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Inheritance of citrus nematode resistance and its linkage with molecular markers

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Abstract Eleven RAPD markers linked to a gene region conferring resistance to citrus nematodes in an intergeneric backcross family were identified. Two sequence-characterized amplified region markers linked to a citrus tristeza virus resistance gene and one selected resistance gene candidate marker were evaluated for their association with citrus nematode resistance. A nematode-susceptible citrus hybrid, LB6-2 [Clementine mandarin (*Citrus reticulata*)×Hamlin orange (*C. sinensis*)], was crossed with the citrus nematode-resistant hybrid Swingle citrumelo (*C. paradisi*×*Poncirus trifoliata*) to produce 62 hybrids that were reproduced by rooted cuttings. The plants were grown in a greenhouse and inoculated with nematodes isolated from infected field trees. The hybrids segregated widely for this trait in a continuous distribution, suggesting possible polygenic control of the resistance. Bulked segregant analysis was used to identify markers associated with resistance by bulking DNA samples from individuals at the phenotypic distribution extremes. Linkage relationships were established by the inheritance of the markers in the entire population. A single major gene region that contributes to nematode resistance was identified. The resistance was inherited in this backcross family from the grandparent *Poncirus trifoliata* as a single dominant gene. QTL analysis revealed that 53.6% of the phenotypic variance was explained by this major gene region. The existence of other resistance-associated loci was suggested by the continuous phenotypic distribution and the fact that some moderately susceptible hybrids possessed the resistance-linked markers. The markers may be useful in citrus rootstock breeding programs if it can be demonstrated that they are valid in other genetic backgrounds.

Key words *Tylenchulus semipenetrans* · Bulked segregant analysis · Linkage map · QTL · Molecular markers

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Introduction

Breeding citrus cultivars for disease and pest resistance is one of the major challenges to citrus improvement. Long periods of juvenility, large plant size, high heterozygosity, nucellar embryony, and the quantitative inheritance of most characters have greatly impeded progress toward genetic improvement in citrus by conventional breeding methods (Gmitter et al. 1992). Citrus nematode [CN, *Tylenchulus semipenetrans* (Cobb 1914)] is one of the major parasitic pests of the plant rhizosphere that causes serious damage to citrus roots and long-term production loss throughout the citrus growing areas of the world (Duncan and Cohn 1990). Although an understanding of the mode of inheritance of citrus nematode resistance is important for breeding citrus nematode-resistant rootstock cultivars, this information is not yet available. Most of the previous reports have described only the response of a number of citrus species and varieties to citrus nematode infection (Baines et al. 1968, 1973; Hutchison and O'Bannon 1972; O'Bannon et al. 1977; O'Bannon and Ford 1977; Kaplan 1981). Some fertile intergeneric hybrids have inherited citrus nematode resistance from *Poncirus trifoliata* (L.) Raf., a close relative of *Citrus* (Swingle and Reece 1967). Most clones of *Poncirus* are resistant to citrus nematode (Cameron et al. 1954). Citrus nematodes have been classified into three biotypes (Inserra et al. 1980). The Citrus and Mediterranean biotypes reproduce poorly on *P. trifoliata* but infect all *Citrus* spp. The Poncirus biotype is capable of infecting *Citrus* spp., *P. trifoliata*, and their

hybrids (Inserra et al. 1980). The infection of the Poncirus biotype on *P. trifoliata* and its hybrids with *Citrus* has been reported in Florida, but with lower reproduction levels than on known citrus nematode-susceptible rootstocks (Duncan et al. 1994). The occurrence of the resistance-breaking population of *T. semipenetrans* emphasizes out the importance of understanding fully the genetic control of citrus nematode resistance.

Genetic markers have become very efficient and powerful tools in plant breeding (Lande and Thompson 1990). The use of DNA markers has greatly accelerated the construction of saturated linkage maps in plants (Michelmore et al 1991). A well-defined linkage map can describe the linkage relationships of genetically characterized markers with traits of interest. Once verified, the trait-linked marker genotypes can serve as phenotype predictors, thus providing a timely and less costly way of identifying individuals possessing beneficial alleles without going through field testing for phenotypes. The ability to use marker-assisted selection (MAS) for perennial plant crops is expected to have a profound impact on breeding schemes. Citrus linkage maps have been developed using isozyme and restriction fragment length polymorphism (RFLP) markers (Durham et al. 1992; Jarrell et al. 1992), and some of these maps have been saturated further with random amplified polymorphism DNA (RAPD) marker loci (Cai et al. 1994). Several loci of horticultural significance have been tagged with RAPDs and sequence-characterized amplified regions (SCARs), including genes for resistance to citrus tristeaza virus (*Ctv*) (Gmitter et al. 1996; Deng et al. 1997; Mestre et al. 1997; Fang et al. 1998), cold acclimation (Cai et al. 1994), and rootstock-mediated tree size control (Cheng and Roose 1996). Bulked segregant analysis (BSA) (Michelmore et al. 1991) has been described as an efficient way to locate molecular markers linked to disease resistance genes. Recently, resistance gene candidate-derived markers (RGC) have been used to tag disease resistance genes in plants (Whitham et al. 1994; Mindrinos et al. 1994; Lawrence et al. 1995; Salmeron et al. 1996; Aarts et al. 1998; Parker et al. 1997; Shen et al. 1998; Simons et al. 1998). RGC markers can be used in MAS or to provide a basis for map-based cloning of important genes (Martin et al. 1993). The objective of the study presented here was to determine the mode of inheritance of citrus nematode resistance in an intergeneric backcross family [*Citrus*×(*Citrus*×*Poncirus*)] and to identify associated molecular markers.

Materials and methods

Plant materials and experimental design

Sixty-two intergeneric hybrid plants were obtained by controlled hand pollination of LB6-2 [Clementine mandarin (*C. reticulata*)×Hamlin orange (*C. sinensis*)]×Swingle citrumelo (*C. paradisi*×*P. trifoliata*). LB6-2 produces monoembryonic seeds resulting in zygotic hybrid offspring and is susceptible to citrus nematode. Swingle citrumelo is known to be highly resistant to citrus nematode (Kaplan and O'Bannon 1981). Stems were cut selectively and rooted on a water mist bed filled with Aero-soil horticulture perlite (Chem-Rock Co. Jacksonville, Fla.) in the greenhouse to produce six uniform replications of each hybrid and Swingle citrumelo. Each cutting was treated with HORMODIN 2 (0.3% indole-3-butyric acid, MSD AGVER, Division of Merck & Co, N.J.) to improve stem rooting. Six comparable seedlings from open-pollinated seeds of LB6-2 were selected for testing because rooted cuttings were unavailable. The rooted cuttings were planted 7.5 cm×10 cm in a soil mix consisting of two parts sandy soil (97% sand, 2% silt, 1% clay) and one part of organic supplement (PRO-MIX, BX, Primer Brands, Stanford, Canada) by volume. The mix was fumigated with methyl bromide prior to planting. All plants were grown in a greenhouse, irrigated according to the moisture of the soil mix, and fertilized once weekly with 25% Peters (20-20-20) solution. The experiment was arranged in a randomized complete block design.

Phenotyping for citrus nematode resistance

Live citrus nematodes of the Citrus biotype were collected from heavily infected citrus roots harvested from the field. A modified sugar extraction method (Hussey and Barker 1973) was used in the nematode extraction. The citrus nematode inoculant was sterilized with a $CuSO_4$ solution (1000 ppm) before being used to inoculate plants to prevent *Phytophthora* infection. The number of citrus nematode eggs and juveniles was estimated for each extraction by sample counting. Plants were inoculated five times over 2 months. An equal amount of citrus nematode inoculum (approximately 9060 juveniles and 61280 eggs) was injected into two 5-cm-deep holes in the soil on two sides of each plant using a 25-ml glass syringe. In addition to the hybrids and parental plants, ten sour orange (*Citrus aurantium*) seedlings and six rooted cuttings of LB7-3 (Clementine mandarin×Valencia orange) were inoculated at the same time to monitor the nematode population development. After inoculation, the plants were grown under normal management for two months at temperatures of 26°C–32°C, which are favorable to citrus nematode activity. Infection rates of the sour orange and LB7-3 plants were examined three times after the initial inoculation to monitor nematode population development.

Two months after the initial inoculation, roots of each plant were harvested and weighed. A maximum of 2 g of fibrous roots was collected from each plant for nematode extraction by the bleach extraction method (Baines et al. 1968). Nematode extracts were taken three times from each replicate, and the female citrus nematodes, eggs, and juveniles were counted. The mean number of female nematodes per gram of fresh root (female/g root) was calculated for each hybrid. All the hybrids and parental plants were evaluated for their response to citrus nematode inoculation by counting the female nematodes in each of the root samples. Data were subjected to analysis of variance using the MSTATC computer program. Duncan's multiple range test was used to separate mean values of female/g root. A simple linear correlation analysis was conducted to estimate the degree of association between the number of females/g root of each plant and the extraction root weight of each plant. The hybrids were classified for citrus nematode resistance on the basis of the mean number of females/g root. Numerous citrus rootstock varieties tested for nematode infection under the greenhouse conditions have positively reflected the result of evaluations in the citrus orchard (Duncan et al. 1994).

DNA extraction, marker identification and linkage analysis

Genomic DNA was extracted from healthy, newly expanded tender leaves of the hybrids and parents of the backcross family using the standard procedure of Dellaporta et al. (1983) or by a quick method (modified from Edwards et al. 1991). Fragments associated with citrus nematode resistance were identified using the bulked segregant analysis (BSA) approach (Michelmore et al.

1991). Bulk DNA samples were constructed by pooling the DNA from the 6 individuals comprising each extreme of the phenotype distribution. A second pair of bulks was produced by pooling DNA samples of 15 individuals from the extremes of the phenotype distribution curve.

RAPD fragments were amplified by the polymerase chain reaction (PCR) using single, randomly selected decamer primers from Operon Technologies (Alameda, Calif.) and bulked DNA as the template. A programmable thermal controller, MJ PTC-100, Model 96U (MJ Research, Watertown, Mass.) and the previously described reaction mixture and temperature cycling profiles (Gmitter et al. 1996) were used for all RAPD amplifications. The SCAR fragments were amplified using the procedure of Deng et al. (1997). The amplification products were separated by electrophoresis as pairs of resistant/susceptible bulks in adjacent lanes on 1.8–2.0% agarose gels with 1× TAE buffer and were detected with ultraviolet light after ethidium bromide staining. The bulk screening results were compared with those polymorphisms generated by parental DNA templates to be certain that markers selected for further study were inherited from the resistant parent.

The RAPD markers associated with citrus nematode resistance, as determined by screening bulks of 6 and 15 individuals, respectively, were then used to determine the marker genotypes of all individual hybrids in this backcross family. The presence and absence of these markers in each of the hybrids were recorded for subsequent linkage analysis. The resulting fragment patterns of each individual were compared with those revealed by BSA to confirm the selection of appropriate RAPD markers (those markers capable of repeatedly revealing polymorphism between resistant and susceptible bulks, and individuals). Two *Ctv* associated SCAR markers, $SCA07_{650}$ and $SCAD08_{1100}$, derived from another intergeneric backcross population (Deng et al. 1997) were used to directly screen individuals in this population. The PCR amplifications were carried out according to previously described reaction conditions (Deng et al. 1997). A selected RGC marker, $Pt8a_{440}$ (Deng et al. submitted), was also tested in this population. The RGC marker $Pt8a_{440}$ was derived from PCR amplification of USDA 17–47 (hybrid of *Citrus grandis* and *P. trifoliata*) DNA with degenerate primers designed from the conserved NBS motif of the *RPS2* gene in *Arabidopsis* (Mindrinos et al. 1994). Amplifications of the RGC marker were performed on a MJ PTC-100 thermal cycler (MJ Research) in 25-µml reaction volumes; each reaction contained 50 m*M* TRIS-HCl, pH 8.3, 2 m*M* $MgCl_2$, 800 µm*M* dNTPs, 25 µm*M* forward and reverse degenerate primers, 150 ng genomic DNA, and 1 U *Taq* polymerase. The initial denaturation was at 93°C for 2 min, followed by 42 cycles of 1 min at 92°C, 1 min at 50°C, and 2 min at 72°C. PCR products were separated on agarose or polyacrylamide gels. The segregation ratios for all markers were tested for goodness-of-fit to predicted Mendelian inheritance ratios by the chi-square test. MAPMAKER 3.0 (Lander et al. 1987; Lincoln et al. 1992) was used to conduct linkage analysis with a LOD value threshold of 3.0 and a map distance threshold of 25 cM. MAPMAKER/QTL (version 1.3) was used subsequently to map possible citrus nematode resistance quantitative trait loci (QTLs).

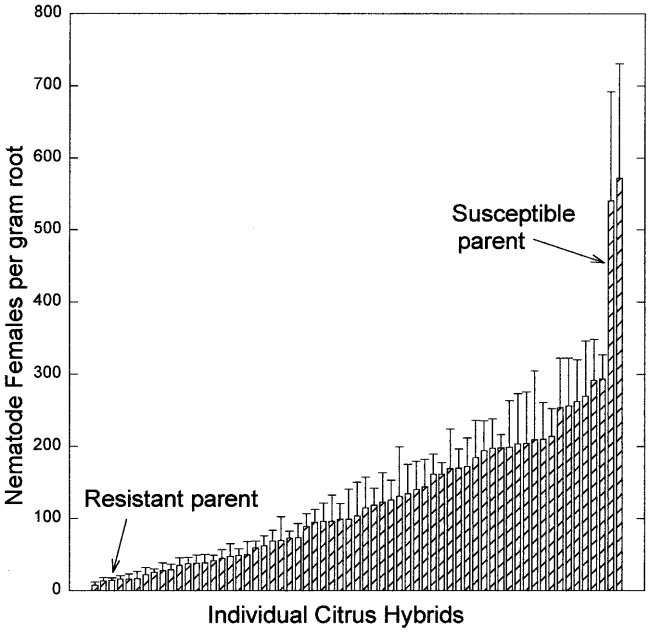

Fig. 1 The phenotypic distribution (mean and standard deviation from 6 replicates) of 62 hybrids in response to citrus nematode inoculation. The infestation levels of the resistant (Swingle citrumelo) and susceptible parent (LB6-2) are indicated

Results

Inheritance of citrus nematode resistance

Analysis of variance indicated that there were significant differences among the hybrids for the numbers of females per gram of root ($P<0.001$). The distribution of the mean values among the hybrid individuals was continuous and wide-ranging. The number of female nematodes/g root varied from as low as 8 (resistant parent Swingle citrumelo=15) to as high as 580 (susceptible parent LB6-2=541)(Fig. 1). The phenotypes of these hybrids were classified as resistant, susceptible, or intermediate. The simple linear correlation coefficient value between the numbers of females/g root and the extraction root weight was r =–0.228, with 376 degrees of freedom, which was significant at the 1% level. Only 5.2% of the variation in females per gram of fresh root was accounted for by extraction root weight.

The most resistant and susceptible individuals were initially assorted into groups by mean number of females/g root. The mean values of the parental plants were used as references. The resistant bulk (n=6) had a mean of 16 females/g root; the susceptible bulk (n=6) had a mean of 335 females/g root; the mean for the total population was 131 females/g root. Bulks of 15 individuals also had mean values that were substantial deviations from the population mean; the resistant bulk had a mean of 27 females/g root, and the susceptible bulk mean was 260 females/g root.

Identification of citrus nematode resistance linked-RAPD fragments by BSA

The screening of 580 arbitrary decamer primers against resistant and susceptible bulks resulted in the identification of 11 RAPD markers putatively linked to the citrus nematode resistance gene. In general, each primer generated 9–12 bands of variable intensity. The 11 amplified polymorphic fragments were named according to Operon kit and primer number and approximate fragment size (bp), and they were designated $OPC17_{750}$, $OPW14_{800}$, $OPO07_{650}$, $OPO04_{950}$,

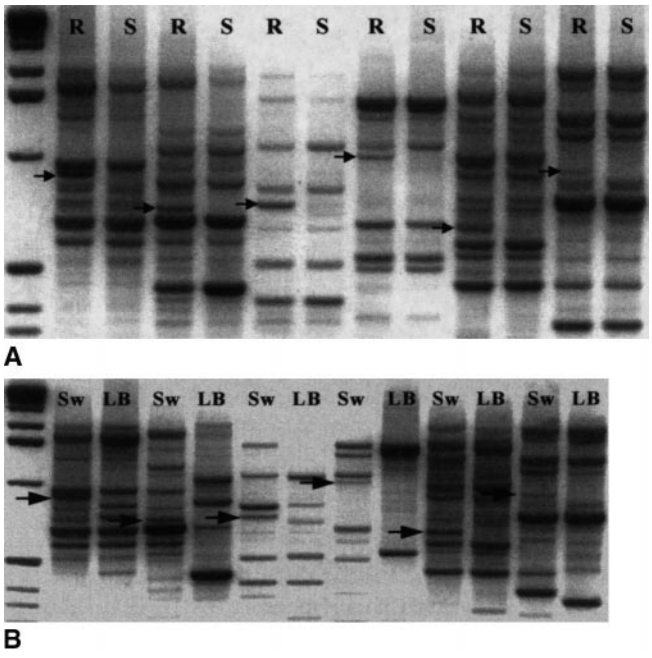

Fig. 2 A RAPD markers detecting polymorphisms between bulks of 15 individuals made with DNA from the most nematode-resistant (*R*) and -susceptible (*S*) individuals in an intergeneric backcross family. Each set of two lanes resulted from PCR amplification with different 10-mer oligonucleotide primers: (from *left* to *right* they were OPW14, OPU06, OPX10, OPC17, OPO07, and OPO04). The polymorphisms distinguishing the bulks are indicated by *arrowheads*. **B** RAPD markers amplified from the resistant parent [Swingle citrumelo (Sw)] and the susceptible parent [LB6-2 (LB)] by 10-mer oligonucleotide primers, as control. The *first lane* is a standard 1 Kb DNA ladder

$OPX10_{1000}$, $OPU06_{700}$, $OPAE02_{1600}$, $OPAG13_{800}$, $OPAJ04_{1400}$, $OPAJ05_{780}$, and $OPAL11_{950}$. Polymorphisms were confirmed by repeated amplifications and comparisons with the two parents (Fig. 2A, B). The same 11 polymorphic RAPD fragments were produced when the larger bulk samples (composed of DNA from 15 individuals each) were tested (data not shown). The individual hybrids were also screened with a newly developed RGC marker, $Pt8a_{440}$, and SCAR markers $SCA07_{650}$ and $SCAD08_{1100}$. All except 2 of the resistant individuals (1G and 1D) within the larger bulk possessed the fragment of the RGC marker $Pt8a_{440}$, but none of larger susceptible bulk had it. $SCAD08_{1100}$ was absent in 2 of the resistant individuals (5F and 1D) in the larger bulk, and it was present in 2 individuals of the larger susceptible bulk, 2H and 1 E.

The results of individual hybrid screening by the above RAPD, SCAR, and RGC markers have closely reflected the phenotype of those resistant and susceptible individual, and of those ranked in the middle of the phenotype curve. Upon screening the individuals in the second susceptible bulk (with 15 individuals), 3 susceptible hybrids (6C, 1E and 6A) were found with resistance-associated markers ($OPW14_{800}$ and $OPC17_{750}$), and another susceptible hybrid (2H) was also found with resistant-associated markers $OPU06_{700}$, $OPX10_{1000}$, and $OPO04_{950}$. Further, some individuals in the resistant bulk were found missing one fragment (5L; $OPC17_{750}$), three fragments (1G; $OPU06_{070}$, $OPX10_{1000}$, and $OPO04_{950}$), and all fragments (1D). The markers $OPU06_{700}$, $OPO04_{950}$, and $OPX10_{1000}$ consistently co-segregated with each other, as did marker $OPAL11_{950}$ and $OPC17_{750}$ in this population. There was 1 hybrid (5L) in the resistant bulk that was recombinant for markers $OPW14_{800}$ and $OPC17_{750}$. $OPW14_{800}$ and $OPC17_{750}$ were defined with tight linkage but not co-segregation in this mapping population. Markers $OPO07_{650}$ and $SCA07_{650}$ were produced by all individuals in the larger resistant bulk except hybrid 1D, which lacked all the markers, and were not produced by any individual in the larger susceptible bulk. Therefore, these 2 markers were predicted to be the most closely linked markers to the resistance region. This prediction was confirmed by the subsequent linkage analysis. There were 2 individuals (5L and 1C) recombinant for markers $Pt8a_{440}$ and $SCA07_{650}$, while 7 individuals (5F, 5C, 5N, 1E, 2H, 1G and 1C) were recombinant for markers $SCAD08_{1100}$, $Pt8a_{440}$, and $SCA07_{650}$. The relative map position was determined in the subsequent linkage analysis.

Linkage analysis and construction of a localized linkage map

The segregation ratios of all 11 RAPD markers, two SCARs and the RGC marker in this intergeneric backcross family fit the 1:1 inheritance mode (data not presented). Linkage analysis results from MAPMAKER revealed that these molecular markers were linked to each other, in the best order defined as: (1) $OPAE02_{1600}$, (2) $OPAJ04_{1400}$, (3) $OPAL11_{950}$, (4) $OPC17_{750}$, (5) $OPW14_{800}$, (6) $OPAG13_{800}$, (7) $SCAD08_{1100}$, (8) $SCA07_{650}$, (9) $OPO07_{650}$, (10) $Pt8a_{440}$ (11) $OPO04_{950}$, (12) $OPU06_{700}$, (13) $OPX10_{1000}$, (14) $OPAJ05_{780}$. The genetic distances and LOD scores were calculated based on the best order marker sequence by multipoint analysis (3-point analysis) command. The map distances were expressed as Kosambi functions.

The results from MAPMAKER/QTL analysis indicated that the gene(s) responsible for nematode resistance are most likely located in the 3.3-cM interval defined by RGC marker $Pt8a_{440}$ and co-segregating markers $OPO07_{650}$ and $SCA07_{650}$. Since a major QTL peak was defined by the confidence interval of $OPO07_{650}$-$SCA07_{650}$, which explained 53.6% of the phenotypic variance, it indicates that this gene region is very close to the loci of $OPO07_{650}$ and $SCA07_{650}$. The maximum likelihood value for the target region interval was 10.3, which was highly significant. This major CN resistance gene has been designated as *Tyr 1*. The genetic distances of the 14 marker loci and the citrus nematode resistance QTL are presented on the localized map (Fig. 3).

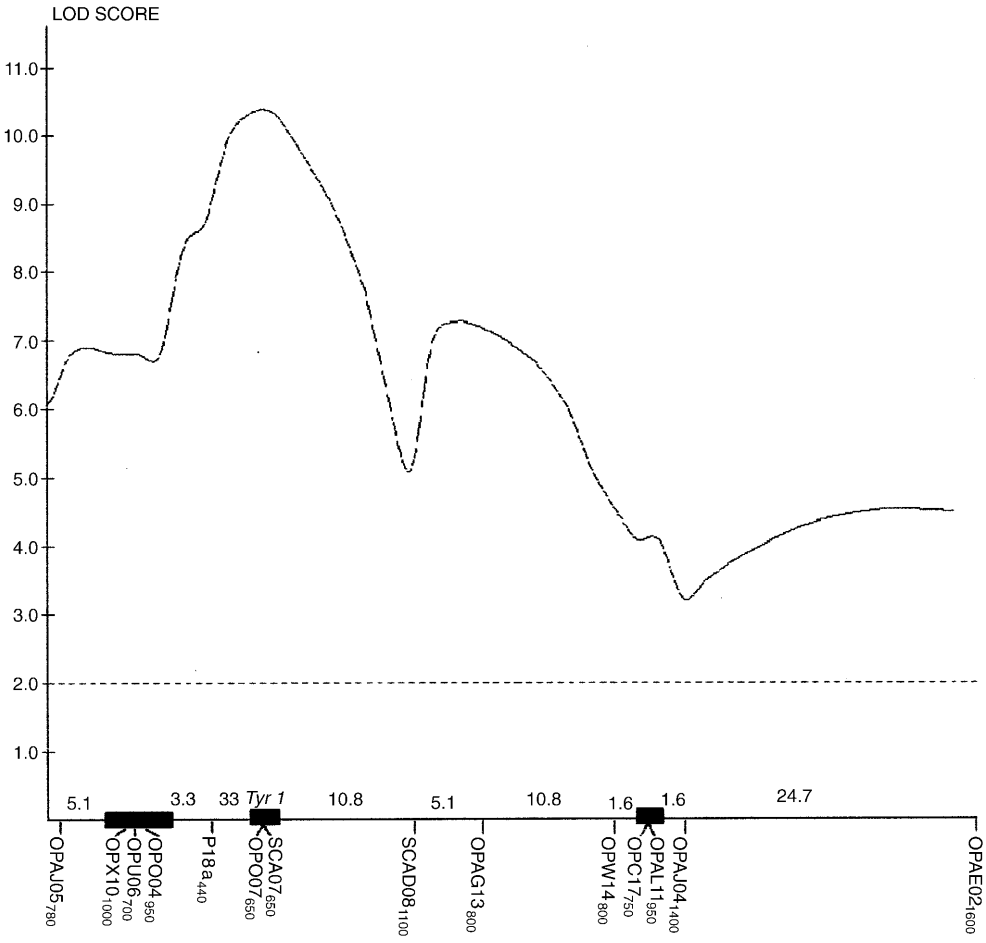

Fig. 3 The localized linkage map of the citrus nematode resistance gene region. The map distances (cM) were calculated using Kosambi function and indicated between markers. The major CN resistance gene (*Tyr 1*) region corresponded with highest QTL peak. LOD score value indicated that the *Tyr 1* region was most closely associated with marker loci $OPO07_{650}$ and $SCA07_{650}$. The co-segregating marker loci were indicated by *shading*

Discussion

An efficient and reliable technique for nematode recovery is essential in a comprehensive screening program (Hutchison and O'Bannon 1972). The extraction method and the inoculation procedures used in this study were well-suited for the genetic study of citrus nematode resistance. Evaluation of resistance was based on the number of female nematodes per gram of roots, which is indicative of the level of nematode infection in the citrus rhizosphere (Duncan and Cohn 1990). Similar conclusions regarding the resistance and susceptibility of individual hybrids were reached when the number of the nematode eggs and juveniles were used to indicate the nematode infection level. Six uniform replicates and their average density were used to determine the phenotype of each individual hybrid, thereby minimizing the influence of the developmental status of plants to the nematode population density on individual hybrids. It is possible that some genetic characters other than the resistance genes might have some influence on phenotype, such as root growth ability or growth habit.

Immune individuals were not observed in this family, and citrus nematodes completed their life cycle on the resistant parent Swingle citrumelo and other resistant individuals. These results suggest the gene(s) associated with resistance may contribute to resistance indirectly rather than directly. However, the population densities of citrus nematodes were low on Swingle citrumelo and some of the hybrid plants. Moreover, the female nematodes observed on Swingle citrumelo and those individuals with low nematode density were mostly immature females; eggs and juveniles were observed in much lower numbers. Those observed on the highly susceptible parent LB6-2 and hybrids with high densities of nematodes were mostly mature females, eggs, and juveniles. These observations support previous findings that the reproduction of *Tylenchulus semipenetrans* on Swingle citrumelo is limited (Kaplan and O'Bannon 1981).

It is clear that those hybrids with lower nematode population densities inherited nematode resistance-associated genetic information from the resistant parent. The continuous variation curve and the mean distribution frequency histogram imply that the nematode resistance trait in citrus is probably conferred by multiple genes in a dominant fashion and/or influenced by some other minor factors.

DNA bulks were constructed from the two extreme phenotypes and used to identify markers for a quantitative trait as reported previously by Mansur et al. (1993). By bulking DNA from those selected individuals (i.e., the extremes of resistance and susceptibility), it was pos-

sible to eliminate the randomized genetic background of unlinked loci (Michelmore et al. 1991); therefore, the particular genomic region(s) associated with nematode resistance could be studied by detecting DNA fragment polymorphisms between these two bulks. Michelmore et al. (1991) pointed out that only a small number of individuals in each bulk can provide great enrichment for the markers linked to the target region. In this study, each bulk with 6 individuals was considered to be sufficient for the first round of RAPD marker screening. Because the actual genotypes of the individuals in the two extreme bulks were unknown, it was considered necessary to increase the selection pressure for markers associated with nematode resistance. Some individuals may have been "over-inoculated", whereas others may have escaped infection. Therefore, the number of individuals in each bulk was increased to 15 individuals to form a second pair of DNA bulks to confirm the validity of markers selected by the first bulk screening of primers. When this was done, primers OPW14 and OPC17 generated much weaker homologous RAPD fragment bands from the susceptible bulk than that produced by the resistant bulk. These weak bands were not observed when screening with the bulks of 6 individuals, and they resulted from including recombinant individuals in the susceptible bulk. These results implied that those markers were farther away from the target region than other markers that were found.

As the individuals toward the middle of the phenotype curve were studied, more hybrids were found that were recombinant between any 2 markers. This result agreed with the expectation that as the phenotypic distinction between the two bulks was diminished, the linkage between the target region and linked RAPD markers would diminish gradually, resulting from more recombination between the resistance region and the markers. In the course of screening bulks, there were several polymorphisms found to be associated with only the susceptible bulk, but further investigation of these fragments has not yet been conducted. Such fragments might be useful for marker-assisted selection in breeding programs if linkage to susceptibility-associated alleles could be demonstrated.

In this study, the proportion of resistance-linked fragments $OPW14_{800}$ and $OPC17_{750}$ in the larger susceptible bulk (n=15) was relatively low (20%), resulting in weak, slightly detectable bands produced from the susceptible bulk template. In the first susceptible bulk (n=6), only 1 recombinant hybrid (6C) was included, so the proportion of resistance-associated fragments was about 16%, which may have been too low to be visualized after amplification. For the same reason, markers $OPU06_{700}$, $OPX10_{1000}$, and $OPO04_{950}$ were not observed in the susceptible bulks. The proportion of resistance-associated fragments was 16% for $OPU06_{700}$, $OPX10_{1000}$, and $OPO04_{950}$ in the small susceptible bulk (n=6), and only 6.7% in the large bulk (n=15).

MAPMAKER confirmed that all 14 markers were linked to each other and that the target resistance region was within the linkage group. Based on the results of this study, one major genomic region responsible for citrus nematode resistance was found in this family, and QTL analysis has strongly supported this conclusion. It is highly significant that 53.6% of the resistance phenotype variance is explained by one single gene region. The QTL peak has shown that this major gene (*Tyr1*) is located within the 3.3-cM interval of co-segregating loci $OPO07_{650}$ and $SCO07_{650}$ and closely linked RGC marker $Pt8a_{440}$. Marker $Pt8a_{440}$ was reported to have a high level of sequence identity with *Arabidopsis* gene *RPS2* (Deng et al. submitted). The SCAR marker $SCAD08_{1100}$, closely linked to *Ctv* in another backcross family (Deng et al. 1997), was mapped 10.8cM away from the *Tyr 1* region in this backcross family. Whether there is 1 major gene, or multiple tandem genes directly or indirectly contributing to citrus nematode resistance at different levels, could not be determined without additional study. Other QTLs may be revealed when larger families and additional markers are studied. Since *C. paradisi*, the other grandparent contributing to the resistant parent in this cross, is known to be highly susceptible to *T. semipenetrans* (Reynolds and O'Bannon 1963), the molecular evidence supports the conclusion that resistance in this backcross family is controlled by and inherited as a single dominant gene from the grandparent *Poncirus trifoliata*.

The reliability of these molecular markers was confirmed on the basis of their inheritance in a single backcross population, which reflected only four genetic backgrounds. Previous experience with markers linked to a citrus tristeza virus resistance gene demonstrated that although many markers revealed by bulk segregant analysis are family-specific, it is possible to identify more universally applicable markers (Gmitter et al. 1996). Whether these markers linked with citrus nematode resistance in this backcross population will have any relevance in other populations remains to be determined. In the future, it may be beneficial to use markers that are derived from known resistance genes and those selected markers that have been tested across different backcross populations for disease resistance screening. Utilization of these markers by citrus breeders for indirectly selecting nematode resistant hybrids from other families should be delayed until the applicability of these markers in other genetic backgrounds can be demonstrated. Furthermore, the allelic conformation of the resistance-linked RAPD markers should be heterozygous for the loci to be used in the progeny screening; therefore, allelic conformation must be ascertained in *P. trifoliata* clones before these markers can be used to screen intergeneric F_1 families.

The breeding and development of new and genetically improved citrus rootstocks is a challenging task because of a multitude of reproductive impediments, mentioned above. Equally challenging, however, is the lack of efficient, cost-effective screening procedures to use in selecting superior individuals that recombine the multiple traits required of improved cultivars. Currently available

methods of screening new citrus rootstock candidates for resistance to multiple and widespread pathogens (such as CTV, *Phytophthora* spp., and citrus nematodes) are effective, for the most part. However, the number of individual hybrids that can be screened to identify those which possess resistance alleles to several targeted pathogens is far fewer than the number that mathematics dictates. *P. trifoliata* has been shown to transmit resistance or tolerance to CTV, *Phytophthora*, and citrus nematode, and therefore it is an important germplasm resource for citrus rootstock breeding. Markers for the virus resistance gene *Ctv* have been developed and are now being utilized by breeders. Work is under way, not only with nematodes but with *Phytophthora* resistance also, to develop of battery of markers for multiple pathogen resistance that can be used to make the development of improved citrus rootstocks a more feasible and achievable goal. Larger families can be developed and screened by breeders to identify many hybrids with multiple disease resistance. These hybrids can then be planted in field trials to evaluate the other critical aspects of the horticultural and economic performance. In this way, greater incremental progress towards citrus rootstock cultivar improvement can be realized.

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