
D. Q. Fang, C. T. Federici and M. L. Roose

Department of Botany and Plant Sciences, University of California, Riverside, California 92521

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ABSTRACT

Resistance to citrus tristeza virus (CTV) was evaluated in 554 progeny of 10 populations derived from Poncirus trifoliata. A dominant gene (Ctv) controlled CTV resistance in P. trifoliata. Twenty-one dominant PCR-based DNA markers were identified as linked to Ctv by bulked segregant analysis. Of the 11 closest markers to Ctv, only 2 segregated in all populations. Ten of these markers were cloned and sequenced, and codominant RFLP markers were developed. Seven RFLP markers were then evaluated in 10 populations. Marker orders were consistent in all linkage maps based on data of single populations or on combined data of populations with similar segregation patterns. In a consensus map, the six closest marker loci spanned 5.3 cM of the Ctv region. Z16 cosegregated with Ctv. C19 and AD08 flanked Ctv at distances of 0.5 and 0.8 cM, respectively. These 3 markers were present as single copies in the Poncirus genome, and could be used directly for bacterial artificial chromosome library screening to initiate a walk toward Ctv. BLAST searches of the GenBank database revealed high sequence similarities between 2 markers and known plant disease resistance genes, indicating that a resistance gene cluster exists in the Ctv region in P. trifoliata.

Citrus tristeza virus (CTV) is one of the most severe diseases of citrus in the world. It causes rapid decline and death of trees grafted on sour orange (Citrus aurantium L.) rootstocks and stem pitting of grapefruit (C. paradisi Macf.) and sweet orange [C. sinensis (L.) Osbeck] trees, regardless of rootstock. These diseases severely jeopardize the citrus industry worldwide (Bar-Joseph et al. 1989). CTV is transmitted by several species of aphids. Of them, Toxoptera citricida Kirk, (the brown citrus aphid) is the most efficient CTV vector (Rocha-Pena et al. 1995). The combined presence of CTV and T. citricida resulted in the loss of an estimated 30 million citrus trees in South American countries in the 1930s and 1940s. The spread of T. citricida to Central American countries caused a CTV outbreak in the 1980s, leaving more than 20 million dead or unproductive trees (Rois- tacher et al. 1991; Yokomi et al. 1994). T. citricida has already reached Florida and threatens California, Texas, and Arizona, the major citrus-growing states in the United States. Because CTV represents a serious problem to the citrus industry in the United States and other countries, development of rootstock and scion cultivars resistant to a broad range of CTV strains is a priority.

Most citrus species are susceptible to CTV, although some pummelo [C. maxima (Burm.) Merrill] accessions were recently found to be resistant to certain CTV strains (Gar nsey et al. 1997). It remains unclear whether pummelo accessions will provide durable resistance to a broad range of virus strains. However, Poncirus trifoliata (L.) Raf. (trifoliate orange), a close relative of citrus, is resistant to all CTV isolates tested (Yoshida 1985; Gar nsey et al. 1987; Gmitter et al. 1996; Mestre et al. 1997a), and this resistance is controlled by a single dominant gene called Ctv (Gmitter et al. 1996).

Development of citrus scion cultivars with Ctv is likely to be extremely difficult using conventional hybridization-selection approaches. Citrus breeders have attempted to develop cold-hardy scion cultivars using P. trifoliata as a parent since 1897 (Hodgson 1967). However, no commercially useful scion cultivar originating from P. trifoliata has been developed because undesirable traits of P. trifoliata remain after several generations of back crossing with citrus. Likewise, it would be impossible or at best require several decades to breed a marketable CTV-resistant scion cultivar by crossing P. trifoliata with a citrus variety using conventional breeding techniques. Incorporation of Ctv from P. trifoliata into a citrus variety by genetic transformation should enable us to develop a CTV-resistant cultivar without altering other desirable traits present in citrus.

Molecular markers provide efficient and powerful tools for constructing genomic maps and tagging genes of interest for map-based cloning or marker-assisted selection. In citrus, several genes controlling important horticultural traits, including CTV resistance (Gmitter et al. 1996; Deng et al. 1997; Mestre et al. 1997a), dwarfing (Cheng and Roose 1995), and fruit acidity (Fang et al. 1997a) have been tagged by molecular mark-
ers using bulked segregant analysis (Michelmore et al. 1991). Of them, CTV resistance genes have attracted the most attention. Gmitter et al. (1996) first identified 8 random amplified polymorphic DNA (RAPD) markers linked to Ctv, and 2 of them cosegregated with this gene in a 65-plant population. However, when these two markers were extended to 5 other populations with 136 progeny in total, 8 and 22 crossovers were observed between Ctv and them. Deng et al. (1997) later incorporated 12 more RAPD markers into this linkage map and found that OPA081100 cosegregated with Ctv in this 65-plant population. Moreover, Deng et al. (1997) converted 3 RAPD markers into codominant sequence characterized amplified region markers. Mestre et al. (1997a) identified 7 RAPD markers linked to Ctv in 2 populations with a total of 90 progeny. However, only 64 progeny were evaluated for CTV resistance, and all of these markers were at least 5 cM away from Ctv. In brief, though many markers have been developed to tag Ctv, few of them are close enough to be useful in map-based cloning. Furthermore, because the population sizes used in these studies were relatively small and most markers were dominant, it was difficult to map them precisely. Correct marker order is crucial for map-based cloning because any errors in placing markers will mislead subsequent chromosome walks. Thus, construction of a high-resolution map around the Ctv region is prerequisite to the isolation of Ctv using a map-based cloning strategy. Toward this goal, we report herein the following: (1) identification of RAPD and inter-simple sequence repeat (ISSR) markers linked to Ctv, (2) conversion of dominant markers to codominant RFLP markers applicable to diverse populations, (3) development of a high-resolution map around the Ctv region in 10 populations with 554 progeny trees combined, and (4) identification of single copy markers that can be used to screen a bacterial artificial chromosome (BAC) library.

**MATERIALS AND METHODS**

**Plant materials:** Ten populations segregating for CTV resistance were used (Table 1). Zygotic seedlings in populations 7-10 were distinguished from apomictic ones by isozyme analysis (Khan and Roose 1988). In the remaining populations, seedlings with trifoliolate or difoliolate leaves were identified as zygotic. Rubidoux and Flying Dragon are small-flowered trifoliate oranges, and they are indistinguishable after scoring >1000 marker bands. Webber Fawcett and Pomeroy are large-flowered trifoliate oranges that have identical molecular marker fingerprints but are distinguishable after scoring small-flowered ones (Fang et al. 1997b). Carrizo and Troyer citranges are hybrids of *C. sinensis* cv. Washington navel × *P. trifoliata*, while C-35 citrange is a hybrid of *C. sinensis* cv. Ruby × *P. trifoliata* cv. Webber Fawcett. Sacaton citrumelo is a hybrid of *C. paradisi* × *P. trifoliata*.

**Determination of CTV resistance:** Two inoculation methods were used in this experiment. The first method was only applied to the 60 Sacaton citrumelo × Troyer citrange progeny trees. Each progeny was vegetatively propagated in triplicate.

<table>
<thead>
<tr>
<th>Population</th>
<th>Parentage</th>
<th>Segregation ratio</th>
<th>Expected ratio</th>
<th>No. observed</th>
<th>R</th>
<th>S</th>
<th>U</th>
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<tbody>
<tr>
<td>1</td>
<td>Citrus taiwanica × Poncirus trifoliata cv. Webber Fawcett</td>
<td>Rr R2r2</td>
<td>3:1</td>
<td>17 12 1:1</td>
<td>0.862 NS</td>
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<td>2</td>
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<td>Rr R2r2</td>
<td>3:1</td>
<td>15 20 1:1</td>
<td>0.600 NS</td>
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<tr>
<td>3</td>
<td>C. maxima cv. Tahitian × P. trifoliata cv. Flying Dragon</td>
<td>Rr R2r2</td>
<td>3:1</td>
<td>41 13 1:1</td>
<td>2.351 NS</td>
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<tr>
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<td>C. sinensis cv. Ruby × P. trifoliata cv. Flying Dragon</td>
<td>Rr R2r2</td>
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<td>Sacaton citrumelo × Troyer citrange</td>
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<td>3:1</td>
<td>62 17 12</td>
<td>0.082 NS</td>
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<td>Rr R2r2</td>
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<td>Rr R2r2</td>
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<td>Rr R2r2</td>
<td>3:1</td>
<td>17</td>
<td>0.620 NS</td>
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and kept in a temperature-controlled greenhouse (16-28°C). Day maxima were higher in the summer and sometimes exceeded 35°C for a couple of hours at midday. These progeny trees were inoculated in June 1990 by grafting either two buds or two pieces of bark from a Ruby blood sweet orange tree infected by CTV strain T-514 (a moderate strain in California). One year later, leaves or bark from above the inoculation points were sampled for enzyme-linked immunosorbent assay (ELISA) to determine their CTV resistance according to the method of Mathews et al. (1997). In a few cases, three samples of the same progeny gave inconsistent results. Therefore, these grafted trees were retested for CTV resistance, and consistent results among the three samples were obtained after prolonged CTV challenge.

For the remaining 494 progeny trees and their parents, the following inoculation method was used. Seedlings of Pineapple sweet orange were grown in pots in a temperature-controlled greenhouse as described above. When ~8 mo old, these seedlings were inoculated with CTV strain T-514 by grafting into the trunk a piece of bark tissue or a bud from CTV-infected Ruby blood sweet orange trees. Four weeks later, four buds from each progeny tree were grafted onto the CTV-infected Pineapple sweet orange rootstocks. Buds were forced to grow by pruning and treatment with 1.8% 6-benzylaminopurine dissolved in dimethyl sulfoxide. Three months after the buds grew, leaves from both progeny shoots and rootstocks were harvested for ELISA evaluation as described above. All sweet orange rootstocks gave high ELISA titers, indicating that they were successfully infected by CTV. In most cases, all four samples of the same progeny tree gave the same ELISA results. However, leaves from the ELISA-negative progeny and those that gave ambiguous ELISA results were harvested again after 6, 12, and 15 mo for ELISA evaluation. This prolonged CTV challenge was essential for accurate evaluation of those progeny that showed weak positive in ELISA test. The CTV resistance of the 30 C. tawii a n i c a Tan. & Shim. × P. trifoliata progeny that were used in bulked segregant analysis was also evaluated by reverse transcriptase PCR according to the method of Mathews et al. (1997).

**DNA extraction and bulked segregant analysis:** Total DNA was extracted from leaves using 1.5% hexadecyltrimethyl amonium bromide according to the method of Fang et al. (1997b). To prepare bulks for bulked segregant analysis, only DNA samples of progeny from population 1 were used. For the CTV-resistant bulk, DNA samples of 10 progeny that were CTV resistant by both ELISA and reverse transcriptase PCR were pooled at equal ratios and diluted to 10 ng μl. A similar bulk of DNA samples from 10 CTV-susceptible progeny was made. Primers that generated polymorphic patterns between bulks and for which the band of interest derived from P. trifoliata were retested using another two bulks, each composed of five progeny individuals not included in the first two bulks. Those primers that still gave polymorphisms were further tested using the 30 individual DNA samples that were included in the bulks. The markers linked to Ctv in both coupling and repulsion phases were analyzed in all 116 progeny of population 1. Finally, the markers within 2.0 cM of Ctv were tested in the other nine populations.

**PCR amplification and electrophoresis:** RAPD-PCR amplification was carried out using the reaction mixtures and temperature profiles described by Cheng and Roose (1995). A total of 620 decamer primers (Kits A to Z, AA, AB, AC, AD, and AF) were purchased from Operon Technologies (Alameda, CA). Amplification products were resolved by electrophoresis through a 1.8% agarose gel in 1× TBE buffer (89 mm Tris-borate plus 2 mm EDTA, pH 8.2). A marker was named after the primer used and the estimated product length. For example, OpC1990 was a 960-bp product generated by Operon primer OpC19. ISSR-PCR amplification, polyacrylamide gel electrophoresis, and silver staining were conducted as described in Fang et al. (1997b). The primers HVH (TG) T (H = A + C + T; V = A + G + C) was synthesized by Cruechon et al. (Dulles, VA).

**Cloning and sequencing RAPD and ISSR products:** The following 11 marker bands that were closely linked to Ctv were cloned: OpC1990, OpE1690, OpE2090, OpE20920, OpE70370, OpE0490, OpP01990, OpP1690, OpP301100, and OpP301100. The probes were the cloned RAPD or ISSR products from 34 individuals of population 1, 2, or 7 plus their parents. Southern transfer, probe labeling, hybridization, and washing were performed as described in Tang et al. (1997). The probes bound only to individuals that had the marker bands. The cloned marker bands were sequenced using a Li-Cor (Lincoln, NE) automatic sequencer.

**Development of RFLP markers:** The probes were the cloned marker bands described above. To identify informative restriction endonucleases, genomic DNA samples from seven parental cultivars, one CTV-resistant progeny, and one susceptible tree of population 1, were digested with 16 restriction endonucleases according to the manufacturers’ recommendations with the addition of 3 mm spermidine. Each reaction had 4 μg of DNA and 30 units of restriction endonuclease. Digestion was conducted at 37°C for 14 hr. DNA electrophoresis, Southern transfer, probe labeling, hybridization, and washing were described above. Membranes were exposed to Phosphorimage screens for 12-18 hr. Autoradiographic images were obtained by scanning the screens with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Dral-digested blots were used to develop RFLP markers for OpE2090, OpE20920, OpP1990, OpP1690, and HVH 690. and Hspl-digested blots were used only for OpC1990. EcoRI was used to digest genomic DNA samples from populations derived from small-flowered trifoliate oranges to reveal RFLPs for OpE21690. HindIII and HaeIII were used to digest DNA to develop an RFLP marker for OpAD0890. The RFLP markers obtained were named Rf primer name, except RF2090 and RE2090. In which the fragment sizes were also included for identification. For example, the RFLP marker derived from OpC1990 was called RFC19. RFLP marker genotypes were always consistent with those of their progenitor RAPD or ISSR marker. Therefore, we used a single name to represent a RAPD or ISSR marker and the RFLP marker derived from it in constructing maps. For example, C19 stood for both RFC19 and OpC1990. E20 included OpE2090, OpE20920, RE2090, and RF2090.

**Linkage analysis:** Segregation data for CTV resistance, RAPD, ISSR, and RFLP markers of each population were analyzed using MAPMAKER 3.0 (Lander et al. 1987) to view the consistency of marker orders across populations. Maps using the combined data from populations showing similar segregation patterns were also constructed by MAPMAKER 3.0. A consensus map of Ctv and the six closest marker loci was constructed using JoinMap 2.0 (Stam and van Ooijen 1995) based on the data of all 554 progeny individuals. The Kosambi mapping function (Kosambi 1944) was used to convert recom
bination frequency into genetic map distance. Linkage was considered significant if the LOD score was ≥3.0. In most cases, the LOD scores were >100.0.

RESULTS

Inheritance of CTV resistance in P. trifoliata: The inheritance of CTV resistance in P. trifoliata was studied in 554 progeny individuals belonging to 10 populations. Segregation in 7 populations was consistent with 1:1 or 3:1 ratios (resistant:susceptible), as expected if the CTV resistance in P. trifoliata was controlled by a single dominant gene (Table 1). The segregation for CTV resistance in population 8 deviated significantly from a 3:1 ratio. Populations 2 and 3, derived from C. maxima Chandler × P. trifoliata, segregated in 3:1 ratios. However, analysis of marker genotypes showed that the resistance gene in C. maxima Chandler was inherited independently from Ctv (D. Q. Fang and M. L. Roose, unpublished results).

Identification of RAPD and ISSR markers linked to Ctv: A total of 620 random decamer primers were screened against the resistant and susceptible bulks. Most primers generated 1–13 scorable bands, but 17 primers produced no products. Within the first two bulks, 146 primers generated polymorphisms for a band derived from P. trifoliata. When these primers were tested with the second two bulks, only 29 primers generated polymorphic patterns. These 29 primers were then analyzed in the 30 progeny included in the bulks. Sixteen primers amplified 17 markers linked to Ctv: OpC19960, OpJ07570, Op20150, Op12430, OpO05400, Op012100, OpW1850, OpD02300, OpAF05100, and OpAF07200 were linked to Ctv in the coupling phase, while OpC17760, OpE1650, OpF19960, OpG06900, OpO04700, OpT09390, and OpZ16650 were in the repulsion phase with Ctv.

We also tested the markers identified by Gmitter et al. (1996) and OpAD081100, discovered by Deng et al. (1997) in these 30 progeny. OpAD081100 cosegregated with Ctv, but all others either did not segregate in this population, had more than five recombinants with Ctv, or were difficult to score. Because OpB11100 was identified as closely linked to Ctv by Gmitter et al. (1996), we further tested it in 60 Sacaton citrumelo × Troyer citrange progeny. We observed 10 recombinants between OpB11100 and Ctv, indicating that this marker was of little value in map-based cloning.

We analyzed our 17 markers and OpAD081100 in all 116 progeny of population 1. These 18 RAPD markers covered 41.3 cM (map not shown). OpC17760, OpE1650, OpJ07570, OpO04700, and Op0121000 were located at one side of Ctv, OpC19960, OpZ16650, and OpAD081100 at the same location as Ctv, and the remaining markers were mapped on the other side of Ctv. Only 8 markers, OpC19960, OpE1650, OpJ07570, OpO04700, Op0121000, OpT09390, OpZ16650, and OpAD081100, which were within 2 cM of Ctv in population 1, were studied in the other populations.

In a separate experiment, we generated >400 molecular markers to construct a linkage map in a population of 60 Sacaton citrumelo × Troyer citrange progeny. After a preliminary linkage map was constructed, 2 additional markers, i.e., RAPD marker OpE20920 and ISSR marker HVH600, were found to be closely linked to Ctv in the coupling phase.

We analyzed all 10 markers that were closely linked to Ctv in 10 populations. Only OpJ07570 and OpAD081100 segregated in all populations. Markers OpC19960 and OpZ16650 segregated in populations derived from large-flowered trifoliate oranges, but not in those derived from small-flowered trifoliate oranges. ISSR marker HVH600 only segregated in populations 5 and 7, indicating that all trifoliate oranges tested were homozygous for this marker.

OpE20920 did not segregate in populations 1, 3, and 8, suggesting that large-flowered trifoliate oranges were homozygous for this marker. In addition to OpE20920, a new marker, OpE20920, was identified as tightly linked to Ctv in the repulsion phase in populations 2, 4, 6, 9, and 10. After studying these two markers in 217 progeny of the above five populations, it was concluded that these two markers were the products of two alleles at the same locus in small-flowered trifoliate oranges, and they were inherited codominantly. This was the sole codominant RAPD marker we identified in our experiment.

In brief, although 11 RAPD and ISSR markers were identified as closely linked to Ctv, most were population specific. Moreover, most of them were dominant markers that provided limited information in certain crosses, and, consequently, it was difficult to map them precisely when many populations with different mating schemes were used. To overcome the drawbacks of these markers and to facilitate their uses in all populations, their conversion to codominant RFLP markers was necessary.

Development of RFLP markers and identification of single copy markers for BAC library screening: The bands for cloning were 11 RAPD and ISSR markers that were closely linked to Ctv described above. With the exception of OpJ07570 and Op0121000, all were cloned without difficulty. OpJ07570 was cloned after five attempts, but a clone of Op0121000 was not obtained after three attempts.

To further elucidate whether the markers OpE20920 and OpE20920 were codominant alleles, we hybridized each separately to the same Southern blot of RAPD products generated by primer OpE20. Both probes hybridized to the same bands, implying that they had high sequence homology as expected for codominant alleles.

Identical patterns were obtained when cloned OpE20920 and OpE20920 bands were hybridized separately to the Southern blots of genomic DNA digested by Dral, further indicating that they were allelic. Four to eight frag-
oranges, but only one was revealed in large-flowered Markers C19, Z16, and AD08 were present as single

Figure 1.—RFLP patterns. (A) RFC19 pattern of 15 progeny plants from C. sinensis cv. Ruby × P. trifoliata cv. Flying Dragon and their parents. DNA was digested with restriction endonuclease MspI and hybridized with the cloned OpC19_960 band. Lanes: 1, Flying Dragon trifoliate orange; 2, Ruby blood sweet orange (note that it is heterozygous for a null allele); 3–17, progeny plants. (B) RFZ16 pattern of parent cultivars and 17 progeny plants from C. taiwanica × P. trifoliata cv. Webber Fawcett. DNA was digested with restriction endonuclease DraI and hybridized with the cloned OpZ16_650 band. Lanes: 1, Webber Fawcett trifoliate orange; 2, Rubidoux trifoliate orange; 3, C. taiwanica; 4–20, progeny plants. Note that no fragment was observed in C. taiwanica.

ments were observed in each progeny, suggesting that several copies of these sequences existed in the Citrus or Poncirus genomes. Differences between two groups of trifoliate oranges were observed at this marker locus.

Each progeny had one or two fragments for RFC19 (Figure 1A) and RF T09, indicating that single copies of these markers were present in the Citrus or Poncirus genomes. Though small-flowered trifoliate orange cultivars were homozygous for OpC19_960, they were heterozygous for RFC19. A total of eight alleles at the RFC19 locus and seven at the RFT09 were observed in the populations studied. Sweet orange, grapefruit, and Chandler pummelo were heterozygous for a null allele at the RFC19 locus.

Development of RFZ16 was more complicated than developing the RFLP markers mentioned above. Although eight restriction endonucleases revealed polymorphisms between P. trifoliata and Citrus, revealing heterozygosity at this locus required using different restriction endonucleases for different trifoliate orange cultivars. No sequence homology for this marker was detected in Citrus. Fragments were only observed in trifoliate oranges and their hybrids. Two fragments were observed in large-flowered trifoliate oranges, but only one was observed in small-flowered ones when DNA samples were digested using seven restriction endonucleases. When DNA samples were digested using EcoRI, two fragments were observed in small-flowered trifoliate oranges, but only one was revealed in large-flowered ones. Thus, to reveal RFLPs for the cloned OpZ16_650 band, DNA samples of populations 1, 3, and 8 were digested with Dral, while EcoRI was used to digest the DNA samples from the remaining seven populations. Moreover, Dral was also used to digest DNA samples of population 10 because both groups of trifoliate oranges were involved in this cross. Because there is no sequence homology in Citrus, only one fragment was observed in each progeny of populations 1–7 (Figure 1B). In populations 8–10, each progeny had one or two fragments.

Different restriction endonucleases were also required to reveal two alleles at the locus RFAD08 for two trifoliate orange cultivar groups. HaeIII digestion revealed two fragments in large-flowered trifoliate oranges, while HindIII digestion was required to view two alleles in small-flowered ones. RFAD08 was present as a single copy in the Citrus or Poncirus genomes.

By combining data of RFLP and RAPD or ISSR markers, we were able to evaluate markers OpJ07_570, C19, E20, T09, Z16, and AD08 in all 554 progeny, OpE16_570 in 407 progeny, OpO04_700 in 284 progeny, and HVH in 151 progeny.

Linkage maps of the Ctv region: A linkage map for each population was constructed using MAPMAKER 3.0. Because they segregated for two resistance genes, the Ctv locus was not mapped in populations 2 and 3. The marker orders were consistent in all maps. A map constructed with combined data of the F1 or backcross-type populations included eight marker loci that spanned 7.0 cM of the Ctv region (Figure 2A). The second map (Figure 2B) was constructed using the combined data of populations 7–9. These three populations represented F1 or trifoliate orange selfed-type crosses. The marker order in this map was the same as that in the first map; however, more recombinants between marker loci and Ctv were observed. The third map (Figure 2C) was the consensus map constructed by JoinMap 2.0 using data of all 554 progeny. The six closest marker loci spanned a 5.3-cM region flanking Ctv. No crossovers were observed between Z16 and Ctv. C19 and AD08 flanked Ctv at distances of 0.5 and 0.8 cM, respectively. Markers C19, Z16, and AD08 were present as single
In the present experiment, we identified eight dominant markers linked to Ctv in the repulsion phase. These markers were as informative as those linked in the coupling phase in F1 populations, but had limited or no value in F2 type or certain backcrosses. However, these markers allowed us to develop more informative codominant markers that segregated in many populations. Our closest marker, RfZ16, was developed from OpZ16, which was linked to Ctv in the repulsion phase. Identification of markers closely linked to a target gene is more efficient if conducted in a population in which both coupling and repulsion phase markers can be scored.

If a high-resolution map cannot be developed in a single large population (as may often be the case in tree crops), then it is important to convert dominant markers to codominant ones to improve the markers' applicability across populations. For instance, RAPD markers OpC19960 and OpZ16 were dominant and only segregated in populations involving large-flowered trifoliate oranges. However, RfC19 and RfZ16 segregated in all populations used.

The order of the nine closest marker loci was essentially the same in all populations. We also constructed maps using combined data from some or all populations, and the marker order remained unchanged in all maps generated by either JoinMap 2.0 or MAPMAKER 3.0. This indicated that our marker order in the Ctv region was very reliable. However, distances between markers varied among populations. Previous studies in tomato (Paterson et al. 1990) and maize (Williams et al. 1995) suggested that recombination was sometimes suppressed in wide hybrids. Therefore, we expected that map distances in the Ctv region might be smaller in population 7 (a cross of two intergeneric hybrids) than in those populations in which recombination between two P. trifoliata chromosomes was estimated. However, two-point map distances in population 7 were greater than those in the other populations for 18 of 21 marker pairs and were significantly (P < 0.05) greater for 5 pairs. The map of the wide cross-population was three times as long (Figure 3) as that of the same loci in the other populations. These results clearly showed that recombination in the Ctv region was enhanced in a hybrid of Citrus × P. trifoliata. The cause of this remains unknown to us. Mestre et al. (1997a) reported higher recombination when a trifoliate orange was used as female parent. However, we did not find this direction in the present experiment.

A resistance gene cluster has been found in many plants (Hammond-Kosack and Jones 1997). In citrus, Deng et al. (1997) speculated that Ctv might be linked to the major quantitative trait locus for citrus nematode (Tylenchulus semipenetrans Cobb) resistance. Our computer database searches revealed that 2 of 10 markers had high sequence similarities to many known disease resistance genes in plants. This indicates that a resistance gene cluster (or R gene-rich region) is present in the Ctv region in P. trifoliata. The functions of the other
resistance genes are unknown. Future work will evaluate the functions of the chromosome segments containing OpJ07\textsubscript{570} and OpC19\textsubscript{960}. An R gene cluster has been postulated to have arisen from recombination/duplication and rearrangement events to produce novel specificities (Pryor 1987; Hammond-Kosack and Jones 1997). This might be the case for the origin of Cf genes in tomato (Hammond-Kosack and Jones 1997). The citrus R gene cluster apparently includes more divergent genes. The cloned OpJ07\textsubscript{570} band had high sequence similarities to RPS2, RPM1, and many other resistance genes (or putative resistance genes) that had nucleotide-binding sites and leucine-rich repeat domains. In contrast, the sequence of the cloned OpC19\textsubscript{960} band was highly similar to Cf2, Cf4, and Cf9, which lack a nucleotide-binding site, and also to genes encoding receptor kinase-like proteins. No single sequence in GenBank database was similar to both OpJ07\textsubscript{570} and OpC19\textsubscript{960}. Hammond-Kosack and Jones (1997) classified all R genes into five classes in which members of the same class had similar features. They placed RPS2, RPM1 in class 2, while the Cf genes were placed in class 4. In consideration of the sequence divergence, it is unlikely that the closely linked chromosome segments containing OpJ07\textsubscript{570} and OpC19\textsubscript{960} have evolved from the same ancestral genomic segment by recombination/duplication or rearrangement events. Clustering of two or more classes of R genes in a region is particularly provocative and may provide insight to a novel model of R gene evolution.

Four trifoliate orange cultivars used in this experiment were classified into two groups: large-flowered (Webber Fawcett and Pomery) and small-flowered (Rubidoux and Flying Dragon; Fang et al. 1997b). It was possible to distinguish between groups but not within a group using RFLP or ISSR markers. In this experiment, we found that the four closest marker loci (Z16, C19, AD08, and E20) to Ctv differed between groups, indicating that a mutation or recombination event occurred in the Ctv region in at least one group. Consequently, a plant homozygous for a dominant marker close to Ctv will not necessarily be homozygous for Ctv itself. Thus, it is essential to test heterozygous codominant markers flanking Ctv to identify a Ctv-homozygous plant. We identified a Ctv-homozygous trifoliate orange progeny for BAC library construction using this strategy.

Our final objective of this project is to clone Ctv using a map-based cloning strategy, and to transform it into susceptible citrus cultivars to breed CTV-resistant ones. Moreover, our markers can also be used for selection of CTV-resistant hybrids at the seedling stage in a breeding program.

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