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Molecular markers flanking citrus tristeza virus resistance gene from *Poncirus trifoliata* (L.) Raf.

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Abstract Two segregating populations for citrus tristeza virus (CTV) resistance derived from *Poncirus trifoliata* var 'Flying Dragon' by self-pollination and pollination to *Citrus medica* L. var ethrog 'Arizona' were inoculated with a common CTV isolate. The presence of virus was checked by the Double Antibody Sandwich Enzyme-Linked Assay and Direct Tissue Blot Inmunoassay at 3, 6, and 12 months after inoculation. Seven RAPDs were found linked to the CTV resistance gene by bulked segregant analysis. The closest linked RAPDs were cloned to obtain linked codominant RFLPs and to increase the precision of the genetic distance estimation. The CTV resistance gene seems to be located between cW18 and cK16. Differences in genetic distances among progenies are large and can be explained by genome-wide reduction in the recombination of progeny derived from male versus female gametes.

Key words CTV resistance · Molecular markers · Recombination · Marker-assisted selection · Fruit breeding

Introduction

Citrus tristeza virus (CTV) is the causal agent of one of the most important diseases of citrus (Bar-Joseph et al. 1989). This phloem-limited closterovirus exists in a great variety of isolates that differ in biological properties such as symptoms in the field (Aubert and Bové 1984; Da Graça et al. 1989; Roistacher and Moreno 1991), reaction on indicator plants (McClean 1974; Ballester-Olmos et al. 1988, 1993) and aphid transmissibility (Roistacher and Bar-Joseph 1984; Hermoso de Mendoza et al. 1988). Since its outbreak in the early 1930s, tristeza has caused the dead of millions of trees grafted on sour orange all around the world (Bar-Joseph et al. 1989). The establishment of quarantine regulations and the use of tristeza-tolerant rootstocks have contributed to a diminishing of the damage produced by the disease (Navarro et al. 1988), but it is still common in most of the citrus-growing areas of the world (Bar-Joseph and Lee 1989; Marais 1991). And the use of only tristeza-tolerant rootstocks has greatly narrowed the genetic diversity of rootstock cultivars (only two genotypes are used in Spain) thereby increasing the vulnerability of our citriculture. In addition, virulent isolates of CTV with destructive effects have been reported (Bar-Joseph and Lee 1989; Roistacher and Moreno 1991), and some of them are able to attack the scion regardless of the tolerance of the rootstock. This makes the citrus industry doubly threatened: first, there are destructive isolates which can spread by natural vectors or by illegal introductions of plant material; second, new virulent isolates can arise by mutation, recombination or just separation of strains from the same isolate (Moreno et al. 1993).

Natural virus resistance genes are common among plants (Fraser 1990; Mansky and Hill 1993). Most, if not all, citrus species and cultivars are hosts of CTV (Garnsey et al. 1987; Bar-Joseph and Lee 1989; Bar-Joseph et al. 1989), but some citrus relatives (*Severinia buxifolia*, *Swinglea glutinosa*, and *Poncirus trifoliata*) have been reported to be resistant to CTV (Yoshida et al. 1983; Garnsey et al. 1987; Bar-Joseph et al. 1989).

Genetic markers have become very efficient and powerful tools in plant breeding (Lande and Thompson 1990), especially for traits that are tedious and expensive

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to evaluate such as CTV resistance. Resistance against CTV in progenies derived from *Poncirus trifoliata* (the only resistant species sexually compatible with citrus) behaves as a monogenic trait where the resistance allele is dominant (Mestre et al. 1994; Yoshida 1985; Gmitter et al. 1995; Fang and Roose 1996). Bulked segregant analysis (Michelmore et al. 1991) has been described as an efficient way to locate molecular markers linked to disease resistance genes. Its monogenic control along with the fact that hybrids between *P. trifoliata* and *Citrus* spp. can be obtained makes the resistance to CTV a suitable trait to be introduced into the cultivated citrus, not only by sexual hybridization but also by genetic transformation after cloning of the resistance gene. Map-based cloning experiments require the finding of markers that are very closely linked to the target gene; therefore, the use of experimental populations in which genetic recombination is at a maximum is critical.

In this paper we describe the identification of genetic markers linked to the CTV resistance gene of *P. Trifoliata*. Two very different segregating populations were used to compare the genetic location of the gene and to study the effect of the genetic background on the resistance.

Material and methods

Plant material consisted of two populations: one population was obtained by open pollination of *Poncirus trifoliata* var 'Flying Dragon' (FD population); the other population resulted from an intergeneric cross between *Citrus medica* var ethrog 'Arizona' (female parent) and *P. trifoliata* var 'Flying Dragon' (CxP population). Seeds from the FD population were collected over 2 successive years (1992 and 1993). Seedlings from the 1992 seeds were obtained at two planting dates. Isozyme analysis of plants from the FD population was carried out in order to distinguish zygotic from nucellar seedlings (Torres et al. 1982) and to confirm the self-pollination origin of the plants. Eleven enzymatic systems were studied as described by Asíns et al. (1995): phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), aconitase (ACO), esterase (EST), malic acid dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), peroxidase (PRX), isocitric acid dehydrogenase (IDH), and leucine aminopeptidase (LAP).

Inoculation was made by propagating buds of each plant on rootstocks infected with CTV isolate T-346, a common tristeza isolate (Ballester-Olmos et al. 1993). 'Rough Lemon' was generally used as a rootstock, although some inoculations were done on *C. sinensis* or *C. excelsa* as rootstocks.

The presence of CTV in plants was checked by DAS-ELISA (Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay) using monoclonal antibodies 3CA5 and 3DF1 together as described in Sanchez-Vizcaino and Cambra (1987). A reaction was declared to be positive when the value of its optical density at 405 nm was at least twice that of the healthy (control) samples. Additionally, the detection of CTV by direct tissue blot immunoassay (DTBIA) was also performed for all the samples following the procedure described in Garnsey et al. (1993). Analysis of virus presence was performed at least three times, at 3, 6 and 12 months after inoculation. Infection of the rootstocks was also checked in the same way.

Pools of resistant and susceptible plants of eight individuals of each type were obtained by weighting equal amounts of leaves of each individual. Genomic DNA extractions followed the method of Dellaporta et al. (1983) with some modifications. Random amplified polymorphic DNA (RAPD) analysis of the pools was done with 260 random primers (Operon kits A, B, C, D, E, F, G, H, I, J, K, O, W, Operon Technologies, Alameda, Calif.). Polymorphisms found in the bulked segregant analysis were confirmed by the analysis of individual plants from the segregating populations. Primers OPE20 and OPW18, which had been reported to yield polymerase chain reaction (PCR) products linked to the CTV resistance gene in other populations (M. Roose, personal communication), were also analyzed in our segregating populations. Amplification reactions consisted of buffer (10 m*M* TRIS-HCl, 50 m*M* KCl) (Eurobiotaq); 1.5 m*M* $MgCl_2$ (Eurobiotaq); 100 μ*M* dNTPs (25 μ*M* each); 0.2 μ*M* primer; 1 U *Taq* (Eurobiotaq); 150 ng of DNA and sterile water up to 25 μl. The PCR was conducted in a MJ-PTC-100™ thermal controller with 96 wells under the following conditions: an initial step at 95°C for 5 min; 45 cycles of 1 min. at 95°C, 1 min at 44°C, and 2 min at 72°C; and a final extension step at 72°C for 8 min. Following electrophoresis in 2% agarose TAE gels, the results of the amplifications were visualized with ethidium bromide staining.

RAPD products found to be linked to CTV resistance were cloned using the "TA cloning kit" from Invitrogen. All of the amplification products obtained in the PCR reaction were used in the ligation step. Clones whose inserts appeared to be the same size as the marker were selected and used as probes in hybridizations to Southern blots of RAPDs from individuals that segregated for the marker in order to verify the identity of the cloned product. Cloned RAPD products and clones gp47 and pgcit52 were used as probes in hybridizations to Southern blots of digested genomic DNA from plants from both segregating populations. Clones gp47 and pgcit52 were provided by Dr. M. Roose, University of California and Dr. G. Moore, University of Florida, respectively. Restriction fragment length polymorphism (RFLP) analysis was done as described in Monforte et al. (1996).

MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992) and JOINMAP 2.0 (Stam 1993; Stam and Van Ooijen 1995) were used for linkage analysis. A linkage criteria of LOD 3.0 and recombination fraction of 0.5, and a Kosambi mapping function were used in both programs. In JOINMAP 2.0 both populations were analyzed as the "Cross pollinator" population type, where no knowledge of the parental genotypes nor of the linkage phase of the markers is needed. When using MAPMAKER 3.0, we considered, the FD population to be a F_2 intercross population, and the CxP population was analyzed as a F_2 backcross population. Given that MAPMAKER does not allow mapping without the knowledge of the linkage phase, it was inferred from the results of the analysis with JOINMAP 2.0.

Results

Of the 11 isoenzymatic systems assayed 4 were polymorphic in both segregating populations, providing five segregating loci (*Pgi-1*, *Got-1*, *Got-2*, *Mdh-2*, *Prx*). These loci were used to distinguish zygotic from nucellar seedlings (five loci allow 97% of the zygotic seedlings to be determined) in the FD population. Of the seedlings 23.2% (57/246) were zygotic when the seeds were collected in 1992 and only 8.4% (19/227) when collected in 1993; no significant differences were found between the two planting dates of seeds collected in 1992. All five loci behaved in a Mendelian way, and no deviation from independent segregation was found (data not shown). The segregating alleles and their inheritance confirmed the self-pollination origin of the FD population.

Given that *C. medica* is monoembryonic, all seedlings from the CxP population were zygotic but they showed very low viability. The plants had difficulty in developing a proper radicular system, and it was necessary to graft them on healthy 'Rough Lemon' and sweet orange "pineapple" rootstocks. Nevertheless, 34 out of 175 plants survived and were suitable for marker analysis.

Not all of the plants gave consistent results regarding virus presence over time and space. In some plants, the presence of virus was detected in the first analysis but not in subsequent ones; in other cases, some susceptible plants showed an erratic distribution of the virus. Hence, two different criteria were used to classify plants into susceptible and resistant classes: (1) a plant was considered susceptible when the virus was detected at least once ($CTV_{r\text{-}a}$), and (2) only when the virus was always detected ($CTV_{r\text{-}b}$). Segregation data attending to both criteria are listed in Table 1, and both are consistent with a monogenic control of the trait where the resistance allele is dominant.

Table 1 Segregation data of loci included in the CTV resistance linkage group. See text for marker loci description

Loci[a]	FD population		χ^2
	Expected	Observed frequencies	
$OPA15_{120}$	3/1	36 P/20 A[b]	3.43
$OPO07_{065}$	3/1	43 P/13 A	0.10
$OPE20_{060}$	3/1	40 P/16 A	0.38
$OPW18_{045}$	3/1	40 P/16 A	0.38
$OPG18_{100}$	3/1	38 P/18 A	2.56
$OPO10_{140}$	3/1	43 P/12 A	0.30
cE20	1/2/1	16 FF/28 FS/12 SS	0.57
cW18	1/2/1	14 FF/28 FS/12 SS	0.22
cG18	1/2/1	6 FF/22 FS/18 SS	6.35*
cK16	1/2/1	12 FF/27 FS/17SS	0.96
gp47	1/2/1	3 FF/27 FS/21SS	12.88**
pgcit52	1/2/1	19 FF/32 FS/3 SS	11.33**
CTVr-a	3/1	33 R/17 r	2.16
CTVr-b	3/1	42 R/8 r	2.16
CxP population			
$OPA15_{120}$	1/1	17 P/17 A	0.00
$OPO07_{065}$	1/1	18 P/16 A	0.12
$OPE20_{060}$	1/1	16 P/18 A	0.12
$OPW18_{045}$	1/1	14P/20 A	1.06
$OPG18_{100}$	1/1	18 P/16 A	0.12
cE20	1/1	18 F/16 S	0.12
cW18	1/1	20 F/14 S	1.06
cG18	1/1	18 F/16 S	0.12
cK16	1/1	17 F/17 S	0.00
gp47	1/1	2 F/32 S	26.47**
pgcit52	1/1	9 F/25 S	7.53**
CTVr-a	1/1	4 R/10 r	2.57
CTVr-b	1/1	6 R/8 r	0.28

* Significant at $P < 0.05$; ** significant at $P < 0.005$

[a] Marker loci beginning with OP are RAPDs. Sizes in basepairs, reduced tenfold, are indicated as subindexes

[b] P, Presence of RAPD band; A, absence; R, resistant; r, susceptible; F, S, Fast and slow migrating RFLP alleles

The screening of 260 random primers against susceptible and resistant pools resulted in the identification of five new RAPD markers linked to the CTV resistance gene. Markers were obtained with primers OPO7, OPK16, OPG18, OPA15, and OPO10 (Fig. 1). Segregation data for these linked loci and for those previously known as linked is shown in Table 1.

Markers obtained with primers OPW18, OPE20, OPK16, and OPG18 were successfully cloned and named cW18, cE20, cK16, and cG18, respectively. Hybridization of these clones to Southern blots containing digestions of genomic DNA from plants of the FD population revealed a single-copy banding pattern for cW18 and low-copy banding patterns for cE20, cK16, and cG18. All four clones provided hybridization patterns that allowed the identification of the two alleles at the RFLP locus linked to the CTV resistance gene (Fig. 2), converting the dominant into a codominant marker. Segregation data for these clones are also presented in Table 1.

Linkage analysis resulted in the genetic maps presented in Fig. 3. These maps show the most likely orders obtained at LOD = 3.0, considering as susceptible plants those under the second criterium. Distances obtained with JOINMAP were slightly shorter than those obtained with MAPMAKER. The orders of the loci agreed in both populations although it must be noted that, in the CxP population, no significant differences were found among the best order and some other orders when MAPMAKER was used. Distances obtained from the CxP population are shorter than those from the FD population, even when only codominant markers (the most informative ones) were used in the analysis. Linkage analysis using the first criterium of susceptibility resulted only in slight changes in the distances between the CTV resistance gene and the markers, except for the CxP population and JOINMAP. In this case, the resistance gene was positioned between markers $OPA15_{120}$ and cG18 (data not shown).

Discussion

The percentage of nucellar versus zygotic seedlings is one of the most important traits in citrus rootstock breeding. The frequency of zygotic seedlings in the FD population varied from 23.2% in 1992 to 8.4% in 1993, whereas no significant difference was found between the two planting dates in 1992. Changes in the frequency of zygotic seedlings among years but not among planting dates have been reported by Khan and Roose (1988). These authors also reported the self-pollinating origin of *P. trifoliata* seeds. The important effect of the year in the percentage of zygotic seedlings suggests that the accumulation of a substance in the seeds or their teguments during seed development might be involved in this variation.

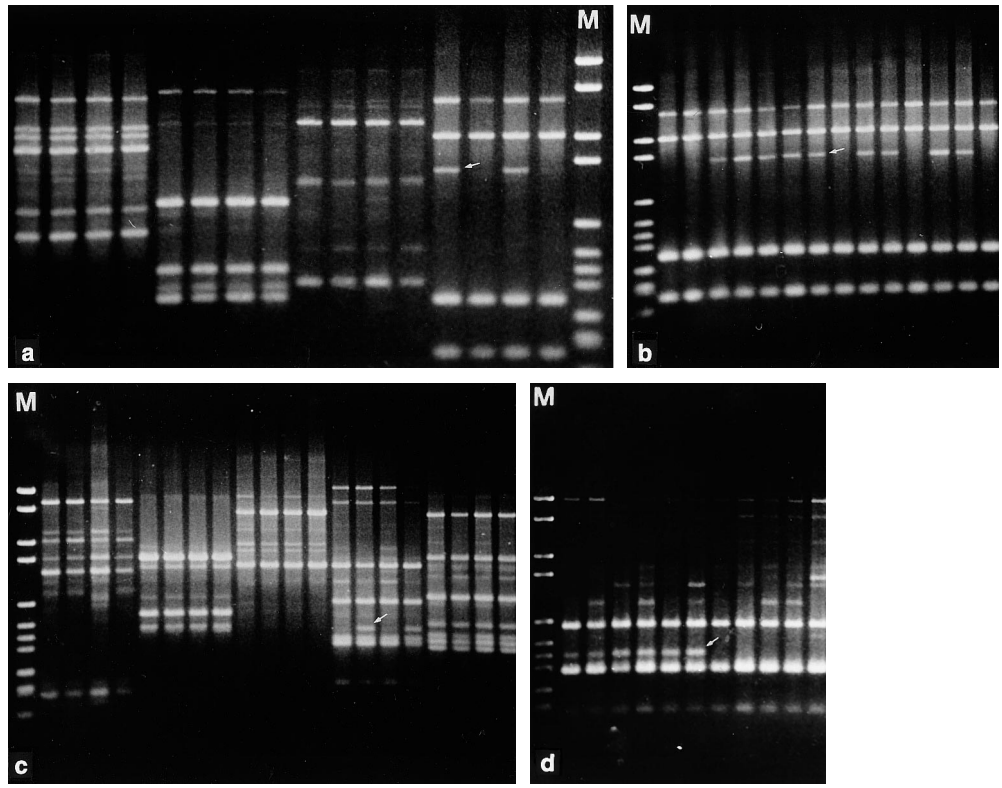

Fig. 1a–d RAPD markers detecting polymorphisms between bulks made for alternate susceptible/resistant CTV phenotypes from *left* to *right* (**a** and **c**). Each set of 4 lanes results from PCR amplification with a different 10-mer oligonucleotide primer. *Arrows* show differential RAPDs from OPG18 (**a** and **b**) and OPK16 (**c** and **d**) primers. **b** and **d** are amplification of genomic DNA from individual plants with OPG18 and OPK16 primers; respectively. *Lane M* shows molecular weight markers VI from Boehringer Mannheim

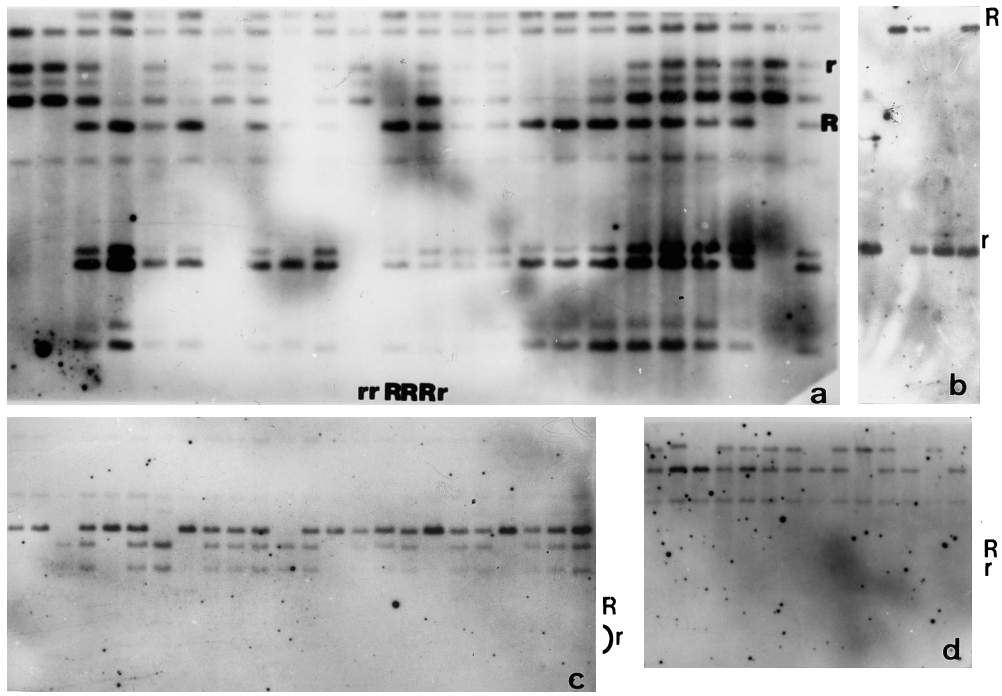

Fig. 2a–d Southern analysis of clones cE20 (**a**), cW18 (**b**), cK16 (**c**), and cG18 (**d**). DNA samples from individual plants were digested with *Bgl*II (**a**, **b** and **c**) or *Eco*RI. *R* bands correspond to the RFLP allele (fast or slow) linked in coupling phase to the CTV resistance allele

Hybrid plants derived from the cross between *C. medica* and *P. trifoliata* presented very low viability. Even when grafted on healthy rootstocks, most of them died, and marker analysis could only be performed in 34 plants, some of which died during the experiment. This low viability can be explained by the high phylogenetic distance between the two species (Herrero et al. 1996). Therefore, phylogenetic studies provide valuable information by which to predict the mean viability of the progeny, which is specially important in planning genetic studies in perennial crops.

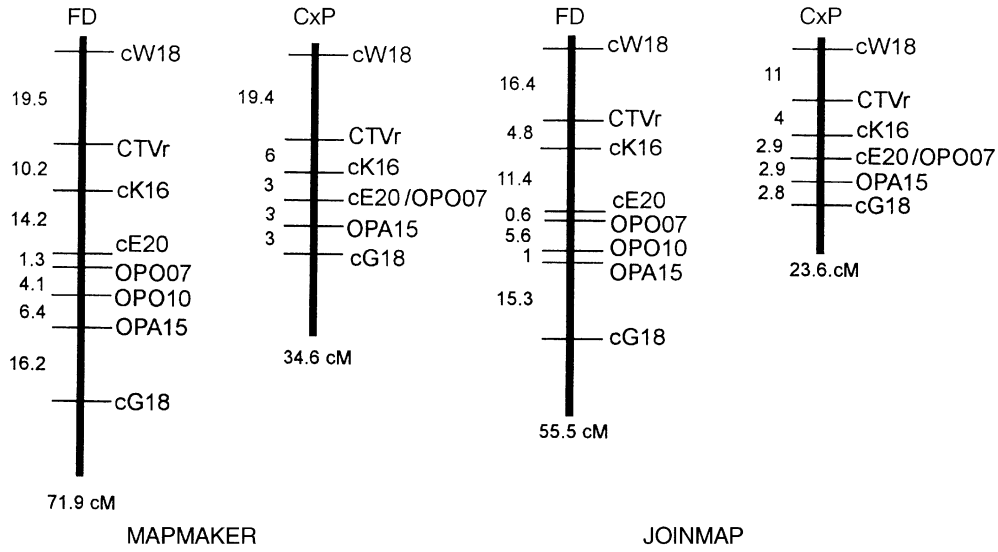

Fig. 3 Linkage maps obtained using LOD = 3 and a maximum recombination fraction of 0.5. Distances are in centiMorgans

C. medica ethrog var 'Arizona' was homozygous for all of the loci scored; therefore, segregation always came from *P. trifoliata* meiosis. Linkage analysis in both populations resulted in the same gene order, but genetic distances inferred from CxP population were significatively shorter. These differences between populations can not be attributed to differences in the population size or population type (intercross versus backcross), as deduced from the theoretical study reported by Säll and Nilsson (1994). Differences in the genetic recombination frequency between male and female gametes may be the main factor involved in the differences in genetic distances between the progenies. Progenies only differ in the source of the female mother cells (*P. trifoliata* var 'Flying Dragon' or *C. medica* var 'Arizona'); hence, similarly to what was observed in *Lycopersicon* by de Vicente and Tanksley (1991), significantly less recombination has been found for male gametes in *Poncirus trifoliata*. Sex differences in recombination have been described in a broad spectrum of plant and animal species (Burt et al. 1991). The fact that no significant difference was found among different gene orderings in the CxP population can be explained by the short distances estimated here along with its small progeny size.

Both mapping approaches located the CTV resistance gene in the same linkage group, with good LOD scores (Table 2). A detailed analysis of the MAPMAKER output for the FD population revealed little differences in probability regarding the location of the CTV resistance gene in other intervals of the linkage group. For the same population, JOINMAP showed that the inclusion of the resistance gene in the map modified the original distances among the marker loci. As the estimated distances among the loci are not small enough to cause this effect, it may be due to the definition of CTV resistance itself. Resistance results from factors that retard or prevent replication and systemic infection by a specific virus (Dawson and Hilf 1992). We have found plants in which the CTV multiplies near the inocolum, but it does not spread or spread very irregularly through the plant, regardless the rootstock. Analyses were carried out up to 12 months after inoculation; perhaps more plants could have become susceptible at a later date. Therefore, although the segregation data for CTV resistance, based on one or another criteria, behaves as though the resistance is monogenically inherited and despite the fact that all markers found associated with the trait are in the same linkage group, something else could still be missing. A better knowledge of the CTV resistance mechanism(s) is needed before proceeding to the map-based cloning of the gene responsible using the FD population.

We have found a linkage group in which the CTV resistance gene of *P. trifoliata* var 'Flying Dragon' is included. That finding can be used to perform marker-assisted selection (MAS), but just, which chromosome contains the whole linkage group is still unknown. RFLP analysis of clones reported to be linked to the CTV resistance gene in other populations (M. Roose, personal communication) resulted in strong

Table 2 Two-locus segregation for markers linked to CTV resistance gene at highest LOD in the FD population. Distances are in centiMorgans

Loci	MAPMAKER		JOINMAP	
	Distance	LOD	Distance	LOD
CTVr-a/cK16	16.3	5.48	14.3	6.41
CTVr-a /.cE20	25	3.53	20.8	3.92
CTVr-a/OPO07$_{065}$	23.2	3.38	19.5	3.69
CTVr-b/cK16	13.7	4.56	12.2	3.98
CTVr-b/cE20	20.4	3.21	17.5	2.61
CTVr-b/OPO07$_{065}$	24.5	2.40	20.5	2.53

segregation distortion in ours, preventing us from using a common reliable marker.

The existence of CTV-resistant individuals in the CxP population shows the effectiveness of the *P. trifoliata* resistance gene in the genetic background of *Citrus*. This fact reinforces the effectiveness of this CTV resistance gene when introduced in *Citrus* by sexual hybridization (in rootstock breeding) or genetic transformation (in both rootstock and cultivar breeding). Markers listed in Table 2 seem to be good candidates for MAS in *Citrus* rootstock breeding, especially when *P. trifoliata* is used as the male parent due to the low recombination level found in pollen. Besides, this is the usual direction of crosses involving *P. trifoliata* given its much earlier flowering than any *Citrus* species. The transformation of the RFLPs into (Sequence Characterized Amplified Regions) (Paran and Michelmore 1993) by sequencing analysis of the corresponding clones will enable a faster screening of the populations. The map-based cloning of a gene requires the precise genetic location of the gene and the presence of markers tightly linked to it; for this purpose, a population in which the genetic and physical distances are well-correlated is a more than just a desirable feature, it is essential. Thus, FD is the most convenient population for map-based cloning of the CTV resistance gene because of the larger recombination frequency of 'Flying Dragon' female gametes in spite of its low percentage of zygotic seedlings. However, a much larger progeny size and new markers more closely linked to the resistance gene should be necessary requirements for attempting its map-based cloning.

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