Theor Appl Genet (1996) 92:688–695

F. G. Gmitter Jr · S. Y. Xiao · S. Huang · X. L. Hu
S. M. Garnsey · Z. Deng

A localized linkage map of the citrus tristeza virus resistance gene region

Received: 27 April 1995 / Accepted: 6 October 1995

Abstract A localized genetic linkage map was developed of the region surrounding the citrus tristeza virus (CTV) resistance gene (designated *Ctv*) from *Poncirus trifoliata* L., a sexually compatible *Citrus* relative. Bulked segregant analysis (BSA) was used to identify potential resistance-associated RAPD fragment markers in four intergeneric backcross families that were segregating for CTV resistance. Eight RAPD fragments were found that were consistently linked to *Ctv* in the four families. Map distances and locus order were determined with MAPMAKER 3.0, using the results obtained from 59 individuals in the largest family. Also, a consensus map was constructed with JOINMAP 1.3, using pooled results from the four backcross families. Marker orders were identical, except for 1 marker, on these independently developed maps. Family-specific resistance-associated markers were also identified, as were numerous susceptibility-associated markers. The identification of markers tightly linked to *Ctv* will enable citrus breeders to identify plants likely to be CTV-resistant by indirect, marker-assisted selection, rather than by labor-intensive direct challenge with the pathogen. These markers also provide a basis for future efforts to isolate *Ctv* for subsequent genetic manipulation.

Florida Agricultural Experimental Station Journal Series No. R-04491

Communicated by F. Salamini

F. G. Gmitter Jr (✉) · S. Huang
Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

S. Y. Xiao · Z. Deng
Huazhong Agricultural University, Citrus Research Institute, Wuhan, Hubei 430070, PRC

X. L. Hu
Yunnan Agricultural Extension Center, Kunming, Yunnan, PRC

S. M. Garnsey
USDA/ARS, Horticultural Research Laboratory, 2120 Camden Road, Orlando, FL 32803, USA

Key words *Citrus* L. · *Poncirus trifoliata* L. · Random amplified polymorphic DNA · Bulked segregant analysis · Genome mapping

Introduction

Citrus tristeza virus (CTV), the most significant and cosmopolitan viral pathogen of citrus, can induce several disease syndromes. The most debilitating are "quick decline" [death resulting from phloem necrosis below the graft union of infected scions on sour orange (*C. aurantium* L.) rootstocks] and "stem-pitting" of scions (which decreases tree vigor, fruit size and quality, and productivity) (Bar-Joseph et al. 1989). CTV is vectored by several species of aphid, including *Aphis gossypii* and *Toxoptera citricida* (Garnsey and Lee 1988). *T. citricida* (the brown citrus aphid or BCA), the most efficient known CTV vector (Yokomi 1993), is endemic to many of the world's citrus growing regions (excluding the USA); it has caused widespread destruction when quick decline or severe stem-pitting strains of CTV have been present (Lee et al. 1992). While CTV-induced quick decline can be controlled simply by grafting scions on resistant or tolerant rootstocks rather than on sour orange, control of stem-pitting poses additional problems. There are no known genetic sources of resistance to CTV among *Citrus* species, although generally mandarins are considered to be highly tolerant. *Poncirus trifoliata* (the trifoliate orange), a sexually compatible genus related to *Citrus*, is the only known source of resistance, which appears to be controlled by a single, dominant gene (Yoshida 1985, 1993). This gene prevents viral proliferation *in planta* by an undetermined mechanism, essentially resulting in immunity. CTV-resistant citrus rootstocks have been developed from *Citrus* × *Poncirus* hybrids, and resistance can be sexually transmitted into new rootstock hybrids; however, evaluation and selection for resistance remain tedious, time-consuming, and costly. While resistance to CTV can be determined in inoculated seedling populations by

ELISA (Garnsey et al. 1981), the tolerance of the grafted combinations should be determined and the growth of inoculated and virus-free trees on candidate rootstocks should be compared. The number of hybrid plants that can be assayed by this method restricts citrus breeding efforts because of time, space, and labor requirements.

The development of CTV-resistant scion cultivars presents an even greater challenge. Fruit of CTV-resistant *Citrus* × *Poncirus* hybrids are unpalatable, although an intergeneric backcross hybrid scion breeding parent (US 119) with CTV resistance and edible fruit (of unmarketable quality) has been released (Barrett 1990). Several subsequent backcross generations may be required to recover hybrids that produce fruit with a fully acceptable quality while still retaining CTV resistance. Furthermore, the two most important cultivar groups grown in Florida [sweet orange (*C. sinensis* (L.) Osb.) and grapefruit (*C.* x*paradisi* Macf.)] are not amenable to cultivar improvement by sexual hybridization (Barrett and Rhodes 1976; Gmitter et al. 1992), and introgression of CTV resistance into sweet orange and grapefruit by breeding seems practically impossible. Genetic transformation offers another approach for the development of CTV-resistant orange and grapefruit cultivars.

Plant genome maps may include important linkage relationships among molecular markers and genes that breeders wish to manipulate for cultivar improvement. Michelmore et al. (1991) were the first to describe bulked segregant analysis (BSA), which is a versatile and useful approach to detect molecular markers possibly linked with plant genes. Various kinds of molecular markers have been mapped along with qualitative or quantitative genetic traits for many plants (Tanksley et al. 1982; Edwards et al. 1992; Haley et al. 1993; Mansur et al. 1993; Miklas et al. 1993; Paran and Michelmore 1993; Penner et al. 1993; Weisemann et al. 1992). The applicability of molecular markers to genome mapping and characterization of *Citrus* and *Poncirus* has been demonstrated by Cai et al. (1994), Durham et al. (1992), and Jarrell et al. (1992). We report here the development of a genetic linkage map of the CTV-resistance gene (designated *Ctv*) region that is defined by random amplified polymorphic DNA (RAPD) fragment markers. Our objectives were to identify markers for use in a marker-assisted selection (MAS) protocol, to select hybrid seedlings with a high probability of possessing *Ctv*, and to lay the groundwork for future efforts at isolating *Ctv* by map-based cloning strategies.

Materials and methods

Plant materials

Six families were used for screening RAPD fragment markers in order to identify those linked to CTV resistance. These included four intergeneric backcrosses and two intergeneric F_1 families, as follows:

1) 65 BC_1 hybrids of 'Thong Dee' pummelo (*C. grandis* L.) × USDA 17–40 ['Thong Dee' × Pomeroy trifoliate orange (*P. trifoliata*)], designated the R family;
2) 47 hybrids of 'Ambersweet' (a complex interspecific hybrid of *C. reticulata*, *C.* x*paradisi*, and *C. sinensis* × US119 [(*C.* x*paradisi* × *P. trifoliata*) × *C. sinensis*], designated the U family;
3) 20 hybrids of LB7–5 (*C. reticulata* × *C. sinensis*) × USDA 17–47 ('Thong Dee' × Pomeroy), designated the LB1 family;
4) 22 hybrids of LB1–21 (*C. reticulata* × *C.* x*paradisi*) × Carrizo citrange (*C. sinensis* × *P. trifoliata*) designated the LB2 family;
5) 29 F_1 hybrids of pummelo × English large trifoliate orange, designated the M1 family; and
6) 40 F_1 hybrids of 'Foster' grapefruit (*C.* x*paradisi*) × Kryder trifoliate orange, designated the M2 family.

All of the seed parents used were susceptible to CTV, and all pollen parents were known to be CTV-resistant.

The distribution of some *Ctv*-associated markers was determined among the *Citrus* seed parents and different *P. trifoliata* clones and hybrids in addition to the segregating families. Fourteen typical *P. trifoliata* clones (isozymically identical) were studied, as well as Flying Dragon trifoliate orange (morphologically distinct, but isozymically indistinguishable from other *P. trifoliata*) and an introduced seedling selection designated DPI 9-6 (morphologically indistinguishable, but isozymically distinct from other *P. trifoliata*). Five seedlings from open-pollinated *P. polyandra* S.Q. Ding et al., a purported new species of *Poncirus* (Ding et al. 1984), were also screened for *Ctv*-linked markers. Finally, two F_1 *Citrus* × *Poncirus* hybrids were tested, CTV-resistant citrumelo 80–9 (*C.* x*paradisi* × *P. trifoliata*) (seed parent of US 119) and CTV-resistant Carrizo citrange.

CTV resistance evaluation

Seedlings of rough lemon were grown in a temperature-controlled greenhouse (between 15°–28 °C) and inoculated with Florida CTV strains (either T-66 or T-4) by grafting leaf pieces from infected source trees into the trunk. Buds of the individual hybrids or selections to be tested were grafted to the CTV-infected rough lemon rootstocks. Rootstocks and grafted hybrid scions were allowed to produce one or two growth flushes (Fig. 1) before the foliage was harvested for evaluation by ELISA (Garnsey et al. 1981, 1987). Either a monoclonal antibody (MCA-13 for T-66) or polyclonal antibodies (1052 and 1053 for T-4) were used to detect the virus in rootstocks and in the grafted hybrids under test. ELISA tests of the R family were run independently at the University of Florida (UF) and USDA laboratories to ensure identical results for individuals in this family. Families LB1 and LB2 were tested only at UF, and M1, M2, and U were tested only at USDA. Hybrids designated ELISA-positive (i.e., susceptible) tested strongly positive at least twice, and hybrids designated ELISA-negative (i.e., resistant) tested negatively a minimum of four times (up to ten times for some plants) after confirming each time that the rootstocks were infected.

DNA extraction, amplification, and electrophoresis

DNA extraction and purification was performed as described by Durham et al. (1992). DNA was amplified by polymerase chain reaction (PCR) in 96-well microtiter plates in a programmable thermal controller (PTC-100, Model 96U, MJ Research). All of the decamer primers in Operon kits A-H and O-X were used for screening (360 primers in total). Each 25 µl of reaction mixture contained 50 m*M* Tris-HCl pH 8.3, 250 µg/ml bovine serum albumin, 2% Ficoll, 1 m*M* Tartrazine, 2 m*M* $MgCl_2$ 800 µ*M* dNTPs, 0.8 µ*M* primer, 1 unit *Taq* polymerase (Promega), and 25–50 ng of genomic DNA. Reactions were run at 93 °C for 2 min (initial denaturation); then 1 min at 92 °C, 1 min at 35 °C, and 2 min at 72 °C, for 42 cycles; followed by 10 min at 72 °C. Amplification products were separated in 1.8–2.0% agarose gels with 1 × TAE buffer and were detected with UV light after ethidium bromide staining.

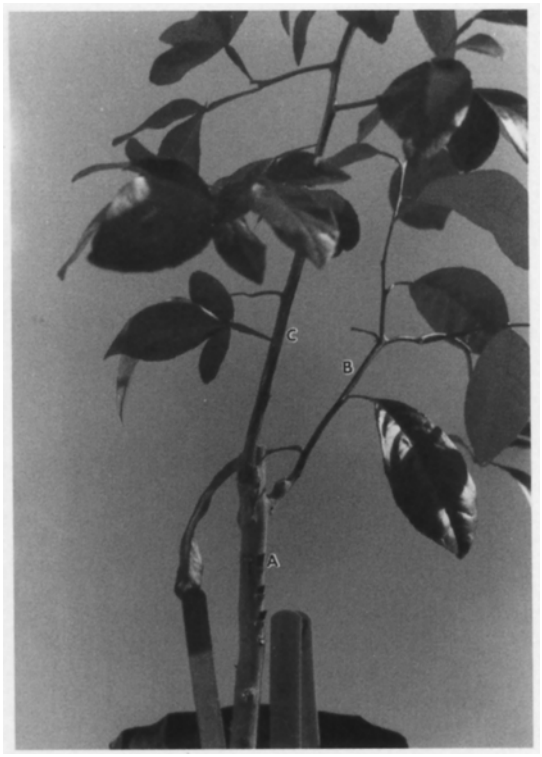

Fig. 1 Inoculation of rough lemon rootstock plant with CTV by grafted leaf pieces and subsequent CTV challenge of individual hybrids by bud grafting. Selection of grafted combinations with high virus titer in the rootstock ensures that the hybrids being tested are challenged by the pathogen. **A** Grafted leaf pieces, **B** rough lemon shoot, **C** hybrid shoot

Bulked segregant analysis (BSA), linkage analysis, and map construction

DNA samples from 8–12 resistant or susceptible individuals from the R and U families were pooled for BSA in order to identify RAPD fragment markers associated with CTV resistance. These bulked samples were utilized for the initial screening of the 320 decamer primers. Then, contrasting bulks of 4–6 individuals from the LB1 and LB2 families were used to test the previously identified promising primers. Finally, all individuals within all six families were examined using the primers considered to be most promising from BSA with the R, U, LB1, and LB2 families to determine the applicability of the CTV resistance-associated RAPD markers in different genetic backgrounds. Results from the largest family (R) were used to determine linkage relationships and to estimate map distances among the linked markers and *Ctv*, with MAPMAKER 3.0b (Lander et al. 1987; Lincoln et al. 1992) and an LOD value threshold of 3.0. Map distances in centiMorgans (cM) were calculated using the Kosambi function and "Error detection on". Consensus maps were developed using JOINMAP 1.3 (Stam 1993), also with an LOD value of 3.0.

Results

Evaluation of CTV resistance

All of the hybrid plants in the six families were evaluated for their reactions to CTV inoculation by ELISA, and the results are summarized in Table 1. Only individual hybrids with consistent ELISA results were classified as resistant or susceptible. Hybrid plants for which ambiguous results were obtained, or those that were not thoroughly tested (in M1 and M2 families), were categorized as uncertain. The results obtained from the U, LB1, and LB2 families fit a 1:1 segregation ratio (data not shown), as expected for a single dominant gene in backcross configuration, although these families were small. However, segregation in the R family was skewed toward susceptibility. The other two families, M1 and M2, were not random because most of the susceptible individuals had been discarded previous to this study. The plants in these families were used only to examine the range of applicability of the selected markers.

Table 1 Segregation of CTV resistance among individuals of six families of intergeneric hybrids produced using a CTV-resistant pollen parent

Family	CTV phenotype[a]		
	Resistant	Susceptible	Uncertain
R[b]	19	40	6
U[b]	17	24	6
LB1[b]	11	6	3
LB2[b]	9	12	1
M1[c]	16	6	7
M2[c]	24	9	7

[a] Determined by repeated ELISA assays of graft-inoculated plants. Resistant hybrids failed to show positive reactions in repeated tests. Susceptible hybrids consistently showed positive reactions similar to those of the susceptible parents. Uncertain plants are those that usually yielded negative reactions but occasionally showed weak or inconsistent positive reactions

[b] Intergeneric backcross

[c] Intergeneric F_1 cross; these families were not random because most of the susceptible individuals had been discarded prior to this study

Screening primers by BSA for RAPD fragment markers linked to *Ctv*

Forty-seven RAPD fragment markers amplified by 39 of 320 primers (excluding kits C and H) appeared to be polymorphic between the contrasting bulked DNA samples in at least one of the four families screened by BSA (i.e., R, U, LB1, LB2) (Table 2). Among these markers, 4 were present in the resistant bulks and absent from the susceptible bulks of all four families, implying that they were possible universal markers linked to *Ctv*. These markers have been named according to Operon kit and primer number and approximate fragment size (kb), and they were designated $B11_{0.70}$, $F06_{0.75}$, $O16_{0.65}$, and $W18_{0.45}$. A fifth marker, $X18_{0.45}$, was found to be unique to the resistant bulks of only three of the four families; because of the small number of individuals (7) in the LB1 susceptible bulk, this fragment was amplified from the one recombinant included in the bulk and thus appeared to be monomorphic by BSA. Marker $O07_{0.65}$ was not detected by preliminary BSA using small bulks from the R and U families. However, as the ELISA evaluations with these families progressed, the number

Table 2 Classification of RAPD fragments that were polymorphic between immune and susceptible bulks of the families used for bulked segregant analysis. The polymorphisms were produced by 39 of 320 decamer primers and excluded kits C and H

Number of families with markers present in:		Number of fragments
Immune bulk	Susceptible bulk	
4	0	4
3	0	1
2	0	5
1	0	13
1	1	5
0	1	19

of individuals in the bulked samples was increased up to 12 individuals, and the polymorphism for $O07_{0.65}$ among the R, U, LB1, and LB2 families became apparent. Kits C and H were screened with the larger bulks of the four families solely to identify fragments that were polymorphic among all families; 1 such marker, $H01_{1.20}$, was found. Marker $T08_{0.59}$ was missed by BSA, perhaps because it is a weak band. After the first 7 markers were integrated into a general map of the R family (see Linkage analysis and mapping *Ctv* with RAPD markers, below), and the close linkage of $T08_{0.59}$ to these markers was indicated, the individuals of all six families were tested for this marker (Table 3).

Five markers were present in the immune bulks of two families, and 13 were immune bulk-specific in only one family. RAPD patterns of a universal marker ($B11_{0.70}$) and a single family-specific marker ($V13_{0.75}$) are shown in Fig. 2. Nineteen other markers were found to be present only in the susceptible bulk of a specific family. Five markers were identified that were present in the resistant bulk of one family and in the susceptible bulk of another family; it was determined that these 5 were all from the *Citrus* parents (data not presented), and though linkage analysis with CTV resistance was deemed unwarranted, they could be useful for other genetic or taxonomic studies. The universal markers detected in the immune bulks of the four families were studied further, as described below.

Linkage analysis and mapping *Ctv* with RAPD markers

Among the six families studied, R and U were the most suitable for linkage analysis (with 65 and 47 individual hybrids, respectively) (Tables 1 and 3). Marker $B11_{0.70}$ appeared to be tightly linked to *Ctv* because no recombinants were detected among the 59 hybrids of the R family, and only 1 among the 41 hybrids of the U family, that were classified unambiguously for resistance or susceptibility. The segregation for this marker among some of the backcross hybrids can be seen in Fig. 3. A second marker, $T08_{0.59}$, also cosegregated with *Ctv* among the hybrids of the R family unambiguously categorized for resistance or susceptibility. More recombinants were found among *Ctv* and the other markers in the R and U families (Table 3), indicating looser linkage relationships. The LB1 and LB2 families were too small for linkage analysis, but it appeared that recombination frequencies in these families were greater than in R and U, except for $F06_{0.75}$ and $O16_{0.65}$ in LB2. Because of the

Table 3 Segregation of eight universal RAPD fragment markers and CTV resistance in six diverse intergeneric families. The M1 and M2 families were non-random because most of the susceptible individual were discarded prior to this study

RAPD Marker	Family	CTV and RAPD phenotype[a]			
		R/+	S/+	R/−	S/−
$B11_{0.70}$	R	19	0	0	40
	U	16	0	1	24
	LB1	11	0	0	6
	LB2	7	2	2	10
	M1	16	6	0	0
	M2	24	3	0	6
$W18_{0.45}$	R	17	0	2	40
	U	16	0	1	24
	LB1	11	1	0	5
	LB2	8	1	1	11
	M1	15	0	1	6
	M2	20	1	4	8
$F06_{0.75}$	R	15	3	4	37
	U	15	2	2	22
	LB1	11	1	0	5
	LB2	8	0	1	12
	M1	14	0	2	6
	M2	20	1	4	8
$O16_{0.65}$	R	16	1	3	39
	U	15	1	2	23
	LB1	11	1	0	5
	LB2	8	0	1	12
	M1	16	6	0	0
	M2	23	3	1	6
$O07_{0.65}$	R	19	1	0	39
	U	17	1	0	23
	LB1	11	1	0	5
	LB2	8	0	1	12
	M1	14	0	2	6
	M2	23	4	1	5
$T08_{0.59}$	R	19	0	0	40
	U	16	2	1	22
	LB1	11	4	0	2
	LB2	7	4	2	8
	M1	16	0	0	6
	M2	17	2	7	7
$X18_{0.45}$	R	17	0	2	40
	U	17	0	0	24
	LB1	11	0	0	6
	LB2	8	1	1	11
	M1	16	6	0	0
	M2	24	3	0	6
$H01_{1.20}$	R	16	1	3	39
	U	17	0	0	24
	LB1	11	0	0	6
	LB2	7	2	2	10
	M1	14	0	2	6
	M2	19	2	5	7

[a] R, resistant based on ELISA assay; S, susceptible based on ELISA assay; +, fragment present; −, fragment absent

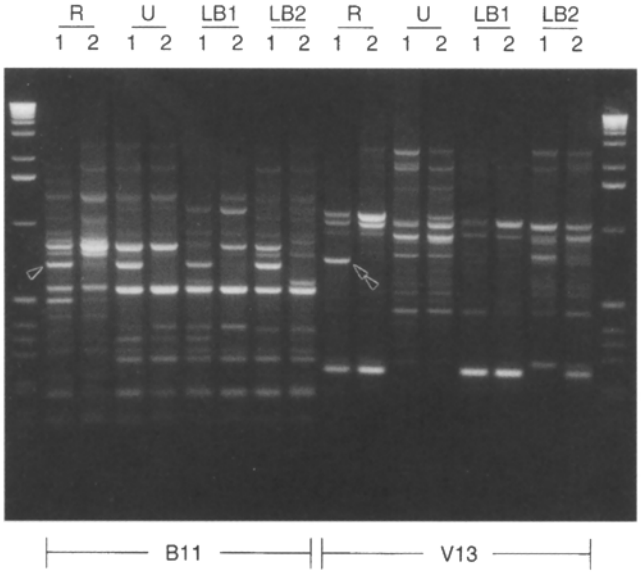

Fig. 2 RAPD fragment patterns amplified from resistant and susceptible bulks of four families segregating for CTV resistance. Fragment $B11_{0.70}$ (*single arrow*) is a universal marker for CTV resistance, and $V13_{0.75}$ (*double arrow*) is a family-specific marker. *R*, *U*, *LB1*, and *LB2* are families. *Lanes 1* are resistant bulks, and *lanes 2* are susceptible bulks. Extreme *left* and *right lanes* are a 1-kb molecular weight marker

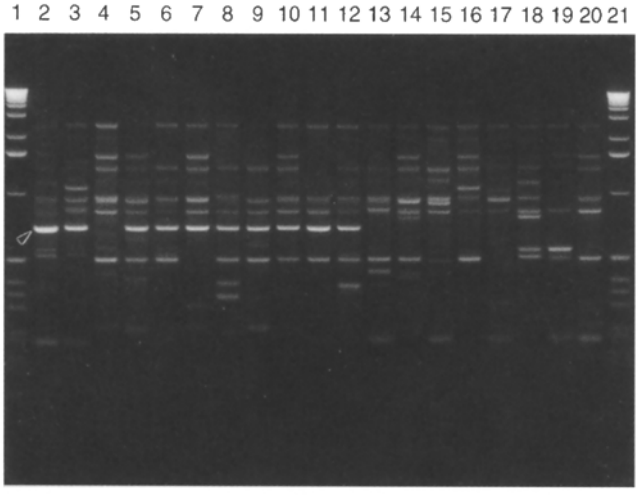

Fig. 3 Segregation of the marker $B11_{0.70}$ among some of the backcross progeny of the R family. *Lanes 1, 21* 1-kb molecular weight marker, *2* Pomeroy trifoliate orange, *3* USDA 17–40, the F_1 intergeneric hybrid used as pollen parent, *4* 'Thong Dee' pummelo, the recurrent *Citrus* seed parent, *5–12* resistant hybrids, *13–20* susceptible hybrids

non-random nature of the M1 and M2 families, they were used only to check the applicability of the markers in other genetic backgrounds. Recombination frequencies in these two families were surprisingly high, except for $T08_{0.59}$ in the M2 family. It is noteworthy that $B11_{0.70}$ $O16_{0.65}$, and $X18_{0.45}$ did not segregate in the M1 family, implying that the *Poncirus* parent, English large trifoliate orange, was homozygous for these markers. In contrast, the *Poncirus* parent of M2, Kryder trifoliate orange, appeared to be heterozygous for these loci.

A localized linkage map was constructed, by means of MAPMAKER 3.0 with the Kosambi function, based on the segregation of *Ctv* and the 8 RAPD fragment markers in the R family (Fig. 4a). The most likely map order spans a genetic distance of 14.1 cM. Seven of the markers were mapped to one side of *Ctv*, but curiously only 1 marker, $O07_{0.65}$, was located on the opposite side. Two consensus maps were constructed by JOINMAP using cumulative data from the R, U, LB1, and LB2 families (Fig. 4b) or R, LB1, and LB2 (excluding U) families. Although map distances varied, the relative order of the markers and *Ctv* were virtually identical on the three maps, except that $T08_{0.59}$ and $B11_{0.70}$ no longer cosegregated with *Ctv*. The most likely consensus marker orders from JOINMAP located $T08_{0.59}$ on the same side of *Ctv* as $O07_{0.65}$. The *Ctv* gene region has also been integrated into a base genomic map constructed with the R family (Cai et al. 1994) and was found to be located on linkage group I of that map.

Distribution of *Ctv*-linked markers in *P. trifoliata* clones and hybrids

All available *P. trifoliata* clones and the intergeneric F_1 hybrids used in hybridization were found to have the 8 markers linked to *Ctv*, with the exception of the Gainesville trifoliate orange, which lacked 4 of the markers (Table 4). The distinctive Flying Dragon and DPI 9–6 trifoliate orange clones possessed all of the linked markers as well, in accordance with their demonstrated resistance to CTV (data not given). Five seedlings of *P. polyandra* unanimously lacked the 8 markers, in accordance with their virus susceptibility (authors' unpublished data). It was not possible to determine whether the *P. trifoliata* clones were homo- or heterozygous for the marker fragments with the available plant material (with exceptions noted above).

Fig. 4a A localized genetic linkage map of the *Ctv* region, based on the R family, containing eight linked RAPD fragment markers. MAPMAKER 3.0 was used, with the Kosambi function and an LOD threshold value of 3.0, to estimate map distances and to determine loci order. Numerals *below* the line represent distances in centiMorgans. **b** A consensus map of the *Ctv* region based on the R, U, LB1, and LB2 families. JOINMAP 1.3 was used, as MAPMAKER in Fig. 4a

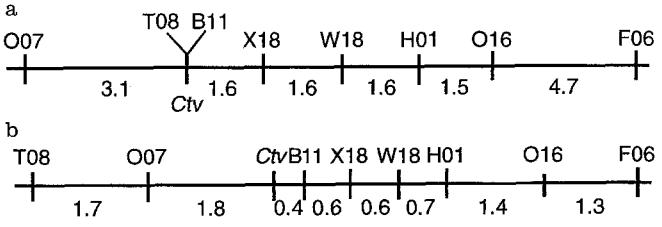

Table 4 Distribution of *Ctv*-linked markers among different *P. trifoliata* clones and hybrids

Plants tested	Markers							
	$B11_{0.70}$	$F06_{0.75}$	$H01_{1.20}$	$O07_{0.65}$	$O16_{0.65}$	$T08_{0.59}$	$W18_{0.45}$	$X18_{0.85}$
P. trifoliata[a]	+	+	+	+	+	+	+	+
Flying Dragon[b]	+	+	+	+	+	+	+	+
DPI 9–6[c]	+	+	+	+	+	+	+	+
Gainesville TO	+	0	0	+	+	0	0	+
P. polyandra[d]	0	0	0	0	0	0	0	0
80–9 citrumelo[e]	+	+	+	+	+	+	+	+
Carrizo citrange	+	+	+	+	+	+	+	+
USDA 17–40	+	+	+	+	+	+	+	+
USDA 17–47	+	+	+	+	+	+	+	+
US 119	+	+	+	+	+	+	+	+

[a] Summary results from 13 typical *P. trifoliata* clones
[b] Morphologically distinct but isozymically indistinguishable from other *P. trifoliata*
[c] Isozymically distinct but morphologically indistinguishable from other *P. trifoliata*
[d] Five seedlings of *P. polyandra* were tested with identical results
[e] Seed parent of US 119, the pollen parent of the U family

Discussion

It was possible to classify most plants as resistant or susceptible to CTV by graft inoculation followed by ELISA, but certain plants yielded ambiguous results with repeated ELISA; these most frequently were manifested as occasional weak-positive ELISA readings interspersed with negative results. It is possible that the occasional weak positives may have been a result of the inoculation method. Given the high virus titer measured in the infected rootstock, some virus may have moved across the graft union and thus have been detected by the ELISA procedures used to test the scion hybrid. The low readings in these cases may indicate the presence, but not necessarily the replication, of CTV in these hybrids. It is also possible that under the severe test methods used, periodic but unsustained replication may have occurred. Plants yielding ambiguous results were not included in the bulked DNA samples, but they were screened for linked markers when all of the individuals within families were studied. In some instances, up to ten repeated ELISA tests were made with a given hybrid in order to determine unequivocally its reaction to CTV inoculation. These experiences underlined the difficulty encountered with standard methods of CTV-resistance phenotyping and the relative ease with which presumed CTV phenotypes can be predicted by *Ctv*-linked RAPD markers.

Yoshida (1985, 1993) reported that CTV resistance inherited from *Poncirus* was controlled by a single dominant allele. This hypothesis was supported by the normal segregation ratios observed in the U, LB1, and LB2 families, although segregation was skewed toward susceptibility in the R family. This result was not surprising, though, because isozyme loci in intergeneric *Citrus* families (Torres et al. 1985) and restriction fragment length polymorphism (RFLP) markers in the R family (Durham et al. 1992) also exhibited distorted segregation. Further, segregation ratios were skewed among nearly 38% of neutral RAPD markers mapped in the R family, with a large block of loci skewed toward *Citrus* (Cai et al. 1994). Skewed segregation of molecular markers is a characteristic associated with wide crosses and is believed to result from abnormal segregation rather than pleiotropy or epistasis (Weeden et al. 1992). In spite of the poor fit to expected 1:1 ratios, all markers associated with CTV resistance mapped to only one genetic region, thus supporting the idea of monogenic control.

It was important to identify *Ctv*-linked markers that were applicable across a range of genetic backgrounds rather than family-specific markers, in order to utilize markers for indirect selection of resistant hybrid offspring. Bulks with few individuals were used to identify markers in some families, but the linkage of these to *Ctv* was loose and such markers should be of limited utility. The universal markers identified were usually detected first when bulks of 12 individuals were used. Several markers were associated with susceptible bulk samples, and others were found in the immune bulk of one family and the susceptible bulk of another. Some of these markers were found in the F_1 backcross parent of the R family (USDA 17–40), but most seemed to come from the recurrent parent, 'Thong Dee' pummelo. Although we did not follow segregation of these markers in the hybrid offspring, the results suggest that the susceptibility-associated markers were of *Citrus* origin and may represent a genomic region homologous to *Ctv* in *Poncirus*.

Recombination frequencies of individual markers varied among the different families. For example, recombinants for $H01_{1.20}$, $W18_{0.45}$, and $X18_{0.45}$ were found in the R family, yet these same markers were observed to cosegregate in the U family. Variable recombination frequencies in different genetic backgrounds have been reported even among individuals within the same cross family (Fatmi et al. 1993). Variable recombination frequencies (and skewed segregation ratios) highlight the importance of using several diverse families to identify markers linked to a specific trait or

gene by BSA, especially if it is expected that linked markers will be used in breeding programs for marker-assisted, indirect selection in different genetic backgrounds.

It was important to identify several linked markers for indirect selection of CTV-resistant offspring. For example, $B11_{0.70}$ was tightly linked to *Ctv* in both R and U families, but more recombinants were observed for this marker in the other families. Selection for resistance based solely on this single marker would have resulted in possible error rates of 10% or more. To improve the precision of selection, several of the most closely linked markers should be used; utilization of markers flanking *Ctv* would be most desirable. For greater confidence, small subsamples from large hybrid families produced by breeding programs should be inoculated and tested by ELISA to confirm marker-predicted resistant phenotypes.

All of the *P. trifoliata* clones tested thus far, including those used by Yoshida (1985, 1993), have been heterozygous for resistance, but it is possible that clones homozygous for resistance may exist. Each fragment linked to *Ctv* was also amplified from the *P. trifoliata* clones screened, except for the Gainesville trifoliate orange, which lacked 4 markers; it is noteworthy that the missing markers were those most distal to *Ctv* according to the map orders determined. Although marker phenotypes were determined, the zygosity of each clone remained unknown, with the exception of Kryder and English large trifoliate oranges. New F_1 families have been produced using 11 different trifoliate orange pollen parents in order to determine the zygosity of linked markers. This step is necessary to identify appropriate markers for selection from F_1 intergeneric families. Hybrids produced from trifoliate parents found to be homozygous for markers may then be inoculated with CTV to identify *P. trifoliata* clones that may be homozygous sources of resistance.

It must be pointed out that the most likely map orders calculated with either MAPMAKER or JOINMAP are tentative, and map distances are biased estimates at this point. The R family-derived map was based on 59 meioses only, but the most likely four-family map (based on 138 total meioses among four intergeneric hybrids) showed the same marker order (excluding $T08_{0.59}$) as the R family map. Nonetheless, the greater map resolution required for the positional cloning (Grill and Somerville 1991; Martin et al. 1992) of *Ctv*-containing fragments should be forthcoming from mapping exercises currently underway with a substantially larger, segregating backcross family. Modifications of primer sequences and amplification conditions are being tested to develop locus- and allele-specific markers, not only to improve hybrid selection efficiency, but to screen genomic libraries for *Ctv*-linked sequences. Intensive efforts are being made to transform citrus cultivars with CTV coat protein (and other) genes (Gutierrez-E et al. 1992; Moore et al. 1993; Niblett et al. 1993), despite the fact that this approach to virus resistance remains unproven in perennial crops. Although *Ctv* appears to be a single gene and the evolution of resistance-breaking virus strains is possible, such strains have yet to be found. From this perspective, *Ctv* is an attractive candidate for transformation to produce CTV-resistant scion cultivars.

The potential value of marker-assisted selection for genetic improvement of citrus cultivars by standard plant breeding methods cannot be overstated. Juvenility and the large size of individual plants at maturity impose tremendous costs on citrus breeding programs. Prior to the availability of PCR technology, there were no selection methods that could be applied effectively at the young seedling stage to predict mature plant performance. Selection methods for citrus disease resistance are costly, time-consuming, and frequently imprecise. As studies of these traits are undertaken and linked molecular markers are identified and utilized, it will become possible for citrus breeders to maximize the frequencies of desirable alleles in hybrid families that are planted in the field and, in turn, to increase the likelihood of producing and identifying recombinants that possess improvements for the multitude of characteristics that are demanded by growers and consumer of citrus.

Acknowledgements The authors thanks Drs. H.C. Barrett and C.J. Hearn (USDA-ARS, Orlando) for providing access to USDA plant materials, Dr. R.F. Lee (UF-CREC) for guidance with CTV inoculation and ELISA procedures, Dr. Q. Cai for map information on the R family, Ms. M.K. Wendell for maintaining plant materials, Ms. T. Zito for assistance with photography, and Ms. P.A. Hicks for manuscript preparation. This research was supported in part by a grant (No. 920-1765) from the NRI Competitive Grants Program/USDA, and by a USDA-UF Cooperative Research Agreement (ARS/IFAS Agreement No. 58-55-1-102).

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