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ORIGINAL PAPER

Root-knot nematode (*Meloidogyne* spp.) *Me* resistance genes in pepper (*Capsicum annuum* L.) are clustered on the P9 chromosome

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Abstract The root-knot nematode (*Meloidogyne* spp.) is a major plant pathogen, affecting several solanaceous crops worldwide. In *Capsicum annuum*, resistance to this pathogen is controlled by several independent dominant genes—the *Me* genes. Six *Me* genes have previously been shown to be stable at high temperature in three highly resistant and genetically distant accessions: PI 322719, PI 201234, and CM334 (Criollo de Morelos 334). Some genes (*Me4, Mech1*, and *Mech2*) are specific to certain *Meloidogyne* species or populations, whereas others (*Me1, Me3*, and *Me7*) are effective against a wide range of *Meloidogyne* species, including *M. arenaria, M. javanica*, and *M. incognita*, the most common species in Mediterranean and tropical areas. These genes direct different response patterns in root cells depending on the pepper line and nematode species. Allelism tests and fine mapping using the BSA-AFLP approach showed these genes to be different but linked, with a recombination frequency of 0.02–0.18. Three of the PCR-based markers identified in several genetic backgrounds were common to the six *Me* genes. Comparative mapping with CarthaGene software indicated that these six genes clustered in a single genomic region within a 28 cM interval. Four markers were used to anchor this cluster on the P9 chromosome on an intraspecific reference map for peppers. Other disease resistance factors have earlier been mapped in the vicinity of this cluster. This genomic area is colinear to chromosome T12 of tomato and chromosome XII of potato. Four other nematode resistance genes have earlier been identified in this area, suggesting that these nematode resistance genes are located in orthologous genomic regions in *Solanaceae*.

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Introduction

Root-knot nematodes (RKN) are polyphagous pests causing severe damage to crops worldwide. The RKN species of economic importance in cultivated areas and greenhouse include *M. incognita* (Kofoid & White) Chitwood, *M. arenaria* (Neal) Chitwood *and M. javanica* (Treub) Chitwood, which may be found in isolation or together. Some species, such as *M. chitwoodi* Golden, O'Bannon, Santo & Finley and *M. fallax* Karssen, were added to the list of quarantine organisms in new phytosanitary regulations 77/93/EG for EU member countries issued in May 1998.

Chemical treatments are currently the most widely used and the reliable way of controlling these pests. However, the increasing use of toxic agrochemicals is being restricted. Plants displaying genetic resistance to these pests are likely to become an efficient, economically competitive and non-polluting method of nematode control. In pepper (*Capsicum annuum*), resistance to RKN is associated with several dominant genes (Hare 1956; Hendy et al. 1985; Djian-Caporalino et al.

1999, 2001) that are thought to act independently in gene-for-gene interactions.

We have described several heat-stable genes (named *Me* genes) from three highly resistant and genetically distant (Lefebvre et al. 1993, 2001) pepper accessions: PI 322719, PI 201234, and CM334 (Criollo de Morelos 334) (Djian-Caporalino et al. 1999, 2001). These small-fruited and pungent inbred lines originate from local open-pollinated cultivars in India, Central America, and Mexico, respectively. Some *Me* genes are highly specific for particular *Meloidogyne* species or populations, whereas others confer resistance to a broad spectrum of RKNs (Hendy et al. 1985; Djian-Caporalino et al. 1999). These genes also induce different response patterns in root cells, depending on the pepper line and nematode species considered (Pegard et al. 2005). Using a bulked segregant analysis (BSA) approach (Giovannoni et al. 1991; Michelmore et al. 1991) together with AFLP methods (amplification fragment length polymorphism; Vos et al. 1995), we were able to identify molecular markers tightly linked to the dominant genes *Me3* and *Me4* carried by the inbred line PM687, derived from PI 322719 (Djian-Caporalino et al. 2001). We showed that these two loci were linked and separated by 10 cM on chromosome P9. Segregation analyses carried out on doubled haploid lines showed that the *Me1* gene and a gene conferring resistance to *M. chitwoodi* (the *Mech2* gene), both of which are carried by inbred line PM217 derived from PI 201234, are linked and have an estimated recombination frequency of 0.13 (Berthou et al. 2003). Two genes conferring resistance to *M. incognita* (the *Me7* gene) and *M. chitwoodi* (the *Mech1* gene) were also found to be carried by inbred line PM702 derived from CM334. However, the genes carried by PM217 and PM702 were not localized on the pepper genetic map.

Classical genetic and molecular studies have shown that pathogen-specific plant *R*-genes are frequently linked within genome regions of various sizes (summarized in Michelmore and Meyers 1998; Grube et al. 2000b; Kruijt et al. 2004). Several clusters of resistance loci (*R*-loci) and QTLs conferring resistance to various pathogens have been located on pepper chromosomes P10, P11, and P12, to which dominant resistances to several viruses and QTLs for resistance to viruses, fungi and oomycetes have also been mapped (summarized in Lefebvre 2004).

We report here (1) the identification, by BSA methods, of AFLP markers linked to the *Me1/Mech2* and *Me7/Mech1* loci, (2) the development of locus-specific PCR-based markers of the pepper RKN *Me* resistance loci, and (3) the confirmation of their linkage and their localization on the genetic map. The *Me* genes were found to be clustered within a 28 cM segment colinear with other nematode resistance loci in *Solanaceae*. The following genes were considered of particular importance in pepper: the broad-spectrum *R*-genes *Me1, Me3*, and *Me7*, the specific *Me4* gene controlling *M. arenaria* AinTaoujdate, and the genes conferring resistance to the quarantine RKN *M. chitwoodi* (the *Mech1* and *Mech2* genes).

Materials and methods

Pepper genetic populations

Resistant progenitors were selected by resistance tests with several populations of *Meloidogyne* from a collection of 1,400 pepper accessions maintained at INRA in Avignon (Djian-Caporalino et al. 1999; Berthou, unpublished data). Three genetically distant inbred lines: PM687 (derived from PI 322719), PM217 (derived from PI 201234), and PM702 (derived from Criollo de Morelos 334) (Table 1), highly resistant to the three main species of RKN (*M. arenaria*, *M. incognita*, and *M. javanica*) and to the quarantine nematode *M. chitwoodi* were used as resistant parents in crosses and as control genotypes in RKN screens. The doubled haploid (DH) line DH330 was obtained by in vitro culture of anthers from F1 plants generated by crossing the donor line PM217 with YW (Yolo Wonder), as described by Dumas De Vaulx et al. (1981). The resistant parents—PM687, DH330, and PM702—were crossed with cultivars susceptible to *M. incognita* and *M. chitwoodi*—DLL (Doux Long des Landes) or YW (Table 1)—to generate F1 progenies and F2 segregating populations for gene mapping. F2 plants were self-pollinated to generate F3 progenies. One DH progeny was obtained from F1 plants resulting from the [PM687 × YW] cross. DH lines and F3 families can be used for the simultaneous testing of each genotype for resistance to different RKN populations and for the mapping of an unlimited number of markers. The number of lines of the various progenies used for RKN screening and gene mapping are indicated in Table 2. Two reference intraspecific *C. annuum* maps were used: PY, constructed from the DH progeny of a [Perennial × Yolo Wonder] cross, and HV, constructed from the DH progeny of an [H3 × Vania] cross (Lefebvre et al. 2002). The parents of these mapping populations are susceptible to all *Meloidogyne* species, with the exception of YW, which is resistant to *M. arenaria* (Djian-Caporalino et al. 1999, 2001).

Table 1 Resistance behaviour of *Capsicum annuum* INRA accessions and the corresponding RKN resistance genes *Me* (according to Hendy et al. 1985; Djian-Caporalino et al. 1999, 2001, and unpublished)

INRA accession code	Origin	*M. incognita*	*M. javanica*	*M. arenaria*	*M. arenaria Ain Taoujdate*	*M. chitwoodi IpoCk*
PM217	Inbred line from PI 201234 (University of California, USA; local population from Central America)	R (*Me1*)	R (*Me1*)	R (*Me1*)	S	R (*Mech2*)
PM687	Inbred line from PI 322719 (University of Punjab, India)	R (*Me3*)	R (*Me3*)	R (*Me3*)	R (*Me4*)	S
PM702	Inbred line from CM334 (Criollo de Morelos 334, INIA Mexico)	R (*Me7*)	R (*Me7*)	R (*Me7*)	ND	R (*Mech1*)
YW	Inbred line Yolo Wonder issued from California Wonder (University of California, USA)	S	S	R (*Me5*)	S	S
DLL	Inbred line from Doux Long des Landes (local population France)	S	S	S	S	S
DH330	Doubled haploid line from intraspecific F1 hybrids [PM217 × YW]	R (*Me1*)	R (*Me1*)	R (*Me1*)	S	R (*Mech2*)

R resistant; *S* susceptible; *ND* not determined

Table 2 *Capsicum annuum* progenies used for allelism tests and mapping the RKN resistance genes *Me*

Cross	Progeny	Size of the progeny	Number of individuals, lines or families screened with RKN/codes of tested RKN	Number of individuals, lines or families screened with DNA-markers	*Me* gene mapped
[PM687 × YW]	DH_{100} lines	118	118/*MiC*	109	*Me3*
			50/*MaAT*	50	*Me4*
	F_2 individuals	163	163/*MiC*	163	*Me3*
[DH330 × DLL]	F_2 individuals	210	99/*MiC*	68	*Me1*
			111/*McIpo*	60	*Mech2*
	F_3 families	98	98/*MiC*	50	*Me1*
			36/*McIpo*	34	*Mech2*
[DLL × PM702]	F_2 individuals	301	193/*MiC*	63	*Me7*
			108/*McIpo*	108	*Mech1*
	F_3 families	59	59/*MiC*	49	*Me7*
			32/*McIpo*	32	*Mech1*

MiC: *M. incognita* (from Calissane, France); *McIpo*: *M. chitwoodi* (population Ipo-Ck from Wageningen, The Netherlands); *MaAT*: *M. arenaria* (from AinTaoujdate, Morocco)

Tests of resistance to *Meloidogyne* for phenotypic assays

Resistance tests were conducted with two *Meloidogyne* species from the collection maintained at INRA Antibes: *M. incognita* (from Calissane, France) (*MiC*) and *M. chitwoodi* (population Ipo-Ck from the Research Institute for Plant Protection, Wageningen, The Netherlands) (*McIpo*). The identification of these RKN populations to species level was checked by PCR (Zijlstra 2000; Zijlstra et al. 2000) before inoculation. For each isolate, hatched second-stage juveniles (J2s) were collected from the infected roots of susceptible tomatoes (cultivar Saint Pierre). They were placed in a mist chamber at 4°C and were used within 24 h.

All experiments were conducted in controlled growth conditions, at a mean temperature of 23°C (±2°C), with an 11-h light/13-h dark lighting regime and a relative humidity of 60–70%. Plants were grown individually in 9 cm plastic pots containing steam-sterilized sandy soil covered by a 1 cm layer of loam. Seven-week-old pepper seedlings (five-leaf stage) were inoculated with a suspension in water of 600 J2s per seedling for *MiC*, and 3,000 J2s per seedling for *McIpo*. This suspension was injected into two holes, each 2 cm deep, close to the roots of the plant (Djian-Caporalino et al. 1999). Resistance/susceptibility was evaluated and observed, and theoretical segregations were compared as previously described (Djian-Caporalino et al. 1999).

Plant DNA isolation and AFLP/BSA analysis

DNA extraction, AFLP analysis coupled with BSA (Michelmore et al. 1991), and data recording were carried out as previously described (Djian-Caporalino

et al. 2001). We produced four bulks, two susceptible (S) bulks and two resistant (R) bulks, by pooling equal quantities of preamplified DNA (1st PCR amplification in the AFLP procedure) from 10 susceptible or 10 resistant lines. Selective amplification was carried out with a 1/10 dilution of preamplified products, using two or three ^{33}P-labeled hexacutter (*Hin*dIII + 3, *Pst*I + 2, *Pst*I + 3) and non-labeled tetracutter (*Mse*I + 3) nucleotide extension primers. Each experiment was repeated at least once.

Conversion of AFLP markers into locus-specific PCR-based markers

The polymorphic fragments generated by AFLP were converted into reliable sequence-characterized amplified regions (SCAR; Michelmore et al. 1991), cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel 1993), or single-strand conformation polymorphism (SSCP; Orita et al. 1989) markers, to facilitate the screening of the progeny for recombination events near *Me* genes. X-ray films were aligned with the dried acrylamide gels and the AFLP products of interest were excised. DNA was eluted in 20 µl of 1× TE buffer, and re-amplification was performed with 1 µl of eluate and the same cycling conditions as for selective AFLP PCR. The re-amplified amplicons were separated by electrophoresis in an agarose gel, excised and purified, using the Qiaex gel-extraction kit (Qiagen, Hilden, Germany). These amplifications were ligated into a pGEMT vector, using the pGEMT cloning kit (Promega) according to the manufacturer's instructions, and the resulting constructs were used to transform *Escherichia coli* DH5α by electroporation. Ten clones were sent to Genome Express (Grenoble, France) for sequencing. The sequences obtained were input into Oligo 4.0 software for the design of specific primers of 17 to 25 nucleotides in length. These primers were synthesized by Oligo Express (Paris, France). Specific PCR amplifications were performed in a total volume of 20 µl containing 25 ng of genomic DNA, 1× Invitrogen buffer, 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM solutions of each nucleotide, 0.5 µM solutions of each primer (Table 3), and 0.5 U of *Taq* polymerase (Invitrogen). The cycling conditions and oligonucleotides used for the locus-specific amplification of five markers tightly linked to the *Me* genes are described in Table 3.

Various methods were used for polymorphism detection. Specific primers were used to amplify SCAR markers (Michelmore et al. 1991), which were visualized by electrophoresis in 1.8 or 3% (w/v) low-melting point agarose gels (Promega). If resistant and susceptible lines gave amplicons of identical size, the PCR products were first digested with a panel of restriction enzymes with four-base recognition sites, at 37–65°C (according to the enzyme). Products were separated by electrophoresis in 3% (w/v) low-melting agarose gels, yielding codominant CAPS markers (Konieczny and Ausubel 1993). If no polymorphism was identified by digestion analysis, we used SSCP (Orita et al. 1989) methods to look for single nucleotide polymorphisms (SNPs). PCR products were denatured by heating at 94°C and then rapidly cooled to 4°C in the thermocycler. The resulting single-stranded nucleic acids were separated by electrophoresis in a neutral 0.7% polyacrylamide gel (Sigma) in 0.5× TAE buffer maintained at 7°C and subjected to silver staining (Melcher 2000). If the AFLP markers were small (less than 160 bp), or if no polymorphism could be detected by the methods described earlier, then we searched for matches with known genes in the GenBank database. We used the nucleotide–nucleotide (blastn) option of the basic local alignment search tool (BLAST) program to identify regions of local similarity in databases. Local alignments were used to design primers flanking the AFLP sequence. BLAST was also used to identify members of gene families. If no sequence similarity was found, primers were first designed based on the AFLP sequence (antisense strand) for a cycle of inverse PCR (I-PCR), as described by Thomas et al. (1994). Sequences flanking the AFLP markers were cloned and sequenced, and the sequence obtained was used to design primers for direct PCR amplification of the AFLP locus.

The sequences of the PCR-based markers were compared with the sequences of the AFLP markers to check that the polymorphisms corresponded to the loci considered. We also checked, in several progenies, that these PCR markers colocalized with the corresponding AFLP markers.

Fine mapping of the *Me* genes and linkage analysis

The three segregating populations [PM687 × YW], [DH330 × DLL], and [DLL × PM702] were screened with the polymorphic AFLP markers and locus-specific PCR-based markers. Marker order and genetic distances were determined using MapMaker/Exp v3.0b software (Lincoln et al. 1993). A minimum LOD score of 3 and a maximum recombination rate of 0.3 were used as the threshold values. Recombination values were converted into map units (cM), using Kosambi's mapping function (Kosambi 1944).

Common PCR-specific markers were mapped with CarthaGene software, version 0.999 (Schiex et al. 2001), which orders a set of markers recorded in multiple

Table 3 Characteristics of the PCR-specific markers linked to the RKN resistance genes *Me*

PCR-specific markers	Corresponding PCR primers	PCR conditions	Corresponding AFLP	Linkage with *Me* genes (Cross where they are polymorphic)	Pepper maps where they can be localized
SCAR_B94 dominant (S) 220 bp	B94 forward: 5′-GCTTATCATGGCTAGTAGGG-3′ B94 reverse: 5′-CGGACCATACTGGGACGATC-3′	94°C for 3 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s	HM6	*Me3–Me4* ([PM687 × YW])	PY; HV
SCAR_CD dominant (R) 107 bp	CD forward: 5′-GAAGCTTATGTGGTAMCC-3′ CD reverse: 5′-GCAAAGTAATTATATGCAAGAGT-3′	94°C for 3 min; 35 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 45 s	PM54	*Me1–Mech2; Me7–Mech1* ([DH330 × DLL]; [DLL × PM702])	HV
SSCP_B54 codominant	B54 forward: 5′-CGGTGGCTGTTACGCTC-3′ B54 reverse: 5′-GCATGTCTTTCTTTACC-3′	94°C for 3 min; 35 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 60 s	HM4	*Me3 – Me4; Me7–Mech1* ([PM687 × YW]; [DLL × PM702])	PY; HV
SSCP_B322 codominant	B322 forward: 5′-GATTCCATAACCTGGAAATTTCTGG-3′ B322 reverse: 5′-CGAACCCGGTCTATTTTC-3′	94°C for 3 min; 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 60 s	PM6	*Me3 – Me4; Me7–Mech1* ([PM687x YW]; [DLL × PM702])	PY; HV
CAPS_F4R4 codominant 750 bp, 500 bp, and 450 bp (R); 330 bp (S)	F4 forward: 5′-AGAACAATAGAATCTCTCTTG-3′ R4 reverse: 5′-CTTCAGGAACCCCTCAGC-3′ Restriction endonuclease: *Tru91*	94°C for 3 min; 38 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 90 s	Obtained from HM58 and corresponding to part of the *Bs2* gene sequence	*Me7–Mech1* ([DLL × PM702])	HV

HV [H3 × Vania]; *PY* [Perennial × YW]

mapping populations, using the maximum likelihood criterion. Data sets from different mapping populations were merged and an integrated map was worked out using the "build" command, which progressively adds markers, always choosing the highest log likelihood and the best insertion point.

Localization of *Me* loci on reference maps for pepper

The positions of the *Me* loci on pepper reference maps were determined by recording *Me*-linked markers in two intraspecific reference mapping populations—PY and HV (Lefebvre et al. 2002). The map positions of the *Me*-linked markers were determined using MapMaker software, as previously described. Markers common to our *Me*-mapping populations and to the reference mapping populations were used to anchor the integrated map of *Me* loci on the pepper genetic linkage map, using CarthaGene software, as described earlier.

Results

Segregation of resistance to *M. chitwoodi* in F2 progenies and F3 families obtained from [DH330 × DLL] and [DLL × PM702] crosses

M. incognita seemed to be more aggressive than *M. chitwoodi* in terms of egg-mass production on the roots of susceptible plants. Almost 25% of the *M. incognita* juveniles produced egg masses on DLL, versus only 3% of *M. chitwoodi* juveniles (mean values for 10 replicates). No egg masses were detected for either of the two RKN species after 6 weeks on the roots of the resistant lines DH330 and PM702.

All plants of the F1 progenies of the [DH330 × DLL] and [DLL × PM702] crosses were resistant to *M. chitwoodi*. We found that 79% of the 111 F2 lines of the [DH330 × DLL] cross and 73% of the 108 F2 lines of the [DLL × PM702] cross were resistant. These results do not differ significantly from the 3R:1S segregation expected for a single gene (χ^2 estimates of 2.9 and 0.2, respectively).

Tests of allelism between *Me1* and *Mech2* carried out on 36 of the 98 F3 families (15 replicates), selected for study on the basis of their homozygosity at the *Me1* locus, revealed that the genes were different but linked, with a recombination frequency of 0.17. This confirms the results of Berthou et al. (2003), who obtained five recombinant individuals from 38 DH lines characterized for resistance to *M. incognita* and *M. chitwoodi* (recombination frequency of 0.13).

Tests of allelism between *Me7* and *Mech1* carried out on 32 of the 59 F3 families (15 replicates), selected for study on the basis of homozygosity at the *Me7* locus, revealed that these genes were also different but linked, with a recombination frequency of 0.15.

Screening AFLP markers linked to the *Me1* and *Mech2* resistance genes from PI 201234

Two susceptible (S) bulks and two resistant (R) bulks of ten lines each for the [DH330 × DLL] cross-characterized for resistance to *M. incognita*, two S bulks and two R bulks for the same cross-characterized for resistance to *M. chitwoodi*, and DNA samples from the parental lines (DLL and DH330) were screened with an average of 200 AFLP primer combinations. We detected 25 AFLPs displaying polymorphism between the R and S bulks. We analyzed these markers in individuals from each pool and found that two AFLPs—PM54 and HM60—displayed complete linkage to *Me1* and *Mech2*. These AFLPs were used for the subsequent analysis of F2 and F3 segregants. Within the 118 lines (68 F2 and 50 F3) characterized for resistance to *M. incognita (Me1)*, we detected 2.5 and 13.5% recombination events with the PM54 AFLP marker and the HM60 AFLP marker, respectively. Within 94 lines (60 F2 and 34 F3) characterized for resistance to *M. chitwoodi (Mech2)*, 19.1 and 13.1% recombination events, respectively, were detected with the same two markers. We used 50 F3 families characterized for resistance to *M. incognita* and *M. chitwoodi* for the mapping of these AFLP markers close to *Me1* and *Mech2* (Fig. 1a). *Me1* mapped 18 cM from *Mech2*, consistent with the recombination rate calculated by Berthou et al. (2003) for 38 DH lines (13%).

Screening AFLP markers linked to the *Me7* and *Mech1* resistance genes from CM334

The same procedure was followed for lines of the [DLL × PM702] cross-characterized for resistance to

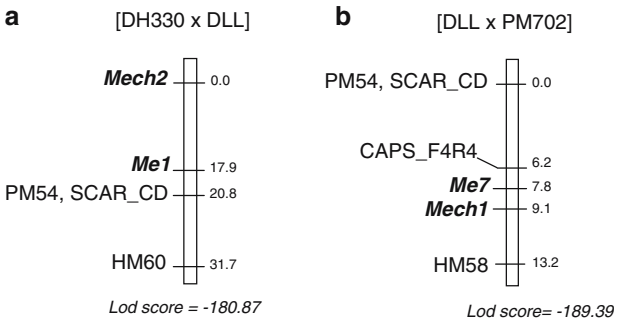

Fig. 1 AFLP analysis of the *Me R*-genes of pepper for the [DH330 × DLL] (**a**) and [DLL × PM702] (**b**) crosses (50 and 49 F3 families analyzed, respectively)

M. incognita (four bulks) and *M. chitwoodi* (four bulks). We detected 31 AFLPs displaying polymorphism between the R and S bulks. Analysis of these AFLP markers in individuals from each pool revealed that two displayed complete linkage to *Me7* and *Mech1*. These AFLPs were subsequently used for the analysis of F2 and F3 segregants. Within 112 lines (63 F2 and 49 F3) characterized for resistance to *M. incognita*, 19.5 and 5.7% recombination events were detected with the AFLP marker PM54, and the AFLP marker HM58, respectively. Within 140 lines (108 F2 and 32 F3) characterized for resistance to *M. chitwoodi*, 4.2 and 0% recombination events, respectively, were detected with the same markers. We used 49 F3 families characterized for resistance to *M. incognita* and *M. chitwoodi* for the mapping of these AFLP markers close to *Me7* and *Mech1* (Fig. 1b). *Me7* mapped 1.3 cM from *Mech1*, confirming that these two loci are different but linked.

Conversion of AFLP markers into locus-specific PCR-based markers

We cloned and sequenced three AFLP markers (PM54, HM60, and HM58) for *Me1/Mech2* and *Me7/Mech1*. Blast2seq (site NCBI) analysis showed that the polymorphic product observed in PM54 for *Me7/Mech1* was the same as that for *Me1/Mech2*. Five other AFLP markers (HM1, HM2, HM4, HM6, and PM6) (Table 4) closely linked to *Me3* and *Me4* (Djian-Caporalino et al. 2001) were also selected for further analysis because they were closely linked to the genes, could easily be converted into PCR-specific markers, or were transferable to the reference maps for pepper.

Six AFLP markers gave matches with YAC, BAC, or EST clones from tomato or pepper in the GenBank database (Table 4). Three markers (HM58, HM60, and PM6) had sequences similar to that of the *R*-gene *Bs2* from pepper. *Bs2* confers resistance to the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (Cook 1984; Cook and Guevara 1984). It belongs to the NBS/LRR (nucleotide-binding site/leucine-rich repeat) class of *R*-genes (Tai and Staskawicz 1999). The sequence of HM58 was similar to that of the conserved NBS, and the sequences of PM6 and HM60 were similar to that of the LRR domain.

Specific primers were designed from the cloned sequence of the AFLP markers (for PM54, HM4, HM6, and PM6), or their flanking sequences (for HM58) (determined by similarity to sequences in GenBank) for PCR amplification of the AFLP locus (Table 3). Polymorphism was clearly seen on agarose gels for SCAR_B94 corresponding to HM6 (Fig. 2a), SCAR_CD corresponding to PM54 (Fig. 2b), and CAPS_F4R4 obtained from HM58 (Fig. 2c). Single nucleotide polymorphisms (SNP) generated by SSCP _B54 corresponding to HM4 (Fig. 2d) and SSCP_B322 corresponding to PM6 (Fig. 2e) were detected on acrylamide gels by the SSCP technique.

Sequence comparisons and analysis in progenies confirmed that the converted PCR markers SCAR_B94, SCAR_CD, SSCP_B54, and SSCP_B322 were equivalent to the corresponding AFLP markers. CAPS_F4R4 could not be sequenced by Genome Express and four recombinant individuals were detected with AFLP-HM58 in 140 F2 and F3 lines, suggesting that CAPS_F4R4, corresponding to part of the *Bs2* gene sequence, was another marker linked to the *R*-genes. Three markers (HM1, HM2, and HM60) could not be converted into PCR-specific markers.

Genetic linkage between *Me* genes and location on the pepper map

As the SCAR-CD marker was linked to *Me1/Mech2* and *Me7/Mech1*, and the SSCP_B54 and SSCP_B322 markers were linked to *Me3/Me4* and *Me7/Mech1*, we hypothesized that the six genes were linked. We aligned the three intraspecific linkage maps of *Me* genes ([PM687 × YW], [DH330 × DLL], and [DLL × PM702]), using shared markers and CarthaGene software. The best putative order of the *Me R*-genes suggested by the software is shown in Fig. 3a. The frequencies of recombination shown between the *Me R*-genes are arbitrary, because they were determined on the basis of linkage to common markers. Nevertheless, the *Me3* gene carried by PM687 was closely linked to the *Me1* gene carried by DH330 (from PM217). This confirms the results obtained by Hendy (1984), who showed that these two genes were not allelic, but linked, with a recombination rate of 9%, estimated by testing 127 F3 families from the [PM687 × PM217] cross for resistance to *M. incognita*. We found that *Me3* and *Me1* mapped nearly 12 and 14 cM from *Me7*, respectively, indicating that all the broad-spectrum *R*-genes were linked rather than allelic.

Moreover, the five PCR-specific markers linked to the different *Me R*-genes were also polymorphic between Perennial and Yolo Wonder or H3 and Vania (Table 3). The molecular size of the polymorphic amplified fragments in these pepper lines was identical to that obtained with PM687 and YW, DH330 and DLL, or DLL and PM702, except for CAPS_F4R4. For this marker, *Tru*91 digestion was required to visualize the

Table 4 Characteristics of the AFLP markers linked to *Me* resistance genes and selected for the present study

Marker code	Corresponding AFLP primers	Length of the PCR product (bp)	Allele linkage	Similarity to sequences in GenBank database	Percentage of identity	References
PM54	*Pst* + GT/*Mse* + CCA	180	DH330 (R), PM702 (R)	No hit found		
HM60	*Hind* + ACA/*Mse* + AAA	342	DH330 (R)	*Capsicum chacoense* disease R protein *Bs2* mRNA (3,099 bp), LRR domain	62% in 103 nt overlap and 50.5% in 319 nt	Tai et al. (1999); Staskawicz (2004)
HM58	*Hind* + ACA/*Mse* + CCG	155	PM702 (R)	*Capsicum chacoense* disease R protein *Bs2* mRNA (3,099 bp), NBS domain	89% in 131 nt overlap	Tai et al. (1999); Staskawicz (2004)
HM1	*Hind* + ACA/*Mse* + AGT	82	PM687 (R)	No hit found		
HM2	*Hind* + ACT/*Mse* + ATG	96	YW (S)	*Solanum lycopersicom*, chrom. 1, clone CO1HBa0163B20 (134,102 bp)	87% in 63 bp	Mueller et al. (2005)
HM4	*Hind* + AGC/*Mse* + ACG	379	YW (S)	*Nicotiana tabacum* mitochondrial DNA (430,597 bp)	94% in 235 bp	Sugiyama et al. (2005)
HM6	*Hind* + ATC/*Mse* + ACG	246	YW (S)	No hit found		
PM6	*Pst* + ATT/*Mse* + ACG	255	PM687 (R)	*Capsicum chacoense* disease R protein *Bs2* mRNA (3,099 bp), LRR domain	81% in 179 bp	Tai et al. (1999); Staskawicz (2004)

polymorphic bands between DLL and PM702, although one band showed polymorphism between H3 and Vania without digestion (the 450 bp fragment was amplified in H3). We used MapMaker software to analyze the segregation of three of these markers (SCAR_B94, SCAR_CD, CAPS_F4R4) in the pepper intraspecific mapping populations 'PY' and 'HV'. One AFLP marker (AFLP_HM1) closely linked to *Me3/Me4* and polymorphic between Perennial and Yolo Wonder was also analyzed. The four markers were all assigned to the P9 chromosome of pepper. Thus, although *Me3/Me4*, *Me1/Mech2*, and *Me7/Mech1* have been mapped in different populations, these loci are probably located at linked positions in the pepper genome. Alignment of the five maps with the CarthaGene software defined a 28 cM genomic region and anchored this *Me* loci cluster on P9 (Fig. 3a), in the vicinity of the RFLP marker CT135, which is located 4.5 cM from *Me1* on our model integrated map. In a recent study, Sugita et al. (2006) detected the *Bs2* gene on the P9 chromosome of pepper, using primers based on the sequence of the gene. This marker was not polymorphic between the parental lines used in this study. However, we defined another marker in the *Bs2* sequence—CAPS_F4R4—which mapped to P9, suggesting that this gene was located close to the *Me* R-genes.

Discussion

We have identified dominant *R*-genes in DH330 and PM702 that completely control the *McIpo* population of *M. chitwoodi*: *Mech1* and *Mech2*. Dominant resistance to *M. incognita* in *C. annuum* PM702 and DH330 (*Me7* and *Me1*, respectively) did not segregate independently of resistance to *M. chitwoodi* (*Mech1* and *Mech2*, respectively), when F3 progenies from segregating populations were screened with both nematode species. Similar observations have been reported for the *Me3* and *Me4* *R*-genes (Djian-Caporalino et al. 2001). The existence of linked dominant alleles in each genotype was demonstrated by recombination between resistance to different RKN species and common AFLP and PCR-specific markers for the six *Me* genes. We constructed an integrated map from the three crosses based on shared markers, providing information about the organization of these six *Me* *R*-genes (Fig. 3a). The recombination frequencies shown are arbitrary and the order of the loci is putative, but the composite map nonetheless shows that the six *Me* *R*-genes are clustered in a single 28 cM genomic region. Moreover, three markers found in the three resistant

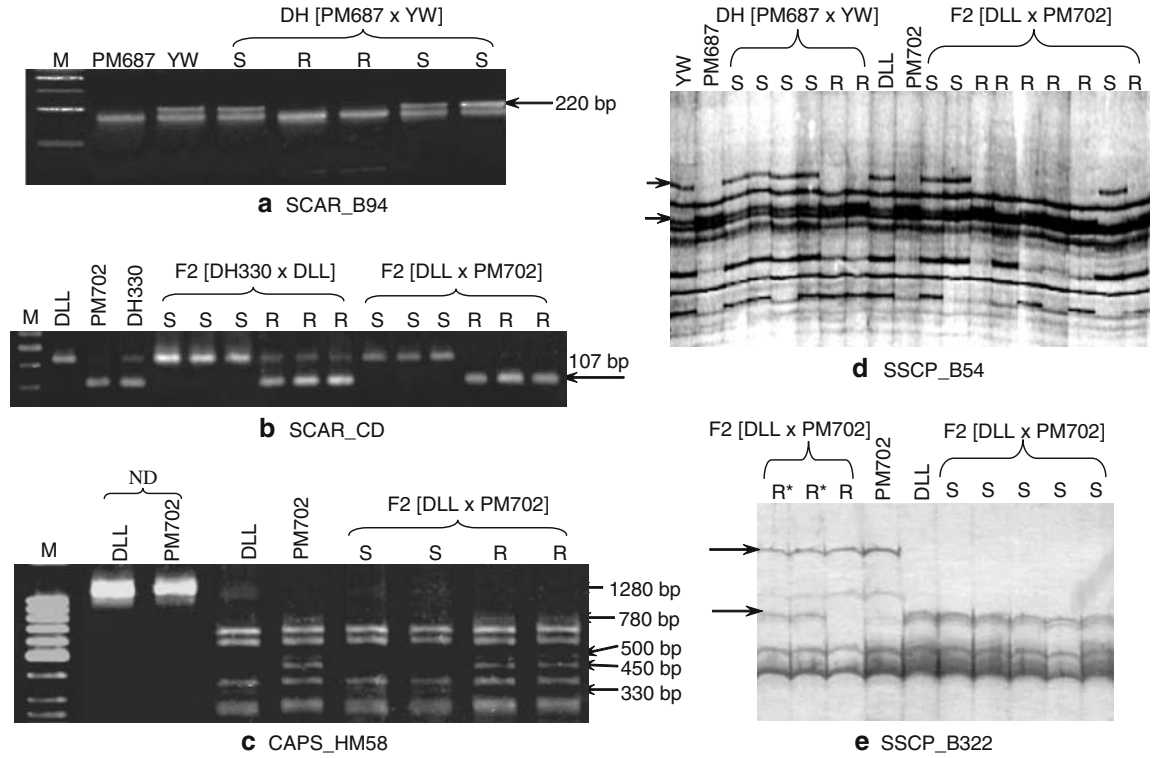

Fig. 2 Example of five locus-specific PCR-based markers linked to *Me* genes. Polymorphisms were visible on agarose gels for SCAR and CAPS markers: DNA from resistant (R) or susceptible (S) lines was subjected to PCR to amplify B94 (**a**), CD (**b**), and F4R4 (digested with Tru91) (**c**). Amplification products were separated by electrophoresis in agarose gels (1.8% for SCAR_B94, and 3% low melting point agarose for SCAR_CD and CAPS_F4R4), and stained with ethidium bromide for visualization. *ND* not digested. Polymorphisms were visible on acrylamide gels for SSCP markers: DNA from resistant (R) or susceptible (S) lines was used for the PCR amplification of B54 (2d) or B322 (2e). The amplicons were separated by electrophoresis in a 0.7× acrylamide gel and visualized by silver staining. *Arrows* indicate polymorphic products linked to S or R alleles. R plants marked with an *asterisk* are heterozygous

lines had sequences similar to that of the *R*-gene *Bs2* from pepper. This is consistent with the six *Me*-genes belonging to the same cluster or genomic region. This cluster was anchored on the P9 chromosome by analyzing shared markers on the reference intraspecific pepper maps developed by Lefebvre et al. (2002). Such *R*-gene clusters are common in many other plant species (Michelmore and Meyers 1998). This study reports the second cluster of dominant *R*-genes to be identified in pepper, after the dominant cluster of potyvirus and tospovirus *R*-genes described by Grube et al. (2000a) on the P10 chromosome.

Two QTLs conferring resistance to *Phytophthora capsici* (Thabuis et al. 2003) and potyviruses PVY (0) and PVY (1,2) (Caranta et al. 1997) have been mapped to the P9 chromosome of pepper in the vicinity of RFLP-CT135 (Fig. 3a). We show here that the cluster of *Me R*-genes maps to chromosome P9 and that the RFLP marker CT135 also maps to the vicinity of this *Me* cluster, further demonstrating the clustering of genetic resistance factors on this chromosome. The *Bs2* gene conferring resistance to the bacterium *Xanthomonas campestris* pv. *vesicatoria* has been cloned (Tai et al. 1999), and shown to be located on the P9 chromosome (Sugita et al. 2006). Three AFLP markers linked to various *Me R*-genes have sequences similar to the NBS or LRR domains of *Bs2*, suggesting that RGA (resistance gene analogs) from the same family as *Bs2* may be located in the same genomic region. We designed primers based on the *Bs2* gene sequence, leading to the identification of a new marker: CAPS_F4R4. This marker is located within the region containing the *Me R*-genes (Fig. 3a), confirming that *Bs2* is located in the same cluster (Fig. 3a).

The clustering of genes conferring resistance to a single pathogen or different pathogens in plants and the broad diversity of these genes are well documented and have been accounted for by an initial tandem duplication event followed by unequal crossing-over, gene conversions, and point mutations (Sheperd and Mayo 1972; Hulbert et al. 1997; Meyers et al. 2005). De Jong et al. (1997) and Van der Vossen et al. (2000) suggested that the clustering of *R*-genes may facilitate the coordination of plant defenses against various

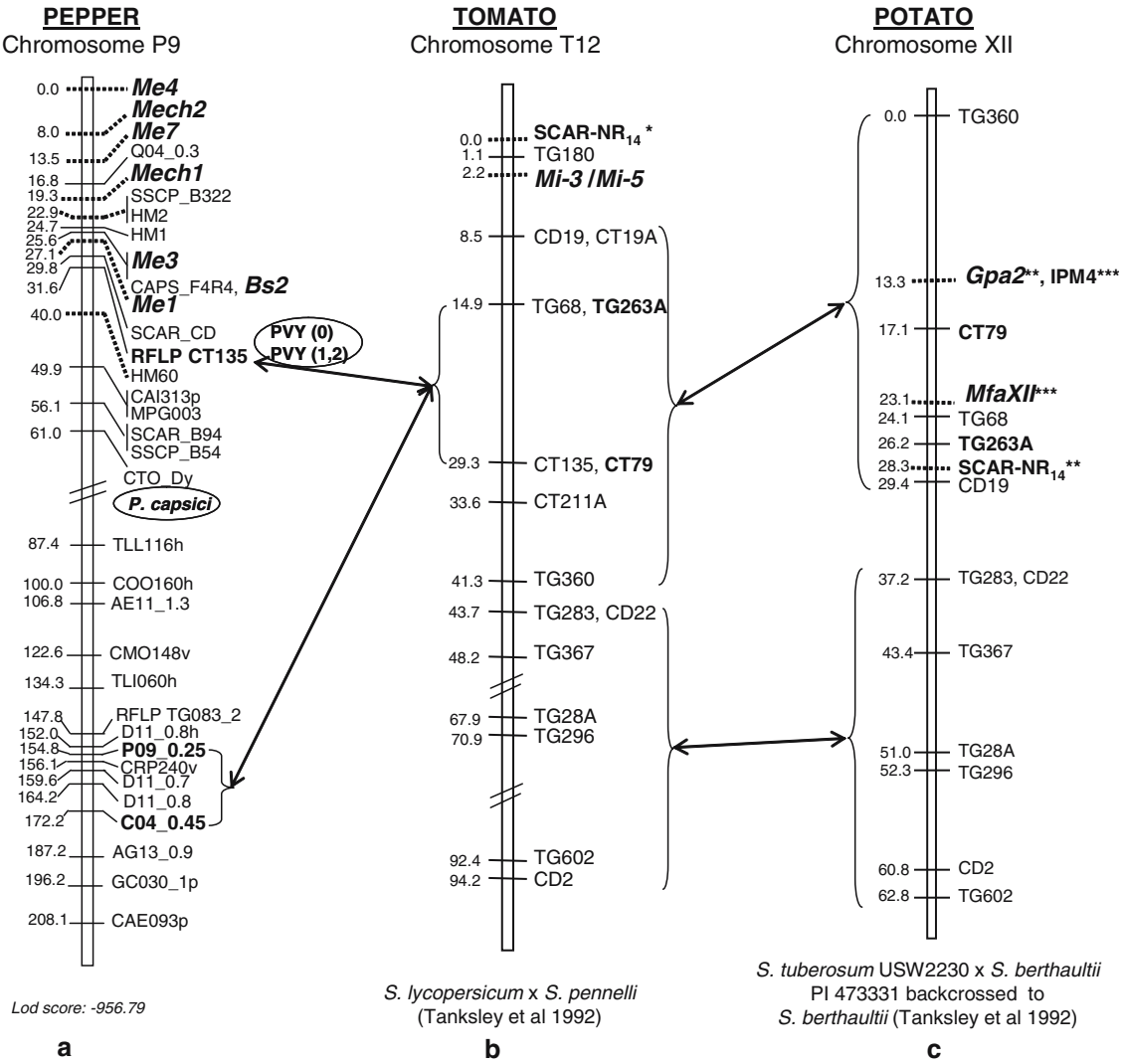

Fig. 3 Comparative mapping of the *Me R*-loci of pepper and the nematode *R*-loci of tomato and potato. **a** Model of an integrated map of chromosome P9 of pepper obtained using mapping markers common to the three *Me*-segregating populations (670 lines analyzed) and to the two reference intraspecific mapping populations PY and HV (215 lines analyzed), and CarthaGene software. Nematode *R*-genes are indicated in *bold*. Their position, determined from linkage to common markers, is putative, as for the markers indicated with *dotted lines*. QTLs for resistance to *Phytophthora capsici* (*P. capsici*) and potyviruses (PVY stains [0] and [1,2]) are *circled*. **b**, **c** Linkage between the markers and the *Mi-3/Mi-5* and *Gpa2/MfaXII R*-loci in tomato and potato, according to Yaghoobi et al. 1995 (*), Rouppe von der Voort et al. 1997 (**), and Kouassi et al. 2005 (***). The putative alignment of markers between **a**, **b**, and **c** is indicated by *arrows*. Distances are given in centimorgans (cM)

pathogens**.** *R*-genes clustering may favor the generation of new specificities sufficiently rapid to deal with an ever-changing array of pathogens. Nevertheless, although many *R*-gene clusters have been isolated, as pointed out by Kruijt et al. (2004), little is known about their evolution in natural populations.

We also compared mapping data in *Solanaceae*. Several studies (Grube 1999; Pflieger et al. 1999; Grube et al. 2000b) have shown that homologs of cloned *R*-genes map to syntenic positions in solanaceaous genomes, suggesting that both the sequence and position of these genes are conserved. The error of estimation of genetic distances from comparisons of maps between genera and the use of different mapping population structures and sizes is unknown; however, Grube et al. (2000b) suggested that it would be reasonable to assume that genetic distances are constant for all molecular maps.

In tomato, the cDNA probe CT135 maps to the telomeric region of the short arm of the T12 chromosome (Tanksley et al. 1992) (Fig. 3b). Yaghoobi et al. (1995) mapped a PCR-specific marker (NR_{14}) of the nematode resistance genes *Mi-3* and *Mi-5* from *Solanum* (formerly registered as *Lycopersicon*) *peruvianum* var. *glandulosum*

PI 126443-1MH to the same genomic region. *Mi-3* confers resistance to some naturally virulent *M. incognita* isolates (Roberts et al. 1990; Veremis et al. 1999). The *Mi-5* gene confers heat-stable resistance to *M. incognita* in *L. peruvianum* PI 126443-1MH (Veremis et al. 1999).

The CT135 region of the tomato genome is colinear with a segment of the distal end of chromosome XII in potato, with these two regions having the TG360, CT79, TG68, TG263, CD19, TG283, CD22, TG367, TG28, G296, CD2, and TG602 markers in common (Gebhardt et al. 1991). This region also contains the nematode resistance genes *Gpa2* and *MfaXII* (Fig. 3c), which control pathotype *Pa2* of the potato cyst nematode *Globodera pallida* (Rouppe van der Voort et al. 1997) and the RKN *M. fallax* (Kouassi et al. 2005), respectively.

Two RAPD markers, P09_0.25 and C04_0.45, were mapped to the P9 chromosome by Lefebvre et al. (2002; Fig. 3a). Paran et al. (2004) showed that they were linked to RFLP CT79 and TG263 on an integrated pepper map. CT79 cosegregates with CT135 on chromosome T12 of tomato, and TG263 is close to *Mi-3* and *Mi-5*. Both RFLP markers are close to *Gpa2* and *MfaXII* on chromosome XII of potato. These findings are consistent with the definitive assignment of the *Me* *R*-genes of pepper to an interval equivalent to that containing *Mi-3* and *Mi-5* in tomato and *Gpa2* and *MfaXII* in potato, although most of the positions concerned were inferred from linkage to reference markers and should be considered a best approximation. These comparative mapping data suggest that the three clusters of *R*-genes conferring resistance to nematodes are located in orthologous genomic regions of pepper, tomato, and potato, and that these regions are conserved within and between species and even between genera. Other cases of plant genes conferring resistance to the same pathogen mapping to syntenic chromosomic regions have been reported. For example, Pflieger et al. 2001 and Thabuis et al. 2004 described a major-effect QTL conferring resistance to *Phytophthora capsisi* on chromosome P5 of pepper in a genomic region colinear to that containing QTLs conferring resistance to *Phytophthora infestans* on chromosome IV of potato and identified by Leonards-Schippers et al. (1994) and Sandbrik et al. (2000). Moreover, the *R2* gene conferring specific resistance to *P. infestans* also maps in the vicinity of this region on chromosome IV of potato (Li et al. 1998).

The presence of genomic regions containing *R*-genes against nematodes in *Solanaceae* provides new insights into defense mechanisms against nematodes. Evidence is accumulating that NBS–LRR motifs are common in nematode *R-genes*. *Mi-1, Hero A*, *Gpa2, Gro1*, and *Ma* have been cloned and all belong to the NBS-LRR gene family (Milligan et al. 1998; Ernst et al. 2002; Van der Vossen et al. 2000; Paal et al. 2004; Claverie 2004). The NBS region resembles an ATPase domain present in proteins regulating programmed cell death (Van der Biezen and Jones 1998; Aravind et al. 1999). The *MfaXII* QTL in potato, the *Mi-3/Mi-5* *R*-genes in tomato, and the *Me* *R*-genes in pepper consistently induce localized cell necrosis in infected plants. Michelmore and Meyers (1998) suggested that *R*-genes containing the NBS-LRR motif may have evolved by divergent evolution of an individual ancestral gene in *Solanaceae*. Grube et al. (2000b) suggested that transposable elements may play a role in the creation and maintenance of such clusters in *Solanaceae.* The presence of transposable elements has been correlated with both large-scale genomic rearrangements (Robbins et al. 1989; Kim et al. 1998) and disease *R*-gene clusters (Meyers et al. 1998; Song et al. 1998).

Breeding for RKN resistance is a major challenge for pepper breeders. The diversity of RKN species infecting pepper plants in several major production areas requires combinations of several *R*-genes. The three pepper lines used in this study are therefore of considerable interest for controlling the main RKN species in various countries. Moreover, the pyramiding of *Me* genes based on the complementarity of their mode of action may make it possible to prevent the breakdown of RKN resistance. This strategy has been successfully used for pepper *pvr* genes with complementary action spectra (*pvr2* and *pvr6*), to control the main potyviruses in tropical countries (Palloix et al. 1998). Castagnone-Sereno et al. (2001) have shown that *Meloidogyne* populations virulent on *Me3* cannot overcome *Me1*. Thus, the combination of a locus controlling the penetration and migration of RKN juveniles in roots, such as *Me3* or *Me7* (Bleve-Zaccheo et al. 1998; Pegard et al. 2005), with a locus inhibiting female development by conferring the development of defective giant cells, such as *Me1* (Bleve-Zaccheo et al. 1998) should strengthen resistance to RKN. Nevertheless, pyramiding several *Me* genes in pepper remains a difficult task for breeders due to the linkage between genes. It will therefore be necessary to screen large segregating populations to identify recombinants occurring at a low frequency. It is also difficult to introduce more than one *R*-gene at a time into breeding material by traditional methods, because of the masking effect of genes and/or interactions between different RKN on the same plant. PCR-specific markers linked to *R*-genes and genetic background will therefore be very useful for screening of recombinant genotypes within the genomic region containing the *Me*

R-genes. We analyzed the *Me*-specific PCR-based markers on a set of eight pepper breeding lines (PM217, PM687, PM702, YW, DLL, H3, Vania, Perennial). Our preliminary results suggest that the markers generated in this study can be used in many susceptible genetic backgrounds. Studies to determine the durability of these *R*-genes (alone or pyramided) in different genetic backgrounds are now underway.

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