Biotechnology for triploid mandarin breeding

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Received 15 January 1998
Accepted 12 May 1998

Fruits, 1998, vol. 53, p. 307–317

RESUMEN ESPAÑOL, *p. 317*

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Abstract — Introduction. As seedlessness is a major criteria for the citrus fresh fruit market, breeding triploid individuals appears promising. Three strategies are currently under development by Cirad-flhor and Inra (France) for triploid citrus breeding with the support of biotechnology. **Materials and methods**. Spontaneous triploids were researched in clementin zygotic embryos collected in mature fruit small seeds (strategy 1). Somatic hybridization was used either to obtain allotetraploids for subsequent hybridization with diploids (strategy 2) or, directly, for haploid and diploid protoplast fusion (strategy 3). Regenerated plants, embryos or calli were studied by flow cytometry and by isozyme analysis to assess their ploidy level and the origins of their nuclear genomes. **Results and discussion**. 1) A total of 85 triploid hybrids is currently being propagated in greenhouse for further evaluation under mediterranean and tropical conditions. As several mechanisms appear to be at the origin of triploid progenies, additional molecular marker studies are necessary to correctly assess the rules of the spontaneous triploid formation in citrus. 2) Tetraploid somatic hybrids were selected from 16 intraspecific, interspecific and intergeneric combinations. They will be used for pollination of diploid monoembryonic mandarins and should generate heterozygous and polymorphic triploid progenies. 3) Somatic hybridization between haploid and diploid protoplasts was achieved with a haploid embryogenic callus line of clementine and several diploid callus lines or mesophyll protoplasts. **Conclusion**. A proper choice between these strategies, according to specific objectives and heterozygosity of diploid genitors, will result in large triploid populations with a high potential for the selection of new easy peeler seedless citrus cultivars. (© Elsevier, Paris)

***Citrus* / plant biotechnology / protoplast fusion / somatic hybridization / tetraploidy / triploidy / diploidy**

Sélection de mandarines triploïdes à l'aide des biotechnologies.

Résumé — Introduction. Les fruits sans pépin étant un atout majeur pour le marché des agrumes frais, la sélection de triploïdes, actuellement poursuivie par le Cirad-Flhor et l'Inra (France), apparaît intéressante. Trois stratégies basées sur les biotechnologies sont utilisées. **Matériel et méthodes**. Des triploïdes spontanés ont été recherchés dans les embryons issus de croisements sur clémentiniers et prélevés dans les petits pépins de fruits mâtures (stratégie 1). L'hybridation somatique a été utilisée soit pour créer des allotétraploïdes pouvant être ensuite hybridés avec des diploïdes (stratégie 2), soit directement par fusion des protoplastes haploïdes et diploïdes (stratégie 3) ; les plants, embryons ou cals régénérés, ont été étudiés par cytométrie de flux et analyse d'isoenzymes afin de déterminer leur niveau de ploïdie et l'origine de leur génome nucléaire. **Résultats et discussion**. 1) Actuellement, 85 hybrides triploïdes sont multipliés en serre pour évaluation ultérieure en conditions méditerranéenne et tropicale. Comme plusieurs mécanismes semblent impliqués dans la formation des individus triploïdes, des études complémentaires par marqueurs moléculaires sont nécessaires pour déterminer l'origine des triploïdes spontanés d'agrumes. 2) Des hybrides somatiques tétraploïdes ont été obtenus à partir de 16 combinaisons intra- ou interspécifiques, ou intergénériques. Ils seront utilisés pour la pollinisation de mandarines monoembryonnées diploïdes et devraient engendrer des descendances triploïdes hétérozygotes et polymorphes. 3) L'hybridation somatique entre protoplastes haploïdes et diploïdes a été effectuée à partir d'une lignée de cals embryogènes haploïdes de clémentiniers et plusieurs lignées de cals diploïdes ou de protoplastes de mésophylles. **Conclusion**. Un choix judicieux de la stratégie utilisée, adapté à des objectifs spécifiques et à l'hétérozygotie des géniteurs diploïdes, devrait aboutir à la création d'une vaste population de triploïdes propice à la sélection de nouveaux cultivars d'agrumes sans pepins, faciles à éplucher. (© Elsevier, Paris)

***Citrus* / biotechnologie végétale / fusion de protoplaste / hybridation somatique / tétraploïdie / triploïdie / diploïdie**

1. introduction

Easy peeling citrus have received increasing attention from the European consumer over the last 20 years [1]. Clementine is presently one of the most popular fresh citrus easy peelers' but there is a consistent demand for earlier and later ripening cultivars of this type of mandarin. As seedlessness is a major criteria for the fresh fruit market, breeding triploid individuals is a promising avenue for easy peeler diversification [2].

In the early 1970s, occasional triploid offsprings had been selected from conventional diploid crossing 2x × 2x [3–5]. The authors demonstrated that triploid progenies are more likely to be found in small seeds of monoembryonic cultivars. This spontaneous natural process, probably at the origin of triploid limes (Tahiti, Bears, etc.), can be worked out in breeding strategies combining the techniques of embryo rescue and flow cytometry [6]

Another possible way is endosperm in vitro culture that has also enabled the recovery of triploid plantlets [7]. However, this technique seems poorly adapted for large scale breeding programs (Gmitter, personal communication).

Recently, protoplast fusion has proven also very promising for ploidy manipulation in citrus. As a matter of fact, the most classic way of breeding triploid citrus was crossing diploid and tetraploid genotypes [8, 9]. But, so far, this strategy has been limited by the scarcity of tetraploid genitors. The latter were generally autotetraploids arising from spontaneous chromosome doubling of nucellar embryos [2, 10]. Today, the pool of tetraploid genitors could be enriched considerably with new highly heterozygous tetraploid genotypes obtained by somatic hybridization [11–16]. Moreover, fertility can be restored in somatic hybrids obtained from sterile cultivars [17], thus enabling the introgression of original traits. Finally, somatic hybridization opens the direct synthesis of triploid hybrids by the fusion of haploid and diploid protoplast.

This paper presents the updated results obtained with three strategies currently being developed by Cirad-Flhor and Inra (France) for triploid citrus breeding with the support of biotechnology:

– the research of spontaneous triploid in progenies of diploid × diploid sexual hybridization,

– the diversification of the allotetraploid gene pool dedicated to further sexual hybridization between diploid and allotetraploid plants,

– the somatic hybridization between diploid and haploid protoplasts.

2. materials and methods

2.1. small seeds embryo rescue

Small seeds were collected from mature fruits of clementine evolving from controlled pollination carried out at SRA, san Giuliano (Corsica), and Pocquereux (New Caledonia) orchards. These small seeds were cultivated in vitro in MT nutrient solid medium [18] added with 30 $g \cdot L^{-1}$ sucrose and 1 $mg \cdot L^{-1}$ GA3. The dishes were maintained at 26 °C with 16 h light daily.

2.2. protoplast fusion and plantlet regeneration

2.2.1. isolation of protoplasts and electrofusion

Isolation of mesophyll or callus protoplasts was achieved according to Grosser and Gmitter [19] and protoplast density adjusted at 3.10^5 protoplasts·mL^{-1} in 0.8 M manitol + 0.5 mM $CaCl_2$ solution. Protoplast suspensions of the two parents were mixed and 1 mL of the mixture was poured into a 55 × 10 mm petri dish for electrofusion. Protoplast suspensions were submitted to an AC electric field (20 V, 1 Mhz) for 10 to 20 seconds. Then two to four pulses (35 µs) of 225 V (DC) were emitted to induce protoplast fusion.

2.2.2. culture medium and conditions

After electrofusion, five volumes of BH3 0.6 M medium [20] modified by Grosser and Gmitter [19] were added to one volume of protoplast suspension. Afterwards 1 mL of this new protoplast suspension was poured into a MT 0.6 M sucrose [18] solid medium in 90 × 15 mm petri dishes. Protoplasts were cultivated under dark condition over 3 weeks at 28 °C. Then, they were transferred in a culture room, at the same temperature, with 12 h of light daily. One month after electrofusion, the proembryos and cell lines were subcultured in MT 0.15 M sucrose or MT 0.15 M galactose solid medium for further development. When cotyledonary embryos were obtained, they were subcultured on the embryo rescue medium for root and shoot elongation.

2.3. analysis of nuclear genome

To assess their ploidy level and the origins of their nuclear genome, regenerated plants, embryos or calli were studied by flow cytometry according to Ollitrault et al. [21], and by isozyme analysis (isocitrate dehydrogenases, malate dehydrogenases, phosphoglucose isomerase and phosphoglucomutases) according to Ollitrault et al. [22]. For flow cytometry, Tahiti lime or petunia were used as internal control.

2.4. acclimatization and grafting

The selected somatic hybrids and alloplasmic in vitro plants have been acclimatized or grafted onto one year *Citrus macrophylla* or *C. volkameriana*. Good results have been obtained with the shoot grafting technique (*figure 1*).

3. results and discussion

3.1. research of spontaneous triploids

Clementine controlled pollinations yield different kinds of seeds (*figure 2*). Seed production and small seed rates are affected by the male and female parents and the environmental conditions. Therefore, the average number of small seeds per fruit varies between 0 and 3.3. As estimated by flow cytometry, about 20% of the plantlets obtained in vitro from the small or atrophied seeds are triploids. If we consider that all the normal seeds would have produced diploid plants (as we confirmed for several progenies; data not shown), the estimated rate of triploids per total progenies is about 0.7%. This result is similar to those of Geraci et al. [5, 23] who found 1% of triploid for clementine crosses conducted respectively in Florida and Sicily. In our experiment, the global yield of triploid progenies per pollinated flower was about 9% which is quite reasonable for managing a breeding strategy.

The triploid in vitro plantlets grafted onto 1 year old *C. macrophylla* or *C. volkameriana* rootstocks are vigorous as previously described for triploids arising from 2x × 2x crosses [2]. A total of 85 triploid hybrids (*table I*) are currently being propagated in greenhouse for further evaluation under mediterranean and tropical conditions.

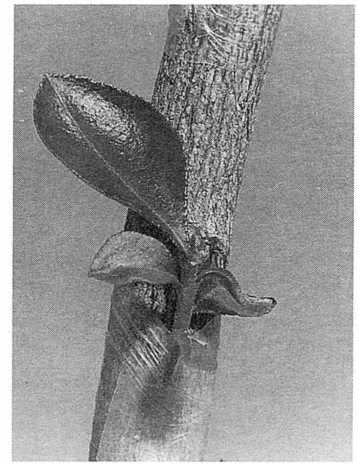

Figure 1.
Haploid clementine + Shamouti sweet orange triploid hybrid shoot grafting.

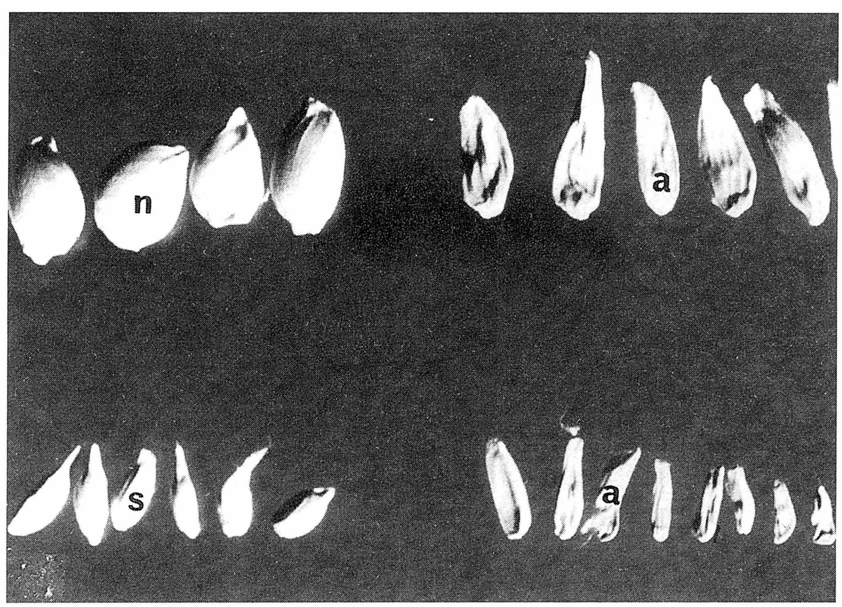

Figure 2.
Different kinds of seeds raised in clementine issued of controled pollinations (n: normal seed; a: atrophied seed; s: small seed).

Table I.
List of spontaneous triploid hybrids propagated for evaluation under mediterranean and tropical conditions.

Parent A		Parent B		Triploid hybrid number
Type	Variety	Type	Variety	
Clementine	Caffin	Mandarin	Hansen	5
			Imperial	2
			De Chios	2
	MA3		Hansen	9
			Anana	19
			Willow leaf	7
			Murcott	7
			Kinnow	1
			Satsuma	8
	Oroval		Hansen	1
Clementine	MA3	Sweet Orange	Sanguinelli	4
			Tarroco Rosso	4
	SRA 63		Sokotoro	1
Clementine	Caffin	Pomelo	Star Ruby	2
	MA3		Star Ruby	1
	SRA 63		Star Ruby	2
Clementine	SRA 63	Lemon Meyer		5
Clementine	MA3	*Fortunella crassifolia*		4

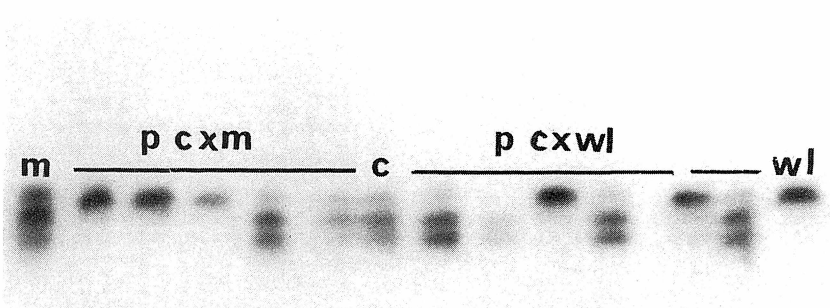

Figure 3.
Study of the origin of spontaneous citrus triploid hybrids by isozymes analysis (m: Meyer lemon; c: clementine; wl: Willow leaf mandarin; p c × m: progeny clementine × Meyer lemon; p c × wl: progeny clementine × Willow leaf mandarin).

Most of the triploid plants were analyzed with isozymes to assess their origin. The data (*figure 3*) clearly indicated that diploid ovules do not result from total diploid nucleus restitution either of clementine or of the male parents. Several mechanisms appear to be at the origin of triploid progenies. As proposed by Esen and Soost [3], the production of diploid ovules is the most frequent event but diploid pollen grains can also occur. The doubling of ovule chromosome set seems to be the major rule, however some cases of heterozygous diploid ovules were also identified. For other crops, several mechanisms have been described concerning the formation of 2n gametes [24]. Moreover, for some species, 2n male and female gametes arising from different mechanisms have been observed [25]. Consequently, additional molecular marker studies are necessary to assess correctly the rules of the spontaneous triploid formation in citrus and then improve the management of this natural phenomena in breeding programs.

3.2. somatic hybridization for triploid easy peeler citrus breeding

3.2.1. establishment of a callus line library

Embryogenic calli play a major role for somatic hybridization [26], since the regeneration from heterofusion protoplasts strongly depends on embryogenic potential of the callus. Moreover, it is easier to isolate protoplasts from callus lines than from leaves of adult trees, while mesophyll protoplasts from in vitro seedlings could be heterogeneous (zygotic). Thus, the establishment of a callus library of the potential genitors is a prerequisite for an efficient somatic hybridization program. The callus induction has been obtained by ovule culture on MT + 1 $mg \cdot L^{-1}$ of kinetine or BAP. The established embryogenic callus lines (*table II*) can be amplified in hormone free MT medium for several years or cryopreserved in liquid nitrogen and efficient regeneration protocols have been developed according to Cabasson et al. [27].

3.2.2. diversification of the allotetraploid gene pool

Electrofusion of mesophyll protoplasts + nucellar callus protoplasts or callus protoplasts + callus protoplasts was used. In the best case, proembryos have been obtained in less than 1 month after fusions (*figure 4*).

Tetraploid somatic hybrids were selected using flow cytometry (*figure 5*) and isozymes (*figure 6*) from 16 intraspecific, interspecific and intergeneric (*Citrus* + *Fortunella)* combinations (*table III*) and the regenerated material of 10 additional combinations will be soon characterized. Most of the regenerated plantlets are diploids with one of the parental nuclear genome, or tetraploid combining two parental nuclear genomes. The major exception is the combination between Mexican lime and Murcott which is displaying a broad range of ploidy variations.

Furthermore, cytoplasmic genome analysis by RFLP of some of these combinations [data not shown] has proved that alloplasmic diploid plants associating the nuclear genome of one species with the mitochondrial and chloroplastic of another one can be obtained by this technique. Similar results have been obtained formerly by Tusa et al. [28], Saito et al. [29] and Moriguchi et al. [30]. Such alloplasmic diploids might have some interest for vigour and tolerance to different types of stress [31].

The selected allotetraploid plantlets or shoots have been grafted (*figure 7*) and the somatic hybrids are currently being propagated under mediterranean and tropical conditions for agropomological evaluation. They will be used for pollination of diploid monoembryonic mandarins as has already been done for lemon breeding by Tusa et al. [13] and for pummelo and mandarin breeding by Deng et al. [32]. This strategy will generate the most heterozygous and polymorphic triploid progenies; however, two cycles of hybridization (one somatic and one sexual) will be required.

Table II.
Citrus embryogenic callus lines with potential interest for triploid breeding, present in the Cirad-flhor and Inra library.

Citrus type	Variety
Mandarin and Tangor	Willow leaf
	Chios
	Cleopatra
	Sunki
	King of Siam
	Beauty
	Murcott
	Kinnow
	Fortune
	Dihaploid clementine
	Haploid clementine H1
	Haploid clementine H2
Sweet orange	Hamlin (from J. Grosser laboratory)
	Valencia late
	Navel late
	Shamouti
Sour orange	Bigaradier (from L. Navarro laboratory)
Grapefruit	Star Ruby
Lemon	Lemon apireno (from J. Grosser laboratory)
Lime	Mexican lime
Limequat	Lakeland

3.2.3. production of triploid citrus by somatic hybridization between haploid and diploid

Somatic hybridization between diploid and haploid protoplasts was achieved with an haploid embryogenic callus line of clementine obtained from haploid embryos generated by induced gynogenesis [33] and several diploid callus lines or mesophyll protoplasts. In both cases, the development of embryos from the protoplast was very fast. Triploid hybrids have been obtained for each combination as well as some tetraploids, pentaploids and aneuploids (*table IV*). Therefore, nuclear genome instability seems to be much more important than for the combi-

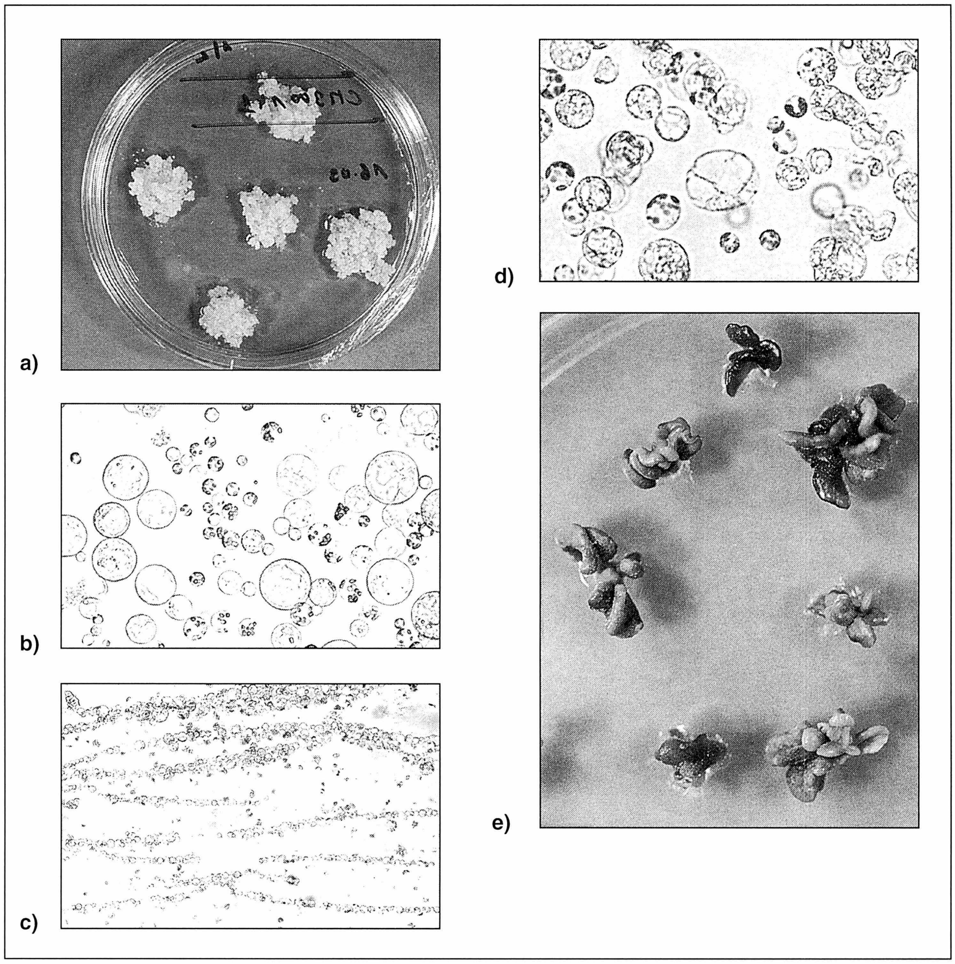

Figure 4.
Protoplast isolation, electrofusion and embryo regeneration.
a) parental callus line;
b) mixture of Willow leaf callus protoplasts and Marumi kumquat mesophyll protoplasts;
c) alignment of protoplasts under electric field;
d) mixture of Willow leaf protoplasts and Marumi kumquat protoplasts after electrofusion;
e) regeneration of embryos 2.5 months after fusion.

nations between two diploids. The production of allotriploid from somatic hybridization between haploid potato and diploid tomato has been previously described [34] while triploid somatic hybrids plants of *Nicotiana* have been obtained by gametic (n) + somatic (2n) protoplast fusion [35].

The polyploid hybrid plantlets have been grafted onto *C. volkameriana* rootstocks and are currently being propagated under greenhouses for further evaluation.

This method enables to add, without recombination, an haploid genome to the whole diploid genome of high organoleptic quality mandarin cultivars. Triploid hybrids should be exploited directly while tetraploid hybrids will join the pool of allotetraploids. Moreover, this strategy opens the perspective of developing a broad range of transgenic triploid cultivars by adding a haploid transgenic genome to the diploid genome of several citrus cultivars.

4. conclusion

Consistent results have been obtained with the three strategies used by the Cirad-Flhor and Inra team. Biotechnological tools have enabled the efficient exploitation of spontaneous rare events but also the development of new ways for the creation of triploid cultivars. The three strategies are complementary with

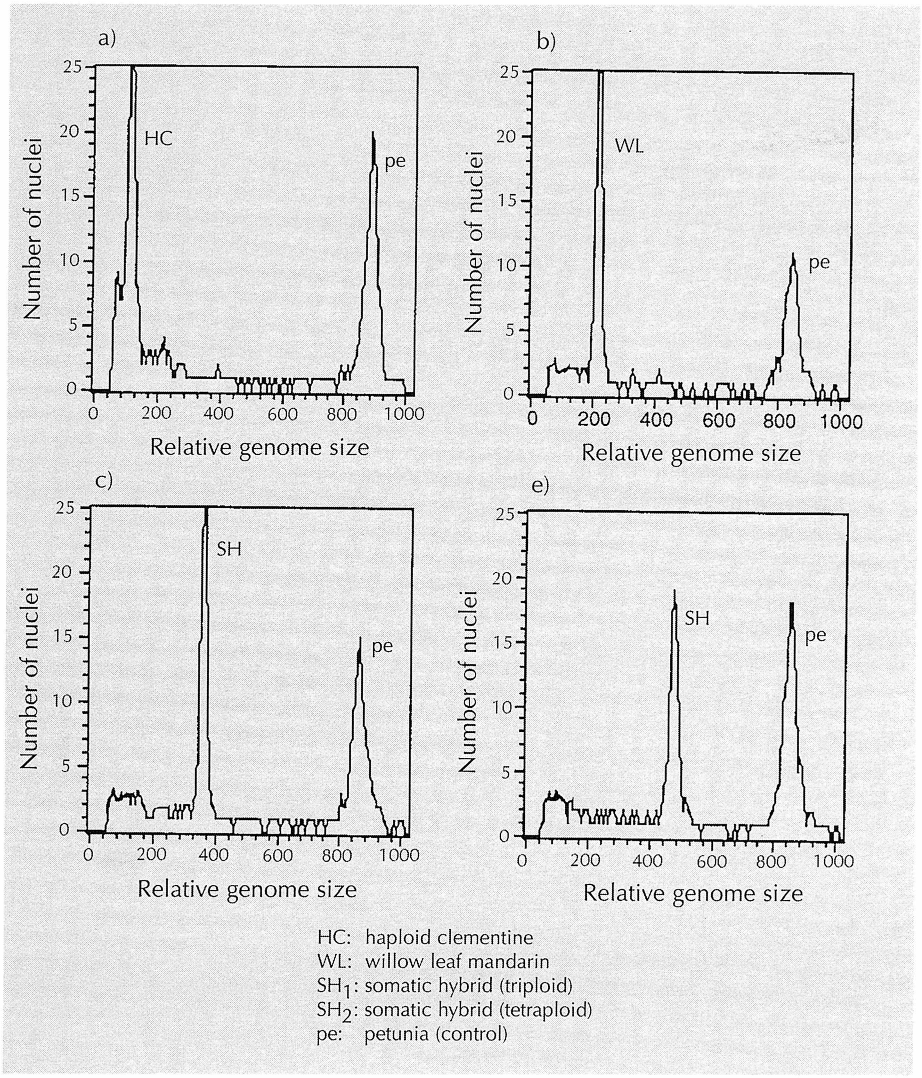

Figure 5.
Study of ploidy level of citrus somatic hybrid by flow cytometry (petunia is used as triploid control).

regards to 1) the usable germplasm, 2) the level of genome recombination, and 3) the level of heterozygosity of the final triploid progenies. A proper choice between these three strategies, according to specific objectives and heterozygosity of diploid genitors, will result in large triploid populations with a high potential for the selection of new easy peeler seedless citrus cultivars. To date, one-hundred triploids have been propagated by our group for evaluation under mediterranean and tropical field conditions.

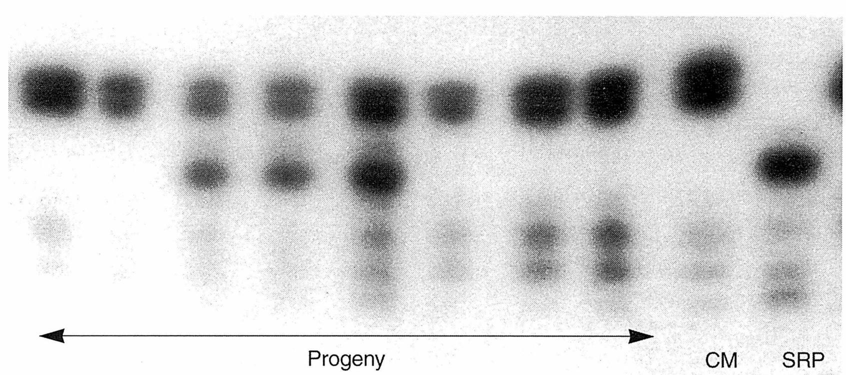

Figure 6.
Isoenzymatic profiles (phosphoglucomutases: PGM) of Star Ruby pomelo (SRP), Commune mandarin (CM) and their somatic progeny.

Table III.
List of citrus tetraploid somatic hybrids with potential interest for triploid cultivar breeding.

Genitors	In vitro plantlets	Grafted plants
Willow leaf mandarin + Eureka lemon	+	+
Willow leaf mandarin + Mexican lime	+	+
Willow leaf mandarin + Star ruby grapefruit	+	–
Willow leaf mandarin + Marumi kumquat	+	+
Willow leaf mandarin + Shamouti sweet orange	+	–
Murcott + Mexican lime	+	–
Star Ruby grapefruit + mexican lime	+	+
Star Ruby grapefruit + Shamouti orange	+	+
Star Ruby grapefruit + Apireno lemon	+	+
Dihaploid Clementine + Willow leaf mandarin	+	–
Dihaploid Clementine + Shamouti orange	+	+
Dihaploid Clementine + Sunki mandarin	+	+
Dihaploid Clementine + Kinnow	+	+
Dihaploid Clementine + Mexican lime	+	–
Dihaploid Clementine + Marumi kumquat	+	+
Shamouti orange + Mexican lime	+	+

+: confirmed allotetraploid.
–: material not available.

Table IV.
Different kinds of citrus individuals regenerated (embryo level) from the fusions between diploid cultivar protoplasts + haploid clementine protoplasts.

Parental diploid	Progeny					
	1 × P	2 × P	3 × H	4 × H	5 × H	AH
Willow leaf mandarin	0	14	19	2	0	5
Sunki mandarin	0	3	8	2	1	4
Murcott	0	0	1	3	1	2
Shamouti orange	0	0	15	2	0	0
Star Ruby grapefruit	0	1	13	35	5	3
Mexican lime	0	0	5	10	0	0
Marumi kumquat	1	3	6	5	0	4
Kinnow	?	?	?	?	?	?
Valencia late orange	?	?	?	?	?	?
Total	1	21	67	59	7	18

1 × P: haploid parental genome; 2 × P: diploid parental genome; 3 × H: triploid hybrid genome; 4 × H: tetraploid hybrid genome; 5 × P: pentaploid hybrid genome; AH: aneuploid hybrid genome; ? : materiel to be tested to assess the genetical origin.

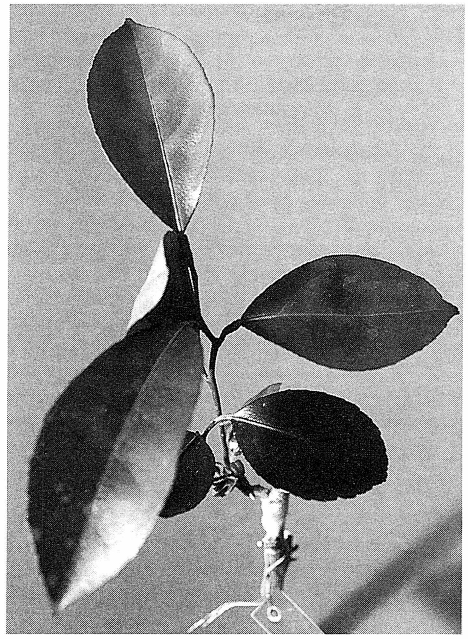

Figure 7.
Shoot grafting of citrus somatic hybrid (Star Ruby pomelo + Mexican lime).

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Selección de mandarinas triploides mediante biotecnologías.

Resumen — Introducción. Las frutas sin pepino siendo un triunfo mayor para el mercado de los agrios frescos, es considerada interesante la selección de triploides, que el Cirad-flhor y el Inra (Francia) llevan ahora a cabo. Se utilizan tres estrategias con bases en biotecnologías. **Material y métodos**. Se buscaron triploides espontáneos en los embriones oriundos de cruzamientos en frutales de clementina y tomados en los pequeños pepinos de frutos maduros (estrategia 1). Se utilizó la hibridación somática, ya sea, para generar alotetraploides pudiendo ser luego hibridados con diploides (estrategia 2), ya sea, directamente mediante fusión de los protoplastos haploides y diploides (estrategia 3) ; se estudiaron las plantas, los embriones o callos regenerados mediante citométria de flujo y análisis de isoenzimas a fin de determinar su nivel de ploidia y el origen de su genoma nuclear. **Resultados y discusión**. 1) Actualmente, se multiplican 85 híbridos triploides en invernadero para su evaluación ulterior en condiciones mediterránea y tropical. Como varios mecanismos parecen implicados en la formación de los individuos triploides, es preciso realizar estudios adicionales mediante marcadores moleculares para determinar el origen de los triploides espontáneos de agrios. 2) Se lograron híbridos somáticos tetraploides a partir de 16 combinaciones intra o inter específicos, o ínter genéricos. Se utilizarán para la polinización de mandarinas monoembrionadas diploides y deberían generar descendencias triploides heterozigotas y polimorfas. 3) Se realizó la hibridación somática entre protoplastas haploides y diploides a partir de una descendencia de callos embriogenos haploides de frutales de clementinas y varias descendencias de callos diploides o de protoplastos de mesofilos. **Conclusión**. Una selección juiciosa de la estrategia empleada, adaptada a los objetivos específicos y a la heterozigotia de los genitores diploides, debería llegar a la creación de una amplia población de triploides propicia para la selección de nuevos cultivares de agrios sin pepinos, fáciles de pelars. (© Elsevier, Paris)

***Citrus* / biotecnología vegetal / fusión del protoplasto / hibridación somática / tetraploidia / triploidia / diploidia**