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Identification and Mapping of Markers Linked to the *Mi* Gene for Root-knot Nematode Resistance in Peach

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Abstract. An F_2 population from a single F_1 plant from the cross of peach [*Prunus persica* (L.) Batsch] rootstock cultivars Harrow Blood (HB) × Okinawa (Oki) was used to locate the *Mi* locus, which conditions resistance to *Meloidogyne incognita* (race 1) (Kofoid and White) Chitwood. These data and comparison of common markers among published genetic linkage maps placed the *Mi* locus on *Prunus* L. linkage group 2. Two restriction fragment length polymorphisms (RFLPs) [linked at 4.8 and 6.8 centimorgan (cM), repulsion phase] and one random amplified polymorphic DNA (RAPD) marker (linked at 9.5 cM, coupling phase) were linked to *Mi*. The RAPD marker was cloned, sequenced, and converted to a polymerase chain reaction (PCR)-based cleaved amplified polymorphic sequence (CAPs) marker. Clones of resistance gene analogs (RGA) developed from Oki were highly polymorphic when used as RFLP probes. The RGA's mapped to four linkage groups but clustered on two of the four linkage groups, providing limited coverage of the genome. Even so, they may be useful as markers for disease resistance genes that occur in other populations. The linkage maps of the HB × Oki F_2 population and a peach × almond (*Prunus amygdalus* Batsch) F_2 population were colinear in certain regions, however, a significant number of markers mapped to different linkage groups among the two populations. The locus for the blood-flesh trait (red-violet mesocarp) mapped to the top of linkage group 4.

Most stone fruit and nut trees grown commercially and in home gardens are compound plants consisting of a genetically distinct scion grafted on a rootstock. Root-knot nematode infestation of peach tree roots lowers yields and shortens the lifespan of an orchard. The most agronomically important root-knot nematodes (genus *Meloidogyne* Goeldi) are *Meloidogyne incognita*, *M. javanica* (Treub) Chitwood, and *M. arenaria* (Neal) Chitwood, which are found in all arable regions of the world (Mai, 1985), with *M. incognita* being most widespread and damaging in California peach orchards (McKenry, 1989).

Genetic resistance of the rootstock, while only one of several methods used to control nematodes, is the most economical, effective, and ecologically acceptable (Mai, 1985; Opperman et al., 1994). Currently, only three peach rootstock cultivars, Nemaguard, Nemared, and Lovell, are used extensively in California (Yoshikawa et al., 1989), with Nemaguard and Nemared being resistant to root-knot nematodes. However, both have limitations, including sensitivity to wet soil conditions and zinc deficiency, susceptibility to damage by root lesion nematode (*Pratylenchus vulnus* Allen and Jensen), ring nematode [*Criconemella xenoplax* (Raski) Luc. and Raski], bacterial canker (*Pseudomonas syringae* van Hall), crown gall [*Agrobacterium tumefaciens* (Smith and Townsend) Conn.], crown rot and oak root fungus [*Armillaria mellea* (Wahl.) Quel], and phytophthora root rot (*Phytophthora* DeBary) (McKenry, 1988, 1989; Yoshikawa et al., 1989). New rootstocks that combine multiple disease and pest resistances with size control, along with the good horticultural traits of currently available rootstocks, are needed wherever peaches are grown.

The *Mi* gene that confers resistance to *M. incognita* race 1 from the peach rootstock Oki has been shown in multiple crosses to be controlled by a single dominant gene (Sharpe et al., 1969). It is a good candidate for developing linked molecular markers because phenotypic screening takes 3–4 months and selected seedlings must be re-propagated because infected plants cannot be planted safely in the field. Alternatively, markers that can be used to distinguish between heterozygous and homozygous plants, or at least identify a susceptible tree, without greenhouse testing will allow a segregating population to be screened for resistance in one growing season, which will save considerable time, space, and money.

Resistance to *M. incognita* may be due to different genes that can affect other root-knot nematode species and show specificity for certain populations of *M. incognita*. Thus, it is desirable to have markers with which to elucidate this problem and to pyramid different resistance genes producing similar phenotypes. Tests for allelism are useful for analyzing interactions among these genes, but they are costly and allow only limited differentiation among different combinations of related genes. To our knowledge, results of allelism tests for *M. incognita* race 1 resistance genes from Nemaguard × Oki have not been published. An F_2 population from Nemaguard × Oki was tested against *M. incognita* race 3 (ssp. Florida) and all progeny were susceptible (McKenry, 1989), but results for screening against race 1 were not reported.

Information from genetic linkage maps from different populations is being used to increase efficiency of developing markers linked to important horticultural traits [e.g., the European *Prunus*

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mapping project (Arús et al., 1994)]. As outlined in Joobeur et al. (1998), full utilization of information in diverse maps requires markers, such as RFLPs, simple sequence repeats (SSRs), or isozymes, that are highly reproducible and transferable to different populations. A set of well-distributed markers can be the basis for a linkage map that allows comparisons among linkage maps of different populations and suggests how other markers can be used to target a specific genomic region.

Several groups are working to develop a saturated genetic linkage map for *Prunus* utilizing diverse markers (AFLP, RAPD, RFLP, microsatellite, morphological, isozyme markers) in interspecific peach × almond (Foolad et al., 1995; Joobeur et al., 1998), peach × *P. davidiana* (Carrière) Franch. (Viruel et al., 1998) crosses, intraspecific peach crosses (Chaparro et al., 1994; Dirlewanger et al., 1998; Lu et al., 1998b; Rajapakse et al., 1995), and an intraspecific almond cross (Viruel et al., 1995). The linkage map published by Joobeur et al. (1998) (European *Prunus* map) has become the standard for numbering the linkage groups in the *Prunus* genome.

RFLP markers using resistance gene analogs (RGAs) (Kanazin et al., 2000) or resistance-gene candidates (Shen et al., 1998) have been used as probes to develop markers that target important areas of the genome. Polymerase chain reaction (PCR) amplification of genomic DNA using degenerate primers derived from the nucleotide binding site (NBS) domain of cloned plant resistance genes has been used to isolate sequences, some of which map to known resistance gene loci, with high homologyto known plant disease resistance genes (R-genes) (Kanazin et al. 2000; Leister et al., 1996; Mago et al., 1999; Shen et al., 1998; Yu et al., 1996). Functional motifs in R-gene protein structures have been shown to be conserved within species and across taxa. Degenerate primers based on the phosphate-binding loop (P-loop) and GLPL motifs from the NBS of the *Arabidopsis thaliana* (L.) Heynh *RPS2* gene conferring resistance to *Pseudomonas syringae* (Bent et al., 1994) were used to amplify peach sequences with homology to soybean [*Glycine max* (L.) Merr.] RGAs and known resistance genes (Bliss et al., 2002; Thormann et al., 1998). This suggests they may be related to, or at least, linked to true resistance genes. Two candidate genes for the *Mi* gene in tomato (*Lycopersicon esculentum* Mill.) were found to be a tandem repeat and almost identical (91% amino acid identity), yet only one conditioned resistance (Milligan et al., 1998).

The possibility of obtaining markers tightly linked to, or identical to resistance genes, is attractive to plant breeders. R-genes are found in clusters in the genome (Kanazin et al., 2000; Leister et al., 1996; Mago et al., 1999; Shen et al., 1998; Yu et al., 1996) and there is genetic evidence that multiple disease resistance genes exist in a single cluster (Michelmore, 2000). Therefore finding a marker linked to a cluster may identify several resistance genes.

Bulked segregant analysis (BSA) (Michelmore et al., 1991) was used successfully with AFLP markers to obtain markers near *Mi* (Lu et al., 1998b) in a Lovell × Nemared peach population (the L×N population). Subsequently, two markers linked to *Mi* in a Lovell × Nemared cross were developed; a cleaved amplified polymorphic sequence (CAPs) marker (Lu et al., 1998a, 1999), which was not polymorphic in our parents (L×N CAPs) and a microsatellite marker designated "pchgms1" (formerly MicB3D3) (Lu et al., 1998b; Sosinski et al., 2000). However, because AFLPs were used extensively in constructing that linkage map, suitable reference markers for locating the *Mi* locus in our population were lacking.

Our experimental approach for obtaining markers linked to the *Mi* gene was to use a segregating population to 1) develop a linkage map of RFLP markers to locate the *Mi* gene, 2) use information from other *Prunus* linkage maps to increase marker development efficiency, and 3) use BSA to target the *Mi* gene for mapping and identifying linked markers in the specific genomic area. Information from our population can be used to compare the loci underlying this trait in other nematode resistant genotypes with different origins, yet similar phenotypes. Other genes and markers (e.g., resistance gene analogs and the blood-flesh locus) were mapped when possible to add useful information for subsequent genetic studies and rootstock breeding.

Materials and Methods

Plant materials. An F_2 population (PMP2) derived from self-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood (HB) × Okinawa (Oki) was studied. The original cross was made in 1990 at the USDA/ARS Horticultural Crops Laboratory Orchard in Fresno, Calif.; the F_1 tree (No. P248-139) was self-pollinated in 1993, and 64 F_2 plants were produced, of which 56 F_2 trees survived. The parents, F_1 and F_2 plants are maintained at the USDA/ARS San Joaquin Valley Agricultural Center, Parlier, Calif. Okinawa is a peach rootstock resistant to *M. incognita* and *M. javanica*, and lacks the blood-flesh trait (red-violet mesocarp). Harrow Blood is a Canadian peach selection that provides size control, has the blood-flesh trait, and is susceptible to root-knot nematode (Layne, 1975).

Phenotypic trait evaluation. Evaluation of the 56 PMP2 F_2 plants for nematode response was done by Dr. Craig Ledbetter at the USDA/ARS Horticultural Crops Laboratory, Fresno, Calif. (Current address: USDA/ARS San Joaquin Valley Agricultural Center, Parlier, Calif.), who found 47 resistant and 9 susceptible plants. Test for goodness-of-fit of 47:9 to an expected ratio of 3:1 gave a chi-square value of 2.38, $P = 0.12$; therefore, the 3:1 ratio was accepted. Also, he evaluated the mapping population for the blood-flesh trait and found a ratio of 43 white-flesh to 12 blood-flesh trees. Test for goodness-of-fit of 43:12 to the expected ratio of 3:1 gave a chi-square value of 0.3, $P = 0.59$; therefore, the 3:1 ratio was accepted. Those data were used to map the locus in the linkage map.

To test applicability of markers linked to the *Mi* gene for marker-assisted selection in peach rootstock breeding, a separate but related population (PMP2 OP-F_2) was formed by open pollination of the same F_1 tree that produced PMP2. The *Mi* genotype of each PMP2 OP-F_2 individual was established by screening their open pollinated progeny (F_3) for resistance to root-knot nematode. Open pollinated F_3 seeds from 49 PMP2 OP-F_2 trees were collected in 1997, stratified and germinated in a greenhouse. Either 15 or 16 seedlings per F_3 family were tested for resistance (i.e., presence or absence of root galls) by inoculation with *M. incognita* eggs obtained from peach roots collected near Fresno, Calif., and eggs from tomato roots infected with the *M. incognita* race 1 isolate 'Beltran' provided by Phil Roberts, Univ. of California, Riverside.

DNA isolation and southern blotting. Total genomic DNA was extracted using a protocol modified from Saghai-Maroof et al. (1984) that used a sodium dodecylsulfate (SDS) extraction buffer [50 mM TRIS base, 10 mM EDTA, 0.7 M NaCl, 1% SDS, pH 9.5; 2% β-mercaptoethanol and ≈1 g of polyvinylpolypyrrolidone per 10 g leaf sample]. Genomic DNA was purified by cesium chloride-ethidium bromide density gradient. Agarose

gel electrophoresis and capillary transfer Southern blotting were performed using standard procedures outlined in Sambrook et al. (1989). From each tree, 5 μg of total genomic DNA were digested separately with one of four restriction endonucleases (*EcoRI*, *EcoRV*, *DraI*, and *HindIII*) according to the manufacturer's directions. The digested DNA was separated by electrophoresis in a 1% agarose gel, blotted onto Zeta-bind nylon membrane (AMF-CUNO Division, Meriden, Conn.), baked for 2 h at 75 to 80 °C and stored until use.

DNA sequencing and sequence analyses. All nucleotide sequence determinations were performed using Big Dye Terminator chemistry (PE/Applied Biosystems Division, Foster City, Calif.) at the Plant Genetics Facility of the Univ. of California, Davis. DNA templates were PCR-generated fragments from either amplification of genomic template with custom primers or cloned PCR fragments using T7 and SP6 primers. Sequences were analyzed using the Sequencher computer program (Gene Codes Corp., Ann Arbor, Mich.).

Marker Development for Linkage Mapping in PMP2

DNA probes for RFLP analyses

Peach cDNA probes. Probes (CPM probes) were generated from the same cDNA library made from mRNA isolated from developing peach mesocarp that was used by Foolad et al. (1995) and the clone designations used in this research are the same as Foolad et al. (1995) and Bliss et al. (2002).

Almond probes. The *PstI* almond genomic clones (AG probes) (Viruel et al., 1995) were provided by Dr. Pere Arús, Institut de Recerca i Technologia Agroalimentàries, Cabrills, Spain. They were the same clones used for genetic linkage mapping in a peach × almond cross (Bliss et al, 2002; Foolad et al., 1995).

RGA and resistance gene analog-related sequence (RRS) probes derived from Oki. The RGA and RRS probes were developed by Thormann et al. (1998). PCR amplification of Oki DNA using degenerate oligonucleotide primers with sequence similarity to the P-loop and leucine rich repeat (LRR) domains of the *RPS2* gene from *Arabidopsis thaliana* and the primers S1 and AS1 from Leister et al. (1996) were used to isolate potential RGAs. Putative RGA clones, which later showed no sequence similarity to cloned plant disease resistance genes or known RGAs were designated as RRS (See Bliss et al., 2002).

Plasmid inserts were amplified by PCR with reaction conditions of 10 mM Tris-HCl, 50 mM KCl, 1.9 mM $MgCl_2$, 0.12 mM each dNTP, 0.2 ng·μL^{-1} each of SP6 and T7 primers (GIBCO/BRL-Life Technologies, Gaithersberg, Md.) for CPM probe amplification or T3 and T7 primers (Promega Corp., Madison, Wis.) for AG probes, or primers M13F and M13R for RGA and RRS clone amplification, 0.04 Units (U)/μL AmpliTaq DNA polymerase (Perkin Elmer, Wellesley, Mass.) and ≈0.6 ng·μL^{-1} plasmid in a 25-μL volume. Thermal cycle of 2 min at 94 °C; 25 cycles of 30 s at 94 °C, 1 min at 52–55 °C, 2 min at 72 °C; 10 min at 72 °C followed by a 4 °C soak on a Perkin Elmer Cetus model 480 thermal cycler was used. Amplified fragments were labeled and detected using the ECL (Enhanced Chemiluminescence) direct nucleic acid labeling and detection system kit (Amersham Biosciences, Uppsala, Sweden).

PCR markers

Except where noted, all primer synthesis was done by GIBCO/BRL-Life Technologies. Agarose gel electrophoresis was performed using standard procedures outlined in Sambrook et al. (1989). A 6% polyacrylamide, 7 M Urea and 1× TBE gel was used with the Bio-Rad Sequi-Gen GT sequencing cell (Bio-Rad Laboratories, Hercules, Calif.). The SILVER SEQUENCE DNA Staining Reagents Kit (Promega Corp.) was used according to the manufacturer's instructions to silver stain the polyacrylamide gels.

Markers derived from the L×N CAPs marker. Lu et al. (1999) described the L×N CAPs marker that is linked to the *Mi* locus in a Lovell × Nemared population. However, it was monomorphic for HB and Oki when primers Mij3F and Mij1R from Lu et al. (1999) were used on Oki, HB, F_1, Nemared, and Lovell. The Mij3F and Mij1R primers were derived from an AFLP marker designated AA/CAT10 on linkage group 1 of the linkage map presented in Lu et al. (1998b), which is reproduced in Fig. 4. Anchored PCR was used to develop the new marker, designated APMij3F, which was produced using primer combination Mij3F and Operon 10 base pair (bp) oligonucleotides (Operon oligos) Kit G (OPG) (QIAGEN Operon, Alameda, Calif.) number 13 with PCR conditions of 1× PE Buffer II, 0.12 mM dNTP, 2.3 mM $MgCl_2$ (total concentration), 12 ng of Mij3F primer, 6 ng OPG primers, 1.0 U AmpliTaq DNA polymerase (Perkin Elmer), 100 ng template DNA in a 25 μL volume. Thermal cycle program was 94 °C for 2 min; 5 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min; 5 cycles of 94°C for 1 min, 35 °C for 1 min, 72 °C for 2 min; 19 cycles of 94 °C for 30 s, 35 °C for 30 s, 72 °C for 1 min; and soak at 72 °C for 15 min

Microsatellite pchgms1. The microsatellite pchgms1 (formerly known as MicB3D5) (Lu et al., 1998b; Sosinski et al., 2000) was mapped. The reaction conditions provided by Dr. A. Abbott, Clemson Univ.) were modified slightly to use 0.5 pM/μL instead of 0.6 pM/μL of each primer. A reaction volume of 10 μL containing 1× PCR Buffer II (Perkin Elmer), 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.5 pM/μL of each primer, 0.05 U/μL of AmpliTaq DNA polymerase (Perkin Elmer), and 0.4 ng/μL template was used. The original thermal cycle program was optimized for the RoboCycler Gradient 96 Temperature Cycler (Stratagene, La Jolla, Calif.) to be 94 °C for 4 min; 32 cycles of 94 °C for 45 s, 66 °C for 30 s, 72 °C for 30 s; followed by 72 °C for 5 min The PCR products were separated by PAGE and visualized by silver staining.

RAPD marker development

BSA performed using random 10-bp oligonucleotides typically requires screening many primers to find linked markers. Rather than screening random primers, information from published *Prunus* maps was used to select certain Operon oligos for RAPD analysis. The only *Prunus* map to indicate location of the *Mi* locus was the L×N population map (Lu et al., 1998b), which consisted primarily of AFLPs. The AFLP primers *EcoRI*+AA / *MseI*+CAT and *EcoRI*+AA / *MseI*+CA produced products linked to the *Mi* locus in this map. The same AFLP primer pairs and many RAPD markers were used by Dirlewanger et al. (1998), but the *Mi* locus was not mapped in that population. From the Dirlewanger et al. (1998) map, RAPD primers that produced markers in the same region as AFLPs generated by *EcoRI*+AA / *MseI*+CAT and *EcoRI*+AA / *MseI*+CA were chosen, as were the Operon oligos shown previously to be polymorphic in peach (Warburton, 1995). The Operon oligos A5, B5, AB10, D3, D5, D7, E7, F16, G16, I6, I7, I18, O2, O3, O4, O6, O7, O8, O19, S18, V1, V2, V12, V18, and W6 were screened on both the resistant and susceptible bulks as defined below.

Plants for susceptible and resistant bulks were chosen from mapping population (PMP2) based on phenotype of the PMP2 F_2 individuals because failure of F_3 progeny screening prevented

unambiguous identification of the genotypes. The susceptible bulk consisted of DNA from PMP2 F_2 plants nos. 3, 6, 7, 8, 10, 15, 16, 17, and 23, and the resistant DNA bulk consisted of PMP2 F_2 plants nos.14, 19, 20, 26, 28, 31, 42, and 60.

RAPD fragments were amplified in a reaction mixture of 1× PCR Buffer I (Perkin Elmer), 120 μM dNTPs, 1.5 mM $MgCl_2$, 1 ng·μL^{-1} Operon oligos as primer, 0.04 U/μL AmpliTaq DNA polymerase (Perkin Elmer), and 2.5 ng·μL^{-1} template in a 10-μL volume. Temperature cycling conditions and thermal cycler (Perkin Elmer 9600) were the same as used by Dirlewanger et al. (1998). Electrophoresis using 1.5% agarose gels in a TAE buffer followed by ethidium bromide staining was used to separate and visualize the RAPD products.

Development of CAP1 marker from RAPD marker and CAP1 related markers

The CAP1 molecular marker linked to *Mi* in PMP2 was developed from a RAPD marker O06-0.2. The RAPD fragment O06-0.2 linked in coupling to the resistant allele was amplified from Oki DNA, isolated, purified, and cloned into the vector pCR 2.1 using the Original TA Cloning Kit (Invitrogen Corp., Carlsbad, Calif.) according to the manufacturer's instructions. Transformations of *E. coli* strain DH5-α competent cells were performed according to the GIBCO/BRL transformation protocol for *E. coli* strain DH5-α competent cells. The insert was PCR amplified, purified, sequenced and found to be 227 bp.

A sequence characterized amplified region marker (SCAR) (Paran and Michelmore, 1993; Staub et al., 1996) based on RAPD marker O06-0.2 was expected to produce a reliable single dominant PCR product in Oki. Primers O6:206L20 (206L) [5′-ACG GGA AGC CAT CAT GTA TG-3′] and O6:2U19 (2U19) [5′-CAC GGG AAG GAG AAA ATC G-3′] (collectively the CAP1 primers) designed from the sequence of the RAPD O06-0.2, produced a PCR product of the expected size in *both* parents. Therefore the product was purified and PCR sequenced using the 206L primer. The sequence data showed that Oki and HB differed for a *PvuII* restriction endonuclease recognition site. These differences were exploited to create a CAPs marker designated "CAP1."

The CAP1 marker was amplified using 1× PCR Buffer II (Perkin Elmer), 0.12 mM dNTP, 2 mM $MgCl_2$ (total concentration), 0.6 pM/μL primer, 0.04 U/μL AmpliTaq DNA polymerase (Perkin Elmer), and 1 ng·μL^{-1} template DNA in a 10- or 15-μLvolume. Thermal cycle of 94 °C for 1 min; 5 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min; 29 cycles of 94 °C for 30 s, 60 or 61 °C for 30 s, 72 °C for 30 s; 72 °C for 3 min followed by a soak at 6 °C was used. After cycling, 10 μL of the PCR reaction was mixed with 5 μL of a digestion reaction mixture of 0.75 μL of 10× Universal Buffer (Stratagene), 5 U *PvuII* restriction endonuclease and water to a volume of 5 μL. The final concentration of the Universal Buffer was 0.5× (0.05 M KOAc, 12.5 mM Tris-Acetate (pH 7.6), 5 mM MgOAc, 0.25 mM β-mercaptoethanol and 5 μg·mL^{-1} BSA). The digestion products were separated and visualized using a 0.7% Agarose / 2.15% Synergel 1× TAE (equivalent to a 5% agarose gel) gel or a 2% agarose 0.5× TBE gel and ethidium bromide staining. The screening of all populations and Nemaguard, Nemared, and Lovell DNA for CAP1 marker segregation was done at least twice.

Primers 2U19 and 206L20 (CAP1 primers), which were used to amplify the CAP1 marker, were used with Operon oligos to develop anchored PCR products used for BSA. The CAP1 primers were used in combination with the following Operon oligos: Kit A – 5; Kit D – 7; Kit E – 7; Kit G – 16; Kit H – 1, 3; Kit I – 6, 7; Kit O – 3, 4, 7; Kit V – 1, 12, 18. The PCR reaction mixture consisted of 1× PCR Buffer II (Perkin Elmer), 0.12 mM dNTP, 2 mM MgCl2 (total concentration), 0.8 ng – 1 ng·μL^{-1} Operon oligos as primer, 0.8 pM/μL 2U19 or 206L20 primer, 0.04 U/μL AmpliTaq DNA polymerase (Perkin Elmer), and 1 ng·μL^{-1} template DNA (HB or Oki) in a 10- to 15-μL volume. Thermal cycle was 94 °C for 2 min; 7 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min; 5 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min; 20 cycles of 94 °C for 30 s, 36 °C for 30 s, 72 °C for 1.1 min; and soak at 72 °C for 20 min. Controls included a reaction with only one primer as well as the standard negative (no DNA) control. For example, a PCR reaction using primers 2U19 and Operon oligo V18 would have as controls a reaction with only V18 and a reaction with only 2U19. Amplification products were size-fractionated by electrophoresis on a 2% 1× TAE agarose gel. Primer combinations 2U19/I7, 2U19/O7, 2U19/S18, 2U19 only, and 206L20 only were polymorphic between the PMP2 parents and were screened using the above conditions on parental DNA, the resistant bulk and susceptible bulk. Only the primer combination of the CAP1 primer 2U19 and Operon oligo I7 gave a product that was able to be mapped in PMP2, and this marker was designated "AP2UI7b9."

Statistical and linkage analyses

Chi-square goodness-of-fit tests of 1:2:1 or 3:1 segregation ratios were done and skewed loci omitted. The map was constructed with MAPMAKER/EXP v. 3.0 (Lander et al., 1987). The "group" command (LOD ≥3 and recombination fraction ≤40) was used to assign linkage groups. Map order was determined using the "compare" command for small groups and the "order" and "ripple" command for larger groups. The "try" command was used to add new loci. The "three point" command was set to LOD ≥3, maximum distance 37.2 and the "multipoint exclusion threshold" of 2.00 and the strict threshold of 3.0 was used. "Error detection" was used to find possible incorrect scorings of markers after the map was constructed.

Results

Genetic linkage mapping. Markers mapped in PMP2 are described in Table 1 and the resulting linkage map is presented in Fig. 1. The locus (Blood) controlling the blood- or red-fleshed fruit trait mapped to the top of linkage group (LG) 4. Resistance to root-knot nematode was dominant and the *Mi* locus mapped to the top of LG 2. We used information from other maps to locate a RAPD marker linked to *Mi*, but only after considerable time spent developing an RFLP map. When we used RAPD markers developed by Lu et al. (1996) they did not give the expected banding patterns, but this was not surprising given the unreliability of RAPDs. The microsatellite, pchgms1, detected a single polymorphic locus that mapped 49.2 cM from *Mi* in PMP2. The L×N CAPs marker developed by Lu et al. (1999) in a Lovell × Nemared population was monomorphic between our parents, and the PCR marker (APMij3F) derived from L×N CAPs marker was initially unlinked to *Mi*. With addition of more markers, it mapped 12.9 cM from *Mi* in our population.

After screening 155 probes for RFLP polymorphism and developing a linkage map using 76 RFLP loci and the APMij3F marker no linkages to the *Mi* locus were found. BSA of plants from PMP2 using 25 oligos for RAPDs detected one marker of 227 bp (RAPD O06-0.2) linked in coupling to the resistant allele 9.5 cM from the *Mi* locus.

Development of CAP1 marker. RAPD O06-0.2 was cloned and converted to a more reliable SCAR marker. Although SCAR

Table 1. Descriptions of markers used for genetic linkage mapping in an F_2 population (PMP2) derived from self-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood × Okinawa.

A. Probes used to detect RFLP markers

Probe origin	Prefix[z]	No. of probes	Probes mapped	No. of loci mapped	Co-dominant loci	Unlinked loci
Almond genomic DNA	AG	14	4 (28.6%)	8	3	n/a
Peach mesocarp cDNA	C	126	33 (26.2%)	43	11	5
Resistance gene analog and resistance gene analog-related	R	15	12 (80.0%)	25	4	n/a

B. PCR markers

Origin	Label or prefix	No. of markers	No. of loci mapped	Unlinked loci
Anchored PCR product using primer Mij3F (CAPs marker linked to *Mi* gene in Lovell × Nemared population) and Operon G13 oligo is ≈860 bp	APMij3F	1	1	0
RAPD band ≈0.2 kb in length from Operon O6 oligo	O06-0.2	1	1	0
CAP1 marker linked to *Mi* gene which was developed from the RAPD O06-0.2	CAP1	1	1	0
Anchored PCR product using the CAP1 primer 2U19 and Operon I7 oligo. The mapped PCR product is ≈930 bp and found in 'Harrow Blood'	AP2-	2	1	1
Microsatellite linked to *Mi* gene in Lovell × Nemared population	pchgms1	1	1	0

C. Phenotypic traits

Description	Label	Scoring of trait in PMP2
Red-flesh fruit color known as "Blood."	Blood	Original PMP2 trees scored by C. Ledbetter
Resistance to root-knot nematode *M. incognita*, race 1	*Mi*	PMP2 F_2 seedlings were screened by C. Ledbetter. Screening of F_3 seedlings was unable to be completed.

[z]Loci detected by RFLPs are designated by a prefix representing the type of probe followed by: the clone number, a code designating the restriction endonuclease used to detect the RFLP (E = *EcoR1*, EV = *EcoRV*, D = *DraI*, H = *HindI*II), and capital letters designating the RFLP band(s) scored from the films.

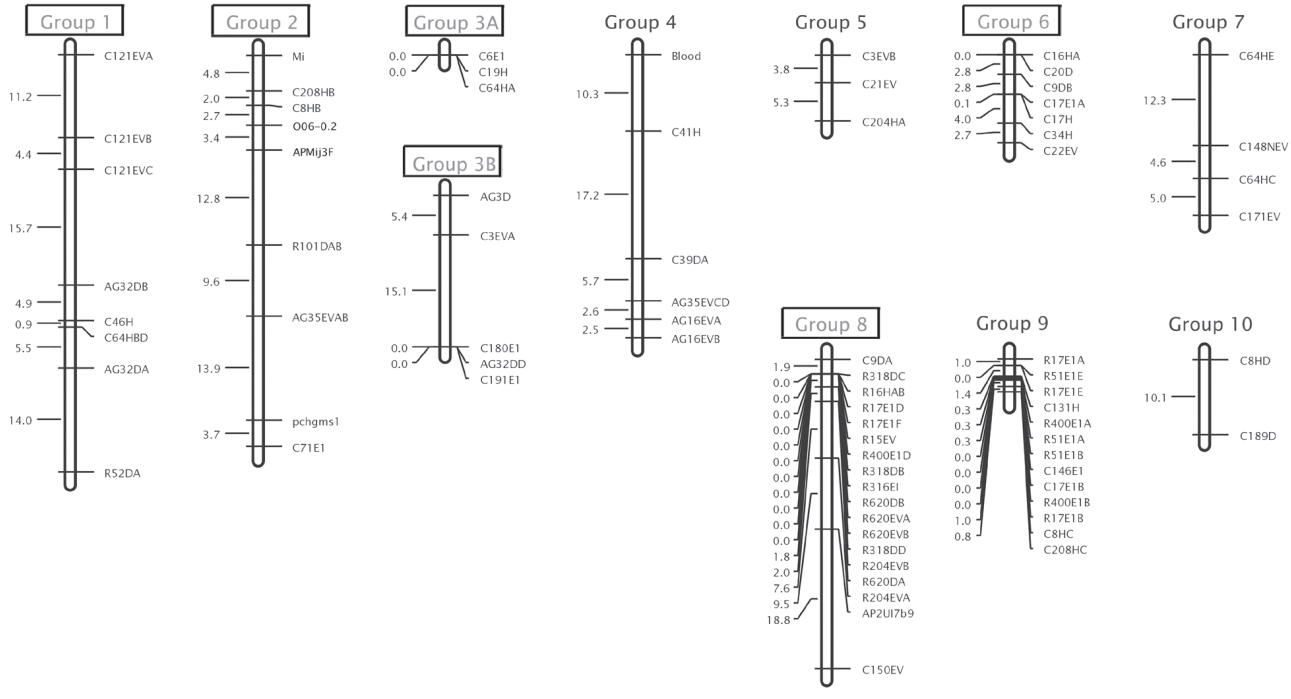

Fig. 1. Genetic linkage map from an F_2 population (PMP2) derived from self-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood × Okinawa. Distance in centimorgans is listed on the left side and marker designation on the right side of each linkage group. Linkage groups with labels in boxes correspond to homologous linkage groups in the European *Prunus* mapping project's map of a peach × almond F_2 population (Joobeur et al., 1998) (the "European *Prunus* map") and are labeled accordingly. Homology was determined by comparing common restriction fragment length polymorphism, isozyme and microsatellite loci between the PMP2 map, the PMP1 linkage map [an almond × peach map (Bliss et al., 2002; Foolad et al., 1995)], and the European *Prunus* map. The relationship of the other linkage groups to other *Prunus* maps is uncertain. RFLP loci are labeled with the source of the probe (AG, C, or R). See Table 1 for descriptions of the markers.

markers have been shown to be reliable and potentially codominant (Paran and Michelmore, 1993; Staub et al., 1996), PCR primers developed from the sequence of the O06-0.2 marker produced only a 224-bp monomorphic fragment in PMP2, Nemaguard, Nemared, and Lovell (data not shown).

Sequencing of the SCAR PCR band produced a chromatogram with clear peaks, except for the first 20 bp, which is expected. This indicated only one product was detected in each parent by the sequencing procedure. Comparison of parental sequences showed differences for *AluI*, *HaeIII,* and *PvuII* restriction endonuclease recognition sites. Digestion of the SCAR products with *PvuII* gave fragments of 224 bp (no recognition sites) in Oki, F_1, and the resistant F_2, and 128 and 96 bp (one recognition site) in HB and the susceptible F_2 at an annealing temperature of 61 °C (Fig. 2). Therefore the CAPs marker (designated CAP1) was dominant. The CAP1 PCR product (not cleaved by *PvuII*) from Oki is referred to as the "Uncut Band" and the PCR product from HB as the "Cut Band." CAP1 co-segregated with the RAPD O06-0.2 marker as expected. These markers are 9.5 cM from *Mi* in LG 2 of PMP2.

Dominant expression of the Oki (resistant) allele of the CAP1 marker allowed susceptible homozygotes to be identified and culled from populations. Attempts to obtain allele-specific PCR primers to amplify both alleles in the F_1 plant were unsuccessful. Production of the CAP1 PCR product was dependent on use of a 61 °C annealing temperature. Use of lower annealing temperatures produced the Uncut Band and Cut Band in all individuals, which can lead to ambiguity in the interpretation of this pattern since differences in band intensity are not reliable in practice. This suggests that the dominant nature of the CAP1 marker is due to competition among primer sites and preferential amplification of certain sequences, which is characteristic of the PCR reaction. The CAP1 primers apparently can amplify multiple species of very similar size. This was not obvious from the sequencing chromatograms of the SCAR products.

Verification of the CAP1 marker in other populations. Linkage of the CAP1 marker to the *Mi* locus was confirmed in the related test population PMP2 OP-F_2 where it showed a co-segregation ratio expected for a marker 9.5 cM from *Mi* (Table 2). The interspecific peach × almond F_2 population (PMP1) (Bliss et al., 2002) was used to evaluate utility of CAP1 in an interspecific population. CAP1 mapped to LG 2 in PMP2 and was expected to map to the homologous LG 2 in PMP1. Instead it mapped to LG3 in PMP1 (Bliss et al., 2002). The peach allele of CAP1 marker was scored as dominant in PMP1.

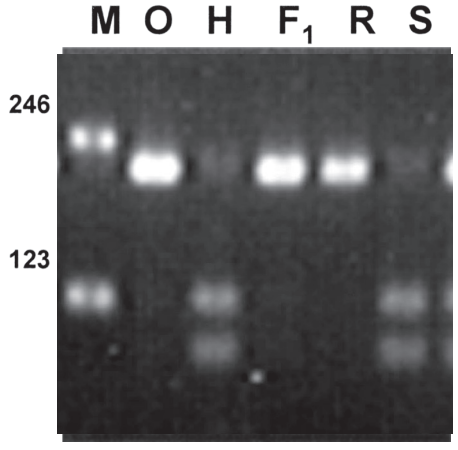

Fig. 2. Results of PCR amplification of CAP1 marker at a 61 °C annealing temperature followed by digestion with *PvuII* restriction endonuclease using Okinawa (O), Harrow Blood (H), F_1 (F_1), resistant F_2 (R), and susceptible F_2 (S) DNA from PMP2 as template for the PCR reaction. Visualized using a Synergel/agarose gel equivalent to 5% agarose with 1× TAE running buffer. The marker was a 123 base pair marker.

Table 2. Test of co-segregation of CAP1 marker and resistance to *Meloidogyne incognita* race 1 in an F_2 population derived from open-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood × Okinawa (PMP2 OP-F_2). Expected co-segregation results based on recombination fraction of 9.5% obtained from the F_2 population (PMP2) derived from self-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood × Okinawa that was used for genetic linkage mapping in this study (see Fig. 4). Nematode reaction phenotype was the expected phenotype of the PMP2 OP-F_2 individuals based on the observed segregation for resistance of their open-pollinated F_3 progeny when inoculated with *M. incognita* race 1.

Nematode reaction	CAP1 marker phenotype observed (expected)	
phenotype	Resistant	Susceptible
Resistant	33 (33)	2 (2)
Susceptible	1 (2)	11 (10)

Commercial rootstocks Nemaguard, Nemared, and Lovell were screened several times to evaluate the repeatability and broad applicability of the CAP1 marker for rootstock breeding. Nemaguard and Nemared were expected to have the resistant pattern of one Uncut Band because both are resistant to *M. incognita*, whereas Lovell is susceptible to *M. incognita* and was expected to have the susceptible pattern. The results using an annealing temperature of 61 °C were repeatable, but Nemaguard, Nemared, and Lovell had the susceptible pattern of three bands (Fig. 3). Since CAP1 is 9.5 cM distant, it is possible that recombination and/or sequence divergence resulted in resistant individuals that display the susceptible marker pattern.

Map Placement of Other Markers

RFLP markers: CPM 208 and CPM8 clones. After addition of the CAP1 marker to the linkage analysis it became clear that RFLPs C208HB and C8HB were closer to *Mi* than the CAP1 marker. Both loci had been scored as dominant bands from the susceptible parent. The complex banding patterns detected by the CPM208 and CPM8 clones suggested that these cDNA clones might belong to a multi-gene family.

CPM probes. CPM probes consisted of two groups: "A," which contained 63 probes that were mapped in a peach × almond cross (Foolad et al., 1995), and "B," an additional 90 clones isolated from the same CPM library. Group A produced 18 (28.6%) potentially mappable probes, but one deviated from the segregation ratio of 3:1 and was not used. Group A had 35 (55.6%) monomorphic and 10 (15.9%) illegible films (usually too much signal) and/or inconsistent results and one probe that did not amplify.

Among the 63 Group B clones that produced a PCR product, 11 (17.5%) generated mappable loci. Group B had 30 (47.6%) monomorphic clones, 16 (25.4%) illegible blots (faint, dark, or only faint unreliable bands were polymorphic), four (6.3%) clones that gave no hybridization signal, and one (1.6%) that had two amplification products and was discarded. Most of the illegible blots were monomorphic, or they were polymorphic when the signal was faint.

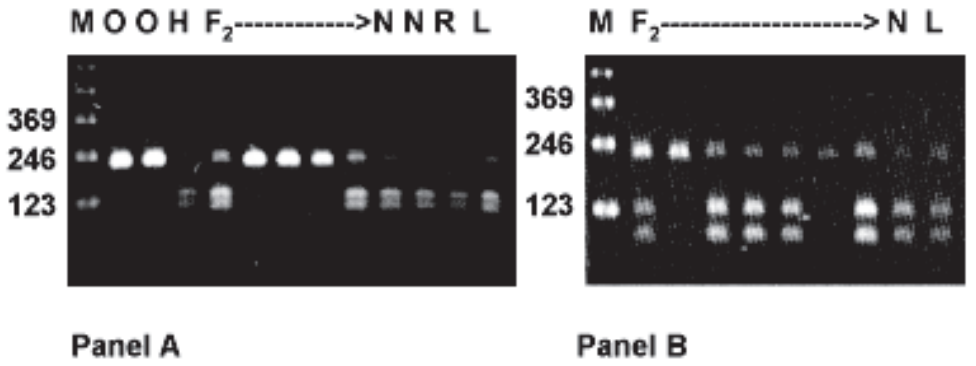

Fig. 3. Test of CAP1 marker in the peach rootstocks Nemaguard (N), Nemared (NR), and Lovell (L). (**Panel A**) Okinawa (O) (two samples), Harrow Blood (H) and six individuals (PMP2 OP-F_2) from an F_2 population (PMP2 OP) derived from open-pollination of a single F_1 plant of the peach intra-specific hybrid Harrow Blood × Okinawa, compared to N, NR, and L. L clearly had the susceptible pattern of three bands, whereas N and NR had the susceptible pattern of 3 bands but the larger undigested band is faint. (**Panel B**) Seven PMP1 F_2 individuals [an almond × peach F_2 population (Bliss et al., 2002; Foolad et al., 1995)], N and L. The susceptible pattern of three bands is segregating with the resistant pattern of one band in the F_2. N and L have the susceptible bands. Visualized using an agarose/Synergel gel equivalent to 2% agarose with 0.5× TBE running buffer. "M" was a 123 base pair marker.

Our main reason for using probes already mapped in other populations was to compare genetic linkage maps and because they might be more easily scored than probes from random screening of a cDNA library. However, there was only a small improvement in detected polymorphisms in Group A over Group B. The figure of 28.6% of clones detecting polymorphisms is in line with other reports (Quarta et al., 1998; Rajapakse et al., 1995).

AG PROBES. Only 26.6% of the almond genomic clones were mappable, which is comparable to the overall percentage of mappable CPM probes. Three probes detected seven loci on four linkage groups and despite some clustering gave a somewhat dispersed distribution of markers.

RGA AND RRS PROBES. Eighty percent of the RGA and RRS probes developed using the resistant parent Oki were mapped. In most cases, Southern hybridization produced a multi-banded profile with several dominant loci scored per probe. These probes did not provide broad coverage of the genome, but rather clustering of loci in two main regions. Seventeen loci produced by 14 RGA and RRS probes mapped within 3.8 cM of each other in LG8, with a singleton of one dominant locus being 7.6 cM from that cluster. A second cluster of eight dominant loci produced by two RGA and one RRS probes and five loci detected by 5 CPM probes mapped within 5.1 cM of each other on LG9. Singleton loci produced by two different probes were detected in each of LG1 and LG2.

Discussion

LOCATION OF THE *MI* LOCUS AND LINKED MOLECULAR MARKERS. For comparisons among different linkage maps, the most useful markers are RFLPs, isoenzymes, and microsatellites (Cipriani et al., 1999; Sosinski et al., 2000). However, differences in recombination frequencies among the same markers in different populations are common. In PMP2, APMij3F and the microsatellite pchgms1 were 12.9 and 49.2 cM from *Mi,* respectively. By comparison, the L×N CAPs marker (from which APMij3F was derived via Anchored PCR) and pchgms1 were, respectively, 19.9 and 40.5 cM from the *Mi* locus in the N × L population map (Lu et al., 1998b). This good correlation increases confidence that they are detecting the same locus and will be generally useful in *Prunus*.

Comparison of LG2 of PMP2 to other *Prunus* maps showed that *Mi* was placed on LG2 of the European *Prunus* map (saturated peach × almond linkage map) (Fig. 4). This was determined using RFLP, isoenzyme, PCR, phenotypic, or microsatellite markers that are highlighted in Fig. 4. Based on the pchgms1 microsatellite and the APMij3f marker, we concluded that *Mi* maps to the same linkage group in PMP2 and in the L×N population (Lu et al., 1998b). Therefore, linkage group 1 from Lu et al. (1998b) probably corresponds to LG2 in maps obtained from PMP1, PMP2, T×E, and others that can be aligned with them using common markers.

Addition of the CAP1 marker allowed *Mi* to be placed in LG2 ("group" command LOD≥ 3, recombination fraction ≤45). At LOD 4, *Mi* was linked only to CAP1. Analysis of the order of loci in LG2 showed that dominant RFLP markers C208HB and C8HB were more closely linked to *Mi*, 4.8 and 6.8 cM, respectively, than CAP1. These two markers were linked in repulsion to the resistant allele of *Mi*, whereas CAP1 was linked in coupling. It would be useful to convert the RFLP markers C208HB or C8HB to more economical PCR-based markers, especially since they are linked to the susceptible allele. A single dominant marker linked closely to the susceptible allele is as efficient as a single codominant RFLP in selection during early generations (Haley et al., 1994; Kelly, 1995). Both C208HB and C8HB were part of a complex, multi-banded profile that could inhibit conversion to PCR-based markers.

UTILITY OF CAP1 IN OTHER POPULATIONS. The CAP1 dominant band from the resistant parent behaved as expected in the PMP2 OP-F_2 population, which is closely related to the mapping population. Therefore, it is useful for identifying and eliminating most homozygous susceptible plants from segregating populations derived from HB × Oki. Effective use of CAP1 in other populations was limited because it was not polymorphic among commonly used peach rootstocks Nemaguard, Nemared, and Lovell. The lack of a strong Uncut Band, as in the resistant parent Oki, suggests the source of resistance to *M. incognita* in Nemaguard and Nemared may be of different origin than Oki, or if of similar origin, a recombination event or sequence divergence has occurred. The L×N CAPs marker differentiated Oki vs. Nemaguard and Nemared, but not Oki and HB (Lu et al., 1999). On the contrary, the CAP1 marker differentiates Oki vs. HB, Nemaguard, Nemared, or Lovell, but not HB vs. Nemaguard, Nemared, or Lovell. Data of Lu et al. (1999) suggest that Nemaguard and Nemared are different from HB and Oki, but it also supports the conclusion that HB is more similar to Nemaguard and Nemared than Oki. The only firm conclusion based on the pchgms1 microsatellite and APMij3f markers, is that *Mi* is in the same linkage group in Oki, HB, Lovell, and Nemared (Fig. 4).

PYRAMIDING RESISTANCE GENES. If different genes and/or alleles for resistance to *M. incognita* exist in peach, it would be desirable to combine them into one genotype to increase the range and the durability of resistance (Lecouls et al., 1997, 1999; Roberts, 1995). The *Mi* locus mapped to LG 2 of the *Prunus* genome in

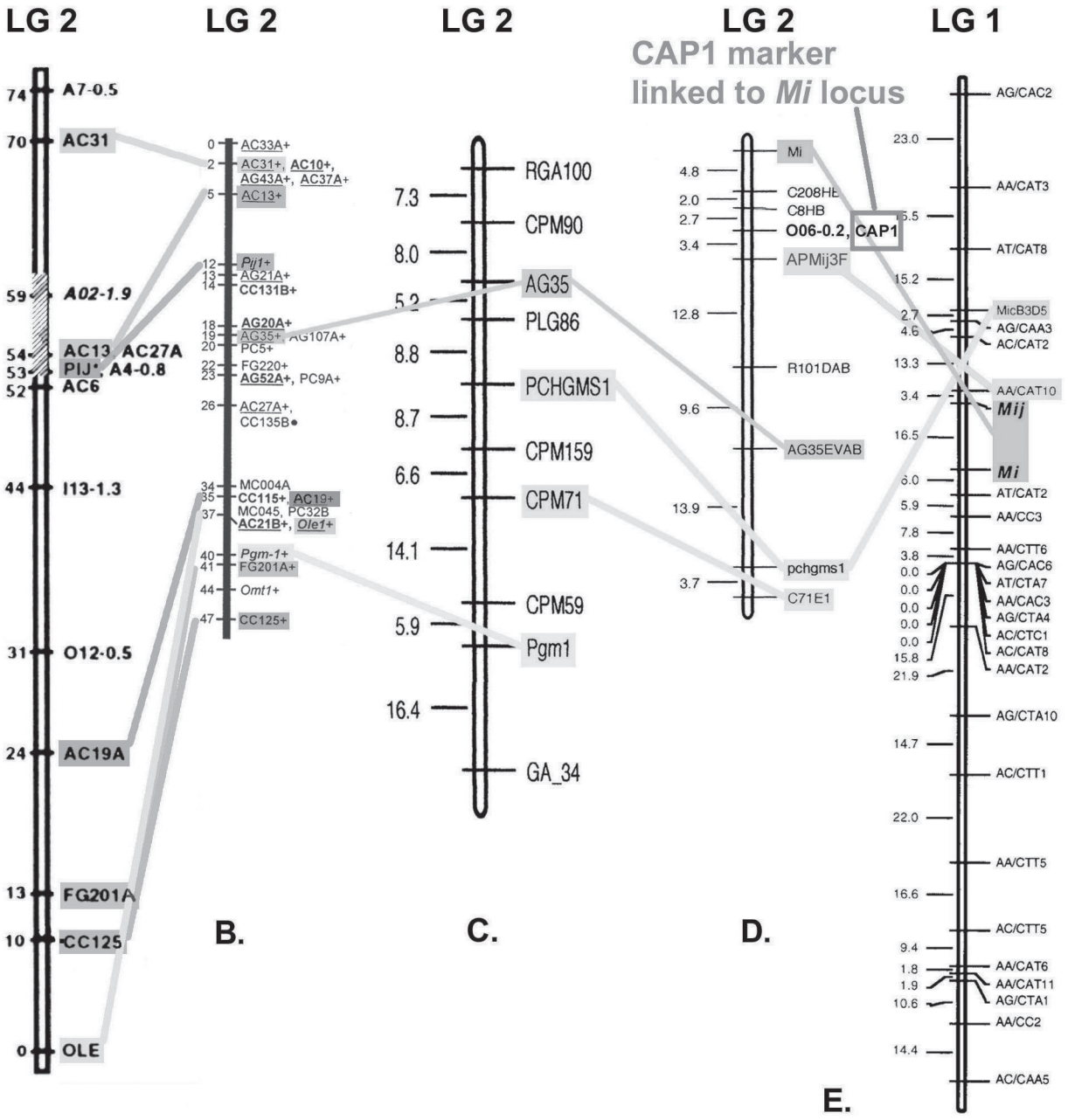

Fig. 4. Relationship of the Harrow Blood × Okinawa linkage group (LG) that contains the *Mi* locus to linkage groups in other *Prunus* genetic maps. Loci detected using the same RFLP probes, PCR primers, isozyme assay, or phenotype are connected. (**A**) *Prunus davidiana* (Viruel et al., 1998); (**B**) almond × peach, linkage group 2 (European *Prunus* map) (Joobeur et al., 1998); (**C**) almond × peach, linkage group 2 (PMP1) (Bliss et al., 2002); (**D**) peach (Harrow Blood) × peach (Okinawa) linkage group 2 (PMP2, this study); (**E**) peach (Lovell) × peach (Nemared) (L×N population) linkage group 1 (Lu et al., 1998b).

PMP2 and the L×N population. Based on F_2 segregation in the L×N population, Lu et al. (1998a) proposed that two dominant genes, *Mi* and *Mij* (a proposed, but not verified, shared gene for resistance to *M. incognita* and *M. javanica)*, control resistance to *M. incognita* in Nemared (*MijMijMiMi*), and that Oki carries the resistance allele at locus *Mi* only (*mijmijMiMi*). Lu et al. (1998a) suggested that *Mi* and *Mij* are either independent or separated by 40 cM (Lu et al., 1998a) but a genetic linkage map based on the same data (segregation of resistance to *M. incognita* and/or *M. javanica* in the L×N population) placed *Mi* 16.5 cM from *Mij* (Lu et al., 1998b). CAP1 and L×N CAPs markers can facilitate selection of hybrids that contain both Oki and Nemared *Mi* resistance gene(s) from crosses among the PMP2 individuals and Nemared.

Colinearity of genes. Usually, within a species there is a high degree of colinearity of genes, while among closely related species it decreases but many chromosomal regions are conserved (Devos and Gale, 2000; Paterson et al., 2000). RFLPs detected by the CPM and RGA probes were expected to have significant regions of synteny between PMP1 and PMP2, but loci located on a particular PMP1 linkage group were frequently found in several PMP2 linkage groups and probes sometimes mapped to different linkage groups among the two populations (Table 3). Probes frequently detected multiple bands, including polymorphic

Table 3. RFLP loci detected by common RFLP probes but mapped to different linkage groups (LG) in the PMP1 (almond × peach F_2 population) (Bliss et al., 2002; Foolad et al., 1995), PMP2 [peach (Harrow Blood) × peach (Okinawa) F_2 population, this study] and a European *Prunus* linkage map (almond × peach F_2 population) (Joobeur et al., 1998). See Table 1 for a description of the PMP2 loci.

RFLP probe	PMP2 LG	PMP1 LG	European *Prunus* map[z]
AG16	4	6	4
AG32	1	1	1, 3
AG35	2, 4	2	2
C148	7	8	
C17	6, 9	6, 7	
C189	10	5	
C3	3B, 5	3	
C34	6	4	
C39	4	6	
C64	1, 3A, 10	7	
C8	2, 9, 10	6	
C9	6, 8	5, 6	
R400	8, 9	1	

[z]Linkage group in the European *Prunus* map (Joobeur et al., 1998) that contained a homologous locus in all three maps.

and monomorphic bands, in PMP2. There are some scenarios where a single copy probe may detect a nonorthologous sequence (Devos and Gale, 2000).

Segmental chromosome duplications, inversions, and reciprocal translocations may cause a lack of colinearity (Devos and Gale, 2000; Paterson et al., 2000). Recently a reciprocal translocation between LG6 and LG8 was found in a 'Garfi' almond and Nemared peach population (Jáuregui et al., 2001). There is evidence for important duplications in the *Prunus* genome, especially between LG2 and LG6 in the European *Prunus* linkage map (Pere Arús, personal communication; Graziano et al., 2001). This agreed with the finding that clone CPM 8 detected RFLPs in LG6 in PMP1 and LG2, LG9, and LG10 in PMP2. Given that there are only 8 chromosomes in *Prunus*, it is possible that either LG9 or LG10 in PMP2 corresponds to LG6 in other *Prunus* maps. However, this is complicated by the mapping of the CAP1 marker to LG3 in PMP1, rather than LG2 or LG6.

Chromosomal duplications may explain why the anchored PCR product APMij3F, which is in LG2 in PMP2, is not located in the corresponding LG2 in PMP1. The anchored PCR product AP2UI7b9 was also expected to map near *Mi* in LG2 in PMP2, yet it mapped to LG8 near a cluster of RFLPs detected by RGA and RRS probes. It is also possible the anchored PCR products are detecting totally different loci, not duplicated regions.

RGA AND QTLs in *Prunus***.** A more complete range of RGA markers would be useful because some RGA that were mapped in PMP1 align with known disease resistance quantitative trait loci (QTL) in the *Prunus* genome. For example, QTLs associated with resistance to powdery mildew and/or peach leaf curl in *P. davidiana* (Viruel et al., 1998) aligned with the locus RGA400d3 at the top of LG1 of PMP1; and with LG2 of PMP1 (RGA100) and PMP2 (R101DAB). However, no RGA were detected in LGs 3, 4, 5 and 6 of PMP1 or PMP2, although QTLs were detected in these regions of *P. davidiana*.

Sequences of R101 (CT100) and R400 (CT400) were included in a phylogenetic analysis of R-gene and R-gene homologs (Meyers et al., 1999) wherein they were found to belong to the TIR-class of R-genes. This was unexpected because they were amplified using degenerate oligonucleotides based on the P-loop and GLPL motifs of the *RPS2* R-gene that belongs to the Non-TIR class. It is possible that another set of degenerate oligonucleotides derived from other R-gene motifs will detect more markers in different regions of the peach genome. This may be useful for developing markers closer to *Mi* because none of the RGA or RRS used in this study detected RFLPs in the vicinity of *Mi* and the only RGA that mapped to LG2 was R101 (loci R101DAB). Meyers et al. (1999) determined that degenerate primers derived from P-loop and GLPL sequences produce both TIR and non-TIR group RGA's. A range of primers derived from different motifs could be analyzed to isolate a more complete set of RGA sequences (Meyers et al., 1999).

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