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P. F. Mestre · M. J. Asíns
J. A. Pina · L. Navarro

Efficient search for new resistant genotypes to the citrus tristeza closterovirus in the orange subfamily Aurantioideae

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Abstract Virulent isolates of the citrus tristeza virus (CTV) are continuously arising and their spread threatens the world citrus industry. Methods for effective utilization of material conserved in germplasm banks are needed in plant improvement. Two objectives are pursued in the present paper: a search for new CTV-resistant genotypes and tests of two strategies for this search. One of these tests is based on a study of genetic relationships among genera and species of the orange subfamily and the other on scores of molecular markers known to be linked to the CTV-resistant locus.

Sampled plants were graft-inoculated with a mild CTV isolate (T-346) and two virulent ones (T-388 and T-305). Susceptible plants were those where CTV multiplication was detected beyond 4 months after inoculation. All cultivars of *Poncirus trifoliata* tested, as well as *Severinia buxifolia* and *Atalantia ceylanica*, were resistant to the three CTV isolates; *Fortunella crassifolia* (Meiwa kumquat) resists two of them. The finding of CTV resistance in this species, closely related to cultivated *Citrus* species, opens a new arena for CTV-resistance improvement of oranges and mandarines by sexual hybridization.

The searching strategy based on phylogenetic data has been successful, whereas the other one may be worthwhile only when the search is restricted to the species where linkage analysis is available. A good documentation system that allows quick sampling of accessions to build up core collections and where the location of new and useful genes could be easily worked out, is suggested to enhance germplasm utilization.

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P. F. Mestre · M. J. Asíns (✉) · L. Navarro
IVIA, Apartago Oficial, 46113 Moncada, Valencia, Spain

J. A. Pina
Servicio de Sanidad y certificación vegetal,
Conselleria de Agricultura y Pesca

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Introduction

The genus *Citrus* belongs to the subtribe Citrinae, tribe Citreae, subfamily Aurantioideae of the family Rutaceae. All Aurantioideae species are trees or shrubs with persistent leaves except for the three monotypic genera *Poncirus*, *Aegle* and *Feronia*, three species of *Clausena* and one of *Murraya*. *Citrus* is one of the major fruit crops in the world. Hybridization, apomixis and many centuries of cultivation have complicated *Citrus* taxonomy with the result that very different numbers of species have been proposed. Genetic diversity and relationships have been recently reviewed in the orange subfamily Aurantioidea (Herrero et al. 1996 a,b). Although the genus *Citrus* has many species (Herrero et al. 1996 a) the main cultivated species are sweet oranges [*Citrus sinensis* (L.) Osb], tangerines [*C. clementina* Hort. ex Tan. and *C. unshiu* (Mak.) Marc., mainly], grapefruits (*C. paradisi* Macf.) and lemons (*C. limon* L. Burm. f.). The cultivars of these species are always propagated vegetatively by bud-grafting onto a seedling rootstock in order to obtain a more uniform and early yielding tree.

In commercial citriculture, sour orange (*C. aurantium* L.) has been a universal rootstock that is well known for many attributes related especially to yield, fruit and juice quality as well as tolerance to cold temperatures and various soil conditions. However, it has one major weakness, it is highly susceptible to decline by isolates of the citrus tristeza virus (CTV).

Citrus tristeza virus (CTV) is an aphid-borne, phloem-limited closterovirus. Its virions are long flexuous particles about 11×2000 nm in size which contain a single-stranded RNA (approximately 20 kb) (Bar-Joseph et al. 1989). CTV is the causal agent of one of the

most important diseases of citrus. The total worldwide damage until 1989 was estimated to be about 50 million trees lost or unproductive as a result of CTV. Based on a conservative estimate of the average cost of a tree (US $ 25), which is very variable among countries, the economic losses could be estimated in hundreds of millions of dollars (Bar-Joseph et al. 1989). Despite efforts to diminish the damage caused by the disease (Navarro et al. 1988) it is still common in most citrus-growing areas (Bar-Joseph and Lee 1989; Marais 1991). Virulent isolates of CTV with destructive effects have been reported (Bar-Joseph and Lee 1989; Roistacher and Moreno 1991). The possibility of these severe isolates to spread, and the fact that new ones could arise by mutation, recombination, or just separation of more severe strains from a less virulent isolate (Moreno et al. 1993 a), makes CTV a constant threat to the citrus industry in the world.

CTV infects all *Citrus* species and varieties, most hybrids, and some *Citrus* relatives. There are only three *Citrus* relatives which have been reported to be resistant to CTV (Yoshida et al. 1983; Garnsey et al. 1987; Bar-Joseph et al. 1989): *Severinia buxifolia* (Poir.) Tenore, *Swinglea glutinosa* (Blanco) Merr. and *Poncirus trifoliata* (L.) Raf. The resistance to CTV in *P. trifoliata* is conferred by one dominant gene and molecular markers linked to this gene have been reported (Gmitter et al. 1996; Mestre et al. 1997). However this reported CTV-resistant species (the only sexually compatible species of the three with *Citrus* spp.) is distantly related to citrus (Herrero et al. 1996 b). This has negative repercussions on the viability of some genetic combinations in progenies of crosses involving *Citrus* and *Poncirus*, and on the ratio of genetic/physical distance between the CTV resistance gene and marker loci. Therefore, the search for new CTV-resistant genotypes more closely related to the cultivated *Citrus* species would provide genetically diverse sources for durable resistance and allow citrus breeding programs and map-based cloning experiments to be more efficient.

Germplasm collections of major crop plants continue to grow in number and size around the world. To-day, better access to and use of the genetic resources in collections have become important issues. However, the very large size and heterogeneous structure of collections have hindered efforts to increase the use of germplasm-bank material in plant improvement. Identification of different resistance genes requires allelism tests which involve extensive crossing and progeny evaluation after inoculation. Strategies to efficiently find resistant genotypes would enhance the use of genetic resources.

Two objectives are pursued in the present paper: to find new CTV-resistant genotypes and to evaluate two searching strategies. One of these is a sampling strategy based on choosing only those species related to previously known CTV-resistant species following a study of genetic relationships among *Citrus* and *Citrus*-related species (Herrero et al. 1996 b). The other is a marker-assisted screening using molecular markers known to be linked to the CTV-resistance locus of *P. trifoliata* (Mestre et al. 1997).

Materials and methods

Names of species and cultivars that were sampled are shown in Table 1 and belong to the Citrus Germplasm Bank at IVIA (Valencia, Spain). They are mature, virus (and virus-like)-free plants grown in containers kept in a screenhouse (Navarro et al. 1988). The choice of this material followed the relationships deduced from a minimum spanning tree based on the chord distance of Cavalli-Sforza and Edwards (1967) that included the main species of the orange subfamily Aurantioideae (Herrero et al. 1996 b). Hence, the nearest species to previously described resistant one (*S. buxifolia*, *S. glutinosa* and *P. trifoliata*) were sampled to build up a putative CTV-resistant core collection (Fig. 1). Thus, *Microcitrus australis* (Planch) Swing. (MIS), the closest species to both *S. glutinosa* (WIN) and *S. buxifolia* (SEV), was sampled; similarly, *Atalantia ceylanica* (Arn.) Oliv. (ACE) that is the closest to SEV, and *Fortunella crassifolia* Swing. (FCR) and *F. hindsii* (Planch) Swing. (FHI), the most closely related species, in this order, to *P. trifoliata* (PON), were also

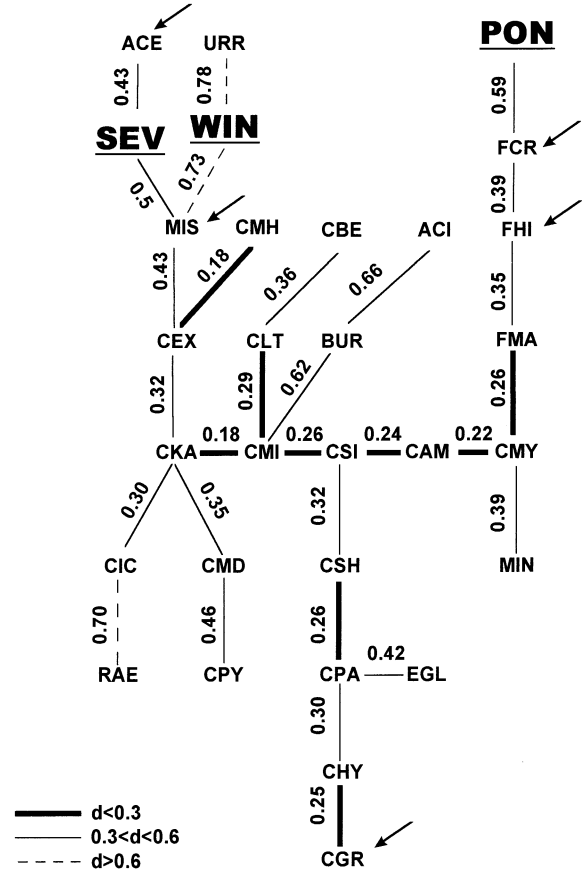

Fig. 1 Relevant part of the minimum spanning tree of *Citrus* species based on the chord distance of Cavalli-Sforza and Edwards (1967) from Herrero et al. (1996). *Arrows* point to species sampled in the present survey and *large fonts* indicate previously reported CTV-resistant species. Relevant abbreviations are found in Materials and methods

sampled. Although *Murraya paniculata* (L.) Jack (URR) is also close to *S. glutinosa* it was not included in this survey because it is so distantly related to *Citrus* that certain clones, like the one we have, are not graft-compatible with citrus. *Citrus grandis* (L.) Osb cv "Cuban Shaddock" (CGR) was included because it had been previously reported as CTV resistant (Yoshida et al. 1983) and is closely related to the cultivated *Citrus* species (although it is distant from SEV, WIN or PON in Fig. 1).

Graft propagations of all plants were done on healthy "Rough Lemon" (*Citrus jambhiri* Lush) as rootstock and then cultured in containers in a greenhouse at 18–26°C. Each plant was graft inoculated with two patches of infected tissue and pruned to force new growth. Three CTV isolates were used: T-346, T-388, and T-305. T-346 is a mild isolate, while both T-388 and T-305 are virulent ones (Ballester-Olmos et al. 1988, 1993; Moreno et al. 1993 b). Not all accession-isolate combinations were employed. Thus, most genotypes known to be CTV resistant were not inoculated with the mild isolate and some accessions that were known to be susceptible to the mild isolate were not inoculated with severe isolates (symbol □ in Table 1).

Multiplication of CTV in plants was checked by DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay) using the mix of monoclonal antibodies 3CA5 and 3DF1 as described in Sánchez-Vizcaíno and Cambra (1987). A reaction was declared positive when the value of its optical density at 405 nm was at least twice that of the healthy (control) samples. Additionally, 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). These analyses of virus multiplication were carried out at four different times: 4, 6, 10 and 12 months after inoculation.

Genomic DNA extractions followed the method of Dellaporta et al. (1983). The clones cE20, cK16, cW18 and cG18, which reveal RFLP (restriction fragment length polymorphism) loci linked to the CTV resistance gene in *P. trifoliata*, were used as non-radioactive labelled probes in the RFLP analysis, as described by Mestre et al. (1997).

Results

The hybridization of the different clones to Southern blots containing digestions of genomic DNA from species included in Table 1 are shown in Fig. 2.

Clones cE20 and cK16 yielded a wide spectrum of multi-banding patterns. Although *P. trifoliata* cultivars showed very similar patterns, both clones distringuished "Flying Dragon" and "Hiryu" from "Pomeroy" and "Benecke" [the last two cultivars differ in marker alleles linked to the CTV-susceptibility allele (*r*)]. Only *A. ceylanica* presented a band (using cE20) of identical mobility as the band associated with the resistance in *P. trifoliata* (*R*).

Clone cW18 produced a simple band pattern in all the species studied. *P. trifoliata* cultivars were all heterozygous. *C. grandis*, *M. australis* and both *Fortunella* species were homozygous for an RFLP allele with a similar size to that associated with the putative susceptibility allele. *S. buxifolia* and *A. ceylanica* had different two-band patterns and differed from *P. trifoliata*.

All *P. trifoliata* cultivars tested with clone cG18 were heterozygous for the same alleles. The other species showed bands similar in size to those of the alleles of *P. trifoliata*, ranging from the same size as the putative susceptibility allele (*F. crassifolia*) to almost the same size as the putative resistance allele (*F. hindsii*).

The detection of CTV by DAS-ELISA agreed with that of DTBIA. The results are shown in Table 1. All rootstocks ("Rough lemon") where species and cultivars were grafted to be challenged with CTV gave a clear positive reaction, demonstrating the effectiveness of the inoculation method. Differences in response were found only in *F. crassifolia*, where T-346 was not detected while the T-388 CTV isolate, was, although at a low titer. Almost all plant-isolate combinations gave consistent results regarding virus multiplication through time. The exception was again *F. crassifolia* which, when inoculated with isolate T-305, gave a clear positive reaction in the 4th month after inoculation, but a negative reaction in all subsequent analysis. Analysis of *F. hindsii* inoculated with isolate T-346, and *C. grandis* with any isolate, gave low virus titers in DAS-ELISA and weak and scattered marks in DTBIA, when compared to rootstocks or other susceptible plants. This restriction of virus accumulation was more evident in mature areas of the plant, where, in some analyses, virus could not be detected (Fig. 3).

Discussion

Searching strategies

The use of genetically diverse resistance sources is important in breeding for durable disease resistance; however, detection and evaluation of resistance genes by conventional inheritance experiments require laborious screening and genetic testing. The existence of a long juvenile period makes such objectives almost unapproachable in citrus. Two searching strategies have been considered in this paper to find new sources of CTV resistance. One is a sampling strategy based on choosing only species related to previously known, CTV-resistant, species following a study of genetic relationships among *Citrus* and *Citrus*-related species (Herrero et al. 1996 b); the other is marker-assisted screening using molecular markers known to be linked to the CTV resistance locus in *P. trifoliata* (Mestre et al. 1997). Only the first strategy proved to be successful; it was based on a phylogenetic analysis by Herrero et al. (1996 b). Two accessions sampled using this strategy were found to be CTV resistant. This, additionally, confirms the relationships between *A. ceylanica* and *S. buxifolia* and among *P. trifoliata*, *F. crassifolia* and *F. hindsii*, and suggests that resistance genes from *A. ceylanica*, *S. glutinosa*, and *P. trifoliata* were lost from *M. australis* and *F. hindsii* (see Fig. 1). Confirming the efficiency of this searching strategy, Yoshida (1996) has recently found no evidence of CTV infection in

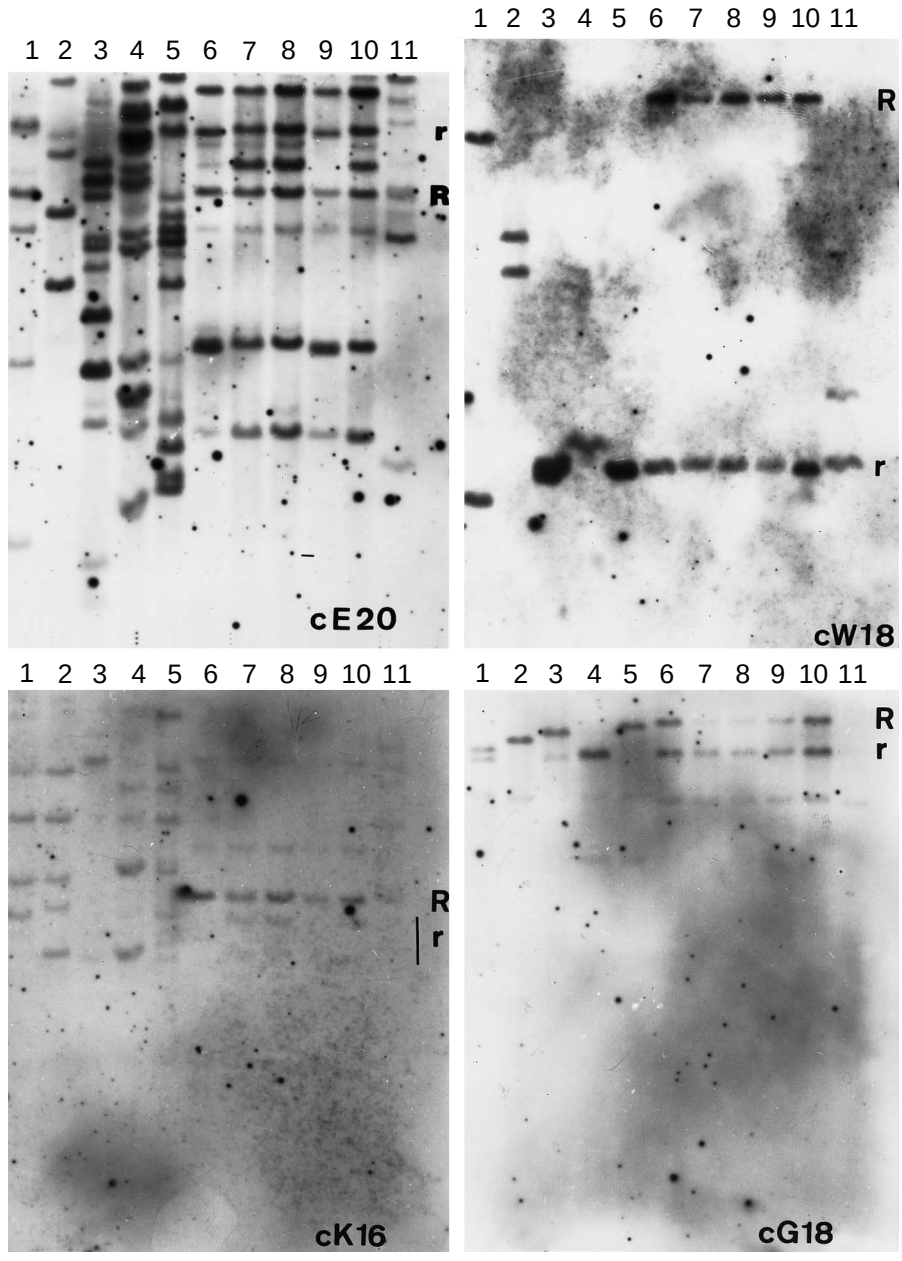

Fig. 2 RFLP analysis of accessions using DNA clones cG18, cE20, cW18 and cK16 as non-radioactive labelled probes. *R* is the RFLP allele associated with the CTV resistance response in *P. trifoliata* and *r* is its alternative allele, associated with the susceptible response. *1*: *A. ceylanica*; *2*: *S. buxifolia*; *3*: *M. australis*; *4*: *F*, *cassifolia*; *5*: *F. hindssi*; *6*: *P. trifoliata* cv "Benecke"; *7*: *P. trifoliata* cv "Hiryu" clone 1; *8*: the same as *6* but clone 2; *9*: *P. trifoliata* cv "Pomeroy", *10*: *P. trifoliata* cv "Flying dragon"; *11*: *C. grandis* cv "Cuban shaddock"

M. paniculata (URR in Fig. 1), which agrees with its phylogenic grouping with *S. glutinosa* (WIN).

The failure of the marker-assisted screening strategy must be interpreted with caution. All *P. trifoliata* cultivars tested behave as resistant ones and carry RFLP alleles linked, in coupling phase, to the resistance allele *R* in all the linked markers assayed. The only difference among these accessions concerned the RFLP allele putatively linked to the susceptibility allele *r*. Among other cultivars tested, only *A. ceylanica* shows a RFLP allele (with cE20 only) identical to that linked, in coupling phase, to the resistance gene in *P. trifoliata*. This coincides with the resistance of this accession to all CTV isolates employed. However, following this criterion (presence of the *R* allele, at least at one RFLP locus) *F. crassifolia* would have been rejected. Should a more flexible criterion be used as, for instance, to show a band with similar mobility to the one associated to the resistance in *P. trifoliata*, *F. crassifolia* (which is resistant against two CTV isolates) would still have been rejected, while accessions of *F. hindsii* and *M. australis* (both susceptible) would have been selected as new putative resistant genotypes due to their RFLP genotype for cG18. It is clear that mere similarities in the size of the RFLP alleles are misleading. Two different explanations are possible: first, new CTV-resistant genotypes may differ in the locus (loci) controlling resistance from that mapped in *P. trifoliata*; second,

Table 1 Results of the CTV-resistance response beyond 4 months after inoculation of the species and cultivars included in the survey

Species	Accession number	CTV isolates [a]		
		T-346	T-388	T-305
Atalantica ceylanica (Arn.) Oliv.	IVIA C-172	−	−	−
Severinia buxifolia (Poir.) Tenore	IVIA C-147	□	−	−
Microcitrus australis (Planch) Swing.	IVIA C-313	+	+	+
Fortunella crassifolia Swing.	IVIA C-280	−	+	−
Fortunella hindsii (Planch) Swing.	IVIA C-281	+	□	□
Poncirus trifoliata (L.) Raf. cv Benecke	IVIA C-376	□	−	−
Poncirus trifoliata (L.) cv Hiryu	IVIA C-373	□	−	−
Poncirus trifoliata (L.) cv Pomeroy	IVIA C-374	□	−	−
Poncirus trifoliata (L.) cv Rich 7-5	IVIA C-236	−	−	−
Citrus grandis (L.) Osb. cv Cuban Shaddock	IVIA C-428	+	+	+

[a] □: not analyzed, +: CTV detected; −: CTV not detected

allelic relationships may differ in different species. Structural chromosomal changes (inversions and translocations) have frequently been found in citrus (Iwamasa and Nito 1988; Guerra 1993). In fact, differences regarding the genetic maps of *Citrus* and *Poncirus* have already been reported (Durham et al. 1992; Jarrell et al. 1992). Therefore, this strategy might be very useful when the search is carried out within the species where the linkage analysis was developed, although it may fail due to recombination or sequence changes as reported by Dax et al. (1994) and Yu et al. (1996).

New CTV-resistant genotypes

All cultivars of *P. trifoliata* tested, as well as accessions of *S. buxifolia* and *A. ceylanica*, showed resistance to all CTV isolates. These results agree with previous reports (Garnsey et al. 1997; Bar-Joseph et al. 1989) for the two former species, but this is the first time that resistance to CTV has been reported for *A. ceylanica*. A second *S. buxifolia* accession (IVIA C-282) was found to suffer CTV infection when inoculated with the mild CTV isolate T-300. This result agrees with Yoshida (1996). Given the distant relationship between *P. trifoliata* and *S. buxifolia* or *A. ceylanica* (Herrero et al. 1996 b), the genes involved in CTV resistance most probably are different in origin, although equally effective. The effectiveness of the resistance against a wide spectrum of isolates of the virus makes the genes suitable candidates for breeding or genetic engineering purposes.

M. australis was very susceptible to the CTV isolates tested, which agrees with previous reports (Bar-Joseph and Lee 1989; Yoshida 1996); it even showed clear symptoms such as "vein clearing" and "stem pitting". *F. hindsii* and *C. grandis* cv "Cuban Shaddock" showed relatively low levels of virus multiplication (see Fig. 3). Recently, Garnsey et al. (1997) have reported that the level of CTV infection in *C. grandis* varied according to the cultivar and CTV isolate used, suggesting that *C. grandis* presents resistance different from that existing in *P. trifoliata*. The restriction to virus accumulation that we found in *C. grandis*, might be an expression of such resistance. Analysis of *F. crassifolia* resulted in clear differences against isolates T-346 (never detected), T-388 (always detected) and T-305 (detected at first analysis and not detected at the three subsequent ones). Given that virus isolates differ in genotypes composition (Moreno et al. 1993 a), an explanation for this behavior might be that a certain level of some virus component might be necessary in order to trigger the resistance response by *F. crassifolia*. Then, isolate T-346 would be over the threshold level of this component, isolate T-388 would lack it while isolate T-305 would have it but at a very low level.

To our knowledge, this is the first time that CTV resistance has been reported in *F. crassifolia*. The plant-pathogen interaction between *F. crassifolia* and CTV seems to be complex and very variable; thus, Yoshida et al. (1983) also reported an accession of *F. crassifolia* as susceptible to a severe CTV-SY (seedling yellows) strain. Although the resistance found in the accession we have used seems not to be effective against all severe CTV isolates, there may be other accessions that resist a wider spectrum of CTV isolates, like the variability reported for CTV resistance among accessions of *S. buxifolia*. Up to now no citrus cultivar with edible fruits has proven to be resistant to CTV. Although *P. trifolialata* is commonly used in breeding programs for citrus rootstock as a CTV-resistance donor, it can not be used for variety (sweet orange or tangerine) improvement because it is a wild species with not edible fruits, and fruit characters would be lost by recombination and hybridizaton. In contrast to *P. trifoliata*, *F. crassifolia* (Meiwa kumquat) yields edible fruits. Since the genus *Fortunella* is more closely related to *Citrus* than *Poncirus*, and is the closest genus to the most important scion cultivars, sweet orange and mandarins (Herrero et al. 1996 b), a new possibility is open for their CTV-resistance improvement by means of sexual hybridization. Additionally, large segregant families where genetic and physical distances are likely

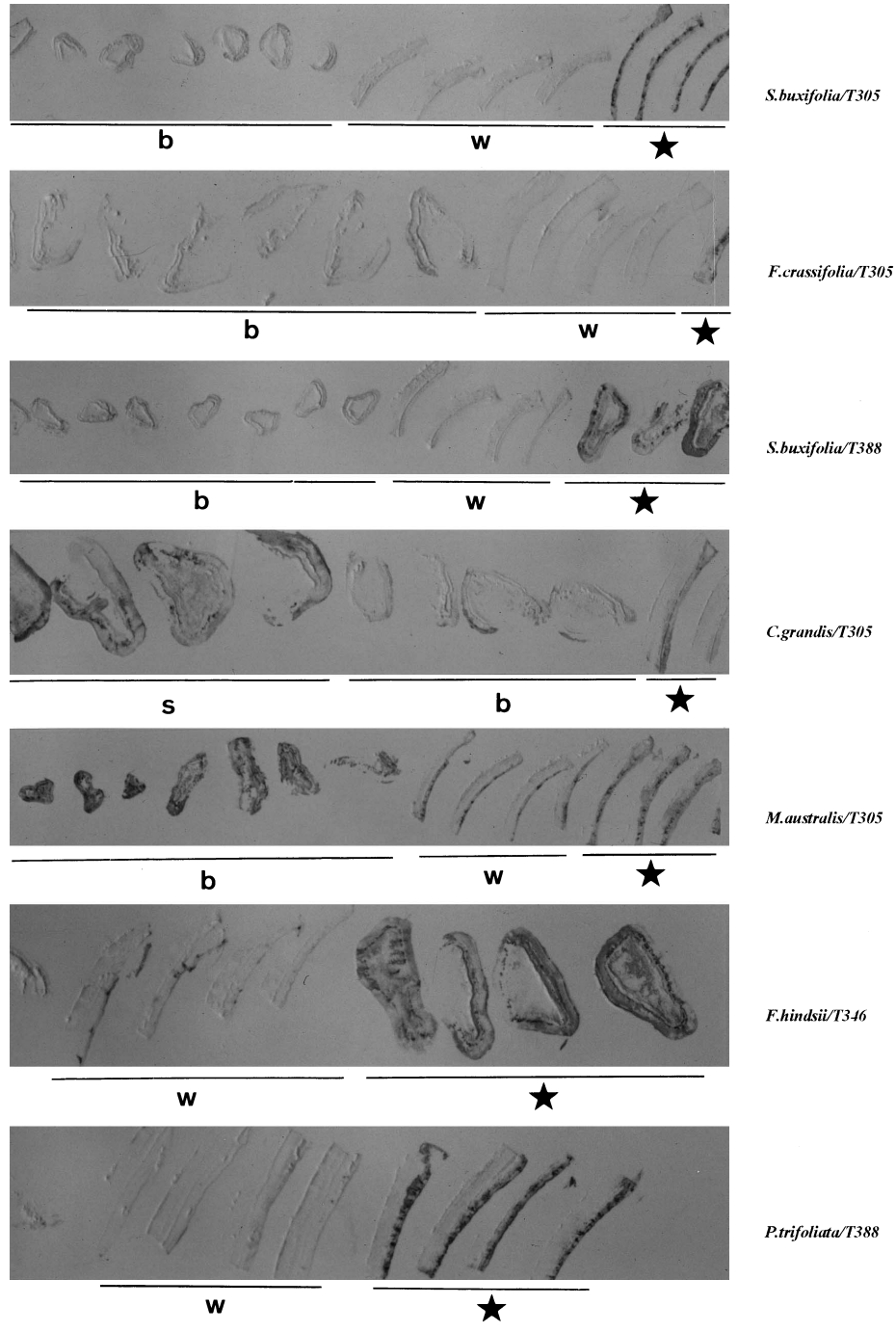

Fig. 3 Direct tissue-blot immunoassays for some of the species/CTV isolate combinations. * means rootstock (always infected). The other prints correspond to different parts of the challenged plant. *w*: bark rectangle; *b*: branch and *s*: shoot

to be more coincident should be possible, given the relatedness of *Fortunella* to *Citrus*. This would represent a very favorable situation for fine mapping of the CTV resistance gene in order to attempt its map-based cloning. Efforts should now be focused on the evaluation of more accessions of *F. crassifolia* for CTV resistance and on an analysis of the resistance, including its inheritance, genetic location, functioning and efficiency against different CTV isolates.

Finding new resistant genotypes, very different from each other as revealed by isozyme (Herrero et al. 1996 a) and RFLP analysis (Fig. 2), will help in the fight against CTV. New resistant genotypes may differ in resistance alleles and could be used to control the continuously growing genetic variability of the virus. This strategy is especially important in the case of an RNA virus because of its high mutation rate. Cases of resistance-breaking isolates have been reported for several virus-host systems (Fraser 1990), and some authors have suggested the combined use of different resistance sources as an efficient way to combat RNA viruses (Hull 1994; Scholthof et al. 1993). Finding new

resistant genotypes will also help in designing more experiments to study the virus-plant interaction comparatively and unveil how the resistance gene of the plant makes the virus unable to multiply or to move through the plant.

Information on phylogeny has been shown to be very useful for establishing sampling strategies to quickly find new genetic sources for plant breeding and achieve an efficient use of germplasm banks. Our results suggest that phylogenetic analysis should be included in the documentation system of germplasm banks and that they should be up-dated when accessions are added.

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