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New gene(s) involved in the resistance of *Poncirus trifoliata* (L.) Raf. to citrus tristeza virus

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Abstract Citrus tristeza virus (CTV) causes important economic losses in the citrus industry worldwide. Resistance to CTV is present in *Poncirus trifoliata* and is known to be controlled by a dominant gene at the *Ctr* locus. Short-distance movement of CTV around the inoculum, as well as passive movement through the phloem vessels, were studied in segregant plants derived by self-pollination from *P. trifoliata* var. "Flying Dragon" in order to genetically analyze the mechanism of CTV resistance. Accumulation of CTV in the vicinity of the inoculum and in new flushes was studied by means of a direct tissue-blot immunoassay (DTBIA). CTV is able to passively move with the phloematic flux from inoculated resistant genotypes *Ctr-Rr* and *Ctr-RR* up to a susceptible scion cultivar (*Ctr-rr*). Differences regarding CTV accumulation around the inoculum were found among *Ctr-Rr* individuals of the progeny. Bulked segregant analysis identified five RAPD markers linked to a locus (*Ctm*), or a genomic region, involved in short-distance accumulation of CTV but located in a different linkage group from *Ctr*. This result indicates that *Ctr* is not the only locus responsible for resistance to CTV in *P. trifoliata*, and that at least one other gene is involved. Given that citrus is a perennial crop, breeding for durable disease resistance should take into account selection at both the *Ctr* and *Ctm* loci.

Key words Closterovirus · Cell-to-cell movement · Molecular markers · Marker-assisted selection · Fruit-tree breeding

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Introduction

Citrus tristeza virus (CTV) is a phloem-limited closterovirus which infects plants belonging to the genus *Citrus* and some citrus relatives (Bar-Joseph and Lee 1989). Since its outbreak in the early thirties, CTV has caused widespread and important economic losses, and is common in most of the citrus-growing areas of the world (Bar-Joseph et al. 1989; Marais 1991).

Monogenic resistance to plant viruses can be classified into induced resistance, with a resistant response of the plant after a gene-to-gene recognition of the pathogen, and constitutive resistance, where non-induced resistance occurs by interruption of the virus infection cycle at some stage (mainly at replication, short-distance movement or long-distance movement) (Mansky and Hill 1993). While induced resistance is usually expressed as a hypersensitive response with a localization of the virus (Dawson and Hilf 1992), constitutive resistance results in suppression or reduction of virus replication, accumulation or movement.

Poncirus trifoliata (L.) Raf. is a citrus relative which has been reported to be resistant to CTV (Garnsey et al. 1987; Bar-Joseph et al. 1989; Yoshida 1996). This resistance is controlled by one dominant gene, *Ctr-R*, that has been recently located by means of molecular markers (Gmitter et al. 1996; Mestre et al. 1997), but nothing is known about the mechanism underlying it. CTV was not detected in new flushes of plants bearing *Ctr-R* when grafted onto infected rootstocks, whereas it was detected in plants lacking *Ctr-R*. Moreover, CTV resistance in *P. trifoliata* is efficient not only against common but also against aggressive isolates. The dominance of this gene and its efficiency against CTV aggressive isolates makes *P. trifoliata* a suitable candidate for citrus breeding. However, since citrus is a perennial crop, resistance genes causing just a reduction in virus multiplication or movement might not be as suitable in breeding for a durable CTV resistance as those other

genes causing a complete suppression of virus multiplication or movement. A better knowledge of the mechanism of resistance is needed to ensure the long-term protection of citrus against CTV.

The main purpose of this paper is to study the long- and short-distance movement of CTV in a segregating family derived from *P. trifoliata* in order to genetically analyze the mechanism of its resistance.

Materials and methods

Plants used in the experiment belong to a family derived by the self-pollination of *P. trifoliata* var. "Flying Dragon" (FD) (Mestre et al. 1997). A total of 24 plants, 17 bearing *Ctr-R* (CTV resistant) and 7 lacking it (CTV susceptible), were analyzed. *P. trifoliata* var. "Flying Dragon" (the mature parental plant) and two nucellar plants (i.e. with the same genotype as *P. trifoliata* var. "F. Dragon", but juvenile) were also included in the study.

Plants were grafted in 1993 onto "Rough lemon" (*Citrus jambhiri* Lush) rootstocks infected with the CTV common isolate T-346. All plants have shown consistent results regarding virus detection through time. Two-years later, plants were graft-inoculated with two patches (1 × 2 cm) containing the isolate T-346 and pruned to force new growth (Fig. 1 a). Virus-free buds of the cultivar "Pineaple" of sweet orange, where CTV reaches high titers, were grafted onto nine of these plants (Fig. 1 b) differing in genotype at *Ctr* in order to study passive movement of the virus. Plants were always grown in a greenhouse at 18–26°C.

One year after the inoculation, the presence of CTV was studied in three patches (1 × 2 cm) of bark surrounding the inoculum (see Fig. 1). A plant was considered to allow short-distance movement of CTV when the virus was consistently found in all three patches. Analysis of new flushes were carried out in all plants in order to confirm the CTV resistance phenotype (long-distance movement).

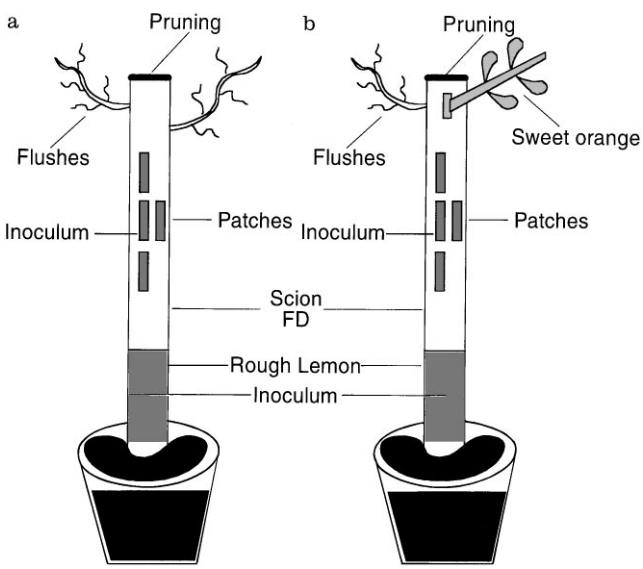

Fig. 1 Schematic representation of the experimental procedure. *Dark rectangles* are inoculum patches; *gray rectangles* are patches used for CTV detection. *Scion FD* segregant plants derived by self-pollination of *P. trifoliata* var. Flying Dragon (Mestre et al. 1997). **a** FD plant grafted on Rough lemon rootstock. **b** Virus-free sweet orange grafted on type (**a**) plants

Sweet orange new flushes were analyzed following the same procedure in order to detect passive movement (Fig. 1 b). CTV was detected by direct tissue-blot immunoassay (DTBIA) using a mix of the monoclonal antibodies 3DF1 and 3CA5 and following the procedure described in Garnsey et al. (1993). Results were confirmed by a double-antibody sandwich enzyme-linked immunosorbent Assay (DAS-ELISA) using the same antibodies as described in Sanchez-Vizcaino and Cambra (1987).

Bulked segregant analysis (Michelmore et al. 1991) was carried out with five individuals of each pool differing in short-distance accumulation of CTV. Genomic DNA extraction followed the method of Dellapporta et al. (1983) with some modifications. RAPD analysis of the pools was done with 180 random primers (Operon kits D, E, F, G, I, J, K, O, W; Operon Technologies, Alameda, Calif.). Polymorphisms found in the pools were confirmed by the analysis of all individual plants in the segregating population. Clone gp47 was provided by Dr. M. Roose, University of California. Amplification reactions and RFLP analysis were carried out as described in Mestre et al. (1997).

Linkage analysis was performed with Joinmap 2.0 (Stam 1993; Stam and Van Ooijen 1995), using the Kosambi mapping function. The population was analyzed as a "cross pollinator" population type, where no knowledge of the parental genotypes or of the linkage phase of the markers is needed.

Results

As expected, analysis of the new flushes of the FD plants (Fig. 1 a) revealed CTV presence only in those plants lacking *Ctr-R*. Analysis of sweet orange grafted onto the FD segregants at *Ctr* (Fig. 1 b) always revealed CTV presence. Therefore, the presence of one, or even two, gene doses of *Ctr-R* does not avoid the passive movement of CTV, through the phloem, from the inoculum up to a susceptible genotype where it unloads, replicates and spreads uniformly.

Analysis of patches surrounding the inoculum in FD plants showed short-distance virus accumulation in all seven *Ctr-rr* plants and in 5 out of 17 *Cr-R* plants: in the remaining 12 plants, CTV was never detected, or else was barely detected in only one of the three patches studied. CTV was never found anywhere in the parental or in the two nucellar plants studied.

From the group of plants bearing *Ctr-R*, pools of plants differing in CTV short-distance accumulation were made. Plants lacking *Ctr-R* (i.e. *Ctr-rr* plants) were not included since they have been infected for 2 years, consequently the bark surrounding the new inoculum could have been infected prior to the second inoculation. Screening of 180 random primers resulted in the identification of five RAPD markers linked to a putative locus that we have called *Ctm* and which seems to be involved in the short-distance movement of the virus. All these markers showed skewed segregation (Table 1) and were strongly linked to each other. In addition to them, gp47, a RFLP marker which had been previously studied (Jarrel et al. 1992; Mestre et al. 1997) was found to be linked to this group. All marker loci included in this linkage group show an important deviation from expected Mendelian segregation ratios (3 : 1 or 1 : 2 : 1) when the whole segregant population

Table 1 Segregation data of marker loci associated with *Ctm*

Locus[a]	Expected	Observed frequencies[b]	χ^{2c}	Linkage to *Ctm*	
				% Recombination	LOD score
$OPD07_{800}$	3/1	26 P/21 A	9.71*	0.20	1.79
$OPE04_{750}$	3/1	31 P/23 A	8.91*	0.20	1.79
$OPG09_{1200}$	3/1	27 P/27 A	18.00**	0.25	1.46
$OPG19_{1000}$	3/1	31 P/23 A	8.91*	0.20	1.79
$OPK16_{800}$	3/1	26 P/26 A	17.33**	0.21	1.63
gp47	1/2/1	3 FF/27 FS//21 SS	12.88*	0.10	1.85

[a] RAPD markers are indicated as "OP" (Operon Technology) followed by the letter and two numbers specific for the primer used. Sizes in bp are indicated as subindexes
[b] P: presence of RAPD band; A: absence. F, S: fast- and slow-migrating RFLP alleles. Markers have been studied in all individuals of the segregating population
* Significant at $P < 0.005$; ** significant at $P < 0.0001$

Table 2 Marker genotypes, putative *Ctr* and *Ctm* genotypes, and resistance pattern of selected individuals. Markers are listed in the same order as they are in the genetic map of each genomic region. *Ctr* positioned between cW18 and cK16 (Mestre et al. 1996). The position of *Ctm* could not be determined (see text). F : RFLP allele associated with resistance; S: RFLP allele associated with susceptibility. P: presence of RAPD marker; A: absence. RR: homozygous for *Ctr*; Rr: heterozygous for *Ctr*; rr: lacking *Ctr*. Mm: heterozygous for *Ctm*; mm: lacking *Ctm*. Plants of nucellar origin are indicated with an *n* as super-index

Plant	*Ctr*	*Ctr*-linked markers				*Ctm*-linked markers						*Ctm*	Resistance pattern
		cW18	cK16	cE20	cG18	OPG09	OPE04	OPD07	gp47	OPG19	OPK16		
22	rr	FF	FF	FF	FS	A	A	A	FS	P	P	Any	Susceptibility
27		FF	FF	FF	FF	P	P	P	FS	P	A		Virus everywhere
148		FF	FF	FS	FS	A	A	A	SS	A	A		
71	Rr	FS	FS	FS	FS	A	A	A	SS	A	A	mm	Resistance but short
156		FS	FS	FS	FS	A	A	A	SS	A	A		distance movement
173		FS	FS	FS	FS	A	A	A	SS	A	A		
25^n	Rr	FS	FS	FS	FS	P	P	P	FS	P	P	Mm	Resistance at short
47^n		FS	FS	FS	FS	P	P	P	FS	P	P		and long distance
163		FS	FS	FS	FS	P	P	P	FS	P	P		
99	RR	SS	SS	SS	SS	A	A	A	SS	A	A	mm	Resistance at short
110		SS	SS	SS	SS	A	A	A	SS	A	A		and long distance
149		SS	SS	SS	SS	A	A	A	SS	A	A		

(54 plants, Mestre et al. 1997) is genotyped and scored (Table 1). LOD scores of pairwise data (marker locus–*Ctm*) are near 2.0 (especially the one involving the co-dominant marker). Using a χ^2 distribution, all linkage between the marker locus and *Ctm*, except for that with $OPG09_{1200}$, are significant at an overall protection level of 5% using the Bonferroni (1937) correction. Thus, pairwise data suggest an association of *Ctm* to the markers obtained by bulked segregant analysis (Table 1).

Table 2 shows the CTV resistance patterns of plants whose marker genotypes had been used to infer their genotypes at the *Ctr* and *Ctm* loci. It should be pointed out that individuals heterozygous *Ctr-Rr* and homozygous *Ctm-mm* showed short-distance movement of the virus, while those which were *Ctm-Mm* did not support it; interestingly, individuals homozygous *Ctr-RR* did not show short-distance accumulation of the virus although all of them were *Ctm-mm*.

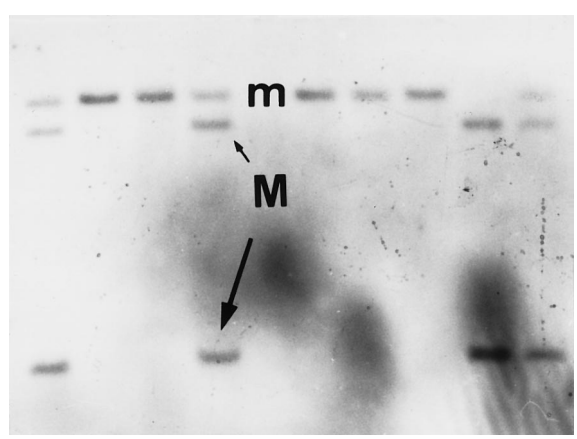

Fig. 2 Southern analysis of clone gp47. DNA samples from individual plants were digested with *Hin*dIII. *M* fast RFLP allele linked in coupling phase to *Ctm-M* (resistance to CTV short-distance movement)

Discussion

Short-distance accumulation of CTV has been found in some *Ctr-Rr* plants. This result can not be explained by a gene-dosage dependence of the resistance because short-distance accumulation of CTV has never been found in *P. trifoliata* var. "F. Dragon" (the heterozygous parental) or in its two nucellar plants. Rather, this results suggests that *Ctr* is not the only gene responsible for resistance of CTV in *P. trifoliata* var. "F. Dragon" but that at least one other dominant gene, which we have called *Ctm-M*, must be involved in this resistance. *P. trifoliata* var "F. Dragon" would then be heterozygous at both *Ctr* and *Ctm.*

Bulked segregant analysis of individuals differing in short-distance accumulation of CTV revealed a group of six markers covering 25 cM. All six markers showed a pronounced skewed segregation. A very low frequency of individuals were homozygous for the F allele (3 out of 54) at marker gp47 (Table 1), which suggests that this group of markers are linked to a gene that would greatly reduce viability or zygote formation in homozygosis. Although pairwise data suggest an association between the markers and *Ctm*, we have not attempted either an estimation of the genetic distances involved, or a mapping of the data, due to the small sample size. Nevertheless, LOD scores suggest that the precise location of *Ctm* could be assessed by increasing the family size (Table 1). It could be argued that the small size of pools ($n = 5$), along with the highly skewed segregation showed by the markers, would result in about a 2% probability of obtaining false positives (Michelmore et al. 1991). Although this fact must be taken into account, we think that deviation from Mendelian segregation ratios along with interactions between *Ctr* and *Ctm*, which mask the phenotype for *Ctm*, are, as explained below, responsible for the lack of statistical power to estimate the genetic distances between the markers and *Ctm.*

Data presented in Table 2 suggest that *Ctr-R* is incompletely dominant: homozygous plants (*Ctr-RR*, *Ctm-mm*) are absolutely resistant, while short-distance movement of the virus is found in (*Ctr-Rr*, *Ctm-mm*) plants. This could explain the low LOD scores obtained between the markers and *Ctm*; in fact, *Ctr-RR* plants would always be classified as absolutely resistant regardless their *Ctm* genotype, leading to a lack of knowledge on the behaviour of *Ctm* for short-distance movement. On the other hand, our results suggest that *Ctm-M* in a single dose is not effective in individuals lacking *Ctr-R*. Because of the skewed segregation showed by the markers around *Ctm*, no *Ctr-rr* plant putatively homozygous for *Ctm-M* was found, which has prevented us from determining the effect of *Ctm-M* when in homozygosis. The inefficiency of *Ctm-M* in *Ctr-rr* plants could indicate that the step(s) governed by *Ctr* occurs prior to that (those) governed by *Ctm.*

A hypersensitive reaction was never observed in plants bearing *Ctr-R*. Although hypersensitive responses are not always visible, this finding, along with the fact that gene-dosage dependence normally underlies constitutive resistance to viruses (Fraser 1992), suggests that the resistance provided by *Ctr* would be constitutive, and that *Ctr-R* would interrupt some initial step of the virus infection cycle. Additionally, the fact that plants heterozygous for *Ctr-R* but differing in genotype at *Ctm* also differ in short-distance movement of CTV, indicates that *Ctm* would be involved in the short-distance movement of the virus. Results on the passive movement of CTV illustrated in Fig. 1 b clearly suggest that the virus is able to move passively through the phloem vessels in (*Ctr-Rr*, *Ctm-Mm*) and (*Ctr-RR*, *Ctr-mm*) plants. Is *Ctr-R* blocking virus replication in a gene-dosage dependent manner and/or is it blocking phloem loading/unloading? Knowledge of CTV is rapidly growing (Karasev et al. 1995), and the infection of citrus protoplasts with CTV has been recently reported (Navas-Castillo et al. 1997). Inoculation of protoplasts from individuals homozygous for *Ctr-R* will allow us to distinguish between *Ctr* being involved in CTV replication or in phloem loading/unloading.

Our results suggest that at least two genes are responsible for CTV resistance in *P. trifoliata* var. "Flying Dragon". The more resistance genes a plant has, the more unlikely the virus will overcome the resistance. CTV isolates able to multiply in *P. trifoliata* have never been found; consequently, our results open up the question of whether this broad-spectrum resistance is due to the interaction of a very conserved viral domain with *Ctr*, or is due to the inability of CTV to overcome two or more different resistance genes present in *P. trifoliata.* The possibility of short-distance accumulation of the virus must be taken into account in breeding for disease resistance, because citrus is a perennial crop and, if CTV is able to move over short distances (cell-to-cell movement), it would infect the whole plant after a more or less long period of time. In a previous paper (Mestre et al. 1997) we reported molecular markers linked to *Ctr*, which makes marker-assisted selection available in citrus rootstock when breeding for CTV resistance. Hence, the finding of *Ctm* should be taken into account in the breeding programme, and individuals with the appropriate alleles at both regions of the genome should be selected in order to ensure a durable disease resistance. Additionally, genetic transformation of citrus cultivars with *Ctr-R* (once it has been cloned) should take into consideration the *Ctm* genotype of the recipient. If transgenic plants are *Ctm-mm* they might allow short-distance accumulation of the virus and then the spread of the virus would be just a matter of time.

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