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Expressed sequence enrichment for candidate gene analysis of citrus tristeza virus resistance

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Abstract Several studies have reported markers linked to a putative resistance gene from *Poncirus trifoliata* (*Ctv-R*) located at linkage group 4 that confers resistance against one of the most important citrus pathogens, citrus tristeza virus (CTV). To be successful in both marker-assisted selection and transformation experiments, its accurate mapping is needed. Several factors may affect its localization, among them two are considered here: the definition of resistance and the genetic background of progeny.

Two progenies derived from *P. trifoliata*, by self-pollination and by crossing with sour orange (*Citrus aurantium*), a citrus rootstock well-adapted to arid and semi-arid areas, were used for linkage group-4 marker enrichment. Two new methodologies were used to enrich this region with expressed sequences. The enrichment of group 4 resulted in the fusion of several *C. aurantium* linkage groups. The new one A(7+3+4) is now saturated with 48 markers including expressed sequences. Surprisingly, sour orange was as resistant to the CTV isolate tested as was *P. trifoliata*, and three hybrids that carry *Ctv-R*, as deduced from its flanking markers, are susceptible to CTV. The new linkage maps were used to map *Ctv-R* under the hypothesis of monogenic inheritance. Its position on linkage group 4 of *P. trifoliata* differs from the location previously reported in other progenies. The genetic analysis of virus-plant interaction in the family derived from *C. aurantium* after a CTV chronic infection showed the segregation of five types of interaction, which is not compatible with the hypothesis of a single gene controlling resistance. Two major issues are discussed: another type of genetic analysis of CTV resistance is needed to avoid the assumption of monogenic inheritance, and transferring *Ctv-R* from *P. trifoliata* to sour orange might not avoid the CTV decline of sweet orange trees.

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Introduction

Citrus tristeza virus (CTV) is one of the most important pathogens affecting citrus. There is a wide range of CTV isolates. Some isolates are very mild and cause little damage. Others may cause a severe decline of trees grafted on sour orange (*Citrus aurantium*). Some isolates also cause a serious stem-pitting disease in limes (*C. aurantifolia*), grapefruit (*Citrus paradisi*) and sweet orange (*Citrus sinensis*), that limits their commercial production (Garnsey 1999).

CTV is a member of the genus *Closterovirus;* it has a genome of 19.2 kb, which is the largest among RNA plant viruses (Karasev 2000). CTV probably originated in Asia, which is also the center of origin of *Citrus*, and has been disseminated in many countries by movement of infected plant material. Subsequent spread by aphid vectors has created major epidemics. RNA viruses are extremely adaptable and are capable of rapid change. Several factors contribute to the great variability of CTV: RNA polymerase errors in replication building up large viral populations, recombination and being hosted by a perennial crop where multiple CTV genotypes (by repeated aphid inoculations), and other viruses, may evolve together for a long time, increasing the probabilities of reassortment (Roossinck 1997). On the other hand, the vector acts as an evolutionary bottleneck (Karasev 2000).

Most citrus species are hosts of CTV. Up to now, the citrus and related genotypes where CTV resistance has been found are: trifoliate orange (*P. trifoliata*) (Yoshida et al. 1983), the Meiwa kumquat (*Fortunella crassifolia*) (Mestre et al. 1997c) and the pummelo 'Chandler' (*Citrus grandis*) (Fang and Roose 1999). All cultivars of *P. trifoliata* tested have been found resistant to most isolates (Mestre et al. 1997c); however, a New Zealand CTV isolate has been reported to break such horizontal resistance (Dawson and Mooney 2000). The evolutionary diversification of CTV and its quicker spread by efficient vectors such as *Toxoptera citricida* (Garnsey 1999) force farmers to replace sour orange as a rootstock with other rootstocks, sometimes not very well adapted to local

areas. The number of rootstock cultivars that are now available from breeding programs is very low, and trifoliate orange is the only source of CTV resistance used up to now. Citrus breeding takes a long time due to the long juvenility period of these species, and is very expensive because of the long time needed and the huge cultivation costs for maintaining and evaluating large segregating progenies. To alleviate such limitations, marker-assisted selection within the progenies, and genetic transformation of outstanding cultivars to introduce resistance genes, are valuable tools. There are several groups pursuing the isolation of *Ctv-R*, a *P. trifoliata* resistance gene to CTV, by chromosome walking (Deng et al. 2001; Yang et al. 2001). A first step towards obtaining those tools is the genetic dissection and mapping of the resistance gene(s). Several studies have reported markers linked to *Ctv-R* (Gmitter et al. 1996; Mestre et al. 1997a; Fang et al. 1998). Fang et al. (1998) reported a marker that co-segregated with the resistance gene, but later on the same group (Yang et al. 2001) reported that it was not due to some scoring errors. In addition to scoring errors, the estimated genetic distance depends on the family, the software for linkage analysis and how CTV resistance is defined (Mestre et al. 1997a).There are also other features that might be relevant: is CTV resistance a monogenic trait? Mestre et al. (1997b) presented evidence supporting the involvement of more than one gene. Does the time after inoculation affect the classification of plants into resistant and susceptible phenotypes? Would *Ctv-R* be effective within any genetic background, including sour orange, the well-adapted rootstock to arid and semi-arid regions?

Resistance genes controlling extreme resistance are thought to be constitutively expressed, and usually are distributed in clusters along the genome (Hammond-Kosack and Jones 1997; Grube et al. 2000). Besides, some of these gene clusters are involved in the resistance to very different types of pathogens (Van der Vossen et al. 2000). Therefore, whenever a region is known to contain a resistance gene it would be very useful to enrich this region with markers, involving not only anonymous but mainly expressed sequences (localized expressed sequences, LES). Methodologies for LES enrichment would be also important at locations where QTLs of large effect map. Selection of candidate genes by QTL analysis might be a useful strategy to connect research on functional genomics with the metabolic pathways of traits (Asins 2002). However, for most traits biochemical information on metabolic pathways is extremely limited, and one of the main limitations of candidate gene analysis is the low number of genes to be used as candidates.

The objectives of this work are: (1) the enrichment of the region containing *Ctv-R* with anonymous markers and localized expressed sequences by using two new strategies, that combine bulk segregant analysis and differential display analysis or the construction of a cDNA subtraction library; (2) a comparison of the new linkage groups of sour orange and *P. trifoliata* harboring *Ctv-R*; and (3) the fine mapping of *Ctv-R* under the hypothesis of monogenic inheritance using a progeny of citradias derived from the cross between sour orange and *P. trifoliata*, for which the virus presence has been followed for more than 2 years after inoculation.

Materials and methods

Plants and segregating populations

Two segregating populations were used for mapping. One was derived from the self-pollination of *P. trifoliata* (L.) Raf. var 'Flying Dragon' (Pp×Pp) and had been previously used for mapping *Ctv-R* by Mestre et al. (1997a). It consists of 66 plants. The other segregating population (104 A×Pa hybrids) derives from the cross between *C. aurantium* L. var. 'Afin Verna' (A) and the trifoliate orange 'Flying Dragon' (FD). A sample of 63 hybrids had been used previously to build a map for *C. aurantium* and another for FD (Ruiz and Asins 2003).

Enrichment strategies based on pools of resistant and susceptible plants consisted of seven individuals of each type (*RR* versus *rr*) from the Pp×Pp population, and homozygotes for the region between two RFLP markers (cG18 and cK16) on linkage group Pp4.

The presence of *Ctv-R* in the A×Pa progeny was inferred from the presence of FD alleles at the flanking marker loci pY65 and pY28 (Yang et al. 2001). To do this, specific primers were designed to develop the corresponding SCAR markers (Fig. 1). Disregarding the heteroduplexes, only one band was visualized whose size exactly corresponds to the expected amplification product in both SCARs. Moreover, the pY65 SCAR from FD was cloned and sequenced, presenting a 99% similarity to the corresponding sequence of the Genbank accession (AF278857).

Evaluation of CTV resistance and accumulation

Data on CTV resistance of the Pp×Pp population was taken from Mestre et al. (1997a, b).

The A×Pa hybrids and parents (sour orange and FD) were propagated on sweet orange rootstocks. Three months after this propagation, the rootstocks were inoculated by grafting patches of infected sweet orange. The CTV isolate was T-346, a common Spanish isolate from the bank of CTV isolates at IVIA.

A first lot of 66 A×Pa hybrids was inoculated at the beginning of 1999, and the rest 2 years later. Control and inoculated plants were grown in a greenhouse with temperature control (25°C±10°C). The presence of the virus was checked every 6 months (Spring and Autumn) at the shoots, and at the inocula as a positive control of each challenged plant, by Direct Tissue Blot Immuno-Assay (DTBIA) following the procedure described in Garnsey et al. (1993) (Fig. 2). CTV accumulation was also evaluated by a semi-quantitative double-antibody sandwich ELISA (DAS-ELISA) using monoclonal antibodies 3CA5 and 3DFI together, as described in Cambra et al. (1993).

At Spring of both 2001 and 2002, at least four branches of each inoculated plant were analyzed by DTBIA and DAS-ELISA. Those hybrids where the virus was detected in at least one branch, but not in others, were declared susceptible with an irregular distribution of the virus.

Following Mestre et al. (1997a) two different criteria were used to classify plants into susceptible and resistant classes (Fig. 2): a plant was considered susceptible (a) when the virus was detected at least once during the experiment and (b) when the virus was detected at the end of the experiment (year 2002). Therefore, some few resistant plants for criterion (b) might be considered susceptible for criterion (a).

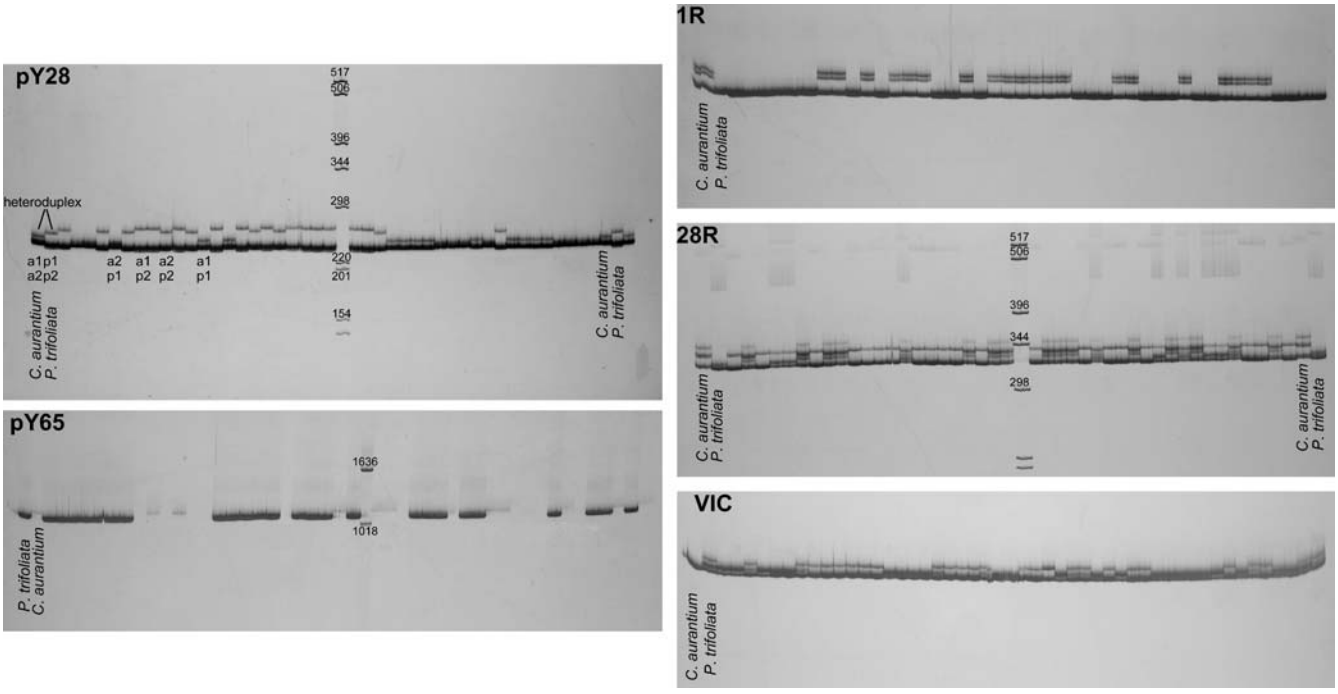

Fig. 1 Silver-stained acrylamide gels showing SCARs for clones pY28, pY65, 1R, 28R and VIC. Differences in heteroduplex mobility allowed the genetic interpretation (Ruiz and Asins 2003) of genotypes at the pY28, 1R, 28R and VIC loci

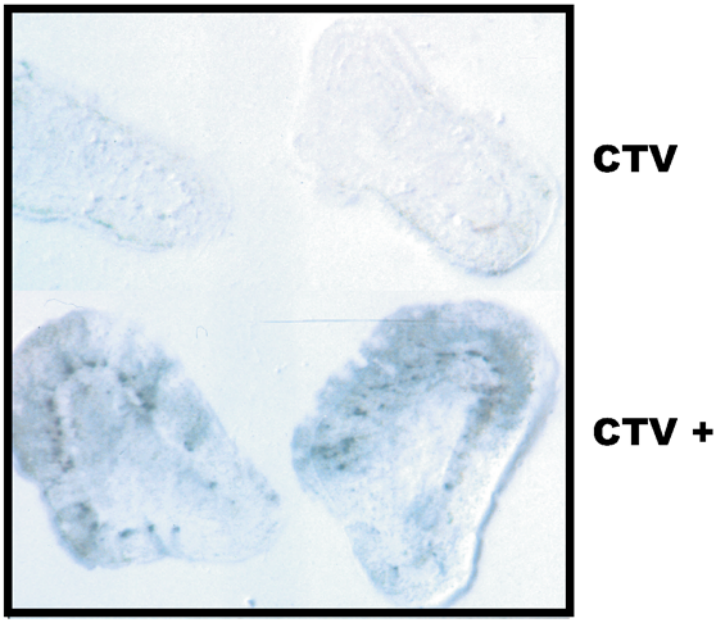

Fig. 2 Direct tissue blot immuno-assays of susceptible (CTV+) and resistant (CTV−) plants

Enrichment strategies and isolation of candidate resistance genes

DNA from the parents and members of A×Pa was extracted according to Dellaporta et al. (1983) with minor modifications. Total RNA was isolated from shoots or leaves of all plant materials using the MPG Total RNA Isolation Kit (CPG inc.). For accurate mapping, those amplification products that were of interest were extracted from gels, eluted in sterile water and re-amplified to verify their presence and purity. Purified PCR products were then cloned into the pGEM-T Easy vector system (Promega) and sequenced.

Five methodologies to enrich *Citrus* and *Poncirus* linkage group-4 with markers and LES (Table 1) were followed using bulk segregating analysis (Michelmore et al 1991):

(1) Primers of random sequence. Operon primers O7, A15, D7, G9, G19, K16, O10, W16, B10, O16, I1, E4 and Z16, and ISSR primers 840 $(GA)_8YT$, 835 $(AG)_8YC$ and 857 $(AC)_8YG$ were also used and correspond to USB set # 9 from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia (Canada). At least one of the corresponding PCR products was specific to the *RR* or *rr* DNA pools. PCR

reactions, electophoresis and staining conditions are described in Bretó et al. (2001). Segregating bands were mapped in the Pp×Pp progeny.

(2) Degenerate primers described by Mago et al. (1999), that were designed on the basis of nucleotide-binding-site (NBS) motifs, conserved among resistance genes. Amplified fragments were mapped previously by Ruiz and Asins (2003). One of them showing linkage to *Ctv-R* and others of similar size were cloned and sequenced for accurate mapping. Electrophoresis and staining conditions were mentioned as before. Segregating bands were mapped in the Pp×Pp and A×Pa progenies.

(3) Degenerate primers were designed from conserved regions of known resistance genes; CTVA: RAGAYRAGCATTGNTA, and CTVB: WTNCTTTGCAAAATRH. These primers were used separately as primers of random sequence. Segregating bands were mapped in the Pp×Pp progeny.

(4) LES that corresponds to differentially expressed sequences were obtained through differential display analysis (Hieroglyph mRNA profile kit, Genomyx corporation) of RNA pools from *RR* vs *rr* plants. In brief, first-strand cDNA synthesis from RNA samples was accomplished with different 3′ oligo(dT) anchored primers (AP1 to AP12), and then double-stranded cDNA fragments labeled with α^{33}P-dATP were synthesized by pairwise combinations of AP and 5′ arbitrary primers (ARP). As arbitrary primers we used them from ARP5 to ARP8, primers Z16, CTVA, CTVB, K16, O7, 857$(AC)_8$YG, and primers derived from cloned bands that map closely to *Ctv-R* in the Pp×Pp family (cK16, cZ16 and D857). Resulting fragments were electrophoresed using the Genomix LR sequencer (Genomix corporation) in a 200 μ thick denaturing 5% polyacrylamide–7 M urea gel. After a 40°C, 800 V, 14-h electrophoresis, gels were rinsed, dried and exposed to Biomax MR film (Kodak) for 2 days at room temperature. Bands differentially present in one of the two pools analyzed were recovered from gels and PCR re-amplified. In most cases, an additional purifying step (10% polyacrylamide-gel electrophoresis and band excision) was necessary to isolate the desired fragment.

(5) LES from the construction of a cDNA subtraction library used mRNA pools from *RR* vs *rr* plants (PCR-select cDNA subtraction kit, Clontech laboratories). mRNA pools from five resistant (*RR*) and five susceptible (*rr*) plants of the Pp×Pp family were used for tester and driver cDNA preparation, respectively. Poly A^+ RNA was extracted from five CTV-resistant (*RR*) and five CTV-susceptible (*rr*) members of the Pp×Pp family. These RNA samples were pooled separately and a SMART PCR cDNA Synthesis Kit (Clontech) was used for cDNA synthesis in order to avoid excess ribosomal RNA and a low concentration of cDNA corresponding to the poly A^+ fraction, which results in inefficient subtractive hybridization. Once ds cDNAs were obtained, column chromatography, *RsaI* digestion and purification of digested cDNAs were done according to the manufacturer's instructions, resulting in cDNAs suitable for subtractive hybridization with the PCR-Select cDNA subtraction kit (Clontech). A subtracted cDNA library was established by cloning the resulting subtracted mixture into the pGEM-T Easy vector system (Promega). A total of 135 clones were randomly chosen, and PCR was amplified with universal primers and blotted onto two identical nylon membranes (Hybond-N+, Amersham pharmacia biotech). For 54 clones, two replicates were blotted on both membranes. Total cDNA from resistant and susceptible pools was then digoxigenin-labeled using the DIG DNA labeling kit (Roche) and hybridized to the filters separately. The blots were pre-hybridized for 2 h and hybridized for 14 h in 1% blocking reagent, 0.1% N-Lauroylsarcosine, 0.2% SDS and 5 × SSPE at 68°C. Filters were finally washed twice in 2 × SSPE, 0.1% SDS for 10 min at 68°C, and twice again in 0.5 × SSPE, 0.1% SDS for 10 min at 68°C. The hybridization signals were subsequently detected with the CSPD chemiluminescent substrate (Roche). Thirty eight clones including all the combinations of hybridization signals for both filters (++, +−, −+, −) were selected for sequencing. According to their abundance in the subtracted library and sequence homologies, ten sequences were finally chosen for specific primer designing and mapping in the A×Pa family. In some cases where no heterozygosity in the parents could be detected, the SCAR bands corresponding to both resistant and susceptible individuals were excised from the gel, purified and sequenced to search for single nucleotide polymorphisms.

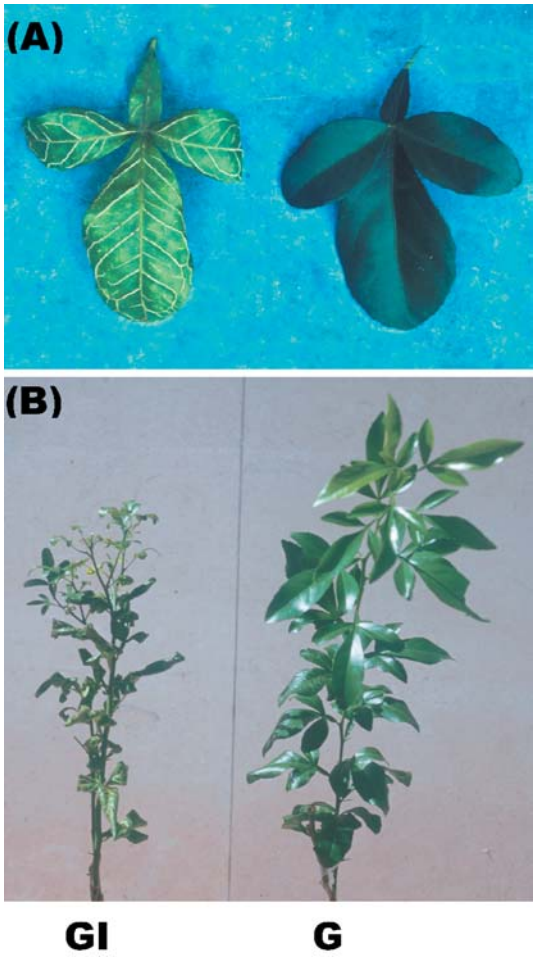

Fig. 3 'Vein corking' (*A*) and 'stunting' (*B*) symptoms of some inoculated resistant hybrids. *GI*: inoculated, propagated plant *G*: non-inoculated, propagated plant

The A×Pa hybrids a110 and a19 presented 'vein corking' (Fig. 3) in less than 3 months after inoculation, but not when they were just propagated on sweet orange. RNA from the a110 mother plant, the a101 propagated on sweet orange but not inoculated, and the a110 propagated on sweet orange and inoculated (showing 'vein conking' and 'stunting' symptoms), were used separately for differential display analysis. Methodology was as described in strategy 4.

JOINMAP 3.0 (Van Ooijen and Voorrips 2001) with a linkage criterion above LOD 4, a recombination fraction of 0.5 and the Kosambi mapping function was used for linkage. The Pp×Pp population was analyzed as a "self-pollination" population type and the A×Pa population as a "cross-pollinator" population type, with no previous knowledge of the linkage phase of the markers. Two separate maps were developed for each parent of the A×Pa family by using the pseudotestcross coding form at each "loc" file. The nomenclature used for linkage groups is described in Ruiz and Asins (2003). Some inter-retrotranposons amplified polymorphisms (IRAP) derived from gypsy-like retrotransposon sequences (C1, C2, C8, C11) were also included in the map (Bernet and Asins 2003).

Results

Enrichment strategies

Five strategies were followed to enrich linkage group 4 of *P. trifoliata* (where *Ctv-R* is located) with new markers and expressed sequences: "random" DNA markers, analogues of resistance genes and cDNA clones from the differential display analysis and a subtraction library between *RR* and *rr* plants (Table 1). Most problems encountered using the differential display strategy were related to the cloning of differential bands, while in the case of the subtraction library, searching for polymorphism (heterozygosity) in the parents for the cDNA clones, was the limiting step. The most profitable enrichment strategies in terms of the number of markers mapped on linkage group 4 and the effort involved, were those based on primers for analogues of resistance by Mago et al. (1999) and the new one to obtain LES from the subtraction library.

Each DNA sequence, or its translation into the amino-acid sequence, was used to search for orthologous genes at the NCBI (Tables 2, 3 and 4), and some might correspond to genes involved in the plant-defence response. Three bands of similar size, two specific of *P. trifoliala* (S2AS2_550 and S2AS2_519) and one specific of *C. aurantium* (S2AS2_520), were cloned. No sequence of significant similarity was found using the sequence of S2AS2_550 as a query sequence; however, the sequence of the others (S2AS2_519 and S2AS2_520) were highly similar to the NBS-LRR type disease-resistance gene analogues of apple tree (AAM77246, 5e-34), *P. trifoliata* (CTV.4 AAN62348, 3e-33; CTV.11 AAN62352 8e-29; CTV.17 AAN62353, 6e-28) and *C. grandis* × *P. trifoliata* (Pt8 AAN08176, 3e-30; 16R1-19 AAN08160, 5e-27; 16R1-13 AAN08159, 3e-26, Pt11 AAN08178, 3e-26; Pt9 AAN08177, 4e-26).

The marker and expressed sequence (ES) enrichment of linkage group 4 resulted in the fusion of several *C. aurantium* linkage groups. Thus, previous groups A7abc, A3 and A4 joins at LOD 6 in the new linkage map (Fig. 4). Marker correspondences with *P. trifoliata* "Flying Dragon", maps from A×Pa and Pp×Pp families and are also shown in this figure. Unfortunately, the high density of markers at A(7abc+3+4) might make gene ordering not very reliable in some regions. Although the corresponding homoeologous *P. trifoliata* linkage groups do not join in a single one; the order of common markers is quite conserved except for CR22_180 and VIC that are in the other *C. aurantium* linkage group (AI+AVII).

Table 1 Marker types used to saturate linkage group 4 (LG4). Selection of bands was carried out by comparing *RR* versus *rr* pools of individuals. Between brackets, the number of differential bands at strategy 4 and initially selected clones at strategy 5. The proportion of polymorphisms that map on linkage group 4 is indicated in the last column

Code	Strategy	Population	Markers on LG4
1	RAPD, ISSR	Pp×Pp	13/59
2	Mago et al. (1999)	A×Pa	3/6
3	CTV primers	Pp×Pp	4/23
4	Differential Display	Pp×Pp	2/3(12)
5	cDNA subtraction library	Pp×Pp	3/6(10)

Evaluation and mapping of CTV resistance

Seven A×Pa plants (7.28%) died before the first CTV evaluation. DTBIA evaluation agreed with DAS-ELISA data, in general. Only one hybrid showed low DAS-ELISA means, but the presence of the virus had been detected by DTBIA. This plant also showed irregular distribution of CTV among branches, which would explain the disagreement.

Table 2 Characteristics of cloned bands from differential display analysis of the citradia a101 mother plant (not grafted) and propagations on sweet orange that were inoculated ('vein corking' and 'stunting' symptoms) or not. LG: linkage group; un: unlinked; np: non-polymorphic

Clone	Expression specific to:	D.D. primers	Homologues—E value	LG
ANER	Inoculated	AP4-ARP5	Wound-induced protein (WIN1) CAA31851 (*Solanum tuberosum*)-3e-09, Pathogen- and wound-inducible antifungal protein CBP20 precursor AAB29959-4e-09	Pa, un
NER	Inoculated	AP1-ARP7	No significant similarity found	np
B	Not inoculated	AP4-ARP5	No significant similarity found	–
2-1	Mother plant	AP1-ARP7	No significant similarity found	–

Table 3 Characteristics of cloned bands from differential display analysis of *RR* versus *rr* pools from Pp×Pp segregating plants (enrichment strategy 4). Primers derived from cloned markers closely linked to *Ctv-R* in the Pp×Pp family are indicated in bold

Clone	Specific of	Primers	Homologues—E value	LG
VIC	rr	AP10-**DO857**	Hypothetical protein NP_179106 (*Arabidopsis thaliana*)-8e-35	Pa4, A(I+VII)
MON	RR	AP11-**DO857**	Polygalacturonase NP_194113 (*A. thaliana*)-4e-27	A(7+3+4)
SCAB	rr	AP3-**ck16A**	SCARECROW gene regulator-like NP_199626 (*A. thaliana*)-6e-16	Pa5b, A(10+5b)
WIL	rr	AP11-**UO857**	Phosphate/PEP translocator protein-like NP_196201(*A. thaliana*)-6e-11	np

Table 4 Characteristics of cDNA clones initially selected from the subtraction library for mapping analysis (enrichment strategy 5). The presence of introns is indicated by an asterisk. Number of clones with the same sequence is also indicated. Size of clones in base pairs. np: not polymorphic. The last column summarizes results on dot-blot analysis using cDNA probes from *RR* and *rr* pools. *RR* stands for the hybridization signal, only with the *RR* probe. *RR>rr* means a brighter signal with *RR* than with the *rr* probe, and so on. ns: not signalled

Clone ID	Size	Number isolated	Homologies—E value	Linkage Group	Dot-blot signal
1R	818	3	Actin T51182 (*Malva pusilla*)-e-113	A(7+3+4)	*rr>RR*
15R*	1,400	1	Calcium/proton exchanger CAX1-like protein AAF91349 (*A. thaliana*)-3e-27	A11	*RR>rr*
24R*	450	1	Putative GDSL-motif lipase/acylhydrolase AAM64916 (*A. thaliana*)-1e-60	Pa7, A(7+3+4)	*RR>rr*
25R	238	2	Lipid transfer protein NP_188456 (*A. thaliana*)-2e-12	A2	*RR*
27R*	300	1	Putative protein AAM97138 (*A. thaliana*)-9e-33	A11	ns
28R*	379	1	No significant similarity found	Pa un., A(7+3+4)	*RR=rr*
4R	373	4	Glutamate decarboxylase isozyme 4 AAK38667 (*Nicotiana tabacum*) - 4e-58	np	*rr*
6R	168	3	bis(5'-adenosyl)-triphosphatase-like; fHIT NP_200632 (*A. thaliana*)-7e-18	np	*rr*
18R*	545	1	Tubulin alpha-6 chain (TUA6) NP_193232 (*A. thaliana*)-3e-81	np	ns
22R	316	1	No significant similarity found	np	*RR>rr*

Both parental genotypes and 64 A×Pa hybrids were resistant; i.e. CTV was not detected at the end of the 4th/2nd year of evaluation. Eight of them were evaluated as susceptible (by both methods) 3/6 months after inoculation, but later on became resistant, four of which do not carry *Ctv-R* as inferred from the pY65 and pY28 markers (Fig. 1). This putative resistance gene was not present in 15 resistant hybrids.

Thirty three A×Pa hybrids were susceptible. In five of these plants, the virus was not detected 3/6 months after inoculation but later on they became susceptible, although the distribution of the virus was irregular within three of these plants. The heterogeneous distribution of CTV was present in 23 out of the 33 susceptible hybrids. Three of them carry *Ctv-R* as inferred by the pY65 and pY28 markers. Mortality during the experiment, after inoculation, was higher in the group of resistant hybrids (13), especially those without *Ctv-R,* than in the group of susceptible ones (1).

Several hybrids (around 5%) showed 'vein corking' very soon after CTV inoculation. To avoid confounded effects between CTV inoculation and grafting on sweet orange, two of those hybrids showing 'vein corking' (a110 and a19) were chosen. From four virus-free propagations per hybrid on sweet orange, two were inoculated and two were kept virus-free. 'Vein corking' did not appear in non-inoculated propagations of both hybrids; however, inoculated propagations of a19 stopped showing 'vein corking' some time after, at the new development of leaves. It is important to point out that a19 changed from resistant to susceptible at the 2nd year of CTV evaluation. The resistant hybrid a110 quickly developed 'vein corking' and 'stunting' only after inoculation (Fig. 3). Inoculated and non-inoculated propagations of this hybrid, and its mother plant, were used for differential display analysis (Fig. 5). Four differential bands were cloned, sequenced, and one of them mapped (Table 2). The segregation of clone ANER (whose translation product is highly similar to a pathogenic-related protein) could be studied because its digestion with *NlaIII* yielded products that segregated in *P. trifoliata* gametes. Its segregation ratio was highly distorted and linkage analysis did not allow its inclusion in a linkage group, remaining unlinked. The hybrid used in the differential display analysis is homozygous for the highly frequent allele. The other allele is present in only seven plants, three of which (all from the first lot of inoculated plants) died during the experiment.

Considering CTV resistance as a monogenic trait, the responsible gene is placed only in the *P. trifoliata* map, at group Pa4, between 5F-5R_3500 and the *Rps2* homologue '520', and not between pY28 and pY65L (Fig. 4). On re-analyzing the Pp×Pp family, including the new markers, the resistance gene is placed depending on the criteria at O7_2000 (a) or at D857 (b). *Ctm* (Mestre et al. 1997b) is now included in Pp4 at LOD 4.0 but its precise position depends on including, or not including, *Ctv-R*.

Therefore, considering CTV resistance (T-346 isolate) as a monogenic trait as evaluated at the end of, at least, 2 years after inoculation, in a progeny derived from the cross between two resistant genotypes of *C. arantium* and *P. trifoliata,* the putative unique responsible gene *Ctv-R* does not map between pY65 and pY28. Two hypotheses might explain it, the progeny (sour orange background) or CTV resistance is not monogenically inherited in this family.

Discussion

A *Poncirus* resistance gene to CTV (*Ctv-R*) has been located in the *Poncirus* genome by means of molecular markers (Gmitter et al. 1996; Mestre et al. 1997a; Fang et al. 1998). The genomic region where *Ctv-R* is located corresponds to a disease resistance gene cluster that includes many resistance gene analogues and a putative

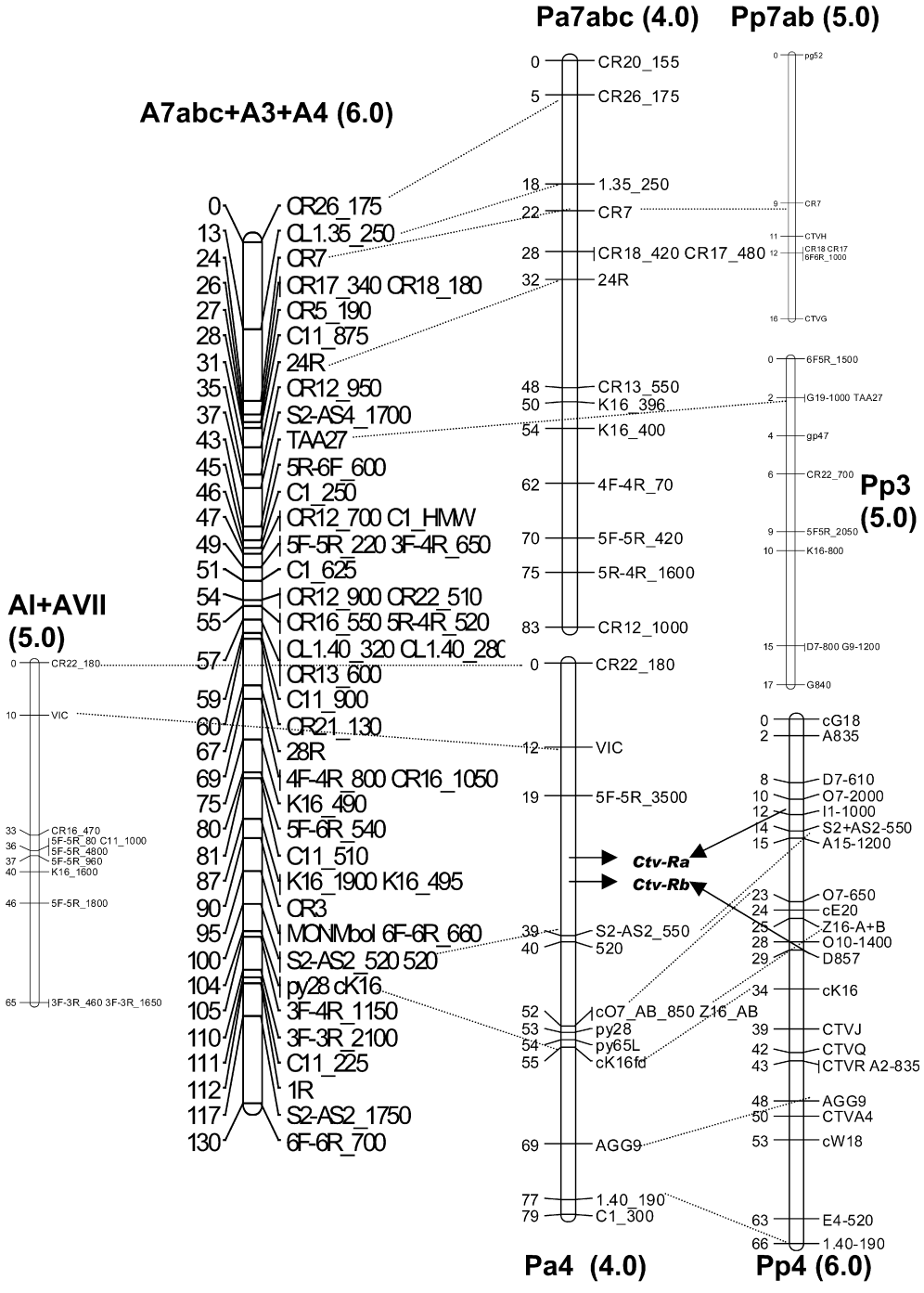

Fig. 4 Enriched linkage groups 4, 3 and 7 from the A×Pa and Pp×Pp families. LOD scores are indicated between brackets for each linkage group. Marker and linkage group nomenclature follow that reported by Ruiz and Asins (2003). Location of Ctv-R following criterion (**a**) or (**b**) is indicated at both Pp4 (from the Pp×Pp family) and Pa4 (from the A×Pa family)

QTL for nematode resistance (Ling et al. 2000; Deng et al. 2001; Yang et al. 2003). Chromosome walking along this region (Yang et al. 2001) localized the *Ctv-R* gene to a region between two resistance gene analogs (pY65 and pY28) spanning approximately 322 kb. Molecular markers linked to *Ctv-R* have been included in linkage group 4 of *P. trifoliata* (Ruiz and Asins 2003). Given that this region is so rich in genes involved in the resistance response to a broad spectrum of pathogens, its enrichment for markers, resistance gene analogs and expressed genes, in general, has been used to identify candidate resistance genes for CTV and other pathogens in the future.

Marker and expressed enrichment strategies

Five strategies to enrich *Citrus* and *Poncirus* linkage maps with markers, resistance analogues and ES linked to *Ctv-R* were attempted. A certain degree of success was reached with any of them, ranging from 16.7% (strategy 4) to 50% (strategy 2). Two of the five strategies are new and

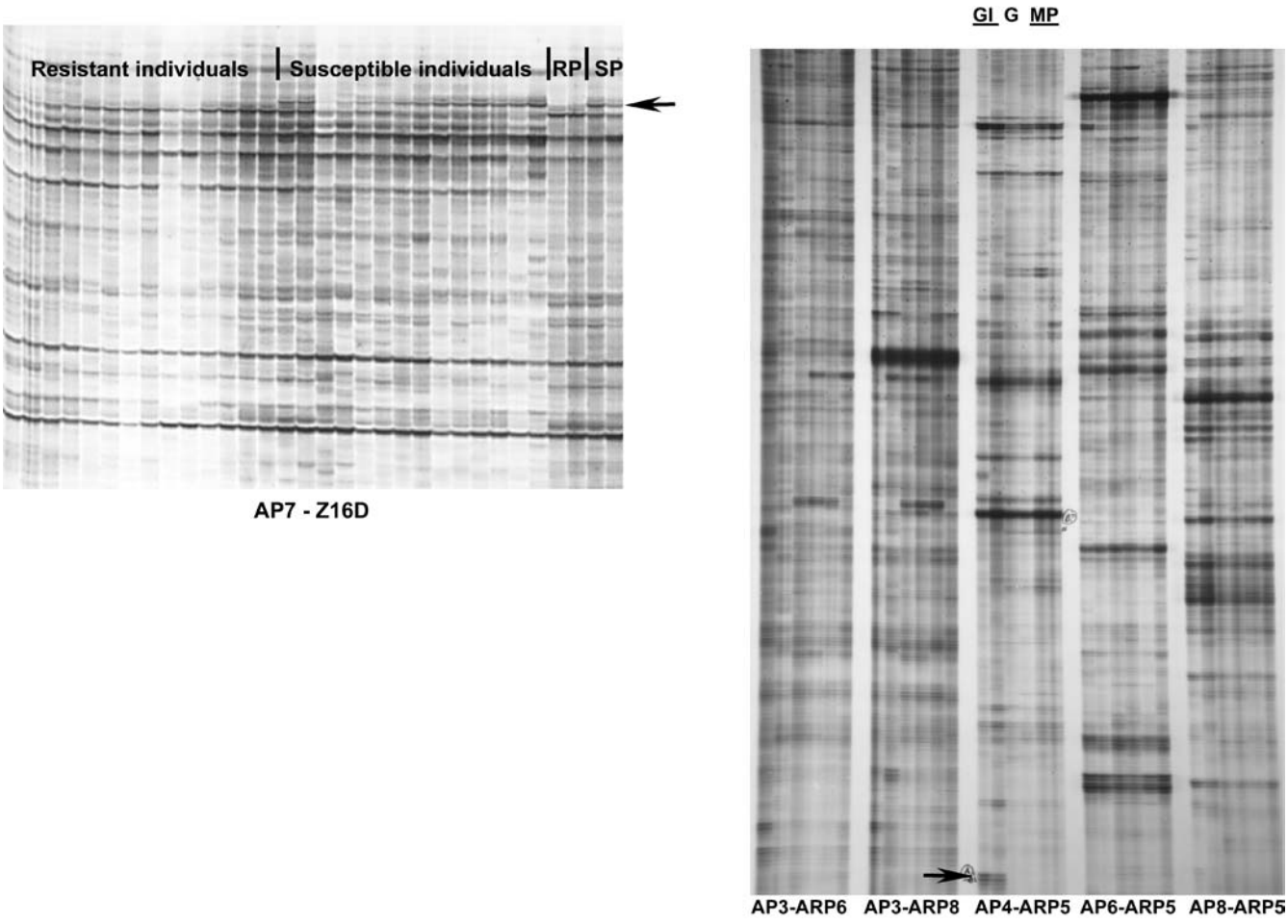

Fig. 5 Examples of differential display analysis for the enrichment strategy 4 (to the left) and for the obtaining of ESTs from the resistant A×Pa hybrid a110. *Arrows* point to differential bands. *RP*: resistant pool; *SP*: susceptible pool. *MP*: Mother plant: *GI*: inoculated, propagated plant, *G*: non-inoculated, propagated plant

combine bulk segregant analysis (Michelmore et al. 1991) (*RR* versus *rr* pools of Pp×Pp individuals) with differential display analysis, or with the construction of a cDNA subtraction library. These strategies were expected to identify genes of constitutive expression whenever those genes are present in a portion of a segregating family, and lacking in the other part. For plants that respond rapidly to microbial attack it was anticipated that R proteins should be present in healthy plants throughout life. RNA gel-blot analyses, using *RPS2*, *RPM1, Pto, Cf-9* and *Cf-2* as gene probes, have revealed the presence of low abundance transcripts in unchallenged plants, indicating that at least the *RPM1* gene and some members of multigenic *R* families are expressed in the absence of the corresponding Avr-expressing pathogen (Hammond-Kosack and Jones 1997). Since CTV resistance seems to be a case of extreme resistance, a constitutive expression of *Ctv-R* was hypothesized and strategies 4 and 5 were especially designed to isolate *Ctv-R*, or at least closely linked expressed sequences for *Ctv-R.* Although the proportion of these genes that could be mapped was low (between 16.7 and 30%), 50% of them mapped on linkage group 4, where *Ctv-R* had been previously mapped. The ES 28R locates at group A(7+3+4) but remains unlinked in the FD map. Another clone, ANER, only segregates in FD but also remains unlinked. Both present distorted segregation ratios, which makes their mapping difficult. An important inconvenience of ES is the lack of polymorphism some of them present, even at the nucleotide level. Why do methodologies exploiting differential expression result in non-segregant sequences? There are two possible explanations: although the amplified fragment does not contain any polymorphism, it may exist upstream or downstream. Alternatively, the segregating gene might be a cis-acting regulatory element of the ES corresponding gene.

The ES enrichment strategies we have presented are methodologically complex, but at least some of the resulting clones have high sequence identities with genes that might be associated with the plant defense response. Further investigations are needed to verify the associated expression of the selected clones with the citrus defense against CTV or other pathogens but, according to their sequence homologies (kinases, phosphatases, ion channel regulators, lipases), some of them could be components of

the downstream R protein-mediated signalling events (Hammond-Kosack and Jones 1997; Glazebrook 2001).

The molecular markers generated have allowed the saturation of the linkage group Pa4 of *P. trifoliata* where *Ctv-R* had been previously positioned. Increasing the number of markers and the progeny size of the A×Pa family has made several linkage groups to merge along with group A4 of *C. aurantium*, the Pa4 homoeologous linkage group. The resultant linkage group A(7+3+4) is now saturated with 48 molecular markers. This saturation that is not balanced with the progeny size has caused the misdetection or detection of very few recombinants between some markers, making their ordering not very reliable. Lower marker heterozygosity and strong segregation distortions, from both gametal and zygotic origin, in *P. trifoliata* (Ruiz and Asins 2003), might be responsible for Pa4, Pa3 and Pa7 remaining separate linkage groups, although synteny of common markers is evident. The Pa4 linkage group includes molecular markers pY28 and pY65, separated by 1 cM, that were reported to flank the *Ctv-R* resistance gene (Yang et al. 2001). Therefore, an approximate equivalence of 322 kb to 1 cM may be established at this mapping position within Pa4, in comparison with the equivalence reported by Yang et al (2001) using a consensus map that is around 472 kb per cM.

Utilization of sequence homology of conserved domains from known resistance genes to amplify analogues of resistance has proven to be useful in many plant taxa, including citrus (Deng et al. 2000). It is a relatively easy technique that has provided excellent results (Table 1) and has allowed the cloning of '520' which, according to its sequence homology and map position, seems to be part of the previously mapped resistance QTL with major gene effects, *Tyr1*, against the citrus nematode (*Tylenchulus semipenetrans*) (Ling et al. 2000). Moreover, '520' presents high similarity to *Rps2*, the resistance gene of *Arabidopsis thaliana* against *Pseudomonas syringe* pv tomato strains that carry *avrRpt2* (in Baker et al. 1997; Pysh et al. 1999). *P. syringe* is also a pathogen of citrus and causes two diseases, blast and black pit, that are important in some areas (Menge 2000). Although resistance genes and their analogous do not usually mark regions of synteny (Grube et al. 2000; Richter and Ronald 2000), it seems to be conserved between *P. trifoliata* and *C. aurantium* at linkage group 4.

Resistance to CTV

For breeding purposes, several types of responses that occur in resistant plants have been reviewed by Solomon-Blackburn and Barker (2001) in potato: extreme resistance (ER) and hypersensitive resistance (HR); resistance to infection (i.e. from vector inoculation); resistance to virus accumulation (RVA) and resistance to virus movement (RVM). ER and HR correspond to the gene-for-gene resistance pathways (Glazebrook et al. 1997). Resistant virus-host interactions have been extensively reviewed. In contrast, little is known about the factors involved in susceptibility. Viruses of plants have limited and specific host ranges. Plants the virus can infect and systemically invade under laboratory conditions are considered the host range. For Dawson and Hilf (1992) susceptibility and resistance occur in various degrees. They describe seven types of virus-host interaction: (1) total susceptibility; (2) initial susceptibility (HR); (3) limited susceptibility due to a reduced level of virus replication; (4) limited susceptibility in which the virus can spread but not systemically infect the plant; (5) limited susceptibility in which the virus replicates but is limited to the initially infected cells; (6) resistance to infection (ER); and (7) true immunity, the genotype is a non-host, even at the cellular level. Since CTV can replicate in *P. trifoliata* protoplasts (Albiach-Martí et al. 1999), this species can not be considered a non-host of CTV. No hypersensitive response has been observed in *Ctv-R*-mediated resistance to virus infection (Yang et al. 2001). Mestre et al. (1997b) speculated that resistance provided by *Ctv-R* would be a constitutive response interrupting the virus infection cycle at initial steps, so 3–4 months after inoculation no virus is detected. Within the resistant Pp×Pp plants, some of them, heterozygous at *Ctv-R*, were graft-inoculated to test the CTV spread (Mestre et al. 1997b). One year after, the virus had spread around the inoculum in some of them, suggesting that the resistance of *P. trifoliata* was due to both lack of CTV unloading from sieve elements (type 4) and lack of cell-to-cell movement (type 5). No type 2 was observed in Pp×Pp plants.

Analyzing the time course of CTV infection in the A×Pa family from 2–4 years after inoculation has shown the appearance by segregation of different types of virus-host interactions. Thus, 15% of the hybrids reverted their capability to control CTV infection during the 1st year after inoculation and 69.7% of susceptible plants showed irregular distribution of the virus among branches. Since 60% of hybrids that reverted to susceptible, and presented irregular distribution of the virus, it can be said that all these hybrids present a certain degree of resistance to systemic infection (type 4). Within the susceptible group, 30.3% of the hybrids presented uniform distribution of the virus. A few of them showed low titers which might be considered as a certain degree of RVA or type 3 interaction.

Within the resistant group of A×Pa hybrids, most of them carry *Ctv-R* (76.6%) but there is a proportion (23.4%) that lacks it. Unexpectedly, sour orange is as resistant to CTV (isolate T-346) as FD, therefore resistance could have been inherited from sour orange in these hybrids. Sour orange resistance to CTV has been observed for certain isolates in the past (Costa and Grant 1951) and is now under study. It is also important to point out that in 12.5% of resistant hybrids, CTV was detected 3–6 months after inoculation, but these plants became resistant later. This behavior is common to hybrids containing or lacking *Ctv-R*, and a similar proportion of both show it. The cure from CTV suggests the presence of a silencing mechanism for becoming resistant (Vance and Vaucheret 2001), genetically different from *Ctv-R* (type 3). Differences in

experimental conditions (CTV isolate, aphid transmission of CTV, and field conditions) will likely provoke differences at the phenotypes found. Nevertheless, since all control and challenged plants were kept under greenhouse conditions, the influence of the environmental variability on phenotypic variation for CTV accumulation and distribution is expected to be low.

Some CTV isolates may cause a severe decline of trees grafted on sour orange. In fact, more than 80 million trees on sour orange have been destroyed to date. The decline results from viral effects on the phloem of the sour orange rootstock just below the bud union or perhaps from the reaction of sour orange to the virus. The decline may occur over a period of several years or very rapidly (quick decline). T-346 was isolated from a 'stunted' satsuma grafted on sour orange that declined (P. Moreno, personal communication). Although the resistance of sour orange to T-346 was surprising, it will not be the first case that the infection of a scion grafted on a resistant rootstock provokes bud-union incompatibility, leading to the death of the tree (i.e. citrus tatter leaf virus and *P. trifoliata*). Moreover, Bar-Joseph et al. (2003) have reported a working hypothesis, according to which, two disease manifestations of CTV (seedling yellows and quick decline) would result from the interaction of CTV avirulence genes (*Avr*) and the resistance (*R*) gene(s) harbored by sour orange. Therefore, it is tempting to speculate that CTV-sour orange presents a type-2 interaction resulting in an hypersensitive response (HR). Then, the continuous defense response of sour orange, when CTV is highly accumulated in the scion, might irreversibly damage its vascular tissues (or their development) under the bud union leading to the death of the tree. The hypersensitive response is an active defense characterized by the appearance of necrosis at the site of infection and the inhibition of further pathogen multiplication and spread. Hypersensitive response includes an oxidative burst leading to the production of reactive oxygen intermediates, an alteration of membrane potentials, an increase in lipoxygenase activity, cell wall modifications, lignin deposition, production of antimicrobial compounds (Baker et al. 1997) and the expression of a characteristic set of genes including pathogenesis-related protein 1 (Glazebrook 2001). Three observations in our data might support the hypothesis of sour orange death through a continuous HR. One corresponds to the differences in mortality index between resistant (8.3%) and susceptible hybrids (0.33%). Within resistant hybrids mortality was higher in hybrids without *Ctv-R* (10.5%) than in those carrying it (2.9%). Therefore, mortality is related to CTV resistance, other than that derived from the *Ctv-R* gene from *P. trifoliata*. The second result concerns the resistant hybrid a110 that showed 'vein corking' a few weeks after CTV inoculation. The third observation suggesting an HR response is the differential expression of a gene coding for a putative pathogenesis-related protein in CTV-inoculated propagations of a110 (Table 2). The translated sequence of this differential EST (ANER) presents high identity with wound-induced protein (WIN 1) in the potato (Stanford et al. 1989), and a pathogen- and wound-inducible antifungal protein CBP20 precursor in tobacco (Ponstein et al. 1994). The CBP20 protein was purified from tobacco leaves inoculated with tobacco mosaic virus and contains an N-terminal chitin-binding domain. Sour orange and a110 are homozygous for ANER (revealed as a co-dominant CAPS) while FD is heterozygous. It has not been possible to place ANER within any *Poncirus* linkage group due to its strong segregation distortion. The other FD allele (not present in a110) is very rare in the progeny and all hybrids that carried it, and were inoculated in the first lot, died during the experiment although FD did not. Not all pathogenesis-related proteins might be harmless plant-defense enzymes depending on the genetic background.

It has been speculated in potato that *R* genes initiate responses that could result in HR, but some *R*-gene responses may prevent disease so effectively that the cell death is not activated (Hammond-Kosack and Jones 1997). However, Bendahmane et al. (1999) showed in both *Nicotiana* spp. and potato that ER is separate and epistatic to necrosis (HR). Gene segregation and epistasis would explain why some A×Pa hybrids might develop HR at the location of phloem-unloading giving rise to 'vein corking', while *P. trifoliata* presents extreme resistance to CTV and not HR. If sour orange (as rootstock) triggers a HR where CTV is unloaded below the graft union, irreversibly damaging its vascular tissue, then receiving *Ctv-R* from *P. trifoliata* might not avoid the decline of sweet orange grafted trees after CTV infection.

The genetic analysis of virus-plant interaction in the A×Pa family after a CTV chronic infection has shown the segregation of five types of interaction, which is not compatible with the hypothesis of a single gene controlling resistance. This would explain why in this population *Ctv-R* does not map between pY28 and pY65, but 15-cM far away. QTL analysis of CTV accumulation may be a more appropriate approach and will be presented in a separate paper (Asins et al. 2003). In brief, a major resistance QTL having its maximum LOD score between pY65 and pY28, and five other QTLs controlling CTV accumulation, have been identified.

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