Molecular markers: a continuously growing biotechnology area to help citrus improvement

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Abstract — Introduction. In order to apply a marker-assisted selection to the breeding programs, a review related to the application of the molecular marker technique, used to study the citrus genetic resources and the resistance to citrus tristeza virus (CTV), was undertaken. **Citrus genetic resources**. The citrus germplasm bank at Ivia (Valencia, Spain) was studied. Six isoenzymatic systems were assayed. A broad spectrum of heterozygosity values was found in the collection. Two principal groups of *Citrus* species are clearly defined: the orange-mandarin group and the lime-lemon-citron-pummelo group. Genetic differences between species and genera are in general high, which suggest that adaptation has played an important role during the evolution of the orange subfamily. **CTV resistance**. There is an urgent need to diversify the genetic basis of CTV-resistant rootstocks by developing breeding programs from only CTV-resistant genotypes. Consequently, the first step was to find molecular markers which allow to discard CTV-susceptible genotypes with a minimum error from large progenies. At least two loci, *Ctr* and *Ctm*, control CTV resistance in *Poncirus trifoliata*. **Utilization of citrus genetic resources for searching new CTV resistance genotypes**. To find out new CTV-resistant genotypes two searching strategies explained in this paper were evaluated, but only one has been truly successful. **Perspectives and further remarks**. The rapid advances of biotechnology give citrus breeders no choice other than to design more efficient breeding programs. (© Elsevier, Paris)

Spain / *Citrus* / plant breeding / plant biotechnology / pest resistances / viroses / virosis / genetic marker

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Utilisation des marqueurs moléculaires : une technique en perpétuelle évolution qui vient en aide à l'amélioration des agrumes.

Résumé — Introduction. Afin d'appliquer une sélection assistée par marqueurs à des programmes d'amélioration, les diverses applications de la technique des marqueurs moléculaires à l'étude des ressources génétiques des agrumes et à celle de la résistance au virus de la tristeza des agrumes (CTV) ont été répertoriées. **Ressources génétiques des agrumes**. La collection d'agrumes d'IVIA (Valence, Espagne) a été criblée à partir de l'utilisation de six sytèmes isoenzymatiques. Un large spectre de structures hétérozygotes a été trouvé. Deux groupes principaux ont été clairement définis au sein du genre de *Citrus* : le groupe des orange-mandarine et celui des lime-citron-cédrat-pamplemousse. Les différences génétiques entre espèces et genres sont en général importantes, ce qui suggère que l'adaptation a joué un rôle capital lors de l'évolution de la sous-famille des oranges. **Résistance au CTV**. Il devient urgent d'élargir la base génétique des porte-greffes résistants au CTV, par développement de programmes d'amélioration à partir de génotypes résistants. Une première étape a donc consisté à trouver des marqueurs moléculaires permettant d'éliminer, sans trop d'erreurs, au sein de vastes descendances, les génotypes sensibles au CTV. La résistance au CTV serait contrôlée au moins par deux loci, *Ctr* and *Ctm*. **Utilisation des ressources génétiques des agrumes pour chercher de nouveaux génotypes résistant au CTV**. Afin de trouver de nouveaux génotypes résistant au CTV, deux stratégies de recherches présentées dans le document ont été évaluées ; seule l'une d'elles a donné des résultats satisfaisants. **Perspectives et remarques**. La rapide progression des biotechnologies oblige les sélectionneurs d'agrumes à prendre en compte ces techniques pour la définition de programmes d'amélioration plus performants. (© Elsevier, Paris)

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1. introduction

The use of polymorphic single genes to facilitate the process of plant breeding was proposed by Sax [1]. The basic principle is that selection for characters with easily detectable phenotypes can simplify the recovery of genes of interest linked to them and more difficult to score such as those governing disease resistance or yield under a stress condition. The first marker loci available were those with an obvious impact on the morphology of the plant, like the trifoliate leaf inherited from *Poncirus trifoliata*. New sources of genetic markers based on the identification of polymorphisms in proteins (isozymes) and DNA have been developed during the last three decades. They have been termed 'molecular markers'. Therefore a molecular marker is a difference, that may exist between two plants, visualized by means of biochemical methods, and is located at a certain chromosomal position.

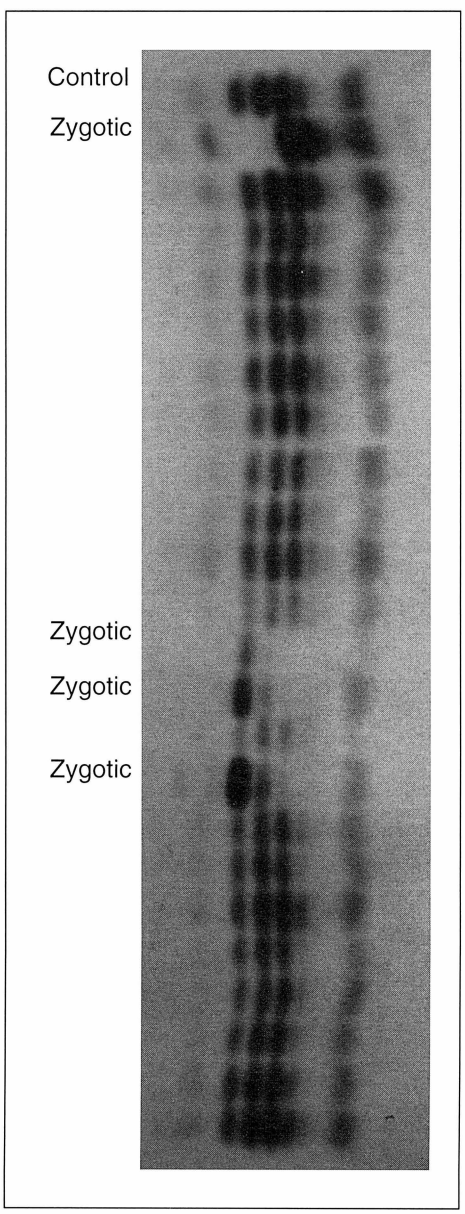

Figure 1.
Phosphoglucoisomerase zymograms to distinguish zygotic from nucellar *Poncirus trifoliata* seedlings.
Control: mother plant zymogram.

When the genotype for one or more marker loci is known for two plants, inferences can be made about the genotypes that might appear in the progeny. Hence, given an individual of dubious origin, it is possible to determine by its marker genotype whether it might derive from a certain cross or not. Many practical problems in citrus breeding respond to situations as simple as this. The most common applications of molecular markers in citrus breeding are: identification of true hybrids or zygotic individuals from apomictic (nucellar) seedlings *(figure 1)*; identification of new varieties (*figure 2*); checking the genetic composition of protoplast fusion products; and checking the stability of cryogenic *calli*.

The quick and wide development of molecular markers has provided very useful tools to help plant breeders. They allow the study of genetic variation, the search of new sources for agronomically important genes, the identification of cultivars and the efficient management of segregant generations during the plant breeding programs through marker-assisted selection.

The purpose of this paper is to review our most important results related to the application of molecular markers for evaluating, among our citrus genetic resources, the resistance to citrus tristeza virus (CTV) in order to apply marker-assisted selection in the breeding programs and the utilization of citrus genetic resources in an efficient search of new CTV resistance genotypes.

2. citrus genetic resources

How much genetic variability should the citrus genetic resources contain? Given that economical changes, evolution of pathogens and environmental changes are unforeseen, there are no clear-cut rules about what should be needed in the future; therefore, the maximum genetic diversity should be conserved and characterized. Some authors (i.e., [2]) suggest that this open-ended task will no longer be important when progress in genetic engineering may allow transfer of useful genes from any species into citrus. However, it is difficult to imagine that techniques used for specific loci might replace the natural association of genes as a source of co-adapted gene complexes. In fact, as genetic engineering progresses, there will probably be an increasing demand for germplasm resources as source material for gene transfer. On the other hand, many important traits in plant breeding exhibit continuous variation (yield, maturity, biotic and abiotic stress tolerance, etc.). For these traits, germplasm resources and established breeding methods, aided at selection by molecular markers, have no current alternative.

After clarifying the need for germplasm conservation the next question is: How much genetic variability is contained in a citrus germ-plasm bank? The answer has been reported recently by Herrero et al. [3, 4].

We have studied the genetic diversity contained in the citrus germplasm bank at IVIA (Valencia, Spain). It consisted of 198 cultivars and accessions of 54 species of *Citrus* and 13 related genera of Aurantioideae subfamily. They are mature, virus and virus-like free plants grown in containers kept under a screenhouse [5]. Six isoenzymatic systems (seven loci as described by Herrero et al. [3]) were assayed following methods described in Asíns et al. [6]. The chord distance [7] and the neighbour-joining method of aggregation [8] were used to obtain the dendrograms where genetic relationships were studied at the genus and species level.

Herrero et al. [3] found that the species with the lowest genotypic variability are *C. myrtifolia, C. deliciosa* (Willow leaf mandarin), *C. paradisi, C. limon* and *C. sinensis* while *Severinia buxifolia* shows the highest value. Not all citrus are highly heterozygous but a broad spectrum of heterozygosity values was found in the collection. Lemons, limes and *C. bergamia* show a very high percentage of heterozygosity which indicates an origin through interspecific hybridization. Regarding the intraspecific variability, its main limiting factor is the apomictic reproduction, where nucellar embryos are much

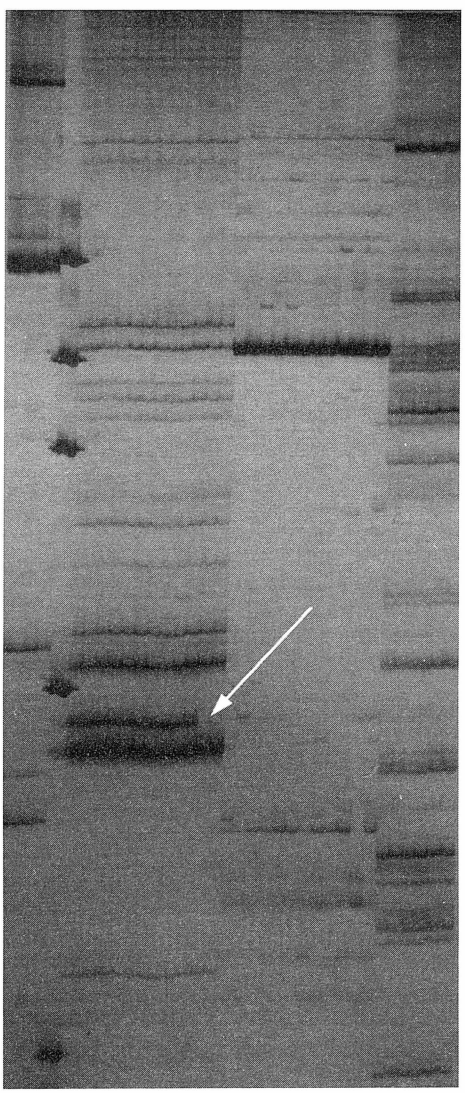

Figure 2.
RAPDs identifying a lemon cultivar. Arrow indicates the genetic difference.

more vigorous than the zygotic ones. Additionally, self pollination appears in some species mainly used as rootstock which would explain their low heterozygosity values.

Two principal groups of *Citrus* species are clearly defined (*figure 3*): the orange-mandarin group and the lime-lemon-citron-pummelo group where the pummelo and the lime-lemon-citron groups cluster. *Microcitrus* is closer to *Citrus* than *Fortunella*, however, a more realistic situation is found when species are considered separately given that the *Citrus* genus is very broad from the genetic point of view. Thus, *Microcitrus* spp. are related to the citron-lime group while *Fortunella* spp. are related to the orange-mandarin group. *Poncirus*, although included in the true citrus group, is located far from the rest of genera of this group. *Atalantia* is one of the most variable genera, even for heterozygosity. Both *Atalantia* species are very distantly placed; thus *A. ceylanica* clusters with *S. buxifolia* while *A. citroides* clusters with *Pamburus missionis*. We consider these three somehow related genera *Atalantia*, *Severinia* and *Pamburus* as important genetic resources for citrus rootstock breeding. Their sexual isolation could be overcome by the use of protoplast fusion methodologies or trying *Microcitrus* spp. as a bridge species towards *Citrus*.

Three subgroups were found in the orange-mandarin group. The sour orange subgroup includes *C. clementina*, *C. tangerina* and *C. nobilis* mandarins together with *C. myrtifolia* and *C. aurantium*. The sweet orange (*C. sinensis*) subgroup includes C. *temple* and *C. unshiu* mandarins. The third subgroup includes the rest of the mandarin species. *C. madurensis* clusters with this subgroup. If sweet and sour orange are considered different species, it does not seem logical to put together all mandarins in a single species, *C. reticulata,* as Swingle and Reece [9] do. The orange-fruited *Citrus* species form a compact group that we have named the orange-mandarin group which is connected with *Fortunella* spp. (also with orange-coloured fruits) and *C. halimii*. This situation closely resembles that found in *Lycopersicon* spp. where the three red-fruited species form a natural assembly within the genus [10]. *A. chevalieri* is closely related to the pummelo subgroup. The citron, the pummelo and the ancient lemon subgroups form a cluster to which the species belonging to subgenus Papeda and the cultivated limes, lemons and bergamots are related. *Microcitrus* spp., to which *S. buxifolia* and *A. ceylanica* seem to be related, are closer to the lime-lemon-citron-pummelo group than to the orange-mandarin one. These relationships suggest the further prospecting of certain key species such as *C. halimii*, *Aeglopsis chevalieri*, *C. ichangensis*, *C. hystrix*, *S. buxifolia* and *C. tachibana*.

In conclusion, genetic differences between species and genera are in general high, which suggests that adaptation has played an important role during the evolution of the orange subfamily. As far as citrus improvement is concerned, a broad distribution of species has been found that should be taken into account to sample genotypes in the search of new genes controlling desired features in order to fully and efficiently use citrus genetic resources.

3. citrus tristeza virus resistance

Citrus tristeza virus (CTV) is the causal agent of one of the most important diseases of citrus [11]. This phloem-limited closterovirus exists in a great variety of isolates differing in biological properties such as symptoms in the field [12–14], reaction on indicator plants [15, 16], and aphid transmissibility [17, 18]. Since its outbreak in the early 1930's, tristeza has caused the death of millions of trees grafted on sour orange all around the world [11]. This disease has forced growers to use only CTV-resistant or tolerant rootstock cultivars which have strongly narrowed their genetic diversity, increasing so the vulnerability of citriculture to future pathogens and environmental changes. Therefore, there is an urgent need to diversify the genetic basis of CTV-resistant

rootstocks by developing breeding programs from only CTV-resistant genotypes. Consequently, the first step was to find molecular markers which allow to discard CTV-susceptible genotypes with a minimum error from large progenies.

Most, if not all, citrus species and cultivars are hosts of CTV [11, 19, 20], but some citrus relatives (*S. buxifolia, S. glutinosa* and *P. trifoliata*) have been reported as resistant to CTV [19–21]. Only *P. trifoliata* is sexually compatible with *Citrus* spp.,

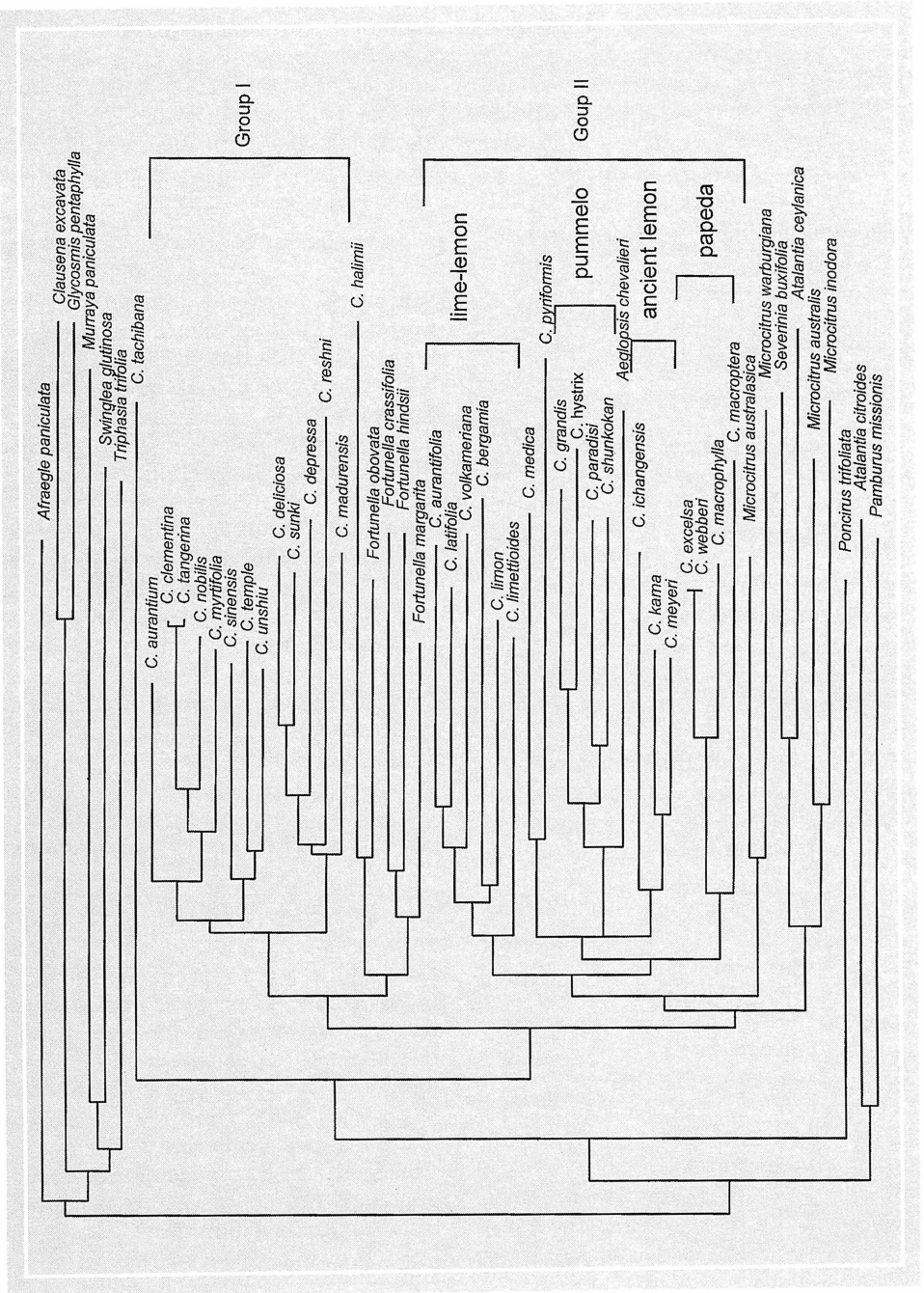

Figure 3.
Dendrogram of Aurantioideae species based on the chord distance and the neighbour-joining clustering method (group I = orange-mandarin group; group II = lime-lemon-citron-pummelo group).

therefore it is the source of CTV resistance in rootstock breeding programs. In order to analyze the inheritance of this character and to find out markers linked to the responsible gene(s), two populations were obtained: one population by open pollination of *P. 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' (C × P population). Isozyme analysis of plants from FD population was carried out in order to distinguish zygotic from nucellar seedlings (*figure 2*). Inoculation was made by propagating buds of each plant on rootstocks infected with CTV isolate T-346, a common tristeza isolate. The presence of virus was checked by Double Antibody Sandwich Enzyme-Linked Assay and Direct Tissue Blot Immunoassay at 3, 6 and 12 months after inoculation.

Segregation data are consistent with a monogenic control of the trait where resistance allele is dominant. The screening of 260 random primers against susceptible and resistant pools resulted in the identification of five new random amplified polymorphic DNA (RAPD) markers linked to the CTV-resistance gene [22]. Markers obtained with primers OPW18, OPE20, OPK16 and OPG18 were successfully cloned and named cW18, cE20, cK16 and cG18. Hybridization of these clones to Southern blots containing digestions of genomic DNA from plants from 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 converting the dominant into a codominant marker. Nowadays, these markers are used to perform marker-assisted selection (MAS) in the seedling progenies derived from *P. trifoliata*, to discard up to a 50% of them, those that lack the CTV-resistance gene (named *Ctr-R*). Therefore, these markers allow us to run field experiments with only elite (putative CTV-resistance) trees.

However, nothing was known about the mechanism underlying this resistance *Ctr-R*. Although the dominance of this gene and its efficiency against CTV-aggressive isolates makes *P. trifoliata* a suitable candidate for citrus breeding, it had to be taken into account that citrus is a perennial crop; then, resistance genes causing just a reduction in virus multiplication or movement might not be so suitable in breeding for a durable CTV resistance as those causing a complete suppression of virus multiplication or movement. Better knowledge of the mechanism of resistance is needed to ensure long-term protection of citrus against CTV.

In order to study the underlying mechanism to *Ctr-R*, the presence of CTV was checked not only in the new flushes but mainly around the inoculation site, 1 year after the inoculation of 24 plants, 17 bearing *Ctr-R* (CTV resistant) and 7 lacking it (CTV susceptible), *P. trifoliata* var. 'Flying Dragon' (the mature parental plant) and two nucellar plants (i.e., with the same genotype as *P. trifoliata* var. 'Flying Dragon', but juvenile). As expected, analysis of the new flushes of the FD plants revealed only CTV presence in those plants lacking *Ctr-R*. Analysis of sweet orange grafted onto the FD segregants at *Ctr* 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 7 plants *Ctr-r* and in 5 out of 17 plants *Ctr-R*; in the remaining 12 plants, CTV was never detected or it was barely detected in only one of the three patches studied. Citrus tristeza virus was never found anywhere in the parental nor in the two nucellar plants studied. This result can not be explained only by a gene-dosage dependence of the resistance, because short distance accumulation of CTV has never been found in FD (the heterozygous parental) nor in its two nucellar plants.

Therefore, this result suggests that *Ctr* is not the only gene responsible for resistance to CTV in FD, but there must exist at least another dominant gene involved in this resistance. The FD population would be heterozygous at both loci, *Ctr* and a new locus we have named *Ctm*. From the group of plants bearing *Ctr-R*, pools of plants differing in CTV short distance accumulation were made. Screening of 180 random primers resulted in the identification of five RAPD markers linked to *Ctm* [23].

Therefore, 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. Our results open the question of whether this broad spectrum resistance is due to the interaction of a very conserved viral domain with *Ctr*, or it is due to the impossibility 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 at short distance (cell-to-cell movement), it would infect the whole plant after a more or less long period of time. The finding of *Ctm* should be taken into account in the breeding program, and individuals with the appropriate alleles at both regions of the genome (*Ctr* and *Ctm*) should be selected in order to ensure a durable disease resistance. The screening of markers linked to these loci will allow now to discard up to 75% of the seedling progenies derived from *P. trifoliata* at an early stage during the rootstock breeding program because they lack *Ctr-R* and *Ctm-M*.

4. utilization of citrus genetic resources for searching new CTV resistance genotypes

Germplasm collections of major crop plants continue to grow in number and size around the world. Today, 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 gene bank material in plant improvement. Efficient searching strategies would enhance the use of genetic resources.

P. trifoliata is far related to citrus [4]. This fact has negative impact on the viability of some genetic combinations in the progenies of crosses involving citrus and *Poncirus*, and on the ratio genetic/physic distance between agronomically important genes and marker loci. The search of 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; therefore, we tried to find out new CTV-resistant genotypes and to evaluate two searching strategies. One 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 [4] and the other is a marker-assisted screening using molecular markers known to be linked to the CTV-resistance locus of *P. trifoliata* [22]. Only the first one has been really shown to be successful and it additionally proves the quality of the previous phylogenetic analysis [4] on which the sampling strategy was based. All cultivars of *P. trifoliata* tested, *S. buxifolia* and *A. ceylanica* behave as resistant against the three CTV isolates and *Fortunella crassifolia* (Meiwa kumquat) resists two of them [24]. Thus, two accessions sampled, using this strategy, were found 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 these resistance genes, from *A. ceylanica* and *S. glutinosa*, and from *P. trifoliata*, were lost at the arising of *M. australis* and *F. hindsii*, respectively. Confirming the efficiency of this searching strategy, Yoshida [25] found

no evidence of CTV infection in *Murraya paniculata* which completely agrees with its location next to *Swinglea glutinosa* in our phylogenetic analysis.

To our knowledge, this was the first time CTV resistance had been reported in *F. crassifolia* [24]. The plant-pathogen interaction between *F. crassifolia* and CTV seems to be complex and very variable; thus, Yoshida et al. [21] 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 to be ineffective 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* [24]. Given that the genus *Fortunella* is more closely related to *Citrus* than *Poncirus* and that it is the closest to most important scion cultivars, sweet orange and mandarins [4], a new possibility is opened for their CTV resistance improvement by means of sexual hybridization, because, unlike *P. trifoliata, F. crassifolia* (Meiwa kumquat) yields edible fruits.

5. perspectives and further remarks

The use of marker loci in plant breeding has evolved mainly on two fronts. First, by devising new applications of these tools to unexplored areas. This is the case of one approach known as 'reverse genetics'. It is a methodology to clone genes involved in the expression of agriculturally important characters such as the CTV-resistance gene, *Ctr-R*. The first step of this method consists in identifying markers flanking the target gene. This step has already been developed by Gmitter et al. [26] and Mestre et al. [22]. The next step is to move from one of the markers to the other through the target gene by means of a 'chromosome walk'. With this technique, partially overlapping DNA probes covering the entire 'physical' distance between both markers are identified. The final step will be the localization of the gene by transformation of CTV-susceptible citrus plants.

The other front of marker research has been towards the improvement of methods in order to obtain either more markers of better quality or more efficient procedures than those previously available. The polymerase chain reaction technique [27] has accelerated the development of new DNA marker systems. As a result, a potentially bewildering array of marker systems, including RAPD, simple sequence repeat pomorphism (SSR or microsatellite), cleavable amplified polymorphic sequences (CAPS), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeats (ISSR) are now available for citrus genetic mapping and identification. Each method differs in application, in the type and amount of polymorphism detected and in the cost and time requirements. The level of polymorphism is an important feature of the marker system of choice for cultivar identification and germplasm management, and when compared [28], wide variations have been found, ranging from a maximum of 100% (SSRs) to only 48.6% (AFLPs). Whenever SSRs have been compared to other systems, they have always revealed the highest levels of polymorphism. However, AFLPs are the most efficient because they have the capacity to reveal many polymorphic bands in a single lane. The ISSRs join both desired features – high level of polymorphism and efficiency of polymorphic bands per lane. Thus, Fang and Roose [29] have recently achieved the identification of closely related citrus cultivars with this system.

In summary, despite the large number of problems that citrus breeding and cultivar identification present – long juvenility period, apomixis, size of the plant, etc. – the rapid advances of biotechnology give citrus breeders no choice other than to design more efficient breeding programs.

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Marcadores moleculares: un área de la biotecnología en continuo desarrollo para la mejora de cítricos.

Resumen — Introducción. Se ha realizado una revisión de la utilización de marcadores moleculares en el estudio de la resistencia de los cítricos al virus de la tristeza (CTV) y el manejo de sus recursos genéticos con objeto de aplicar la selección asistida por marcadores en los programas de mejora de cítricos. **Recursos genéticos de cítricos**. Se estudió el banco de germoplasma de cítricos del IVIA (Valencia, España) mediante el análisis de 6 sistemas isoenzimáticos. Dentro de los cítricos se pueden definir claramente dos grupos de especies: el grupo naranjas-mandarinas y el grupo lima-limón-cidro-pummelo. Dado que en general existe una gran diferenciación genética entre especies y géneros parece que la adaptación a hábitats concretos ha tenido un papel evolutivo un importante. **Resistencia a CTV**. Existe una necesidad urgente de ampliar la base genética de los patrones de cítricos resistentes a CTV para combatir la vulnerabilidad actual del cultivo. El desarrollo de programas de mejora partiendo de sólo genotipos resistentes ayudaría en este objetivo. Consecuentemente, el primer paso fue la búsqueda de marcadores moleculares que permitieran identificar precozmente genotipos susceptibles al CTV, con un error mínimo, dentro de grandes progenies. Al menos dos loci, *Ctr* y *Ctm*, están implicados en la resistencia de *Poncirus trifoliata* al CTV. **Utilización de recursos genéticos de cítricos en la búsqueda de nuevos genotipos resistentes al CTV**. Se probaron dos estrategias de búsqueda de nuevos genotipos resistentes al CTV. Una basada en los marcadores ligados al *Ctr* que fue sólo efectiva dentro de la especie *P. trifoliata* y la otra, basada en el conocimiento de las relaciones filogenéticas ha permitido localizar 2 nuevas especies resistentes al CTV. **Perspectivas.** El avance de la biotecnología es tan rápido que los mejoradores de cítricos deberían aprovecharse de su utilidad en el desarrollo de programas de mejora mas eficientes. (© Elsevier, Paris)

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