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Molecular detection of mutation in apple rootstocks

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ABSTRACT *- This study investigated the molecular characterization of genetic polymorphism in M9 and Marubakaido apple rootstocks propagated by cutting and by micropropagation. Nine isozymic, 39 RAPD, and 10 microsatellite polymorphic markers were identified and considered able to differentiate the rootstocks. Propagated systems could be differentiated by polymorphisms observed in isozyme loci and RAPD in certain genomic regions, and microsatellites were able to detect even the homozygous or heterozygous nature of polymorphic loci. The M9 rootstock, propagated* in vitro, *was more prone to genetically induced variation than the other rootstotock. The choice of the molecular marker is important and depends on the efficiency with which markers detect polymorphism and the different types of ongoing DNA changes.*

Key words: *Malus pumila*, *Malus prunifolia*, polymorphism, isozymes, RAPD, microsatellites.

INTRODUCTION

Vegetative propagation is a valuable strategy for fixing desirable genes in breeding programs, especially to scale up a superior genotype of a species such as apple. More recently, propagation for commercial purposes has been obtained through *in vitro* strategies. In both cases, genotypic fidelity is required (Rout and Das 2002, Feuser et al. 2003). When micropropagation protocols are utilized for cloning, it is expected that all individuals should have the same genotype. However, genetic variants can be induced by the inherent conditions of the culture medium. Some of these variants are epigenetic, while others are hereditary and called "somaclonal variations" (Larkin and Scowcroft 1981). Monitoring the degree of genetic integrity between *in vitro* micropropagated plants by molecular marker techniques is crucial to reduce the chances of variable genotypes (Rout and Das 2002).

In vitro culture techniques have long been recognized as efficient tools for rapid clonal multiplication. In addition, the occurrence of somaclonal variation among micropropagated plants has expanded the application of *in vitro* techniques in crop improvement (Skirvin 1978). Moreover, plant micropropagation can be utilized for *ex situ/ in vitro* conservation or for regeneration and re-introduction into the original or similarly favorable habitats. Furthermore, tissue culture can be used to establish regenerative competence for genetic transformation so as to overcome pre- and post-zygotic barriers and to establish embryogenic cell lines for different purposes.

Cutting propagation and more recently micropropagation have been used in the multiplication of apple rootstocks for the establishment of new orchards or to carry out genetic studies and breeding. However, the proportion of the genotypic fidelity processes is not known. The maintenance

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of the superior performance of a certain clone depends on the maintenance of its allelic association. Thus, the monitoring of the degree of genetic fidelity of different cloning systems (*in vitro* or *in vivo*), using molecular and biochemical markers, is promising as strategy to increase our poor knowledge on this aspect.

The identification of genetic fidelity in apple rootstocks, based on the evaluation of morphological and physiological traits, is not always reliable because these traits are susceptible to interactions with the environment. In addition, certain characteristics are only expressed in the reproductive stage, which is a time-consuming process (Aldwinckle and Norelli 2000). Moreover, not all mutations affect the phenotype.

The best way to detect the existence of variation is to use a progeny test. However, this approach also takes too long if a species is perennial, such as apple. Then, biochemical and molecular markers can be used to detect some of the somaclonal mutations. If a DNA mutation causes a polypeptide alteration, which affects its migration into the gel, it can be detected by comparison with the band pattern of the mother plant.

Thus, the objective of the present study was to evaluate the level of polymorphism among rootstocks and the proportion of genetic variants in apple rootstock, micropropagated (*in vitro*) and propagated vegetatively by cuttings (*in vivo*) using isozyme, RAPD, and microsatellite markers.

MATERIAL AND METHODS

Plant material and propagation method

Two apple rootstocks, M9 (*Malus pumila*) and Marubakaido (*Malus prunifolia*), and two propagation methods (micropropagation and cutting) were used in this study. Juvenile plants of both genotypes were used as original donors of the explants, providing internodal segments (0.5 – 1.0 cm pieces), which were utilized for micropropagation (thereafter *in vitro*) in Murashige and Skoog (1962) medium with benzylaminopurine (BAP) (2.0 mg L^{-1}) and naphtalenacetic acid (NAA) (0.1 mg L^{-1}). The explants were then maintained in a growth room at 25 ± 2 °C during a 16-hour photoperiod and light intensity of 19 mE m^{-2} s^{-1} under white fluorescent light. The plantlets were subcultured at 4-weekly intervals using fresh medium with the same composition. The second propagation method was cutting (thereafter *in vivo*), where the cuttings (10-15 cm) were immersed in an indole butyric acid (IBA) solution (1.000 mg L^{-1}) for 10 seconds to induce rooting. The rooted cuttings had been maintained in the greenhouse for one year before the use in this study.

Isozyme analyses

The isozymes of all analyzed apple plants were extracted by grinding 3.0 g of leaf tissue in an ice-cooled mortar, to which three drops of sodium phosphate 0.034 M extraction buffer had been added (2.5 mM of borate of sodium; 5.8 mM of DIECA; 3 mM of DTT; 0.034 M sodium bi-phosphate; 5.7 mM L-ascorbic acid; 0,2% (v/v) 2-mercaptoethanol; 1% (p/v) polyetilenoglicol 6000; 2.6% (p/v) polyvinylpyrrolidone PVP 40; 20 mM sucrose). Four gel/electrode buffer systems were tested: 1) Morpholine-citrate pH 6.1- CM (Clayton and Tretiak 1972); 2) Tris-citrate pH 7.5 - TC (Selander and Yang 1969); 3) Lithium borate pH 8.1 - LB (Ridgway et al. 1970); and 4) Histidine pH 8.0 - HT (Cheliak and Pittel 1984). Isozyme electrophoresis was performed with starch gel 13% (w/v) of Penetrose 30 (Corn products, Mogí Guaçu-SP), at 5 °C for 4-7 h at 50 mA or 310 V. After electrophoresis, the gels were sliced horizontally into 1.5 mm thick slices and stained for the 24 isozyme systems: PGI, MDH, PGM, SKDH, PRX, SDH, IDH, ADH, ACP, GTDH, G6PDH, to-EST, b-EST, ME, MADH, LAP, CAT, GOT, SOD, GDH, G2DH, ALDH, 6PGDH, and ALP, according to the methodology proposed by Alfenas et al. (1998). The evaluation of the zimograms assessed four parameters: presence or absence, intensity and migration of the different bands, and the best conditions to run each isozyme system and gel/electrode buffer combination.

DNA extraction

The DNA for both RAPD and microssatelite markers was extracted from fresh apical leaf tissue (either *in vivo* or *in vitro* grown genotypes), using the CTAB method (Doyle and Doyle 1987). Sample leaf tissue (1 g) was frozen in liquid nitrogen, pulverized to a fine powder, and transferred to a 2.0 mL eppendorf, to which 700 μl of CTAB extraction solution was added (1% cetyltrimethylammonium bromide; 0.7 m NaCl; 50 mM Tris-HCl; 10 mM EDTA; pH 8.0 and 0.1% 2 mercaptoethanol added before use). The DNA was quantified in 0.8% (w/v) agarose gel and visualized by staining with ethidium bromide (1 mg mL^{-1}) using TBE buffer at pH 8.0 (Sambrook et al. 1989). To estimate the DNA quantity in the sample, the fluorescence intensity of unkown DNA was compared with the DNA standards (DNA Fago Lambda 50, 100 and 200 ng μL^{-1}). Afterwards, each DNA sample was diluted in sterile distilled water adjusted to the concentrations of 3 ng μL^{-1} and 10 ng μL^{-1}, until the use for RAPD and microsatellite analysis, respectively.

RAPD

RAPD amplification was performed in a total volume of 13 μL containing 3 μL DNA template (3 ng μL^{-1}), 1.3 μL 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 1.04 μL BSA (10 mg mL^{-1}), 1.04 μL dNTPs

(2.5 mM each nucleotide), 0.2 μL *Taq* DNA polymerase (5 U μL^{-1}), 3.42 μL sterile water, and 3 μL primer (2.5 μM μL^{-1}). In total, 115 different decamer primers (Operon) were tested. The PCR was run in a thermal cycler (PTC100, MJ Research) for an initial denaturation step of 90 s at 92 °C, followed by 40 cycles of 1 min at 92 °C, 1 min at 35 °C, and 2 min at 72 °C. Afterwards, the reaction was submitted to a final extension step of 5 min at 72 °C. RAPD fragments were resolved by electrophoresis on 1.5% agarose gel with TBE buffer and stained in ethidium bromide (1 mg mL^{-1}). The gels were visualized and photographed under UV light for posterior analysis. A 1 kb ladder (Life Technologies) was used as a molecular weight reference to estimate the amplification product sizes. The DNA amplification was evaluated by the presence or absence of bands. For each sample, the PCR was carried out twice. Only bands reproducible in both runs were scored for analysis.

Microsatellites

Twenty primer pairs were synthesized (Life Technologies) from sequences selected from an apple genomic library (cv. Royal Gala) developed by Gianfranceschi et al. (1998) and Guilford et al. (1997). Amplification reactions were carried out in a final volume of 15 μL containing 3 μL of DNA (10 ng μL^{-1}), 1.5 μL of each foward and reverse primer (2 uM), 3.0 μL 10 X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 0.72 μL $MgCl_2$ (50 mM), 1.5 μL dNTPs (2.5 mM of each nucleotide), 0.2 μL *Taq* DNA polymerase (5 U μL^{-1}) and 5.08 μL sterile water. Initially, DNA was denatured for 2 min at 94 °C followed by 20 cycles of 60 s at 94 °C, 30 s at 50 or 55 °C (primer annealing), and 45 s at 72 °C. The amplified products in the thermal cycler (PTC100, MJ Research) were resolved on 6% polyacrylamide denaturating gel. A 100 bp DNA ladder (Life Technologies) was used as molecular weight reference to estimate the sizes of the amplification products. The gels were stained with silver nitrate, as described by Creste et al. (2001). The presence of the expected alleles and their sizes were evaluated in each of the propagated genotypes through both methods, *in vivo* and *in vitro*.

RESULTS AND DISCUSSION

Isozyme analysis

Considering the quantity of bands and color intensity and resolution, the best buffers were TC and CM for all isozyme systems, but one (6PGDH) presented the best performance in the HT buffer, although this system was not tested in the CM and TC buffers. Seven out of 24 tested isozyme systems (ME, GOT, SOD, CAT, ALDH, ADH, and GDH) did not reveal any bands at all, or presented bands that were very weak. For this reason, these systems were excluded from further analysis. In total, 19 polymorphic loci from nine systems and 10 monomorphic loci from eight systems were detected (Table 1). This degree of polymorphism (65%) was considered satisfactory to perform further genetic analyses, since only two cultivars, belonging to different apple species, were utilized.

The zymograms of the PGI, MDH, PGM, PRX, ACP, and 6PGDH systems showed differences when plants from different propagation methods (*in vivo* and *in vitro*) were compared (Figure 1). In the 6PGDH system, three loci were identified in cultivated apples. However, in the present study, only two out of the three loci were visualized. Three bands were detected in the 6Pgdh-2 locus. As the enzyme's form was dimeric, the M9 rootstock propagated *in vivo* must have been heterozygous, because it exhibited three bands, by contrast to M9 propagated *in vitro*, which is possibly homozygous since it showed only one band (Figure 1a).

Table 1. Enzymatic systems tested in apple rootstocks

Polymorphic system	Number of Loci	Monomorphic system	Number of Loci
6PGDH	2	ADH	1
PGM	3	SDH	2
ACP	3	a-EST	1
MDH	3	b-EST	1
PGI	2	GTDH	2
PRX	2	MADH	1
G2DH	1	ME	1
G6PDH	1	SKDH	1
IDH	2		
Total	19	Total	10

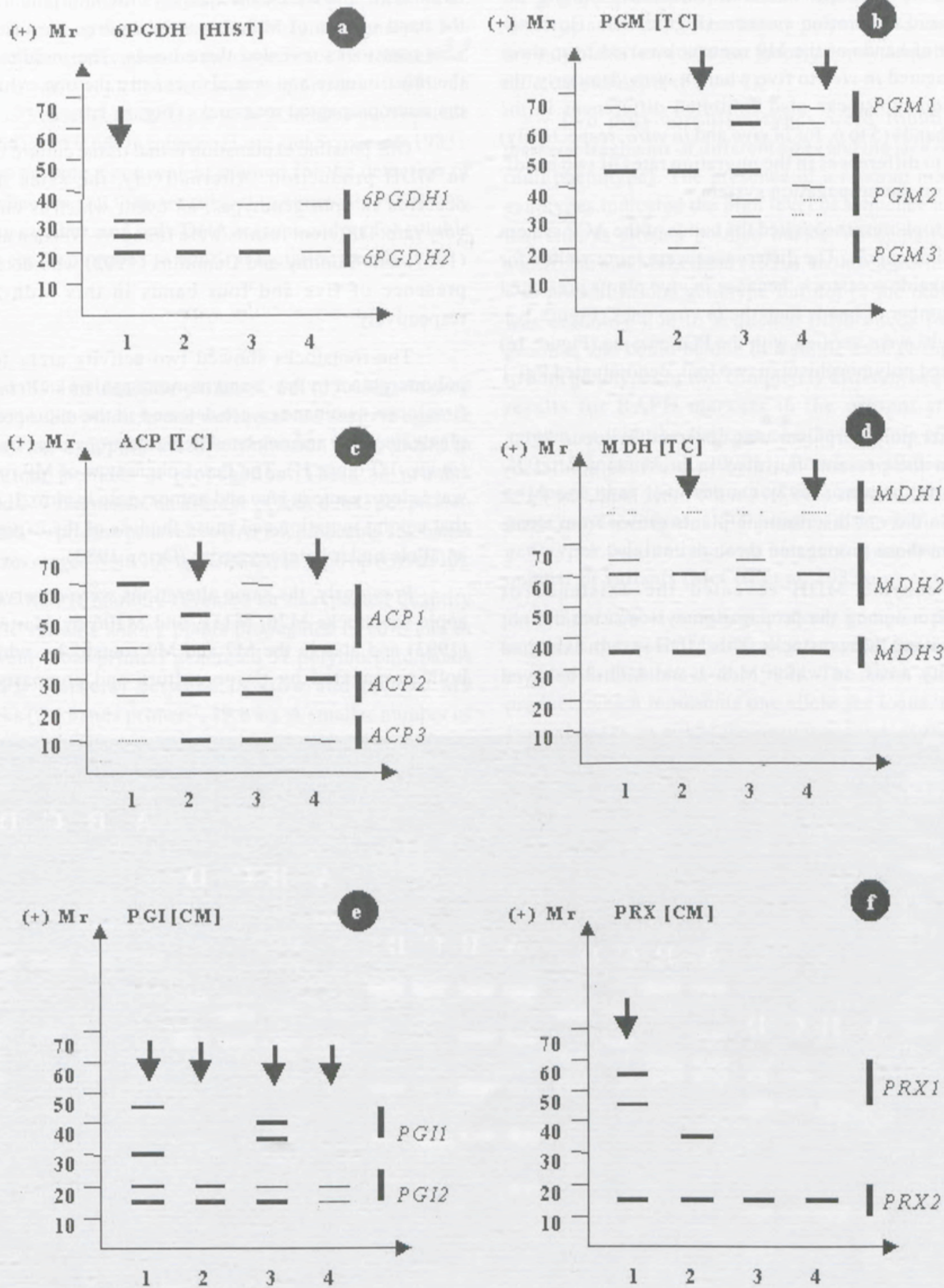

Figure 1. Electrophoretic banding patterns, relative mobility (Rf x 100) of six isozymic loci detected in apple rootstocks. M9 (1) and Marubakaido (3) cultivated *in vivo*; M9 (2) and Marubakaido (4) cultivated *in vitro*

The enzyme PGM detected variability among the rootstocks and cultivation systems (Figure 1b). However, the number of bands of the M9 rootstock varied from three when propagated *in vivo* to five when *in vitro*. Similarly, the Marubakaido rootstocks also exhibited differences in the number of bands (5 to 6, for *in vivo* and *in vitro*, respectively) in addition to differences in the migration rates of two bands, depending on the propagation system.

Electrophoresis separated the bands of the ACP system in three activity areas. The differences were more visible for the Marubakaido rootstock, because *in vivo* plants presented a greater number of bands than the *in vitro* ones (Figure 1c). Similar results were verified with the PGI enzyme (Figure 1e) that exhibited polymorphism in two loci, denominated Pgi-1 and Pgi-2.

Greater polymorphism was observed in locus Pgi-1, not only in the present, but also in previous studies by Samimy and Cummins (1992). On the other hand, the Pgi-2 band pattern did not discriminate plants grown from tissue culture from those propagated through cuttings.

The enzyme MDH revealed the existence of polymorphism among the propagation systems, but did not confirm it among the rootstocks. This MDH system exhibited three activity areas, in which Mdh-1 and Mdh-3 behaved monomeric and were consequently monomorphic. However, the band pattern of Mdh-2 was intriguing, because both *in vivo* rootstocks revealed three bands. The middle one was the most intense and was also exactly the one exhibited by the micropropagted rootstocks (Figure 1d).

One possible explanation is that tissue culture interferes in MDH production. Alternatively, the same mutation occurred in both genotypes, an event which is considered very rare. Different results were found by Weeden and Lamb (1987) and Samimy and Cummins (1992) who detected the presence of five and four bands in this Mdh-2 locus, respectively.

The rootstocks showed two activity areas for PRX: polymorphism in Prx-1 and monomorphism in Prx-2. In the first locus, two bands were detected in the micropropagated plants (*in vitro*) and one band in plants propagated by cuttings (*in vivo*) (Figure 1f). The Prx-1 phenotype of M9 rootstocks was heterozygote *in vivo* and homozygote *in vitro*. It is known that a point mutation can cause the loss of the expression of an allele under heterozygosity (Orton 1983).

Previously, the same alterations were observed in the apple rootstocks M26, M111, and M106 by Martelli et al. (1993) and also in the M7 and M9 rootstocks, which were both propagated by tissue culture and compared to the

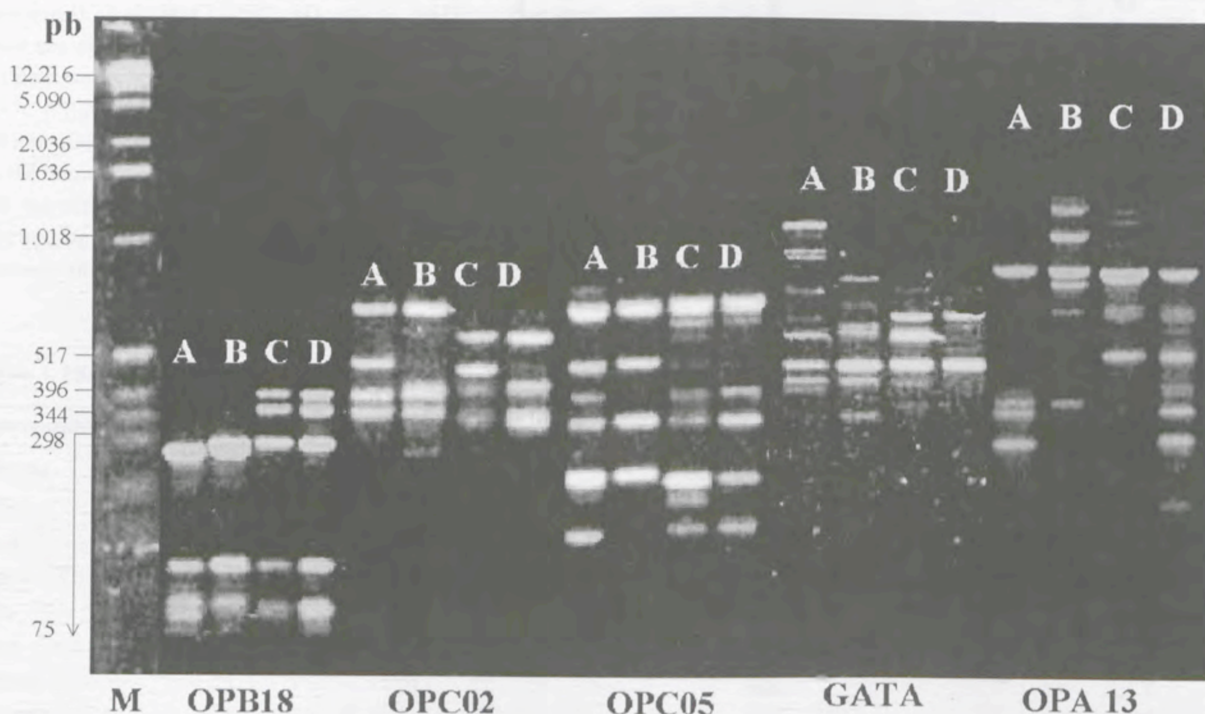

Figure 2 – RAPD products from M9 and Marubakaido apple rootstocks: M) Ladder 1Kb; A) M9 *in vivo*; B) M9 *in vitro*; C) Marubakaido *in vivo*; D) Marubakaido *in vitro*

original material with isozyme analysis by Dantas et al. (2000a). In spite of these difficulties, isozymatic analysis has facilitated the observation of the occurrence of somaclonal variants in different species and proportions: 0.67% in pineapple (Feuser et al. 2003), 2 to 38% in banana (Vuysteke et al. 1988), and 9.6% in tobacco (Lorz and Scowcroft 1983). Isozymes provide a convenient method for the detection of genetic changes, but they are subject to ontogenic variations, limited in number, and only DNA regions coding for soluble proteins can be sampled to confirm mutation events.

RAPD

Out of 115 random primers, 64 (55.6%) showed amplified visible products and well-resolved bands in agarose gels, allowing an appropriate discrimination of rootstocks in the different methods of propagation. These 64 primers amplified 257 fragments, an average of four bands per primer. From these, 39 primers behaved as RAPDs, producing 152 bands whose sizes ranged from 100 bp (OPC05) to 1850 bp (OPAN10).

The RAPD markers revealed an unexpected quantity of genetic variants among plants propagated *in vitro* and *in vivo*. Twenty-four primers generated 51 polymorphic bands (or RAPD markers) between *in vitro* and *in vivo* M9 rootstocks (2.1 bands $primer^{-1}$, 19.8%). A smaller number of primers (18) generated 34 polymorphic bands (1.8 bands $primer^{-1}$, 13.3%) between *in vitro* and *in vivo* Marubakaido (Table 2). These RAPDs were able to discriminate rootstock plants in relation to the propagation system. In addition, the greatest amount of polymorphism was observed in the *in vitro* M9 (Table 2). These results gave rise to the hypothesis that M9 would be more prone to the ocurrence of mutation when cultivated *in vitro* than Marubakaido in the same system. When the two apple rootstocks propagated by cuttings (*in vivo*) were compared, a total of 24 polymorphic bands from 15 primers were detected (1.6 bands $primer^{-1}$, 9.3%).

A greater quantity of polymorphism was detected by the same comparison among *in vitro* plants: 43 polymorphic bands originating from 19 primers (2.3 bands $primer^{-1}$, 16.7%) (Table 2). These data suggested that the micropropagation method would be able to generate more somaclonal variants between the tested rootstock than propagation by cuttings.

Overall, the RAPD primers that detected polymorphism among plants from one propagation system did not detect it among plants from the other system. This trend was also noticed in other comparison types. Five primers (OPA04, OPB18, OPAN07, OPAN11 and OPAN17) were monomorphic for the propagation systems, but they were unable to differentiate the genotypes. On the other hand, the primers OPC02, OPC05, $GATA_{(4)}$, and OPA13 were highly polymorphic, discriminating not only plants from different propagation systems but also from different genotypes (Figure 2).

Two polymorphism types were found in RAPD markers: fragments of different sizes and the lack of fragments (null phenotype). The presence of a random marker in two genotypes indicated the high level of sequence homology at that site, as already pointed out by Williams et al. (1990) and Welsh and McLelland (1990). In the case where a marker was present in one genotype but not in the other, this fact was certainly due to sequence differences found in the genome, and could be due to a single base change common to both genotypes or two completely different sequences. The results for RAPD markers in the present study are in agreement with the findings of other authors (Feuser et al. 2003, Dantas et al. 2000b). Previous studies provided evidence that the frequency of somaclonal variants is genotype dependent. Other studies also verified that plant genotypes originating from tissue culture have a greater number of variants (McCoy et al. 1982).

Somaclonal variation is a very complex problem that requires the use of several strategies for an adequate evaluation (Fourré et al. 1997). The DNA amplification product, which represents one allele per locus, could result from changes in either the sequence sites of the annealing primer or changes that alter the size and present the successful amplification of the target DNA. Because RAPDs are most often expressed in a dominant fashion, it is also possible that for a diploid dominant homozygote at a particular locus, mutations affecting only one of the two alleles would remain undected (Isabel et al. 1993), but could still induce phenotypic alteration if they were expressed as a dominant mutation (Raimondi et al. 2001).

RAPDs are useful analytical tools to genetically characterize clonal material, especially where it is difficult to distinguish clones by their phenotype. In apple, a single DNA primer gives sufficient polymorhism to characterize cultivars and to detect genetic differences among different accessions from the same cultivar that were vegetatively propagated (Mulcahy et al. 1993). When micropropagation is carried out by meristem culture, it is generally assumed that the risk of genetic instability is low. The explant type utilized is generally highly resistant to genetic changes, probably due to better meristematic organization during cell division or differentiation under *in vitro* conditions (Shenoy and Vasil 1992). Even so, different frequencies of variants have been found with meristem culture: 0.045% variance between microcuttings of plants cultivated *in vitro* and *in vivo* clones of *Cedrus libani* (Piola et al. 1999); 6.3% in

Table 2. RAPD primers, sequences, size range of fragments, and numbers of bands in micropropagated plants (*in vitro*) and cuttings (*in vivo*) of M9 and Marubakaido rootstocks

Primers	Sequence (5'→3')	Fragment size range (bp)	Number of bands scored			
			in vivo and *in vitro*		M9 and Marubakaido	
			M9	Marubakaido	*in vivo*	*in vitro*
1. OPA01	CAGGCCCTTC	450	1	0	1	0
2. OPA02	TGCCGAGCTG	500 → 1050	3	5	2	5
3. OPA04	AATCGGGCTG	550 →1600	0	0	2	0
4. OPA05	AGGGGTCTTG'	1100	0	1	0	2
5. OPA09	GGGTAACGCC	980 →1650	0	2	1	0
6. OPA10	GTGATCGCAG	400 →480	0	1	0	0
7. OPA13	CAGCACCCAC	300 →1300	6	3	0	4
8. OPA16	AGCCAGCGAA	800 →1400	1	0	2	2
9. OPA18	AGGTGACCGT	550 →650	3	0	0	4
10. OPB11	GTAGACCCGT	550 →750	2	1	0	1
11. OPB17	AGGGAACGAG	520 →900	2	2	0	1
12. OPB18	CCACAGCAGT	344 →396	0	0	2	2
13. OPC02	GTGAGGCGTC	298 →600	1	0	0	0
14. OPC04	CCGCATCTAC	1500	1	0	1	0
15. OPC05	GATGACCGCC	100 →520	0	2	0	0
16. OPC06	GAACGGACTC	520 →1500	0	2	0	0
17. OPC07	GTCCCGACGA	520	0	1	0	0
18. OPC08	TGGACCGGTG	385	1	0	0	0
19. OPC09	CTCACCGTCC	480 →1500	1	2	0	2
20. OPC10	TGTCTGGGTG	296 →520	3	1	2	5
21. OPE06	AAGACCCCTC	550 → 2000	0	2	0	0
22. OPS30	GCGTAGAGAC	550 → 1080	0	2	0	0
23. OPS36	CTCCAAGGCC	650 →1200	1	0	0	0
24. GATA	$GATA_{(4)}$	350 →1100	4	2	3	4
25. OPF01	ACGGATCCTG	650 →1018	0	1	0	1
26. OPF09	CCAAGCTTCC	550	1	0	0	0
27. OPF10	GGAAGCTTGG	400 → 1080	3	0	0	0
28. OPF13	GGCTGCAGAA	650 → 950	3	0	0	2
29. OPAN03	AGCCAGGCTG	740 →1040	2	0	1	1
30. OPAN07	TCGCTGCGGA	1080	0	0	0	1
31. OPAN08	AAGGCTGCTG	390 → 450	1	0	1	0
32. OPAN09	GGGGGAGATG	396 → 410	2	2	0	0
33. OPAN10	CTGTGTGCTC	350 →1850	3	0	0	2
34. OPAN11	GTCCATGCAG	480 →750	0	0	2	2
35. OPAN12	AACGGCGGTC	1300	1	0	2	0
36. OPAN14	AGCCGGGTAA	290 →510	2	0	0	1
37. OPAN15	TGATGCCGCT	550	1	0	0	0
38. OPAN16	GTGTCGAGTC	396 → 510	2	2	1	1
39. OPAN17	TCAGCACAGG	520	0	0	1	0
Total bands scored			51	34	24	43
Percentage of polymorphism			19.8	13.3	9.3	16.7
Total polymorphic markers			24	18	15	19

pineapple (Feuser et al. 2003), and great genetic instability of *in vitro* apple rootstock (Martelli et al. 1993), Dantas et al. 2000b).

Microsatellites

Microsatellite markers were amplified with 12 primer pairs that produced 27 reproducible fragments ranging in size from 90 bp to 330 bp (Table 3). Between the two rootstocks only the 01E12 microsatellites are monomorphic. In the M9 rootstock, nine microsatellites showed differences between *in vivo* and *in vitro* propagated M9 rootstocks, because the band pattern revealed heterozygosity and homozygosity, respectively. Three microsatellites (01H01, 01E12, and 04H11) showed identical alleles in the *in vivo* and *in vitro* plants (Table 3). In the Marubakaido rootstock, two microsatellites (01H01 and 02D11) revealed differences in heterozygote and homozygote states between *in vivo* and *in vitro* propagated plants. Three microsatellites (01G12, 01E12, and 01A6) were monomorphic.

Table 3. Molecular polymorphism of microsatellite primer pairs in micropropagated plants (*in vitro*) and cuttings propagated (*in vivo*) from the M9 and Marubakaido apple rootstocks

SSR	Primer sequence (5'→ 3')	M9 *in vivo*	M9 *in vitro*	Marubakaido *in vivo*	Marubakaido *in vitro*
01F02	F ACCACATTAGAGCAGTTGAGG	195/185	185/185	200/190	200/190
	R CTGGTTTGTTTTCCTCCAGC				
02D12	F AACCAGATTTGCTTGCCATC	255/205	205/205	270/205	270/205
	R GCTGGTGGTAAACGTGGTG				
01G12	F CCCACCAATCAAAAATCACC	155/142	142/142	140/140	140/140
	R TGAAGTATGGTGGTGCGTTC				
01H01	F GAAAGACTTGCAGTGGGAGC	122/122	122/122	135/135	150/135
	R GGAGTGGGTTTGAGAAGGTT				
02B03b	F ATAAGGATACAAAAACCCTACACAG	110/90	90/90	115/90	115/90
	R GACATGTTTGGTTGAAAACTTG				
02B10	F CAAGGAAATCATCAAAGATTCAAG	148/130	130/130	138/120	138/120
	R CAAGTGGCTTCGGATAGTTG				
23G4	F TTTCTCTCTCTTTCCCAACTC	330/280	330/390	270/270	270/270
	R AGCCGCCTTGCATTAAATAC				
01E12	F AAACTGAAGCCATGAGGGC	122/122	122/122	122/122	122/122
	R TTCCAATTCACATGAGGCTG				
02D11	F AGCGTCCAGAGCAACAGC	215/180	180/180	220/205	205/205
	R AACAAAAGCAGATCCGTTGC				
01A6	F AGGATTGCTGGAAAAGGAGG	190/190	190/210	190/190	190/190
	R TTAGACGACGCTACTTGTCCT				
02B1	F CCGTGATGACAAAGTGCATGA	115/115	135/115	140/115	140/115
	R ATGAGTTTGATGCCCTTG GA				
04H11	F CTTCCATCGAGATTGCATCATA	230/220	230/220	230/220	230/220
	R CGAATTGAGAGGTCGTCGTT				

These microsatellite features confirm the hypothesis that tissue culture techniques induce considerable stress in micropropagated plants (Sibi 1990). Such conditions can also be responsible for changes in the DNA observed in repetitive sequences in the micropropagated plants of the M9 rootstock. The effect induced by the tissue culture in the present study was to change the heterozygous or homozygous state being heterozygous in *in vivo* plants and homozygous *in vitro*. These same variations were also detected with microsatellite markers in *Nicotiana* spp (De Paepe et al. 1982), in cereals (Lapitan et al. 1988), and in *Actinidia deliciosa* (Palombi and Damiano 2002).

The nature of the somaclonal variation observed in the *in vitro* M9 genotypes is important and was confirmed by different marker types. In the case of the microsatellites, the measured genetic variation among the genotypes is dependent on changes in the number of repeats. This type of genetic variation can be detected by microsatellites. They are codominant markers and they are also more polymorphic than RAPD markers (Milbourne et al. 1997).

In the present study, an expressive quantity of RAPD and microsatellite markers was able to amplify the DNA samples from apple rootstock assayed to detect different forms of genetic variations. It is possible to conclude that when molecular markers are used with this goal, the choice of the DNA analysis technique is important and depends on both the efficiency with which the molecular markers detect polymorphism and the different nature of DNA changes occurring in the plants.

The microsatellite markers detect variation at pre-determined sites, such as the DNA repetitive regions. These sites are hypervariable with respect to other regions of the genome, and this hypervariability is due to a particular phenomenon called 'slippage', which can occur more frequently than point mutation or insertion-deletions events and is responsible for the polymorphism that RAPD markers detect (Milbourne et al. 1997).

CONCLUSIONS

This study identified nine isozyme systems (19 loci), 39 RAPDs (24 to 51 polymorphic bands) and 12 microsatellites (17 alleles), which were sufficient to differentiate the two apple rootstocks and were suitable for application in further genetic studies.

RAPD analysis can be used for genetic discrimination and to locate isolated mutations linked to these markers, but the absence of intraclonal RAPD polymorphism cannot ensure genetic stability because genomic and chromosomic mutations may remain undecteted. Vegetative propagation relies on the ability to produce exact genetic copies of the mother plant, and this study has shown that RAPD and microsatellites can be used to assay the fidelity of this process in different genotype sources and propagation by different methods. In our case, the best technique was RAPD, because it was more efficient than the others at detecting changes in regions with small sequences located randomly in the plant genome.

Leaf samples from plants of the same rootstock grown *in vivo* and *in vitro* showed different isozyme, RAPDs and microsatellite banding patterns. These differences are related to the number and the position of bands. The RAPD, microsatellite and allozymic markers can be applied to quickly evaluate the genetic fidelity of the micropropagated plants and the integrity of the genetic resources to be conserved *in vivo* or *in vitro*. Somaclonal variants might be desirable in the extension that they increase the variability for use in plant breeding, but they are not desirable in the mass clonal propagation of a selected superior genotype. Besides knowing the frequency of a somaclonal variant, it is also necessary to know the mutation types it provokes and the effects of these mutations on the phenotype. Finally, it is essential to confirm the variant´s heritability. Once the causes of the somaclonal variation *in vitro* are better understood, protocols can be adjusted to control or to reduce them to the minimum.

Detecção molecular de mutações em porta-enxertos de macieira

RESUMO - *Este estudo envolveu a caracterização molecular do polimorfismo genético dos porta-enxertos M9 e Marubakaido de macieira propagados por estaquia e micropropagação. Foram identificados 9 sistemas isoenzimáticos, 39 RAPD e 10 microssatélites marcadores polimórficos capazes de diferenciar os porta-enxertos. Comparando os sistemas de propagação, enquanto diferenças no número e na posição das bandas foram evidenciadas pelos sistemas isoenzimáticos e RAPD, os microssatélites foram capazes de detectar variação em locos polimórficos homozigotos ou heterozigotos. A presença de variação genética foi maior no porta-enxerto M9* in vitro. *A escolha da melhor técnica de análise de DNA é importante e depende da eficiência com que os marcadores detectam o polimorfismo e os tipos de alterações que ocorrem no DNA.*

Palavras-chave: *Malus pumila, Malus prunifolia*, polymorphism, isozymes, RAPD, microssatéllites.

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