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Use of the multi-allelic self-incompatibility gene in apple to assess homozygocity in shoots obtained through haploid induction

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Abstract To obtain homozygous genotypes of apple, we have induced haploid development of either the female or the male gametes by parthenogenesis in situ and anther culture, respectively. Of the shoots obtained, which were mainly of a non-haploid nature, some could be derived from fertilised egg cells or from sporophytic anther tissue. In order to select the shoots having a true haploid origin, and thus homozygotes, we decided to use the single multi-allelic self-incompatibility gene as a molecular marker to discriminate homozygous from heterozygous individuals. The rationale behind this approach was that diploid apple cultivars contain 2 different alleles of the *S*-gene and therefore the haploid induced shoots obtained from them should have only one of the alleles of the single parent. The parental cultivars used were 'Idared' (parthenogenesis in situ) and 'Braeburn' (androgenesis), and their *S*-genotypes were known, except for 1 of the 'Braeburn' *S*-alleles. To stimulate parthenogenetic development 'Idared' styles were pollinated with irradiated 'Baskatong' pollen, the *S*-alleles of the latter (2n) cultivar were also unknown. The cloning and sequence analysis of these 3 unidentified *S*-alleles, 1 from 'Braeburn' and 2 from 'Baskatong' is described, and we show that they correspond to the S_{24}-, S_{26}- and S_{27}-alleles. We have optimised a method for analysis of the *S*-alleles of 'Idared/Baskatong'- or 'Braeburn'-derived in vitro plant tissues and have shown that this approach can be applied for the screening of the in vitro shoots for their haploid origin.

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Introduction

Fruit trees are characterised by a high degree of heterozygocity, which may hinder the genetic improvement of these crops by breeding. This heterozygocity together with the long generation time of perennial fruit crops imply that classical cross-breeding can only provide solutions on a long-term basis. Biotechnological approaches, like haploid induction, offer new possibilities for both the genetic study and the improvement of these plants. Several methods for obtaining homozygous plants, such as androgenesis in vitro and parthenogenesis in situ, have been described (Jain et al. 1996).

Ten years ago, the in vitro haploidisation technique of parthenogenesis in situ, induced by pollination with γ-irradiated pollen, was developed for *Petunia* (Raquin 1985). More recently, the same technique has been successfully applied to pear (*Pyrus communis* L.; Bouvier et al. 1993) and apple (*Malus domestica* Borkh.; Zhang and Lespinasse 1991; De Witte and Keulemans 1994). In these experiments, the irradiated pollen appeared to stimulate the unfertilised egg cells to develop into embryos, but the precise mechanism of haploid induction still remains to be elucidated (Zhang and Lespinasse 1991). In vitro germination of γ-irradiated pollen generally showed results comparable with the germination of untreated pollen grains (Zhang and Lespinasse 1991; De Witte and Keulemans 1994). Studies of in situ pollen-tube growth using fluorescence microscopy revealed that irradiation decreased the number of pollen tubes reaching the base of the style. Nevertheless, pollen tubes from pollen irradiated with 125–1000 Gy were capable of reaching the embryo sac (Zhang and Lespinasse 1991). During subsequent in vitro culture of isolated embryo sacs, zygotic embryos have shown a higher survival rate than the haploid induced embryos.

In apple, pollen carrying a homozygous dominant marker gene for anthocyanin production was used for parthenogenetic induction by Lespinasse and Chevreau (1984). The expression of the marker gene in seedlings results in red-coloured leaves and stems, allowing

one to visually detect the shoots resulting from fertilised egg cells. In our experiments, the cultivar 'Baskatong' was used, which carries a similar dominant gene for anthocyanin production (De Witte and Keulemans 1993). Control pollinations with untreated 'Baskatong' pollen, however, have shown that some seedlings, while presumably containing the marker gene, do not express the red colour (De Witte, unpublished results). This means that the visual evaluation of anthocyanin production in parthenogenetic shoots is not 100% conclusive, and further analysis should be performed to check the haploid origin of these shoots.

In another haploid induction approach, androgenesis is induced by in vitro culture of whole anthers. The induction of androgenic haploids has been reported for several distinct species (Jain et al. 1996) but appears to be less clear-cut in perennial fruit crops (Seirlis et al. 1979, Höfer and Hanke 1990). Many processes taking place in the in vitro-cultured anthers remain unexplained. Experimental studies have shown that embryos developing in these anthers may originate from haploid microspores, from unreduced diploid microspores or from the diploid tapetum and anther-wall tissue (Zhang and Lespinasse 1992; Meyer et al. 1993; Zhong et al. 1995). Spontaneous polyploidisation often occurs during the development of the embryos. In our experience, most shoots regenerate from meristematic tissue originating from dedifferentiated embryos (De Witte and Keulemans 1993).

Flow cytometrical analysis has shown that most of the shoots obtained through haploid induction in apple appear to be polyploids rather than haploids (De Witte and Keulemans 1993; De Witte et al. in preparation). We were therefore uncertain about the haploid origin, hence homozygocity, of these shoots, and chose to analyse those shoots for genetic markers that are heterozygous in the parental genotypes. One of the best known polymorphic genes in apple is the *S*-gene, which controls self-incompatibility. Self-incompatibility (SI) is a mechanism that has evolved in flowering plants to encourage outbreeding and prevent self-fertilisation. The recognition of *self* pollen versus *nonself* pollen is based on a highly specific mechanism often controlled by a single gene locus with multiple alleles (Newbigin et al. 1993). In apple, several alleles of this gene have been cloned recently, and it has been shown that most apple cultivars are heterozygous for this gene (Broothaerts et al. 1995, 1996; Janssens et al. 1995). Shoots resulting from the (haploid) male or female gametes should only have one of the *S*-alleles of their parents and hence could be identified as such.

Previously, we have described a polymerase chain reaction (PCR)-based method for the rapid determination of the *S*-genotype of apple cultivars (Janssens et al. 1995). As this method is based on the use of allele-specific primers, a prerequisite is to know (at least part of) the nucleotide sequence of the *S*-alleles to be amplified. The nucleotide sequences of the *S*-allele cDNAs termed S_1, S_2, S_3, S_5, S_7 and S_9 have been published (Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996). Among the parental cultivars used for haploid induction, 'Idared' has been shown to contain the S_3 and S_7 alleles, and the S_9-allele has been found in 'Braeburn'. In this paper, we report on the cloning and sequence analysis of the second *S*-allele of 'Braeburn' and of both *S*-alleles of 'Baskatong', the latter being used as a pollen donor for parthenogenesis induction. We show that the cloned sequences correspond to the S_{24}, S_{26} and S_{27} alleles, respectively. The allele-specific PCR amplification technique, described previously, was adapted for analysis of these novel *S*-alleles. We have applied the PCR technique to in vitro shoots obtained by haploid induction in order to determine their haploid origin. This was done by assessing for the presence of the alleles of the original cultivars used in either procedure for haploid induction. We show that analysis of the *S*-genotype of apple shoots, induced by parthenogenesis in situ or by anther culture, is a valuable approach for confirmation of their haploid origin.

Materials and methods

Plant material

Parthenogenesis in situ

Emasculated 'Idared' flowers were pollinated with γ-irradiated 'Baskatong' pollen to stimulate the egg cells to develope into haploid embryos. 'Baskatong' carries the homozygous dominant marker gene *R*, which results in the production of anthocyanins in the stem, leaves and fruits (Lespinasse and Chevreau 1984). The embryos were dissected out of the immature seeds (70 and 140 days after pollination) and cultured in vitro, according to De Witte and Keulemans (1994). The regenerated shoots were multiplied and used for analysis.

Androgenesis

Flower buds of 'Braeburn' apple trees were collected when the petals of the top flower of the cluster (king flower) became visible. Anthers were cultured in vitro according to conditions described in a future paper (De Witte et al. in preparation).

Pistils

Styles were dissected out of flower buds at the "balloon" stage of development, immediately frozen in liquid nitrogen and stored at -80°C.

Ploidy analysis of the different genotypes

This analysis was performed by Plant Cytometry Services (PCS; Schijndel, The Netherlands), generally using a single in vitro leaf (De Laat and Blaas 1984).

Reverse transcription-PCR (RT-PCR)

The method of Logemann et al. (1987) was used to extract total RNA from five mature pistils (without ovary) of the cultivars 'Baskatong' and 'Braeburn', respectively. Reverse transcription (RT) was carried out in a 20-µl reaction containing 0.5 µl of RNA, 50 U MuLV Reverse transcriptase (Perkin Elmer), 0.5 µ*M* *Not*I-$(dT)_{18}$ primer (Pharmacia), 2 U RNAguard (Pharmacia), 250 µ*M* dNTPs and 1 × *Taq* DNA polymerase buffer (Appligene). The reaction was programmed in a thermal cycler (GeneAmp 2400 apparatus; Perkin Elmer) and consisted of an initial period of 10 min at room temperature, 20 min at 42°C, 5 min at 99°C and cooling to 4°C. Five microliters of the RT reaction was used in a subsequent 25-µl PCR amplification reaction with the OWB134 primer (for sequence, see Table 1) and the *Not*I-$(dT)_{18}$ primer. The reaction mixture contained 200 µ*M* dNTPs (Eurogentec), 1 µ*M* of each primer (Eurogentec), 1 × *Taq* DNA polymerase buffer (Appligene) and 0.6 U *Taq* DNA polymerase (Appligene). The "hot start" amplification programme consisted of an initial denaturation period of 6 min at 94°C, 35 cycles

of 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C and a final extension of 5 min at 72°C.

Cloning and DNA sequence analysis

The cDNAs obtained were excised from a 1% agarose gel in TAE, purified by Nucleotrap® (Macherey-Nagel), digested with *Eco*RI and ligated into the plasmid vector pEMBL18$^+$. The recombinant plasmids were transformed to *E. coli* DH5α competent cells. The nucleotide sequences of three clones per allele were determined in both directions with the method of Sanger et al. (1977) on an ALF DNA sequencer (Pharmacia) using an AutoRead Sequencing Kit (Pharmacia).

Isolation of genomic DNA

DNA was prepared from fresh apple leaf tissue (1–100 mg) from in vitro shoots using a method modified from Aldrich and Cullis (1993). Two hundred microliters 2% CTAB-buffer (1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* TRIS-HCl pH 8.0 and 2% CTAB) containing a few sterile sand grains was preheated to 65°C. Just before extraction, 2% β-mercaptoethanol was added. The leaf tissue was added and immediately homogenised with a microfuge pestle and incubated for 10 min at 65°C. The DNA was extracted with chloroform:octanol (24 : 1) and centrifuged for 5 min at 11 000 rpm. The upper phase was added to 120 µl isopropanol to precipitate the DNA (1 h on ice). After centrifugation, the pellet was washed with 200 µl wash buffer (76% ethanol and 10 m*M* ammonium acetate) and then centrifuged for another 5 min. The pellet was air-dried, redissolved in 120 µl distilled water and stored at −20°C until use.

Allele-specific PCR amplification

Allele-specific PCR amplification was performed in a total reaction volume of 25 µl containing 10 µl DNA, 200 µ*M* dNTPs, 1 µ*M* of both allele-specific primers (Table 1), 1 × *Taq* DNA polymerase buffer (Appligene) and 0.6 U *Taq* DNA polymerase (Appligene). The PCR conditions were optimised for each allele individually using DNA from apple cultivars bearing known *S*-alleles (data not shown). The PCR products were digested with an allele-specific restriction enzyme (see Table 2) and analysed by agarose gel electrophoresis according to Janssens et al. (1995). To check on the quality of the genomic DNA isolated, we carried out PCR amplification reactions with the allele-specific primers for the apple *S*-like RNase gene (fragment D, Janssens et al. 1995).

Results and discussion

Cloning of cDNA for the 'Baskatong' *S*-alleles

In the experiments on parthenogenesis in situ , 'Idared', used as the female parent, was pollinated with γ-irradiated 'Baskatong' pollen. While the *S*-genotype of the cultivar 'Idared' (S_3S_7) had been determined before, *S*-genotyping by PCR revealed that the *S*-alleles of 'Baskatong' were different from the 6 already characterised *S*-alleles in apple (data not shown). To clone the 'Baskatong' *S*-alleles, we followed an RT-PCR approach. Pistil total RNA was reverse-transcribed in the presence of oligo(dT), and the single-stranded cDNA was amplified by PCR using the same oligo(dT) primer and the OWB134 primer. The OWB134 primer anneals to a conserved region in all known *S*-alleles, and its 3′-nucleotide triplet corresponds with the translation initiation codon ATG. The RT-PCR product obtained was approximately 850 bp long and was cloned for further analysis. Nucleotide sequence analysis of the cDNA inserts from several clones revealed two sequences that were different from those of the 6 already known *S*-alleles. They were termed the alleles S_{26} and S_{27}, respectively (see below). The cloned sequences were 852 (S_{26}) and 870 base pairs (S_{27}), including a poly(A) tail of 23 and 21 nucleotides, respectively. Both *S*-cDNAs contained an open reading frame coding for a protein of 227 amino acids (27 residues of an amino terminal signal peptide and 200 residues of the mature protein). A comparison of the amino acid sequences for S_{26} and S_{27} with known sequences for *S*-alleles in apple revealed homology percentages varying from 57% to 70%. All cysteine residues and typical conserved regions, which were presumed to be of importance for the activity of this family of proteins, were preserved (data not shown). The sequences of the S_{26}-cDNA and the S_{27}-cDNA were deposited under the GeneBank accession numbers AF016918 and AF016919, respectively.

Table 1 Oligonucleotides used in PCR amplification reactions

Name	Sense/antisense	Nucleotide sequence (5′ → 3′)[c]	Position in cDNA (base numbers)
OWB126	Sense	GCCTTCAGACTCGAATGGACA	108–128 (S_7)
OWB135	Antisense	ATGGATCCGAATTCTTAATACWGAATATKWTTGGTG	585–608 (S_7)
OWB234	Sense	ATGAATTCGGATCCTCCTGACAAGTTGTTTACGGTTC	165–193 (S_{24})
FTC6	Antisense	ATGAATTCTGGGGCAGAAATACCG	667–682 (S_{24})
FTC14	Sense	GAAGATGCCATACGCAATGG	517–536 (S_{26})
FTC9	Antisense	ATGAATTCTTAATACCGAATATTGGCG	684–702 (S_{26})
FTC5	Sense	ATGAATTCCCACAATACAGAACGAGA	378–397 (S_{27})
OWB249	Antisense	ATGAATTCAATCTATGAAATGTGCTCTG	621–641 (S_{27})
OWB134, OWB145[a]			
OWB154, OWB155[b]			

[a, b] Characteristics of these primers were published in [a]Broothaerts et al. (1995) and [b]Janssens et al. (1995)
[c] The underlined sequences are restriction endonuclease recognition sites (*Eco*RI, *Bam*HI), included for cloning purposes

Table 2 Conditions for allele-specific PCR analysis

S-allele	Primers	Annealing temperature (°C)	Band size of PCR product (bp)	Restriction enzyme	Band sizes (bp) after digestion
S_3[a]	OWB134 OWB145	56	375	*Pst*I	226 + 149
S_7[b]	OWB126 OWB135	60	632	*Acc*I	405 + 227
S_9[b]	OWB154 OWB155	62	343	*Eco*RI	212 + 131
S_{24}	OWB234 FTC6	62	850[c]	*Scr*FI	213 + 640[d]
S_{26}	FTC14 FTC9	55	193	*Kpn*I	170 + 23
S_{27}[c]	FTC5 OWB249	58	278	*Taq*I	241 + 37

[a] Previously described by Broothaerts et al. (1995)
[b] Previously described by Janssens et al. (1995). Note: one of the S_7-primers is different from the ones used in the previous paper
[c] Under these conditions, another *S*-allele, presumably S_4, is amplified as well, yielding a PCR product of the same size as S_{27}; after *Taq*I digestion, the S_4-fragment is split into bands of approximately 190 and 90 base pairs, which may be discriminated from the S_{27} digestion products
[d] Size estimation by comparison to DNA markers during gel electrophoresis. The fragment presumably contains an intron of which the length and sequence have not been determined

It is generally accepted that the nomenclature of *S*-alleles in apple goes back to the early investigations of Kobel and co-workers (1939). Based on a microscopical observation of pollen-tube growth after controlled pollination, Kobel succeeded in assigning an *S*-genotype to several cultivars. He identified 11 phenotypically different *S*-alleles, numbered from S_1 to S_{11}, but several additional alleles (named e.g. *Sx* or *Sy*) were inferred from his data. Occasionally, his pollination data were inconclusive as to which *S*-allele should be assigned, and in such a case a less defined *S*-symbol was given, e.g. *Sx* where *x* could be 10 or 11. Kobel's *S*-nomenclature has since been confirmed by both our PCR analysis and by *S*-protein analysis (K. Tobutt, personal communication). The same approaches have revealed that the *S*-alleles of 'Baskatong' are different from each of Kobel's 11 *S*-alleles. They have received the S_{26} and S_{27} numbers, as proposed by Ken Tobutt.

PCR analysis of shoots obtained through parthenogenesis in situ

Shoots resulting from in vitro culture of isolated embryos were divided into three groups according to the colour development in their stem and leaves: red, green and uncertain (De Witte and Keulemans 1994). Shoots expressing the red colour were thought to result from fertilisation with 'Baskatong' pollen because they expressed the *R*-allele of the 'Baskatong' cultivar. Although this visual selection system should theoretically allow us to select the genotypes of haploid origin, i.e. the green shoots, there were several shoots that only had a slightly reddish flush and were classified as "uncertain" or others that expressed the red colour only later in their development. Therefore, we developed a screening method to ensure that the selected green or green-like genotypes were of haploid origin.

We adapted the allele-specific PCR procedure of Janssens et al. (1995) and used it to test the in vitro derived shoots for the presence of the 'Idared' and 'Baskatong' *S*-alleles (S_3/S_7 and S_{26}/S_{27}, respectively). Two allele-specific primers for each allele were designed, and the conditions for PCR amplification were optimised individually for each allele (Table 2). The PCR products obtained were sporadically checked for their allelic specificity by digestion with an allele-specific restriction enzyme (data not shown). As positive control in these analyses, genomic DNA from cvs 'Idared' (positive for S_3 and S_7) and Baskatong (positive for S_{26} and S_{27}) were taken. For the leaves from in vitro shoots, we had to use a genomic DNA isolation method different from the rapid homogenisation method of Wang et al. (1993) used previously, as it yielded inconsistent results when applied to these shoots. This was particularly observed for the red or reddish shoots and may have been caused by the anthocyanins or some by-product of them. We therefore modified the CTAB-method of Aldrich and Cullis (1993) to make it applicable to the in vitro leaves (20–100 mg of in vitro leaf tissue, corresponding to three to four small leaves). An example of the results obtained with this approach is shown in Fig. 1A.

When applied to a random sample of 15 red genotypes, the results showed the presence of either the S_3 or S_7-allele together with either the S_{26}- or S_{27}-allele (Table 3). There was an equal distribution of S_3- versus S_7-bearing shoots, which is consistent with a normal

segregation of *S*-alleles in the progeny of controlled crosses. When the PCR assay was applied to a random sample of 15 genotypes classified as being green, only 11 genotypes appeared to be devoid of both S_{26} and S_{27}, and thus these are likely to be true haploid-derived (Table 4). The remaining 4 shoots contained either S_{26} or S_{27} and should be considered as being derived from zygotic embryos. These results indicate that the visual selection system may generate false positive results and obviates the need to apply our PCR method as a further screening step. One explanation for the absence of anthocyanins in the shoots resulting from fertilised embryos may be that the irradiation of the pollen grains has caused a mutation in the *R* gene, resulting in the failure to express an active protein. The genotypes which could not be clearly assigned to be red or green nearly all appeared to contain one of the 'Baskatong' alleles (data not shown). This result implies that it is justified to apply a more stringent visual selection to the shoots obtained.

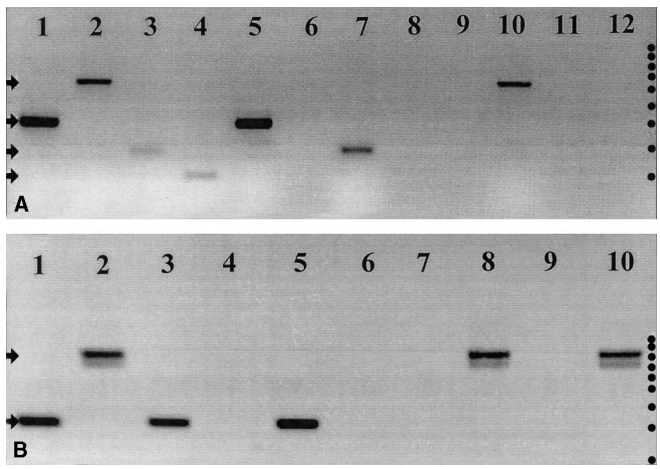

Fig. 1A, B Allele-specific PCR analysis. **A** Analysis of parthenogenetic shoots for the presence of the alleles S_3 (*lanes 1, 5, 9*), S_7 (*lanes 2, 6, 10*), S_{27} (*lanes 3, 7, 11*) and S_{26} (*lanes 4, 8, 12*). *Lanes 1* and 2 'Idared' parent (S_3S_7), *lanes 3* and *4* 'Baskatong' (pollen donor; $S_{26}S_{27}$), *lanes 5–8*: example of a parthenogenetic (red) shoot (S_3S_{27}), *lanes 9–12*: example of a parthenogenetic (green) shoot (S_7) **B** Analysis of androgenic shoots for the presence of S_9 (*odd-numbered lanes*) and S_{24} (*even-numbered lanes*). *Lanes 1* and *2* 'Braeburn' parent (S_9S_{24}), *lanes 3–4, 5–6, 7–8, 9–10* four different androgenic shoots, revealing the presence of either of the parental *S*-alleles. (Note: in the S_{24} *lanes*, a minor band of approximately 700 bp is often observed below the 850-bp band). The *arrows* indicate the position of the *S*-allele-specific PCR products. *Black spots* on the *right side* denote the position of the 100-bp DNA marker bands (200–1000 bp)

Cloning of cDNA for the second *S*-allele of 'Braeburn'

The cultivar 'Braeburn', used for haploid induction by anther culture, was found to contain the S_9-allele and 1 other allele different from the previously characterised *S*-alleles. We used a similar approach as described above to characterise the second allele of this diploid variety. RT-PCR and cloning yielded a high number of S_9-cDNA-containing clones and a few with a different but homologous cDNA sequence. The length of the latter sequence was 870 base pairs, ending in a poly(A) tail. It was shown by allele-specific PCR analysis (see below) that this cDNA corresponded to the second allele of 'Braeburn'. None of the cultivars in Kobel's list (Kobel et al. 1939) seemed to contain this allele, as assayed by allele-specific PCR, except for 'Gravenstein'. The latter 3n cultivar was proposed to have the $S_4S_{10\text{ or }11}S_x$ genotype. In our hands, 'Gravenstein' was found to contain S_4 and not S_{10}, hence the cloned allele

Table 3 Allele-specific PCR analysis of genotypes induced by parthenogenesis in situ and visually classified as being red (*nd* not determined)

Genotype	Ploidy	*S*-alleles derived from Idared[a]		*S*-alleles derived from Baskatong		Genotype[b]	Homozygous
		S_3	S_7	S_{26}	S_{27}		
95-1-38-1.1 1	nd	+	−	−	+	S_3S_{27}	No
95-1-38-1.1 3	nd	−	+	+	−	S_7S_{26}	No
95-1-38-1.1 8	nd	+	−	−	+	S_3S_{27}	No
95-1-38-1.1 9	nd	−	+	−	+	S_7S_{27}	No
95-1-38-1.1 13	nd	−	+	+	−	S_7S_{26}	No
95-1-38-1.1 15	nd	+	−	+	−	S_3S_{26}	No
95-1-38-1.1 16	nd	−	+	−	+	S_7S_{27}	No
95-1-38-1.1 17	nd	−	+	+	−	S_7S_{26}	No
95-1-38-1.1 21	nd	−	+	+	−	S_7S_{26}	No
95-1-38-1.1 24	nd	−	+	+	−	S_7S_{26}	No
95-1-38-1.1 27	nd	+	−	−	+	S_3S_{27}	No
95-1-38-1.1 29	nd	+	−	−	+	S_3S_{27}	No
95-1-38-1.1 31	nd	+	−	−	+	S_3S_{27}	No
95-1-38-1.1 39	nd	−	+	−	+	S_7S_{27}	No
95-1-38-1.1 43	nd	+	−	+	−	S_3S_{26}	No

[a] +, *S*-allele present; −, *S*-allele absent
[b] Probable genotype, as the ploidy level has not been determined

Table 4 Allele-specific PCR analysis of genotypes induced by parthenogenesis in situ and visually classified as being green (*nd* not determined)

Genotype	Ploidy	*S*-alleles derived from Idared[a]		*S*-alleles derived from Baskatong		Genotype[b]	Homozygous
		S_3	S_7	S_{26}	S_{27}		
92-1-2-8	nd	−	+	−	−	(S_7)	Yes
92-1-2-115	2x	+	−	−	−	S_3S_3	Yes
92-1-4-12	2x	−	+	+	−	S_7S_{27}	No
92-1-7-309	2x	+	−	−	−	S_3S_3	Yes
92-1-21-11	2x	+	−	−	−	S_3S_3	Yes
92-1-21-13	2x	−	+	−	+	S_7S_{26}	No
92-1-21-17	3x	+	−	−	−	$S_3S_3S_3$	Yes
92-1-21-18	2x	−	+	−	−	S_7S_7	Yes
92-1-21-20	2x, 3x	+	−	−	−	$S_3S_3(S_3)$	Yes
92-1-21-60	x	+	−	−	−	S_3	Yes
93-1-32-182	nd	+	−	−	+	(S_3S_{26})	No
93-1-32-883	2x	−	+	−	−	S_7S_7	Yes
95-1-8-3	nd	+	−	−	−	(S_3)	Yes
95-1-38-1.1 10	nd	+	−	−	−	(S_3)	Yes
95-1-38-1.1 18	nd	−	+	+	−	(S_7S_{26})	No

[a] +, *S*-allele present; −, *S*-allele absent
[b] In case the ploidy level is unknown, the genotype is indicated between brackets

should be either S_{11} or S_x, where x is > 11 (note: we have additionally tested 4 different accessions of the cultivar 'Adams Pearmain', which is the only other cultivar of Kobel that was reported to bear S_{11}. In our hands, none of these accessions, however, seem to correspond to the genotype assayed by Kobel). We termed this allele S_{24}, following the suggestion made by K. Tobutt that was based on the identification of the *S*-RNases present in 'Braeburn' styles (personal communication). The S_{24}-allele codes for a protein of 226 amino acids (26 residues of an amino terminal signal peptide and 200 residues of the mature protein). The deduced amino acid sequence of S_{24} is 54–61% identical with the other *S*-allele sequences in apple. The Genebank accession number for S_{24} is AF016920.

PCR analysis of shoots obtained through in vitro androgenesis

Shoots originating from anther culture of 'Braeburn' were mainly polyploid (2n, 3n, 4n), possibly as a result of spontaneous polyploidisation during tissue culture. To verify their haploid origin, we examined them for the presence of the parental *S*-alleles (S_9 and S_{24}). An example of this analysis is shown in Fig. 1B. The conditions for PCR analysis of the S_9-allele have already been described (Janssens et al. 1995). For S_{24}, we designed the allele-specific primers shown in Table and optimised the PCR conditions (Table 2) using DNA from cultivars with known *S*-alleles. Table 5 shows the results of the PCR analysis for 30 androgenic genotypes together with their ploidy level. All plants, except one, contained either the S_9- or the S_{24}-allele and are, therefore, homozygous. The genotype bearing both S_9 and S_{24} is diploid and may be derived from an unreduced microspore or from sporophytic anther tissue. Similar observations have been reported for potato (Meyer et al. 1993). Although the frequency of the occurrence of non-haploid derived shoots will be much lower for androgenic shoots compared to parthenogenetic ones, we feel that it is worthwhile to do the analysis in both cases, in particular because the method is easy and fast and may be applied at an early phase of the work programme, i.e. using in vitro shoots.

Table 5 Ploidy analysis and allele-specific PCR analysis of 30 random genotypes induced by anther culture

Ploidy	Number of genotypes	Number of genotypes bearing the following *S*-alleles:		
		S_9	S_{24}	S_9S_{24}
n	1	0	1	0
2n	10	4	5	1
3n	11	7	4	0
4n	7	2	5	0
Uncertain	1	1	0	0
Total	30	15	14	1

In this paper, we have described an original approach to determine whether haploid-derived plants are homozygous. The method proposed is based on the assessment of the homozygocity at a single multi-allelic gene locus, knowing that the plant material from which the obtained shoots are derived is heterozygous at this locus. For ease of analysis, only one gene was envisaged, although it would have been possible to assay the variation at several loci simultaneously using one of

several DNA fingerprinting technologies (e.g. Nybom 1993). It would have taken much more time, however, to optimise DNA fingerprinting assays. The method used here is based on PCR and may be applied to small amounts of starting material, e.g. a few tiny in vitro leaves. In contrast to Southern blotting assays, which would have required several micrograms of adequately purified genomic DNA, our method may be applied at a very early phase of the work programme, i.e. at the tissue culture phase. Although it may be easy in some plant species to isolate considerable amounts of genomic DNA from in vitro shoots, this is not so obvious in apple, as it would require a much longer tissue culture time and several multiplication steps. The choice of the *S*-gene for this purpose was based on the existing molecular knowledge of the *S*-alleles in apple and the availability of a PCR method to genotype plants for the *S*-alleles (Janssens et al. 1995). *S*-alleles are known to have rather divergent sequences, which, obviously, are related to the role of the encoded proteins as recognition molecules. This sequence diversity allowed us to find relatively easily short stretches of polymorphic sequences that could be used for primer design. The primers proposed specifically amplify only a single *S*-allele from the pool of known *S*-allele sequences. However, it may be that some of these primer couples do amplify *S*-alleles of which the sequences have not yet been determined. We recently obtained some evidence that this is the case, and we are analysing this by further nucleotide sequencing. However, this has no consequences for the purpose of this study, which only involves a few alleles.

In conclusion, our data clearly demonstrate that the use of PCR amplification in the presence of *S*-allele-specific primers is a reliable approach for the rapid screening of in vitro-cultured shoots for confirmation of their haploid origin.

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