMia* (*rmia/rmia*). The second F1 cross was Montclar × Nemared between the peach Montclar, susceptible to these RKN species and homozygous recessive for *RMia* (*rmia/rmia*), and Nemared, resistant to *M. incognita* and *M. arenaria* and homozygous

TABLE 1. Status of the resistance spectrum of the three *Prunus* resistance genes (*Ma* from plum, *RMja* from almond, and *RMia* from peach) before the study^a

Resistance source (gene)	Genotype	<i>Meloidogyne arenaria</i>	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. ethiopica</i>	<i>M. enterolobii</i>
P.2175 & P.2980 (<i>Ma</i>)	<i>Ma/ma</i>	R ^s	R ^t	R ^t	–	R ^u
Alnem1 (<i>RMja</i>)	<i>RMja/RMja</i>	–	S ^v	R ^w	–	–
Nemared (<i>RMia</i>)	<i>RMia/RMia</i>	R ^x	R ^y	–	–	S ^z

^a R = resistant, S = susceptible, and – = unknown.
^s Esmenjaud et al. 1996.
^t Lecouls et al. 1997.
^u Rubio-Cabetas et al. 1999.
^v Scotto La Massese et al. 1984.
^w Kochba and Spiegel-Roy 1975; Esmenjaud et al. 2009; Van Ghelder et al. 2010.
^x Claverie et al. 2004a.
^y Claverie et al. 2004a; Esmenjaud et al. 2009; Duval et al. 2014.
^z Khalouk 2013.

Fig. 1. Plant materials from Myrobalan plum, peach, and almond and the different crosses used in the study.

dominant for *RMia* (*RMia/RMia*). The four-way offspring was created for this study by crossing the female hybrid PR140 and a mixture of the pollen from 51 F1 hybrids of Montclar × Nemared. The seedlings assessed were obtained from an F2 progeny of the hybrid number 8 of the PR140 × MN four-way cross (designated PR140MN8) and genotyped *RMia/rmia* using markers developed by Duval et al. (2014). These seedlings belonged to one of the three classes of individuals: *RMia/RMia*, *RMia/rmia*, and *rmia/rmia* (Fig. 1). In this objective, 104 (*M. ethiopica*), 79 (*M. javanica*), and 59 (*M. incognita*) random individuals were genotyped and subsequently phenotyped for resistance to RKNs (Tables 2 and 3).

In almond, an F2 progeny was obtained from the self-pollination of the F1 hybrid (Lauranne × Alnem1)135 (designated F2 LxA). The accession Lauranne is susceptible to RKN species and homozygous recessive for *RMja* (*rmja/rmja*) and the accession

Alnem1 is resistant to *M. javanica* and homozygous dominant for *RMja* (*RMja/RMja*) (Fig. 1). The individuals evaluated are a part of the offspring obtained for another study on the high-resolution mapping of the *RMja* gene (Van Ghelder et al. 2018). The F2 progeny segregated into three different genotypes for resistance (*RMja/RMja*, *RMja/rmja*, and *rmja/rmja*). In all, 89 (*M. ethiopica*), 69 (*M. enterolobii*), and 100 (*M. arenaria*) random individuals were genotyped and subsequently phenotyped for resistance to RKNs (Tables 2 and 4).

Nematode isolates. Five isolates were used: one from each of the three predominant species *M. arenaria*, *M. incognita*, and *M. javanica*; one from the species *M. ethiopica*; and one from the species *M. enterolobii*. For the three predominant RKNs, these were the isolates *M. arenaria* ‘Six-Fours’ from Six-Fours (Provence, France), *M. incognita* ‘Calissane’ from Calissanne (Provence,

TABLE 2. Evaluation of resistance to the root-knot nematode (RKN) species *Meloidogyne ethiopica* (isolate Nancagua, Chile) in reference, parental, and segregating materials for the *Ma*, *RMia*, and *RMja* genes^t

Gene, plant material	Genotype ^u	Segregates ^v	Hybrid	Phenotype ^w	Expected		Observed	
					S	R	S	R
<i>Ma</i>								
P.2175	<i>Ma/ma</i>	...	...	R	...	...	...	...
P.1254	<i>ma/ma</i>	...	...	S	...	...	...	...
P.16.5	<i>ma/ma</i>	...	...	S	...	...	...	...
P.2032	<i>ma/ma</i>	...	...	S	...	...	...	...
P.2980	<i>Ma/ma</i>	...	...	R	...	...	...	...
P.1090 (Myrocal)	<i>ma/ma</i>	...	...	S	...	...	...	...
F1 (P.2980 × P.1090) ^x	...	...	41	...	...	...	...	...
	...	1, <i>Ma/ma</i>	26	...	0	26	0	26
	...	2, <i>ma/ma</i>	15	...	15	0	15	0
<i>RMia</i>								
Pamir	<i>rmia/rmia</i>	...	...	S	...	...	...	...
Rubira	<i>rmia/rmia</i>	...	...	S	...	...	...	...
Montclar	<i>rmia/rmia</i>	...	...	S	...	...	...	...
Nemared	<i>RMia/RMia</i>	...	...	R	...	...	...	...
PR140MN8	<i>RMia/rmia</i>	...	...	R	...	...	...	...
F2 (PR140MN8) ^y	...	...	104	...	...	...	...	...
	...	1, <i>rmia/rmia</i>	32	...	32	0	32	0
	...	2, <i>RMia/rmia</i>	42	...	0	72	0	72
	...	3, <i>RMia/RMia</i>	30	...	...	...	...	...
<i>RMja</i>								
Lauranne (L)	<i>rmja/rmja</i>	...	...	S	...	...	...	...
Alnem1 (A)	<i>RMja/RMja</i>	...	...	R	...	...	...	...
F1 LxA	<i>RMja/rmja</i>	...	...	R	...	...	...	...
F2 LxA ^z	...	...	89	...	...	...	...	...
	...	1, <i>rmja/rmja</i>	18	...	18	0	18	0
	...	2, <i>RMja/rmja</i>	40	...	0	71	0	71
	...	3, <i>RMja/RMja</i>	31	...	...	...	...	...

^t R = resistant and S = susceptible.

^u The genotype of segregating material was evaluated using markers NSCALF2 and CT3-4 N (*Ma*), SNP_APP91 and SNP_APP92 (*RMia*), and LRR65 and kin35 (*RMja*).

^v Segregates into.

^w Phenotype to *M. ethiopica*.

^x Expected data if *Ma* controls this RKN.

^y Expected data if *RMia* controls this RKN.

^z Expected data if *RMja* controls this RKN.

TABLE 3. Numbers of individuals and their resistance phenotype, gall index, collar diameter, BS root and top weights, after evaluation to the root-knot nematode species *Meloidogyne javanica* (*M. jav.* isolate Higuera, Catalonia) using *M. incognita* (*M. inc.* isolate Calissanne, France) as a control in the four-way cross PR140MN8 segregating for the *RMia* gene^y

Class	Genotype ^z	Number of individuals		Phenotype		Gall index (0–5)		Collar diameter (mm)		Root weight (g)	Top weight (g)
		<i>M. jav</i>	<i>M. inc</i>	<i>M. jav</i>	<i>M. inc</i>	<i>M. jav</i>	<i>M. inc</i>	<i>M. jav</i>	<i>M. inc</i>	<i>M. jav</i>	<i>M. jav</i>
1, Homozygous S	<i>rmia/rmia</i>	30	17	All S	All S	1.83 a	3.12 a	8.15 a	7.53 b	54.7 a	24.7 a
2, Heterozygous R	<i>RMia/rmia</i>	30	25	All S	All R	1.67 a	0.16 b	8.26 a	8.60 a	52.6 a	27.2 a
3, Homozygous R	<i>RMia/RMia</i>	19	17	All S	All R	1.89 a	0.18 b	8.23 a	8.88 a	56.2 a	25.5 a

^y R = resistant and S = susceptible. In the same column, numbers followed by the same letter are not significantly different according to the least significant difference Fisher test at $P \leq 0.05$ (analysis of variance statistical analysis).

^z The genotype of segregating material was evaluated using *RMia* markers SNP_APP91 and SNP_APP92.

France), and *M. javanica* ‘Higuera’ from Cabrils (Cataluna, Spain). For *M. ethiopica*, we used the isolate Nancagua from this locality in the VI Region of Chile. This isolate has been evaluated on the peach Nemaguard and Pomona and identified as belonging to the 3 most aggressive and not statistically different isolates out of 15 isolates evaluated (Meza et al. 2016). We also used the isolate *M. enterolobii* ‘Godet’ from Godet in the Guadeloupe Island (French West Indies, France). All five isolates had been reared from a single egg mass. The isolates were maintained on tomato (*Lycopersicon esculentum*) cultivar St. Pierre. Their specific identity was verified before the experiments via their isoesterase profile (Esbenshade and Triantaphyllou 1985, 1990; Janati et al. 1982).

Greenhouse phenotyping method using a high- and durable-inoculum pressure test. Experiments were implemented over 3 years in three independent annual evaluations performed from March to November. Each year, we used a greenhouse phenotyping method based on a high- and durable-inoculum pressure test, as described hereafter. In March, plantlets obtained from 2- to 3-month-old seed (peach and almond) or semihardwood cuttings from the previous autumn (parental and reference materials from plum) were repotted in pairs into 5-liter containers filled with a nematode-free sandy substrate. Evaluations for RKN resistance were performed with a method derived from Esmenjaud et al. (1992). All of the pots were placed on iron benches in the greenhouse, irrigated individually every 2 days, and grown until harvested for rating at a mean temperature of 25°C (extremes of 20 to 30°C). RKN inoculation was performed as follows. At mid-March, tomato plantlets grown in 250-ml plastic pots were inoculated with 500 24- to 72-h-old juveniles (infective stage; J2), obtained from the INRA collection. Each of the RKN isolates was suspended in water and deposited into two holes, 2 cm deep and 2 cm from the stem. At mid-May, the top parts of tomato plants were cut and removed and one soil and root system content was transplanted into each *Prunus* pot. Pots inoculated with the same *Meloidogyne* sp. were grouped on the same greenhouse bench to avoid cross contaminations. Plants of the different genotypes (RR, RS, or SS) for each gene were arranged in a randomized design.

Rating of RKN resistance for R or S phenotyping. In autumn, 5 months after inoculation with galled tomato, *Prunus* plants were harvested, carefully washed free of soil, and given a root gall index rating according to the 0-to-5 scale (Barker 1985), where

0 = no gall and 1 = 1 to 10%, 2 = 11 to 30%, 3 = 31 to 70%, 4 = 71 to 90%, and 5 > 90% of root system galled. This rating was completed with 0.5 steps when galling was intermediate between two categories. This rating procedure has been established in a previous study, which demonstrated that gall index was highly significantly correlated with the $\log_{10}(x + 1)$ transformed numbers of the different nematode stages in the roots (Esmenjaud et al. 1992). When plants exhibited 1 to 5 incipient galls spread throughout the roots, these few galls were dissected to identify the fixed nematode stages (third and fourth developmental stages, female adults) and, thus, state whether the nematodes were the causal agent of the gall symptoms or not. Only in the positive case were they rated 0.1 (= 1 RKN gall visually confirmed) or 0.5 (= 2 to 5 RKN galls visually confirmed) to refine the classification. Individuals from a segregating progeny (together with the parental and reference accessions) were classified into either the R ($0 \leq \text{gall index} \leq 0.5$) or the S (gall index > 0.5) phenotypes.

In the experiment that aimed to evaluate a quantitative effect of the isolate of *M. javanica* on plants carrying the *RMia* peach gene, harvested plants were rated for their collar diameter and their total root and aerial part weights (parts below and above the collar, respectively). In this experiment, additional plants were used as controls for resistance to *M. incognita* (*RMia* gene) and rated for their gall index and collar diameter (Table 3).

Molecular genotyping of plant material using resistance markers for each gene. Young terminal leaves of each seedling were collected as needed in the early spring and kept at -80°C until DNA extraction. Total genomic DNA was isolated using the Dneasy kit (Qiagen). Polymerase chain reaction (PCR) assays were performed in a 12-µl final volume containing 10 ng of genomic DNA, 0.25 U of Taq polymerase (Promega Corp.), 0.2 µM each primer, 0.2 mM each dNTP (Promega Corp.), 1.5 mM MgCl₂, and 1× reaction buffer provided with the enzyme. PCR conditions were as follows: 94°C for 4 min; then, 35 cycles of 94°C for 45 s, annealing temperature of the primers for 45 s, and 72°C for 45 s; and, finally, 72°C for 4 min. Amplifications were performed in a Mastercycler ep gradient thermal cycler (Eppendorf GmbH). Markers were first screened using the parents and then using a set of individuals from the progeny to establish segregation patterns. The PCR products were separated on a 2.5% agarose gel or with a capillary sequencer (Applied Biosystems 3730 DNA Analyzer)

TABLE 4. Evaluation of resistance of parental and segregating materials conferred by the *RMja* gene to the root-knot nematode species *Meloidogyne enterolobii* (isolate Godet, Guadeloupe) and *M. arenaria* (isolate Six-Fours, France)^a

Species, plant material	Genotype ^v	Segregates ^w	Hybrid	Phenotype to ^x	Expected		Observed	
					S	R	S	R
<i>M. enterolobii</i>								
Lauranne (L)	<i>rmja/rmja</i>	...	...	S	...	...	...	...
Alnem1 (A)	<i>RMja/RMja</i>	...	...	R	...	...	...	...
F1 LxA	<i>RMja/rmja</i>	...	...	R	...	...	...	...
F2 LxA ^y	...	...	69	...	...	...	...	...
	...	1, <i>rmja/rmja</i>	20	...	20	0	20	0
	...	2, <i>RMja/rmja</i>	42	...	0	49	0	49
	...	3, <i>RMja/RMja</i>	7	...	...	...	...	...
<i>M. arenaria</i>								
Lauranne (L)	<i>rmja/rmja</i>	...	...	S	...	...	...	...
Alnem1 (A)	<i>RMja/RMja</i>	...	...	R	...	...	...	...
F1 LxA	<i>RMja/rmja</i>	...	...	R	...	...	...	...
F2 LxA ^z	...	...	100	...	...	...	...	...
	...	1, <i>rmja/rmja</i>	30	...	30	0	30	0
	...	2, <i>RMja/rmja</i>	47	...	0	70	0	70
	...	3, <i>RMja/RMja</i>	23	...	...	...	...	...

^a R = resistant and S = susceptible.

^v The genotype of segregating material was evaluated using *RMja* markers LRR65 and kin35.

^w Segregates into.

^x Phenotype to *M. enterolobii* or *M. arenaria*.

^y Expected data if the gene controls *M. enterolobii*.

^z Expected data if the gene controls *M. arenaria*.

using nucleotides labeled with fluorochrome dyes. For the KASP assay, the reaction mixture was prepared according to the protocol described by LGC Genomics (<https://www.lgcgroup.com/products/kasp-genotyping-chemistry/#.XDt67c1MHIU>). Fluorescent end-point genotyping was conducted using the 2103 EnVision Multi-label Reader (Perkin Elmer Company).

In the segregating plum material P.2980 × P.1090, individuals were genotyped and classified into two classes (heterozygous resistant, *Ma/ma*; and homozygous susceptible, *ma/ma*) using the sequence-characterized amplified region markers NSCALF2 and CT3-4 N located within the *Ma* gene genomic sequence (Claverie et al. 2011) (Supplementary Table S1). Similarly, in the segregating peach F2 PR140MN8 involving the *RMia* gene (Tables 2 and 3), individuals were genotyped and classified into three classes (homozygous resistant, *RMia/RMia*; heterozygous resistant, *RMia/rmia*; and homozygous susceptible, *rmia/rmia*) using the flanking markers SNP_APP91 and SNP_APP92 (Duval et al. 2014). Finally, in the segregating almond F2 LxA progeny, individuals were genotyped and classified into the three classes *RMja/RMja*, *RMja/rmja*, and *rmja/rmja* using the flanking markers LRR65 and kin35 (Van Ghelder et al. 2018).

Statistical analysis. A part of the study aimed to evaluate whether the *RMia* gene, that controls the species *M. incognita* in particular, has a quantitative effect on *M. javanica*. In this objective, an analysis of variance using XLSTAT software (version 2014.5.03) was conducted for the quantitative criteria, gall index, collar diameter, root weight, and top weight. Significant differences between individuals were analyzed by the least significant difference Fisher test at $P \leq 0.05$. In this statistical analysis, all the individuals of the same genotypic group (*RMia/RMia*, *RMia/rmia*, and *rmia/rmia*) were considered as replicates (Table 3).

RESULTS

Study of the resistance to *M. ethiopica*. Each of the three genes *Ma*, *RMia*, and *RMja* has been evaluated toward the isolate of this RKN species. Molecular markers of the *Ma*, *RMia*, and *RMja* genes were used to genotype the segregating seedlings of their respective progenies.

Ma markers ranged the seedlings from the F1 cross P.2980 × P.1090 into one of the two genotypic classes, *Ma/ma* or *ma/ma*. The phenotyping evaluation based on gall index ratings allowed distributing both the parental and F1 hybrid individuals into the R or S classes. Gall indices of resistant individuals ranged from 0 to 0.5 whereas gall indices of susceptible individuals were ≥ 1 . From the 41 total individuals of the progeny evaluated to *M. ethiopica*, all 26 *Ma/ma* individuals were resistant whereas all 15 *ma/ma* individuals were susceptible (Table 2). Consequently, individuals classified with *Ma* markers into the R heterozygous class *Ma/ma* were resistant and individuals classified with *Ma* markers into the S genotypic class *ma/ma* were susceptible.

RMia- and *RMja*-specific markers ranged the segregating seedlings from the F2 crosses, PR140MN8 (104 individuals) and LxA (89 individuals), into one of the three genotypic classes *RMia/RMia*, *RMia/rmia*, and *rmia/rmia* or *RMja/RMja*, *RMja/rmja*, and *rmja/rmja*, respectively. In parental and F2 hybrid materials from each cross, the high and durable inoculum pressure provided by the test allowed a clear classification of individuals into either the R ($0 \leq$ gall index ≤ 0.5) or the S (gall index ≥ 1.0) phenotypic groups. From the 104 total individuals of the F2 PR140MN8, all 30 *RMia/RMia* individuals and all 42 *RMia/rmia* individuals were resistant whereas all 32 *rmia/rmia* individuals were susceptible (Table 2). Consequently, individuals classified with *RMia* markers into the two R genotypic classes *RMia/RMia* and *RMia/rmia* were resistant and individuals classified with *RMia* markers into the S genotypic class *rmia/rmia* were susceptible. Similarly, from the 89 total individuals of the F2 LxA progeny evaluated for resistance to *M. ethiopica*, all 31 *RMja/RMja* individuals and all 40 *RMja/rmja* individuals

were resistant and all 18 *rmja/rmja* individuals were susceptible (Table 2). In other words, individuals from the two R *RMja* genotypic classes were resistant and all individuals from the S *RMja* genotypic class were susceptible.

Consequently the complete matching of the genotypic and phenotypic classes obtained for *Ma*, *RMia*, and *RMja* strongly suggests that each of the three genes controls the *M. ethiopica* isolate used.

Study of the activity of the *RMja* gene to *M. enterolobii* and *M. arenaria*. *RMja*-specific markers were used to genotype the 169 total segregating seedlings of the F2 cross LxA intended for a phenotypic evaluation to either *M. enterolobii* (69 individuals) or *M. arenaria* (100 individuals) and ranged them into one of the three genotypic classes *RMja/RMja*, *RMja/rmja*, and *rmja/rmja*. Two phenotypic classes were identified in both parental and F2 materials because individuals were clearly split into either the R or S classes. From the 69 total individuals of the F2 progeny evaluated to *M. enterolobii*, all 7 *RMja/RMja* individuals and all 42 *RMja/rmja* individuals were resistant whereas all 20 *rmja/rmja* individuals were susceptible (Table 4). Thus, individuals from the two R *RMja* genotypic classes were resistant and individuals from the S *RMja* genotypic class were susceptible. From the 100 total individuals of the F2 progeny evaluated to *M. arenaria*, all 23 *RMja/RMja* individuals and all 47 *RMja/rmja* individuals were resistant whereas all 30 *rmja/rmja* individuals were susceptible (Table 4). Therefore, individuals from the two R *RMja* genotypic classes were resistant and individuals from the S *RMja* genotypic class were susceptible. The absence of any recombinant individuals to *M. enterolobii* and to *M. arenaria* strongly suggests that the *RMja* gene controls the isolates of both RKN species.

Evaluation of a putative quantitative effect of the *RMia* gene on *M. javanica*. Because the *RMia* gene confers resistance to *M. incognita*, this RKN species was used as a positive control. We used a total number of 138 individuals from the F2 PR140MN8 progeny, which represents the largest progeny analyzed in this study. Of these, 79 individuals were evaluated for resistance to *M. javanica* and ranged into 30 *rmia/rmia* individuals, 30 *RMia/rmia* individuals, and 19 *RMia/RMia* individuals (Table 3). The 59 other individuals were considered as controls for resistance to *M. incognita* and ranged into 17 *rmia/rmia* individuals, 25 *RMia/rmia* individuals, and 17 *RMia/RMia* individuals (Table 3).

Gall index rating. As expected, the individuals of the three genotypic classes clearly segregated into two phenotypic groups after inoculation with the control species *M. incognita*: the R group (*RMia/RMia* and *RMia/rmia* genotypes) and the S group (*rmia/rmia* genotype). For this RKN species, mean gall index ratings of the two genotypic R classes (*RMia/RMia* and *RMia/rmia*) were not different but both differed significantly from the S class (*rmia/rmia*) (Table 3). By contrast, all 79 individuals evaluated to *M. javanica* were susceptible, whatever the genotypic class they belonged to. Moreover, the mean gall index ratings of the three genotypic classes from the individuals evaluated to this RKN species were not statistically different, which indicated that the *RMia* genetic status has no effect on the RKN galling observed toward this species. Consequently, the *RMia* gene did not confer resistance to the *M. javanica* isolate used.

Collar diameter. This criterion has been retained to illustrate the putative effect of the nematodes on the development of the plants that carried or lacked the *RMia* gene. After inoculation with *M. incognita*, plants with *RMia/RMia* and *RMia/rmia* genotypes displayed a collar diameter statistically higher ($P \leq 0.05$) than plants with a homozygous S genotype, which confirms both the activity of the *RMia* gene on this RKN species and its completely dominant status. By contrast, no significant differences between the three genotypic classes were observed in the plants inoculated by *M. javanica*. This illustrates that plant development was not influenced by the presence of the *RMia* gene, which is in line with its absence of effect toward *M. javanica*.

Root and top weights. These two criteria were measured only on the plants tested to *M. javanica*. For both criteria, our results showed no significant differences ($P \leq 0.05$) between the three genotypic classes of the *RMia* gene. This result supplements previous data on gall index and collar diameter ratings and may be related to *RMia* gene's lack of activity against the *M. javanica* isolate used.

DISCUSSION

We showed that the matching of the phenotypic and genotypic classes of the three genes for resistance to the RKN species evaluated was either complete (*Ma*, *RMja*, and *RMia* with *M. ethiopica* and *RMja* with *M. arenaria* and *M. enterolobii*) or null (*RMia* with *M. javanica*). No intermediate situation (i.e., a partial matching between these classes) occurred. Thus, our evaluation stated unambiguously whether a given gene is active against a given RKN isolate. In some tests, susceptible seedlings may occasionally escape the attacks, which generates false-resistant individuals. The high and durable inoculum pressure provided in our experimental procedure permitted us to avoid this major drawback by reducing considerably the risk of occurrence of misevaluated plants.

Nevertheless, our phenotypic results only allow a relative location of the R factor due to the limited number of segregating individuals (ranging from 41 to 104 for all crosses considered). Thus, theoretically, we cannot exclude the hypothesis that another closely linked factor actually controls the RKN species studied. In the study that confronts the *Ma* gene to the RKN isolates of *M. ethiopica*, the total number of individuals is 41. This implies that the precision of the location of the genetic factor is approximately 2.5 centimorgans (cM) and another R factor located in an interval smaller than 2.5 cM on each side of the gene could control it. Nevertheless, in this case, the *Ma* gene already controls five other RKN species, which is presumably not in line with the existence of an additional factor that would specifically control this species. For *RMja*–*M. ethiopica* and *M. enterolobii*, it had been recently shown that this gene is presumably an ortholog of *Ma* (Van Ghelder et al. 2018), which strongly suggests that no other R factor is involved. Finally in *RMia*–*M. ethiopica*, numbers are the highest of our experiments (104 segregants) (i.e., allowing a precision of ± 1 cM), which limits the possible occurrence of another R factor.

Consequently, our further discussion on the spectrum of the three *Prunus* R genes *Ma*, *RMia*, and *RMja* will be founded on the hypothesis that these genes are alone in their respective chromosomal locations; or, in other words, that no other major R gene closely linked to them is active against the RKN species studied here. Our comments will also support the premise that the isolate tested in each species is representative of the behavior of its RKN species. On this basis, our results are particularly interesting because they bring new information about the activity of all three R genes to the five RKN species considered. The *Ma* gene was already known as controlling the five species *M. arenaria*, *M. incognita*, *M. javanica*, *M. floridensis*, and *M. enterolobii* (Lecouls 2000; Lecouls et al. 1997; Rubio-Cabetas et al. 1999). We showed that it also controls *M. ethiopica*, which confirms its complete spectrum against tested RKNs. This large spectrum tends toward a full resistance to all RKN species, which would be unique in plants thus far. This feature suggests that the *Ma* gene recognizes (directly or indirectly) a component which would be common to most if not all RKN species rather than several specific components. Thus, *Prunus* rootstocks carrying *Ma* may be suitable to use worldwide for

RKN resistance in any stone-fruit-producing regions. Regarding the *RMja* gene, we have completed previous results with the evaluation of its spectrum to three additional RKN species: *M. arenaria*, *M. enterolobii*, and *M. ethiopica*. Previous evaluations had shown that the Alnem sources (Alnem1, Alnem88, and Alnem201) controlled *M. arenaria* (Scotto La Massese et al. 1984) but the genetics of this resistance was ignored. We have established here that this resistance is conferred by *RMja* (i.e., by the same gene initially described as controlling *M. javanica*) (Van Ghelder et al. 2010). Interestingly, the *RMja* spectrum of resistance is close to the spectrum of *Ma*. This enhanced range of resistance conferred by the *RMja* gene increases the global area where rootstocks carrying this almond gene would be able to face RKN attacks. Indeed, the major species *M. arenaria* has a very wide distribution and is present throughout the world in all regions benefitting from a Mediterranean climate. By contrast, the species *M. ethiopica* has a more restricted distribution area but is prevalent in Chile (Carneiro et al. 2007a; Meza et al. 2016) and also widely detected in Brazil (Carneiro et al. 2004; Correa et al. 2014). In addition, *RMja* is the second gene after *Ma* to confer resistance to the emerging aggressive species *M. enterolobii*. Unlike *RMia* or the *Mi-1* gene from tomato (Saucet et al. 2016), we showed that *RMja* fully controls the *M. enterolobii* isolate tested. Finally, our assessment focused on the *RMia* gene and evaluated it to *M. ethiopica* and *M. javanica*. We showed that this gene, like *Ma* and *RMja*, controls *M. ethiopica*. Having available several major R genes to *M. ethiopica* is encouraging for the successful management of this RKN species.

Our study has also unraveled the interaction between the *RMia* peach gene and *M. javanica*. This gene has been shown to confer resistance to *M. incognita* and *M. arenaria* (Esmenjaud et al. 1997, 2009) but its status regarding *M. javanica* was unknown. Studies on the histological mechanisms of resistance in Nemaguard and its derived Nemared rootstock (Ramming and Tanner 1983) established that these plant materials control certain *M. javanica* populations despite the formation of small galls. A “walling-off” process isolating young giant cells from neighboring cells and preventing the maturation of the females in the infection site has been described (Malo 1967; Marull et al. 1994; Meyer 1978). The genetic determinants controlling *M. javanica* in Nemaguard and Nemared remain to be identified. Several genetic hypotheses such as two independent genes (Sharpe et al. 1969) or a single gene (Lu et al. 1998; Yoshida and Seike 1981) have been proposed. Other studies had suggested that R genes for resistance to *M. incognita* might have a partial effect on *M. javanica* (Lu et al. 1998, 1999). Our data showed that the *RMia* gene does not confer resistance to the *M. javanica* isolate used here. This does not allow conclusions about the *M. javanica* species considered as a whole, and other isolates of this RKN species may be completely or partly controlled. Additionally, using *M. incognita* as an RKN species controlled by *RMia* confirmed the phenotypic effect of this gene, as illustrated with the parameter “collar diameter”.

Our results point out the completely dominant status of the three *Prunus* genes, whatever the RKN species they face. This feature is particularly interesting for breeding because it allows growers to obtain completely resistant interspecific rootstock material from a single F1 hybridization between parents carrying different R genes. We confirm that, in future *Prunus* breeding programs, resistance to all predominant species (i.e., *M. arenaria*, *M. incognita*, and *M. javanica*) will be possible by stacking the R genes *RMia* (controlling *M. arenaria* and *M. incognita*) and *RMja* (controlling

TABLE 5. Spectrum for resistance conferred by the three *Prunus* resistance genes *Ma*, *RMja*, and *RMia* to six root-knot nematodes (*Meloidogyne* spp.)^z

Gene (resistance source)	<i>Meloidogyne arenaria</i>	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. ethiopica</i>	<i>M. floridensis</i>	<i>M. enterolobii</i>
<i>Ma</i> (plum, P.2175 and P.2980)	R	R	R	R	R	R
<i>RMja</i> (almond, Alnem1)	R	S	R	R	S	R
<i>RMia</i> (peach, Nemared)	R	R	S	R	S	S

^z R = resistant and S = susceptible.

M. javanica). Thus, using hybrid material Alnem1 × Nemared will guarantee the complete control of all three species. Given that the *Ma* plum gene also confers resistance to all three RKN species, pyramiding the three genes *Ma*, *RMia*, and *RMja* in the same rootstock will provide a double resistance layer to all three prevalent RKN species mentioned previously.

Finally, our results clearly establish the ability of each gene to control the different isolates tested. This allows us to draw a complete picture that summarizes the resistance conferred to six total *Meloidogyne* spp. (Table 5). Interestingly, this updated spectrum of resistance of *Prunus* genes shows that *RMja* differs from *Ma* by being inactive to *M. incognita* and *M. floridensis*. Phylogenetic data on these two parthenogenetic RKN species show that they are evolutionarily quite close (Holterman et al. 2009), even though *M. floridensis* is meiotic whereas *M. incognita* is mitotic. Furthermore *Ma* and *RMja*, located on chromosome 7, are presumably orthologous TNL genes whereas *RMia*, located on chromosome 2 and for which candidate genes are also TNLs (Duval et al. 2014), is quite different. Given their highly probable orthologous status, their differential specificity may be attributable either to the gain of recognition of a ubiquitous effector from *Meloidogyne* spp. in *Ma* or to the loss of recognition of an effector specific to *M. incognita* and *M. floridensis* in *RMja*. Ultimately, understanding the resistance spectrum for each gene is a milestone for future studies aiming to decipher the molecular determinants involved in the resistance to RKNs.

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