Theor Appl Genet (1999) 99:511–518

R. García · M.J. Asíns · J. Forner · E.A. Carbonell

Genetic analysis of apomixis in *Citrus* and *Poncirus* by molecular markers

Received: 30 November 1998 / Accepted: 28 December 1998

Abstract Propagation of citrus rootstocks depends upon the production of clonal plants from nucellar seedlings. This makes apomixis one of the host important traits in breeding programs for citrus rootstocks. The genetic control of apomixis was studied in a 50-tree progeny derived from the cross *C. volkameriana*×*P. trifoliata* using 69 molecular markers and bulked segregant analysis. The proportion of nucellar seedlings was estimated by isoenzymatic analysis of 25 seedlings per tree for 2 consecutive years. The type of embryony (polyembryonic versus monoembryonic seeds) was also determined for fruit-yielding trees. Separate genetic maps for each parental species were developed. The integration and comparison of these maps could be accomplished using common multiallelic segregant loci. Differences in gene synteny between the two species-specific genetic maps were shown. Important distortions in the segregation of markers at several genomic regions, some of them also involving differences in the C-methylation pattern, have been observed, especially for the pollen parent. Analysis of quantitative trait loci (QTLs) revealed the presence of six genomic positions (two in *P. trifoliata* and four in *C. volkameriana*) contributing individually up to 24% of the total variation for apomixis. Within the same species, QTLs with positive and negative allele effects were present, even in the same linkage group. One of the markers associated to apomixis (*Apo2*) is also associated to embryony type. Therefore, the genetic control of apomictic reproduction found in citrus (nucellar embryony) is quite complex compared to what has been reported for gametophytic apomixis. Molecular markers linked to QTLs governing apomixis will be useful to assist selection of future apomictic rootstocks for citrus varieties.

Communicated by P.M.A. Tigerstedt

R. García · M.J. Asíns (✉) · J. Forner · E.A. Carbonell
Instituto Valenciano de Investigaciones Agrarias (IVIA),
Apartado Oficial, 46113 Moncada (Valencia), Spain
e-mail: mjasins@ivia.es
Fax: +34-961390240

Key words Linkage groups · Non-inbred species · QTLs · Polyembryony · Nucellar embryony · Fruit breeding

Introduction

The ability of flowering plants to confine a new individual into a seed is not necessarily linked to sexuality. Some angiosperms commonly reproduce asexually through seed by a process called apomixis (Nogler 1984). Apomixis gives rise to fertile seeds whose embryos derive directly from maternal cells rather than from the fusion of male and female gametes. Therefore, these embryos have a genetic constitution identical to that of the female parent. This feature has been observed in more than 300 plant species belonging to 35 families, the most well-represented including the Gramineae, Compositae, Rosaceae and Rutaceae (Hanna and Bashaw 1987).

Modern agriculture depends on seed uniformity in addition to fruit and vegetable quality. Apomixis allows fixing the genotype of a superior variety, including hybrid cultivars, and hence seed can be produced for many generations without loss of vigor or genotypic segregation, enabling a significant reduction in hybrid seed production costs. Therefore, most research to date has focused on introgressing the trait of apomixis by traditional breeding into agricultural crops of economical importance, such as wheat and maize, from wild, often very distant relatives. However, this is a slow process, and the choice of the type of apomictic mechanism is limited by what is available in close apomictic relatives, which may not be the most optimal mechanism for agricultural purposes. These problems could be overcome if molecular knowledge of the genes involved in initiating and controlling apomixis was available, because they could then be transferred to the crop of interest by molecular transformation (Koltunow et al. 1995).

Apomictic reproduction is generally attributable to two possible mechanisms, adventitious embryony and gametophytic apomixis. In adventitious embryony, embryos are formed directly from nucellar cells. In gameto-

phytic apomixis, the embryo arises from the female gametophyte through displospory or apospory. Many members of the genus *Citrus* and some closely related genera belonging to Rutaceae reproduce apomictically by nucellar embryony (Frost 1943). Since nucellar embryos develop asexually by ordinary mitotic division of cells of the nucellus and the male gamete does not contribute to their formation, nucellar seedlings are identical to the seed parent. The presence of extra (nucellar) embryos in addition to that from sexual origin leads to a closely related term, polyembryony. Polyembryony is the development of two or more embryos in one seed. This is why a polyembryonic variety is commonly considered to be apomictic (yielding apomictic, nucellar, seedlings) and a monoembryonic variety, sexual or zygotic. However, it is well-known that extra embryos are occasionally sexual, produced either from the fission of one fecundated egg or from two or more functional embryo sacs in a single ovule (Bacchi 1943; Cameron and Garber 1968).

Nucellar embryony has very important consequences for the evolution, breeding, and culture of citrus fruits. Budding onto seedling rootstocks almost universally propagates citrus. Therefore, the propagation of citrus rootstocks depends upon the production of clonal plants from nucellar seedlings. Uniformity of the rootstock genotype is esential for reliable plant performance following budding and orchard establishment. Most rootstock cultivars presently grown are polyembryonic, producing seeds that contain both nucellar and zygotic embryos. The proportion of zygotic embryos developing into seedlings varies with both genotype and environment (Cameron and Frost 1968; Cameron 1979), and uniformity is seldom complete. Off-type seedlings are generally rogued in the nursery, but the efficiency of such roguing is not known for many rootstocks. Using enzyme analysis Anderson et al. (1991) determined that roguing based primarily on size and growth habit of seedlings is effective in removing some, but not all, zygotic seedlings. They found that zygotic rootstocks escaped the roguing in two of the three groves studied and, in some instances, an apparent graft-incompatibility developed in young trees. In any case, zygotic seedlings may affect the performance of trees budded on them (Weber 1932; Roose and Traugh 1988). Therefore, one of the most important traits to be considered within a breeding program for citrus rootstocks is apomictic reproduction by seed, i.e. that the new genotype yields the least number of zygotic seedlings. To this end, its genetic control should be first studied. Segregation data suggest that one locus is mainly responsible for the trait "polyembryony – monoembryony" and that a recessive gene is present in the homozygous state in monoembryonic plants (Parlevliet and Cameron 1959; Iwamasa et al. 1967).

To our knowledge, all genetic analysis of the offspring of apomictic versus sexual crosses concern the gametophytic type, providing evidence that both gametophytic mechanisms of apomixis are controlled by single Mendelian factors. Apospory is probably controlled by a single dominant gene in *Panicum maximum* (Savidan 1982), *Ranunculus aricomus* (Nogler 1984), *Cencchrus ciliaris* (Sherwood et al. 1994) and *Brachiaria* (Pessino et al. 1997). Genetic control of displospory in *Taraxacumn* resides on a single chromosome and probably a single locus, as suggested by Mogie (1988). Results provided by Leblanc et al. (1995) with restriction fragment length polymorphism (RFLP) analysis in maize-*Tripsacum* hybrids support the hypothesis of monogenic control. However, nothing is known yet about the genetic control of nucellar embryony.

Molecular markers have become very efficient and powerful tools in plant breeding because they allow the genetic dissection of quantitative traits and the early preselection of desired genotypes. This is especially important for apomixis in citrus where the long juvenile phase (4–7 years) forces citrus breeders to maintain thousands of trees before knowing whether they present apomixis or not, which is highly expensive in terms of land and other resources. The main objective of the investigation presented here was to study the genetic control of the apomictic mechanism of nucellar embryony in citrus and to discover associated molecular markers that might be used to apply marker-assisted selection (MAS) during the first year in rootstock breeding programs.

Materials and methods

Plant material

Parental genotypes and their progeny (80 trees) from an intergeneric cross between *Citrus volkameriana* ('Volkamer' lemon, the female parent) and *Poncirus trifoliata* var "Rubidoux" (C×P) obtained 20 years ago by Dr. J. Forner at IVIA were used. Only 50 trees within the progeny yielded fruits between 1995 and 1996 in spite of their age.

Evaluation of traits

Apomictic reproduction was studied by genotyping 25 random seedlings derived from seeds of each individual fruit-yielding tree for isozymatic loci. Seeds were collected at random from mature fruits of the 50 trees that yielded fruits in autumn 1995 and 1996. Six enzymatic systems were studied, as described by Asíns et al. (1995): phosphoglucoisomerase (PGI), malic acid dehidrogenase (MDH), glutamate oxalacetate transaminase (GOT), isocitric acid dehydrogenase (IDH), leucine aminopeptidase (LAP) and phosphogluco mutase (PGM). Enzymatic loci at which progeny trees were heterozygous were chosen to estimate the percentage of zygotic seedlings per tree. Seedlings that were heterozygous at all those loci were considered nucellar. For some trees, this evaluation was carried out for 2 consecutive years.

Fully developed embryos in eight to ten mature seeds per tree were counted to distinguish between multiple or single embryos. This trait was evaluated in 38 out of the 50 fruit-yielding trees.

Analysis of molecular markers

Pools of 100% apomictic and 0% apomictic (sexual reproduction only) trees of 10 individuals of each type were obtained by weighing equal amounts of leaves from each individual. Genomic DNA extractions followed the method of Dellaporta et al. (1983) with some modifications. DNA samples of the parental trees, their progeny and the pools thereof were individually digested with the

restriction endonucleases *Hin*dIII, *Eco*RI, *Eco*RV, *Xba*I, *Bgl*III, *Dra*I and *Bam*HI for RFLP analysis using non-radioactive probes (Monforte et al. 1996). Seven cDNA clones (pRLc 31, 91, 103, 39, 3, 53, 11) and two genomic clones, gp 47 (provided by M.L. Roose, University of California, Riverside) and CW18 (Mestre et al. 1997a,b), were used as probes.

Random amplified polymorphic DNA (RAPD) analysis of the pools and the segregant population was done with 42 random primers (Operon kits D, E, F, G, O Technologies, Alameda, Calif.) and primers D07, E04, G09, G19 and K16 because they generated RAPDs linked to gp47 (Mestre et al. 1997a,b). Amplification reactions consisted of buffer (10 m*M* TRIS-HCl, 50 m*M* KCl); 1.5 m*M* $MgCl_2$; 100 µ*M* dNTPs (25 µ*M* each); 0.2 µ*M* primer; 1 U *Taq* (Eurobiotaq); 300 ng of DNA and sterile water up to a total volume of 25 µl. The polymerase chain reaction (PCR) was conducted in a MJ-PTC-100 tm thermal controller with 96 wells under the following conditions: 94°C for 2 min; 5 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, 15 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, 94°C for 30 s, 30°C for 30 s; 72°C for 45 s; and an additional extension period at 72°C for 5 min. PCR products were mixed with 0.2 volume of loading dye and analyzed by electrophoresis in a sequencing, but not denaturing, gel with a 6% polyacrylamide stacking phase and a 10% polyacrylamide separating phase (acrylamide: N,N′-methylenebisacrylamide, 29:1) with TBE buffer (90 m*M* TRIS borate; pH 8.3, 2 m*M* EDTA). Electrophoresis was carried out at room temperature at 20 W/700 V for 4 h. DNA bands were visualized with silver staining as described by Ruiz et al. (1998).

Citrus microsatellites TAA1, TAA15, TAA41, TAA45, TAA27, AGG9, TAA52, CAC23, TAA52 and TAA33 were analyzed as reported by Kijas et al. (1997) and visualized as described by Ruiz et al. (1998).

A cleaved amplified polymorphic sequence (CAPs) that corresponds to a citrus ethylene receptor genomic clone, ERS, (A. Granell, personal communication) was used as marker locus. Amplification products were digested with Hpa II for generating restriction fragments that segregate in the progeny. PCR, electrophoresis and visualization methods were the same as for microsatellite analysis.

Statistical analyis

JOINMAP 2.0 (Stam 1993; Stam and Van Ooijen 1995) with a linkage criteria of LOD 6, in general, recombination fraction of 0.5 and Kosambi mapping function was used for linkage analysis. The population was analyzed as the "Cross pollinator" population type with no previous knowledge of the linkage phase of the markers. Segregation data from the whole progeny group (80 trees) was used for the construction of three genetic maps, one for *C. volkameriana*, another for *P. trifoliata* and another with both data files together.

Apomixis was analyzed using two approaches: it was considered either as a categorical (apomixis, at any degree, or sexual) or continuous variable (measured as percentage of apomictic seedlings). For the categorical approach, χ^2 test of independence between each marker locus and phenotypes for "apomixis" versus "sexual" or "polyembryony" versus "monoembryony" was computed. Yate's *P* values were used for the level of significance.

When apomixis was considered to be a continuous variable, putative quantitative trait loci (QTLs) were identified using several statistical methodologies. For the complete set of markers: (1) by single-marker analysis, comparing trait means within male- or female-derived haplotypes; (2) by ANOVA using all genotypes (maximum of four for a $M_iM_j \times M_kM_l$ cross); (3) by specific contrasts within parental types. For only those markers segregating as a backcross (like in the pseudo-test-cross design described by Grattapaglia et al. 1996; $M_iM_j \times M_iM_i$ for the *C. volkameriana* genetic map, or $M_iM_i \times M_iM_j$ for the *P. trifoliata* one) (1) by phenotype on marker regression and (2) by interval mapping using the QTL CARTOGRAPHER computer program (Basten et al. 1997).

Bonferroni adjustment was used to obtain an overall protection level of 0.05.

Results

C. volkameriana×*P. trifoliata* (C×P) progeny was heterozygous at five isozymatic loci: *Mdh-2*, *Got-1*, *Got-2*, *Pgm-1* and *Pgi-2*. Segregation at these loci was studied in 25 seedlings derived from each C×P tree to estimate the percentage of zygotic seedlings in both 1995 and 1996. Progeny trees that yielded fruits in 1995 and/or

Table 1 Number of trees per apomixis class and their embryony type

Apomixis		Embryno type[a]	
Type	Number	Poly-	Mono-
100	12	9	0
70–98	11	8	1
26–3	10	0	8
0	17	0	12

[a] Not all trees evaluated for apomixis were also evaluated for embryong type

Table 2 Segregation data of loci showing distorted segregation

Linkage group	Locus	Observed[a]	Chi-square
V4	OPK16108 (female)	47P:39N	3.28*
V6	TAA 41 (male)	49A:30C	4.57**
V7	*Got 2* (male)	6A:74B	57.8**
	OPE04180 (female)	18P:33N	4.4*
V8	OPF14070 (female)	53P:24N	10.9**
	OPG13110 (female)	24P:54N	11.5**
V2/P2	AGG9 (female)	32B:47C	2.85*
	AGG9 (male)	51A:28B	6.7**
	CW18 (male)	53A:24B	10.9**
V3/P3	*Idh* (female)	30A:50B	5.0*
	OPB05087 (female)	30P:46N	3.37*
	OPG09120 (male)	25P:48N	7.3**
	Egp47 (male)	12C:57D	29.4**
	Egp47 (female)	50A:19B	13.9**
	PRLc 53 (female)	28A:48B	5.26*
V5/P5	TAA 27 (male)	19C:61D	20.1**
	TAA 27 (female)	15A:65B	31.3**
	OPD07087 (male)	12P:68N	39.2**
	OPE04075 (male)	6P:49N	33.6**
	OPG19050 (male)	20P:59N	19.3**
	gp47 (male)	62A:15B	28.7**
Unlinked	OPB05045 (male)	22P:56N	14.8**
	OPG09125 (male)	45P:29N	3.46*
	OPK16120 (female)	48P:30N	4.2*
	OPF14120 (female)	51P:25N	8.9**
	OPO13150 (male)	60P:19N	21.3**
	OPG13090 (male)	49P:26N	7.1**
	OPD07085 (male)	25P:55N	11.3**
	OPE04080 (male)	36P:19N	5.3*
	OPG09060 (male)	46P:22N	8.5**
	OPG13130 (male)	27P:51N	7.4**
	pRLc 103	37A:19B	5.8**
	pRLc 31 (female)	10A:65B	40.3**
	TAA 15 (male)	25A:54B	10.7**
	CAP (female)	12A:30B	7.71*

*, ** Significant at 0.05 and <0.05 levels, respectively (without Bonferroni correction

[a] N, Null; P, presence; A, B, C, D, alleles

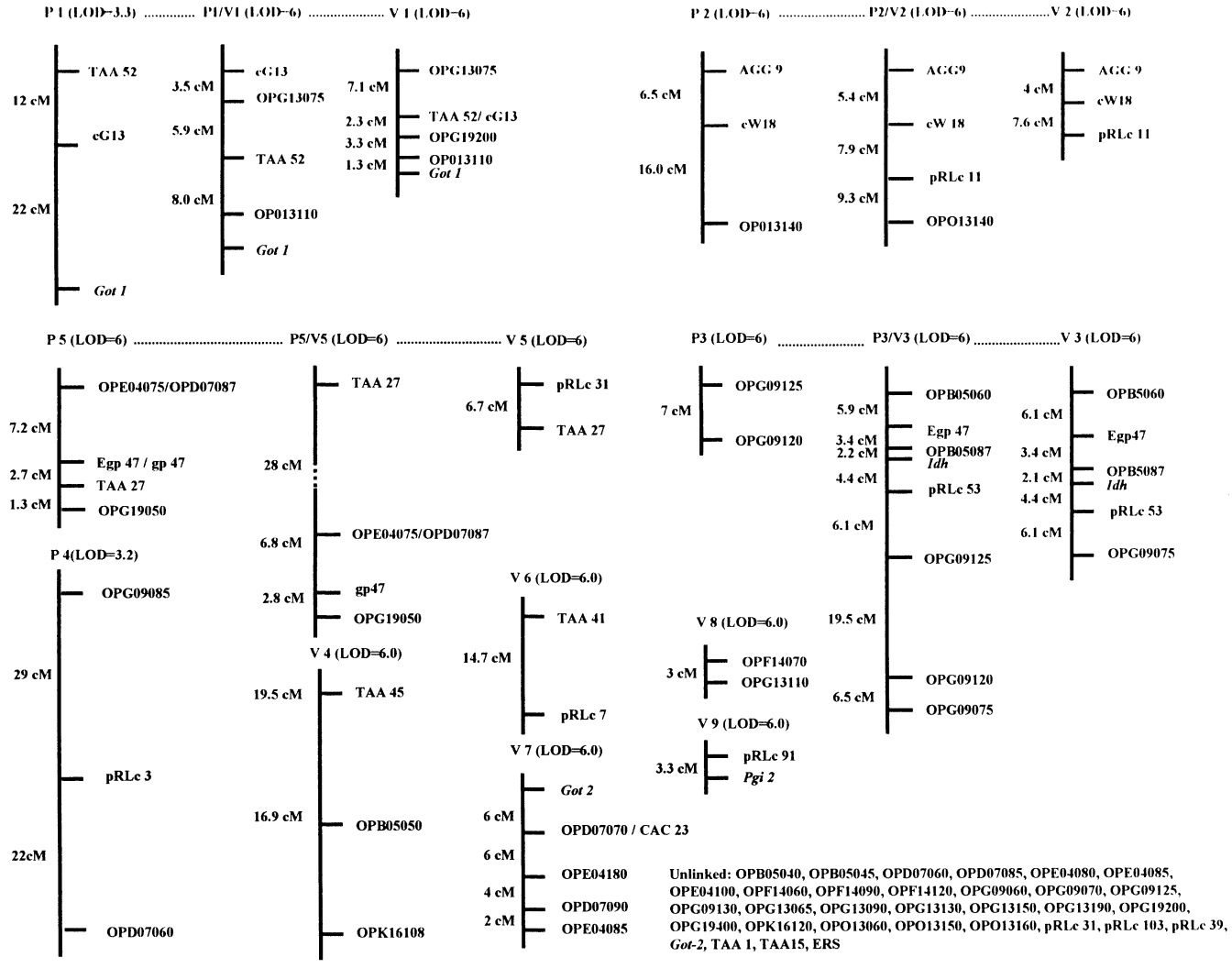

Fig. 1 *Citrus* and *Poncirus* linkage groups. *V C. volkameriana map, P P. trifoliata* var 'Flying Dragon', *V/P* combined map. Most of the groups were obtained at LOD=6

1996 were clearly classified into four classes with respect to percentage of nucellar (apomictic) seedlings and embryony type (Table 1). No major changes in the percentage of nucellar seedlings were observed between years except for 2 trees whose seedlings were all zygotic 1 year, while in the following year some few nucellar seedlings were detected. Seventeen trees yielded polyembryonic seeds (all of them showing high degree of apomictic reproduction) versus 21 that were monoembryonic (9 with apomictic reproduction, 12 with only sexual embryos). Segregation for presence of apomictic reproduction was 17 without apomictic reproduction (zygotic seedlings only) and 33 with some degree of apomixis. This segregation fits the proportion 3 apomixis: 1 sexual, suggesting, at this point, the involvement of a single locus, with the allele determining apomixis being dominant.

Segregation of a total of 69 markers (eight microsatellites, 43 RAPDs, 13 RFLPs, 1 CAPs (cleaved amplified polymorphic sequence) and four isozymatic loci) was analyzed in the whole C×P population. *C. volkameriana* was heterozygous at 45 marker loci and *P. trifoliata* at 38 marker loci.

Segregation data of loci that showed distorted segregation is included in Table 2. In *C. volkameriana* distorted segregation (gametic selection) was found for 22.22% of the markers; in *P. trifoliata*, for 47.37%. Two markers showed distorted segregation in both species. Distorted segregation at the genotypic level could always be explained by gametic selection only.

Linkage analysis of markers segregating in the female gametes, in the male gametes and all markers together resulted in the three genetic maps represented in Fig. 1. Since no complete synteny was found when the *C. volkameriana* (V) and *P. trifoliata* (P) linkage groups were compared, the combined map must be taken with caution (groups 3 and 5).

Based on the fact that monogenic inheritance has been suggested by several authors (gametophytic apomixis) and on the segregation ratio we found, bulked segregant analysis was the strategy chosen for searching for the chromosomal position. The screening of 13 RFLPs against apomictic and sexual pools resulted in the identification of a RFLP marker (gp47) associated with

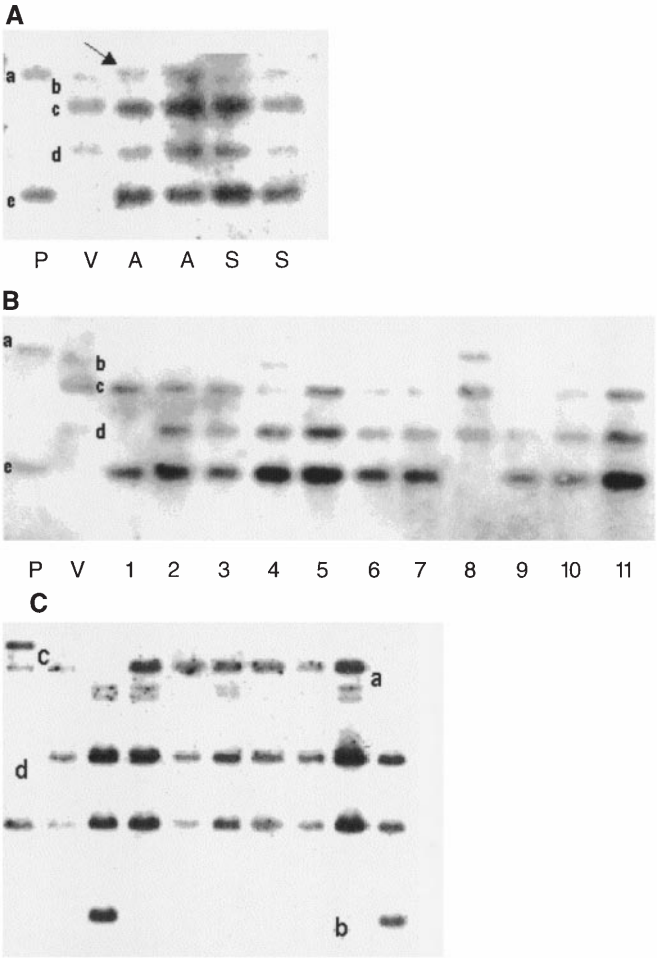

Fig. 2A–C Southern blot analysis of clones gp47 (**A, B**) and cG13 (**C**). *P Poncirus trifoliata, V C. volkameriana, A* pooled DNA of 100% apomictic trees, *S* pooled DNA of trees producing only zygotic seedlings. *Numbers* correspond to progeny trees. **C** Alleles *a* and *b* come from *C. volkameriana*, and alleles *c* and *d* from *P. trifoliata*

apomixis (Fig. 2). Hybridization of *Bgl*II digests of 'Volkamer' lemon DNA with the gp47 probe resulted in three bands (b, c and d) whose segregation in the C×P progeny is interpreted as the segregation of C-methylation among *Bgl*II sites within the gp47 genomic region (Fig. 2B). To study segregation in the *C. volkameriana* gametes at this position we used *Eco*RI digests for gp47 probing (Epg47 marker). The screeing of primers against apomictic and sexual pools resulted in the identification of a RAPD marker, OPG13075, segregating in *C. volkameriana* gametes, that was associated with apomixis. In order to study the segregation at this genomic position in 'Rubidoux' gametes, we cloned this RAPD and used it as a probe for *Hin*dIII digests (Fig. 2C).

Primers that produced RAPDs linked to gp47 (Mestre et al. 1997) were used in PCRs, resulting in 2 RAPDs associated with apomixis. χ^2 tests revealed that three genomic positions were involved (Table 3). Those for embryony type revealed two different positions associated with polyembryony: OPB05040 (χ^2=10.0) in 'Rubidoux' and TAA15 (χ^2=9.2) in *C. volkameriana*.

Results on QTL detection are presented in Table 3. Six putative QTLs controlling apomictic reproduction in the progeny *C. volkameriana*×*P. trifoliata* cv 'Rubidoux' were found and named *Apo1* to *Apo6*. QTLs governing apomixis in 'Rubidous' (*Apo4* and *Apo5*) were different from those segregating in *C. volkameriana* (*Apo1, Apo2, Apo3* and *Apo6*). *Apo1* and *Apo6* are located in the same linkage group, V1, but their gene effects show opposite directions. TAA15 is associated with polyembryony and also with apomixis (*Apo2*). Individual contributions of the QTLs are below 24% of the total variation. Most of the PCR markers associated with apomixis and polyembryony are shown in Fig. 3.

Table 3 Detection of QTLs by several statistical methods: *t*-test mean comparison between marker alleles coming from each parent separately (t); contrast comparisons between genotypes per marker locus when all four genotypes are distinguishable in the progeny (T); and linear regression (LR) and interval mapping (IM) for markers following backcross configuration in both parents

Poncirus linkage group	Marker	χ^2	*t*-test	T[a]	LR	IM	a[b]
P5	E04075	*	[c]	nt[e]			
	D07085	+		nt			
	TAA27 (*Apo4*)	*	* (11.2%)[d]	* (c+)	nt	nt	
	gp47	*	*	nt	*	* (9%)	36.30
	Eg47	*			nt	nt	
P4	OPD07060 (*Apo5*)	*		nt	*	* (11%)	–31.45
Volkameriana linkage group	Marker	χ^2	*t*-test	T	LR	IM	a
V1	OPG13075	**		nt	*	Unlinked	
	cG13 (*Apo6*)	**		* (a+)	nt	nt	
	TAA52	*	* (11%)	* (c+)	nt	nt	
	Got-1	*			nt	nt	
(LOD 3.1)	OPE04100 (*Apo1*)		* (12.2%)	nt	*	* (24%)	–45.44
Unliked	TAA15 (*Apo2*)		* (23.8%)	* (c+, b–)	nt	nt	
Unlinked	ERS (*Apo3*)		* (14.2%)	* (a–)	nt	nt	

*, ** Significance levels as in Table 2
[a] In parentheses, the relevant allele. +, Increases apomixis
[b] The additive valve, a, was estimated by IM
[c] Blank box, Not significant
[d] Values within the parenthesis represent the percentage of variability attributable to the QTL
[e] nt, Not studied because either it was not a backcross type or it did not segregate in 'Volkamer' lemon or 'Rubidoux'

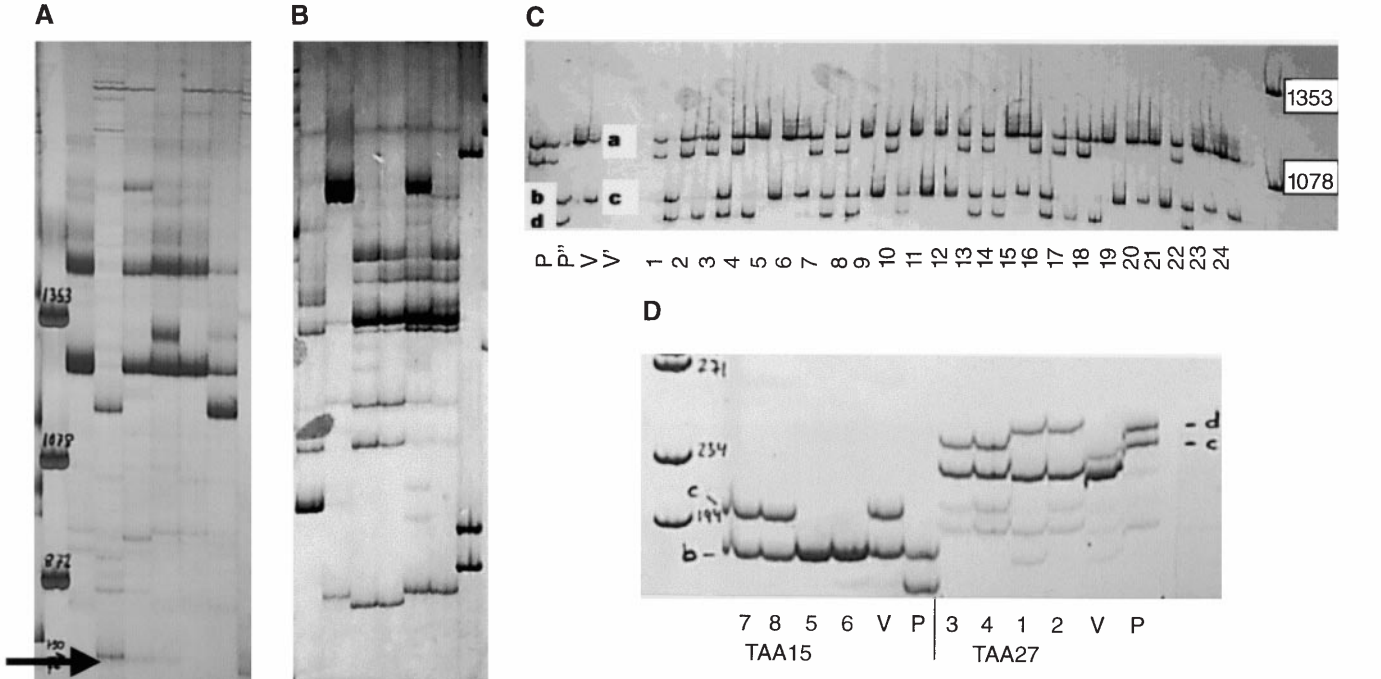

Fig. 3A–D Silver-stained acrylamide gels of random-amplified DNA products (**A, B**), citrus ethylene receptor, ERS, and its cleavaged, products after *Hpa*II digestion (**C**) and citrus microsatellites TAA15 and TAA27 (**D**). *Arrow* in **A** indicates, RAPD OPG13075 linked to Apo6 and inherited from *C. volkameriana*; *lanes 1* and *2* are progeny trees producing only sexual seedlings; *lanes 3* and *4* are progeny trees producing only nucellar seedlings. *Arrow* in **B** indicates RAPD OPB05040 associated with polyembryony; *lanes 5* and *6* are trees producing monoembryonic seeds; *lanes 7* and *8* are trees producing polyembryonic seeds. In **C** *P P. trifoliata, V C. volkameriana, P′* and *V′ Hpa*II digestions of the corresponding amplification products; *numbers* every second lane represent different progeny trees, their amplification products and their *Hpa*II digestions. **A, C** and **D** contain a lane of molecular-weight markers whose sizes are written in base pairs

Discussion

Linkage maps

Five genetic linkage maps are already available in Citrus. Two of these are based on a cross between two intergeneric hybrids (*C. paradisi*×*P. trifoliata*)×(*C. sinensis*×*P. trifoliata*) using codominant markers: isozymes, RFLPs and microsatellites (Jarrell et al. 1992; Kijas et al. 1997). Two others are based on a backcross of an intergeneric hybrid *C. grandis*×(*C. grandis*×*P. trifoliata*) using codominant and dominant markers: isozymes, RFLPs and RAPDs (Durham et al. 1992; Cai et al. 1994). The fifth map is based on the backcross of an interspecific hybrid *C. clementina*×(*C. clementina*×*C. paradisi*) using isozymes and RFLPs (Durham et al. 1992). Structural changes in chromosomes have played an important role in the evolution of Aurantioideae subfamily (Naithani and Raghuvanshi 1958, 1963; Raguvanshi 1962), and no genetic map has yet considered each parent separately. Hence, it is possible that, at least at some extent, some linkage groups are artificial and the genetic linkage map of a citrus species is as yet far from being well developed.

For comparison purposes, it is very difficult to obtain a consensus citrus genetic map from the above maps because: (1) parental genotypes are, at least, interspecific hybrids, making families very complex and different from one another, (2) very few common markers are used and (3) since plant genera and species may differ in gene synteny due to chromosomal translocations and inversions during evolution, genetic maps for each parental species separately should be first studied as in other allogamous species.

As our ultimate objective was to locate genes controlling apomixis using a very common type of family in breeding programs for citrus rootstocks, i.e. *Citrus* spp.×*P. trifoliata*, bulked segregant analysis was chosen to search for markers linked to the putative locus controlling the trait. This approach has been shown to be very efficient for monogenically inherited traits (the observed segregation ratio apomixis: sexual fits the Mendelian proportion 3:1). For this reason, some primers were not used to genotype the whole family, thereby producing a genetic map in which not all linkage groups were equally represented or saturated but mainly those containing genes associated with apomixis.

Given that we have also used multiallelic codominant markers with alleles segregating from both parents, thereby providing a set of common loci (isozymatic loci, CAPs, RFLP and microsatellites), the integration and comparison of linkage maps can be done using those common loci as locus bridges. If 2 linked markers are segregating from both parents, two independent estimates for the marker pair can be determined. An increase in the number of markers segregating from both parents also increases the accuracy of the final combined map. However, if different marker orders in parental genetic maps were due to chromosomal rearrangements, combined maps would be artificial. Egp47 and TAA27 are closely linked in *Poncirus* linkage map (P5) while they are located in two different linkage groups in 'Volkamer' lemon (V3 and V5, respectively) suggesting that a translocation or transposition has occurred between linkage groups 3 and 5 during the evolutive differentiation of *Citrus* and *Poncirus*. These genomic regions marked with Egp47 and TAA27 are strongly affected by segregation distortions (Table 2) not only in the present progeny but also in the progeny derived from *P. trifoliata* 'Flying Dragon' by self-pollination (Mestre et al. 1997). Deviations from expected ratios have been previously reported for molecular markers, specially those analyzed in wide crosses (Weeden 1989; Durham et al. 1992; Jarrell et al. 1992).

Apomixis

Most of the data available on the genetic control of apomixis refer to gametophytic apomixis (diplospory or apospory) and report a single linkage group (Koltunow 1993). Segregation analysis of polyembryonic in terms of proportions of offspring has been previously reported (Parlevliet and Cameron 1959; Iwamasa et al. 1967; Cameron and Soost 1979). However, up to now, no genetic information has been available on the number of loci involved in the genetic control of nucellar embryony in citrus.

When apomixis was considered a categorical variable, independence tests showed that at least three linkage groups are involved (V1, P4 and P5). Since no homology between V1 and P4 or P5 was found, at least 3 independent loci (*Apo4*, *Apo5* and *Apo6*) affecting apomixis are segregating in the progeny. Once we detected the association between OPG13075 and apomixis in 'Volkamer' lemon, we tried to find a marker in the homologous region of *P. trifoliata*. Using that marker as a probe we found no significant association between this RFLP (cG13) and apomixis in *P. trifoliata*. This could be due to (1) allele effects at *Apo6* in 'Rubidoux' not being different enough to find a significant association in our progeny, and/or (2) genetic distances within P1 being much larger than within V1, the distance between cG13 and *Apo6* might also be large enough to have lost the statistical association between apomixis and the marker. Nevertheless, more than 1 locus is related to apomitic reproduction in *Citrus* spp.×*Poncirus trifoliata* progeny, contrary to expectations from the observed segregation ratio 3 apomixis: 1 sexual.

Two molecular markers associated with polyembryony were also found both different from those related to apomixis, and again both parental species differ in the marker locus (TAA15 in 'Volkamer' lemon and OPB05040 in 'Rubidoux'). However, both traits are related since all trees yielding polyembryonic seeds are apomictic trees. The reverse is not true, since not only sexual but also apomictic trees yield monoembryonic seeds. Our interpretation is that citrus seed development must involve at least two steps. A first step where the genotype at some loci controlling apomixis opens, or not, the possibility of developing embryos from the nucellar cells. If no nucellar embryo develops, seeds will be monoembryonic no matter what the genotype at the loci involved in embryony type is. On the other hand, if nucellar embryos finally develop, then it will depend on the genotype at the loci controlling embryony type whether one (monoembryonic) or more (polyembryonic) embryos develop inside the seed. This would explain the existence of monoembryonic trees that produce a certain percentage of nucellar seedlings (9 out of 21).

Apomixis is in fact a continuous variable, and other non-genetic factors, such as the year and the pollen origin, affect the percentage of zygotic seedlings (Cameron and Soost 1969; Mestre et al. 1997). This variation has also been observed in the present study. Thus, we estimated (using a morphological marker, the trifoliate shape of the leaf) that 60% of the seedlings from *C. volkameriana* seeds were zygotic when pollinated with *P. trifoliata* in 1977, while 21.5% were zygotic, as estimated by isozymatic markers, from open-pollination in 1996: Thus, pollination of *C. volkameriana* flowers with *P. trifoliata* pollen must greatly decrease the percentage of nucellar seedlings (from 78.5% to 40%). 'Volkamer' lemon is a polyembryonic rootstock with seeds having both nucellar and zygotic embryos. There must be a competition between the developing embryos, and many do not survive to seed maturity. When the zygotic embryo is an hybrid it could be more vigorous and more competitive to the nucellar embryos as compared with the zygotic embryo derived from self-pollination (all zygotic seedlings found in open-pollination conditions can be explained by self-pollination; no new isozymatic allele regarding the mother tree was observed). Therefore, QTL analysis of apomixis seems the proper approach. Our QTL analysis revealed the presence of six genomic positions (two in *P. trifoliata* and four in *C. volkameriana*) contributing individually up to 24% of the total variation for apomixis. Within the same species QTLs with positive and negative allele effects are present, even in the same linkage group. All genomic positions found to be associated with apomixis by χ^2 analysis contain *Apo* QTLs. However, *Apo1*, *Apo2* and *Apo3*, which contribute most to total apomixis variation, have only been unveiled by QTL analysis. *Apo2* is linked to a marker (TAA15) that was found to be associated with the presence of polyembryony by χ^2 analysis. Therefore, in our case, QTL analysis of the

original variable is statistically more powerful than χ^2 analysis even though the trait is not too normally shaped. Other limitations of the our QTL analysis are the number of fruit-yielding trees of the C×P progeny. This makes us suspect that with more codominant molecular markers and a larger family size where apomixis could be evaluated, more QTLs could be detected.

The genetic control of apomixis we have found in citrus is quite complex compared to our present knowledge of the genetic control of gametophytic apomixis where only one linkage group seems to be involved. The involvement of several QTLs in the genetic control of nucellar embryony in citrus makes this system too complex for gene isolation via map-based gene cloning in order to transfer this type of apomictic reproduction to other crop species.

Although more QTLs might be responsible for the apomictic reproduction of *C. volkameriana×P. trifoliata* hybrids, markers linked to *Apo1* through *Apo6* will be useful in assisting for the selection of apomictic genotypes within breeding programs for citrus rootstocks involving this interspecific cross. Nevertheless, the search for more QTLs affecting this trait in the Aurantioideae subfamily must continue.

Acknowledgements We are grateful to Drs. M. Roose and A. Granell for providing the RFLP clones and the primers to amplify the citrus ethylene receptor sequence, respectively. This work was supported by research grants CICYT (AGF97-0417), INIA (SC95-034) and Conselleria de Cultura y Educación (R.G.).

References

Anderson CM, Castle WS, Moore GA (1991) Isozymic identification of zygotic seedlings in Swingle citrumelo *Citrus paradisi×Poncirus trifoliata* nursery and field populations. J Am Soc Hortic Sci 116:322–326

Asíns MJ, Herrero R, Navarro L (1995) Factors affecting *Citrus* tree isozyme gene expression. Theor Appl Genet 90:892–898

Bacchi O (1943) Cytological observations in *Citrus*. III. Megasporogenesis, fertilization and polyembryony. Bot Gaz 105: 221–225

Basten CJ, Weir BS, Zeng Z-B (1997) *QTL cartographer*: a reference manual and tutorial for QTL mapping. Department of Statistics, North Carolina State University. Raleigh, N.C., USA

Cai Q, Guy CL, Moore GA (1994) Extension of the linkage map in *Citrus* using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-response loci. Theor Appl Genet 89:606–614

Cameron JW (1979) Sexual and nucellar embryony in F_1 hybrids and advanced crosses of *Citrus* with *Poncirus*. J Am Soc Hortic Sci 104:408–410

Cameron JW, Frost HB (1968) Genetics, breeding and nucellar embryony. In: Reuther W, Batchelor LD, Webber HJ (eds) The citrus industry, vol. 2. University of California Press, Berkeley, Calif., pp 325–370

Cameron JW, Gaber MJ (1968) Identical-Twin Hybrids of *Citrus×Poncirus* from strictly sexual seed parents. Am J Bot 55:199–205

Cameron JW, Soost RK (1979) Sexual and nucellar embryony in F_1 hybrids and advanced crosses of *Citrus* with *Poncirus*. J Am Soc Hortic Sci 104:408–410

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Durham RE, Liou PC, Gmitter FG Jr, Moore GA (1992) Linkage of restriction fragment length polymorphisms and isozymes in *Citrus*. Theor Appl Genet 84:39–48

Frost HB (1943) Seed reproduction: development of gametes and embryos. In: Webber HJ, Batchelor LD (eds) The Citrus industry, vol 1. University of California Press, Berkeley, Calif., pp 767–815

Grattapaglia D, Bertolucci FLG, Penchel R, Sederoff R (1996) Genetic mapping of Quantitative Trait Loci controlling growth and wood quality traits in *Eucalyptus grandis* using a maternal half-sib family and RAPD markers. Genetics 144:1205–1214

Hanna WW, Bashaw EC (1987) Apomixis: its identification and use in plant breeding. Crop Sci 27:1136–1139

Iwamasa M, Ueno I, Nishiura M (1967) Inheritance of nucellar embryony in *Citrus*. Bull Hortic Sta Jpn Ser B No. 7

Jarrell DC, Roose ML, Traugh SN, Kupper RS (1992) A genetic map of citrus based on the segregation of isozymes and RFLPs in an intergeneric cross. Theor Appl Genet 84:49–56

Kijas JMH, Thomas MR, Fowler JCS, Roose ML (1997) Integration of trinucleotide microsatellite into a linkage map of *Citrus*. Theor Appl Genet 94:701–706

Koltunow AM, Bicknell RA, Chaudhury AM (1995) Apomixis: molecular strategies for the generation of genetically identical seeds without fertilization. Plant Physiol 108:1343–1352

Leblanc O, Grimanelli D, González-de León, Savidan Y (1995) Detection of the apomictic mode of reproduction in maize-*Tripsacum* hybrids using maize RFLP markers. Theor Appl Genet 90:1198–1203

Mestre PF, Asíns MJ, Pina JA, Carbonell EA, Navarro L (1997a) Molecular markers flanking citrus tristeza virus gene from *Poncirus trifoliata* (L.). Raf Theor Appl Genet 94:458–464

Mestre PF, Asíns MJ, Carbonell EA, Navarro L (1997b) New gene(s) involved in the resistance of *Poncirus trifoliata* (L) Raf. to citrus tristeza virus. Theor Appl Genet 95:691–695

Mogie M (1988) A model for the evaluation and control of generative apomixis. Biol J Linn Soc 35:127–154

Monforte AJ, Asíns MJ, Carbonell EA (1996) Salt tolerance in *Lycopersion* species. IV. Efficiency of marker-assisted selection for salt tolerance improvement. Theor Appl Genet 93:765–779

Naithani SP, Raghuvanshi SS (1958) Cytogenetical studies in the genus *Citrus*. Nature 181:1406–1407

Naithani SP, Raghuvanshi SS (1963) Cytogenetical studies in *Citrus*. Part I. Genetica 33:201–312

Nogler GA (1984) Gametophytic apomixis. In: Jhri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 475–518

Parlevliet JE, Cameron JW (1959) Evidence on the inheritance of nucellar embryony in *Citrus*. Proc Am Soc Hortic Sci 74: 252–260

Pessino SC, Ortiz JP, Leblanc O, do Valle CB, Evans C, Hayward MD (1997) Identification of a maize linkage group related to apomixis in *Brachiaria*. Theor Appl Genet 94:439–444

Raguvanshi SS (1962) Cytogenetical studies in genus Citrus IV. Evolution in genus *Citrus*. Cytologia 27:172–188

Roose ML, Traugh SN (1988) Identification and performance of citrus trees on nucellar and zygotic rootstocks. J Am Soc Hortic Sci 113:100–105

Ruiz C, Breto MP, Asíns MJ (1998) A quick methodology to identify sexual seedlings in citrus breeding programs using SSR markers. Euphytica (in press)

Savidan Y (1982) Nature et hérédité de l'apomixie chez *Panicum maximum* Jacq. PhD thesis. University of Paris XI, Paris, France

Sherwood RT, Berg CC, Young BA (1994) Inheritance of apospory in buffalo grass. Crop Sci 34:1490–1494

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant 5:739–744

Stam P, Van Ooijen JW (1995) Joinmap (tm) version 2.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen

Weber HJ (1932) Variations in citrus seedlings and their relation to rootstock selection. Hilgardia 7:1–79

Weeden NF (1989) Application of isozymes in plant breeding. In: Janick J (ed) Plant breeding reviews, vol 6. Timber Press, Portland, Ore., pp 11–54