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The use of RAPD for verifying the apomictic status of seedlings of *Malus* species

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Abstract The lack of red-purple pigmentation in seedlings obtained from crosses between *M*. cv Baskatong, carrying a dominant homozygous gene for red-purple pigmentation, and other species has been used for the detection of apomictic plants in *Malus* species. RAPD marker techniques were employed to evaluate the genetic similarity between putative apomictic seedlings and their female parents. From the selected set of 20 (OPA) primers about half were able to detect hybrids from the apomictic seedlings, if present. RAPD analyses confirmed the usefulness of the colour-marker gene in detecting the hybrids in vitro for seedlings of *M. toringoides* × *M*. cv Baskatong, but not for crosses involving *M. hupehensis* × *M*. cv Baskatong where in vitro colour-based selection was not possible (due to red stems in all cases). The set of primers (OPA-01, 02, 08, 09, 10, 12, 13, 14, 16, 18 and 20) clearly determined the hybrid nature of seedlings and allowed the selection of apomictic ones. Therefore, although cv Baskatong is useful as an indicator, these data show that this technique is not applicable in all cases.

Key words *Malus* · Apomixis · RAPD · Baskatong · Red-purple pigmentation

Introduction

Different *Malus* species are reported to generate apomictic seedlings facultatively (Sax 1932; Dermen 1936; Schmidt 1970) and to vary in the degree of apomictic seed production (Sax 1959). In the past, apomictic seedlings have been detected among hybrids on the basis of red-purple pigmentation when *M*. cv Baskatong, homozygous for the colour-marker gene, *BB*, (Sampson and Cameron 1965, Sampson 1968/69; later given the notation R_t, Alston and Watkins 1973), was used as a male parent in crosses. Advances in the use of molecular genetic markers now allow research on genetic variation at the DNA level. To assess the validity of the colour-based selection technique (James et al. 1984, 1985) genetic variation between putative apomicts (green seedlings), hybrids (red seedlings) and their female parent were further evaluated in this study using randomly amplified polymorphic DNA markers (RAPD).

Restriction fragment length polymorphism (RFLP) and genetic fingerprinting employing the bacteriophage M13 DNA probe have proved to be time-consuming and require the use of isotopes (Nybom et al. 1990; Parent and Page 1992). RAPD markers have been successfully used for variety identification, mapping, and taxonomic studies (Williams et al. 1990; Graham and McNicol 1995; Demeke et al. 1996), despite the drawback of showing poor 'between laboratory' repeatability. In *Malus*, RAPD has also been used to detect genetic variation and paternity (Harada et al. 1993). Here we evaluate the genetic similarity between the apomictic seedlings and the female parent using RAPD molecular markers in order to validate the set of primers used, and the applicability of the colour-based detection technique for the selection of apomictic seedlings.

Materials and methods

Plant material

The genetic analyses were carried out on 13 seedlings from *M. hupehensis* × *M*. cv Baskatong (flowers were emasculated at the

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Table 1 Code and sequence of primers from OPERON kit A employed in this study

Code	Sequence (5′ → 3′)	Code	Sequence (5′ → 3′)
OPA-01	CAGGCCCTTC	OPA-11	CAATCGCCGT
OPA-02	TGCCGAGCTG	OPA-12	TCGGCGATAG
OPA-03	TGTCAGCCAC	OPA-13	CAGCACCCAC
OPA-04	AATCGGGCTG	OPA-14	TCTGTGCTGG
OPA-05	AGGGGTCTTG	OPA-15	TTCCGAACCC
OPA-06	GGTCCCTGAC	OPA-16	AGCCAGCGAA
OPA-07	GAAACGGGTG	OPA-17	GACCGCTTGT
OPA-08	GTGACGTAGG	OPA-18	AGGTGACCGT
OPA-09	GGGTAACGCC	OPA-19	CAAACGTCGG
OPA-10	GTGATCGCAG	OPA-20	GTTGCGATCC

balloon stage, pollinated with *M*. cv Baskatong pollen and bagged immediately after), and on five putative apomicts (green seedlings) and one hybrid (red seedling) from *M. toringoides* × *M*. cv Baskatong. Seedlings were generated from the embryonic axes dissected at 90-days post-pollination and micropropagated in vitro. The female parents, *M. hupehensis* and *M. toringoides*, one accession of *Malus sargentii*, and the male parent *M*. cv Baskatong used in this analysis were all accessed from the germplasm collection at HRI, East Malling.

DNA extraction

Leaf samples (approximately 30.0 mg) were taken from in vitro grown plantlets and homogenised with a microfuge pestle; 500 µl of pre-warmed (65°C) extraction buffer (Tris 100 mM, pH 8; EDTA 20 mM; NaCl 1.4 M; CTAB 2.0%; PVP-40 2.0% w/v; β-mercaptoethanol 1.0% v/v) were added and the material ground further and incubated for 10 min at 65°C. One volume of dichloromethane : isoamylalcohol (24 : 1 v/v) was added to the tubes and mixed thoroughly. The mixture was centrifuged for 2 min at 13 000 rpm (11 600 g). The top phase was transferred to a fresh microcentrifuge tube and 300 µl of ice-cold isopropanol were added to precipitate the DNA. This was pelleted by centrifugation for 2 min at 13 000 rpm and the supernatant was carefully poured off. The DNA pellet was washed by adding 500 µl of 'wash buffer' (76% ethanol, 10 mM ammonium acetate, water) and centrifuged for 2 min at 13 000 rpm. The supernatant was discarded and the pellet air-dried. The DNA was then re-suspended in 50 µl of double-distilled water (DDW). The DNA concentration was measured fluorometrically as recommended by the manufacturer (Hoefer Scientific Instruments, San Francisco USA) before dilution in DDW to a concentration of 1 ng/µl.

RAPD amplification

Twenty primers from the Operon kit A (Operon Technologies Inc., Alameda, Calif.) were used in this analysis (Table 1). The *Taq* DNA polymerase, the buffer and the $MgCl_2$ were from Gibco-BRL, UK. The nucleotides were from Amersham, UK. The reactions were carried out in 25µ l of *Taq* polymerase (0.025 U/µl); *Taq* buffer × 1 (20 mM Tris-HCl, 50 mM KCl, pH 8.4); dNTPs (0.2 mM each); $MgCl_2$ (2 mM); primer (0.2 µM) and 10 ng of template DNA. The amplifications were achieved in either a PTC 100 thermocycler (MJ Research, Inc. Watertown, Mass., USA) or in a Hybaid OmniGene Temperature Cycler (Hybaid Limited, Middlesex, UK) programmed for a first denaturation step (2 min at 94°C) followed by 55 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. The 55 cycles were followed by a further extension step of 5 min at 72°C and the samples kept at 4°C until loaded on the gel. The amplification products were resolved on 1.5% (w/v) agarose gels in 1 × Tris-Acetic

Table 2 Polymorphic (poly.) RAPD bands shown by *M. hupehensis*, *M. toringoides* versus *M*. cv Baskatong and the primers detecting hybrid seedlings (+) from crosses (*M. hupehensis* × *M*. cv Baskatong ♂., *M. toringoides* × *M*. cv Baskatong ♂). S-seedling number. (–) no polymorphic RAPD bands detected; (+) presence of polymorphic RAPD bands

Primer	*M. hupehensis*		*M. toringoides*		*M. hupehensis* × Baskatong ♂[a]		*M. toringoides* × Baskantong ♂[b]	
	Total bands	No. poly.	Total bands	No. poly.	S-1 to S-4 S-6 to S-13 Red stem	S-5 Red stem	S-1 to S-2, S-4 to S-6 Green stem	S-3 Red stem
OPA-01	12	7	18	6	–	+	–	–
OPA-02	11	6	8	3	–	+	–	–
OPA-03	18	9	21	3	–	–	–	+
OPA-04	10	5	15	6	–	–	–	–
OPA-05	5	2	16	4	–	–	–	+
OPA-06	Failed to give reliable amplification patterns							
OPA-07	14	7	10	4	–	–	–	+
OPA-08	14	6	11	10	–	+	–	+
OPA-09	11	10	12	8	–	+	–	+
OPA-10	10	4	13	5	–	+	–	+
OPA-11	9	9	21	5	–	–	–	+
OPA-12	8	8	10	3	–	+	–	–
OPA-13	12	9	12	3	–	+	–	+
OPA-14	6	4	12	7	–	+	–	+
OPA-15	15	8	16	4	–	–	–	–
OPA-16	20	7	20	6	–	+	–	+
OPA-17	14	9	18	8	–	–	–	+
OPA-18	16	6	16	6	–	–	–	–
OPA-19	8	3	15	8	–	+	–	+
OPA-20	12	8	17	10	–	+	–	+

[a] *M. hypehensis* × *M*. cv Baskatong ♂: seedlings (S-1 to S-13) were not distinguishable in vitro on colour basis all with red stems

[b] *M. toringoides* × *M*. cv Baskatong ♂: seedlings (S-1 to S-2, S-4 to S-6) were detected as putative apomicts in vitro on colour basis (green), and S-3 as hybrid in vitro on the basis of colour (red)

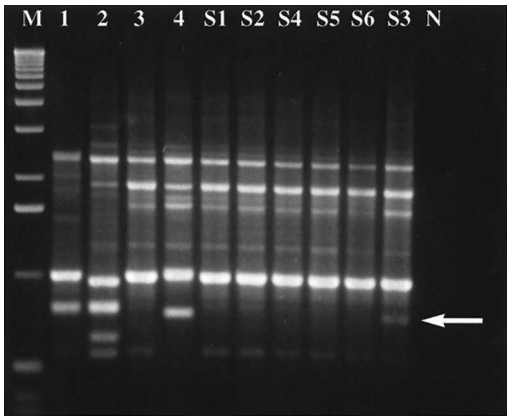

Fig. 1 Amplification profiles of three apomictic *Malus* species, cv Baskatong and six seedlings (*S*) of *M. toringoides* × cv Baskatong with OPA-07 primer. *M* 1-kbp DNA ladder. *Lane 1, M. sargentii*; *2*, *M. hupehensis*; *3*, *M. toringoides*♀; *4*, cv Baskatong♂, *S1 to S6* seedlings, and *N*, negative. *Arrow* indicates shared band

acid-EDTA (TAE) buffer. After ethidium bromide staining, the banding patterns were recorded by photography and analyzed later. Each amplification was repeated three times and only consistent bands were taken into account.

Results and discussion

In order to test the efficiency of the primers in assessing genetic diversity, one other *Malus* species was included in this survey along with the male and the two female parents. From the set of 20 primers used for this study, only one primer, OPA-06, failed to give reliable amplification patterns. Thus 19 out of the 20 primers produced distinct patterns for each of the species (Table 2). Distinctive fingerprints were obtained for 25 apple cultivars using only two primers (Mulcahy et al. 1993) which are both included in our set. By working with 19 primers capable of producing specific fingerprints for each of the parents, we are more likely to detect any genetic variation between the putative apomicts, the hybrids and the female parents. Moreover, we can detect hybrids which are not clearly identifiable by in vitro colour-based selection.

Five of the six *M. toringoides* ♀ × *M.* cv Baskatong ♂ seedlings (S-1, S-2, S-4, S-5, and S-6) were detected as putative apomicts in vitro on the basis of colour (green), and one (S-3) as a putative hybrid (red) (see Fig. 2). Extra RAPD bands, similar to those amplified from the male parent, were observed only in the putative hybrid (red seedling) (Fig. 1) – 13 primers out of the 19 detected this hybrid (Table 2). RAPDs are commonly inherited as dominant markers, where the presence of a particular band is dominant, and its absence is recessive (Tingey and del Tufo 1993). The lower number of primers detecting extra-bands between the hybrid and the female parent could be explained by the dominant nature of the RAPD markers. Similarly, this also explains cases where RAPD bands present in the female are missing in the hybrid. No extra or missing-band was detected in the putative apomicts selected on the basis of their green colour. They always showed the same pattern as the female parent (Fig. 1). This is in accordance with apomicts being derived from somatic cells and so should have the same genotype as the female parent with no segregation of parental alleles.

Fig. 2 In vitro red-purple pigmentation exhibited by *A*: *M. hupehensis*, *B*: hybrid (*M. hupehensis*♀ × *M.* cv Baskatong♂), *C*: *M.* cv Baskatong, *D*: hybrid (*M. toringoides*♀ × *M.* cv Baskatong♂) *E*: *M. toringoides* for comparison

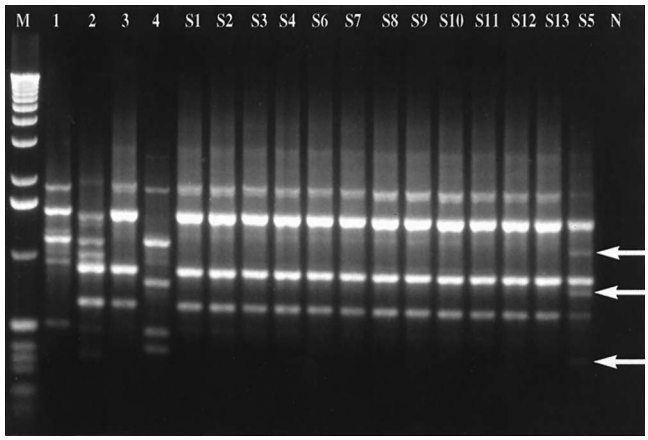

Fig. 3 Amplification profiles of three apomictic *Malus* species, cv Baskatong and 13 seedlings (*S*) of *M. hupehensis* × *M.* cv Baskatong with OPA-09 primer. *M* 1-kbp DNA ladder. *Lane 1*, *M. sargentii*; *2*, *M. toringoides*; *3*, *M. hupehensis*♀; *4*, cv Baskatong♂, *S1 to S13* seedlings, and *N*, negative. *Arrows* indicate shared bands

With the seedlings from the crosses between *M. hupehensis* and *M.* cv Baskatong, all 13 (S-1 to S-13) had red stems and purple pigment on the leaf margins. In vitro identification of the hybrids from *M. hupehensis*♀ × *M.* cv Baskatong♂ was difficult because both the micropropagated female parent and all the seedling clones were of similar colour (Fig. 2). Eleven out of the nineteen primers detected extra RAPD bands in one of the seedlings (S-5) when compared to the patterns of the female parent (Table 2). Bands in identical position on the gel were also present in the male parent and could be considered as derived from *M.* cv Baskatong genetic material (Fig. 3). Twelve of the thirteen seedlings (S-1 to S-4, and S-6 to S-13) having banding patterns similar to the female parent for all 19 primers were regarded as apomicts. This cross represents one of the cases where the RAPD technique could be used to select apomictic seedlings from hybrids while they are in vitro. The colour-marker-based selection in these cases would, in practice, require subsequent transfer of the seedlings to the greenhouse when the red colour disappears from apomictic lines. In fact, the seedling (S-5) with extra RAPD bands, like those in the male parent, was confirmed as a hybrid (on the basis of colour-marker gene) when these seedlings were transferred to the greenhouse.

Conclusion

These results show that the OPERON (kit A) set of primers are suitable for the assessment of genetic variation in *Malus* species and are sufficient to permit the distinction of apomicts from hybrids with the species examined. They could be used in crosses to detect true apomicts where the red-purple colour-based marker could not be employed for in vitro selection, such as in *M. hupehensis*♀ × *M.* cv Baskatong♂. In this cross, the colour-marker gene is not expressed unless the in vitro seedlings are transferred to the greenhouse or planted in the field. The limitations of using the dominant homozygous marker gene R_t as an indicator of hybrid status are revealed by this study and its usefulness and its dependence on the particular cross that is made is clear.

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