Characterization of Fertile Homozygous Genotypes from Anther Culture in Apple

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ABSTRACT. Doubled haploids can improve the efficiency of breeding and genetic study in apple (*Malus × domestica* Borkh.). Seventeen homozygous genotypes were obtained by in vitro anther culture from ‘Senshu’ apple. Flow cytometry analysis revealed that the ploidy level of the anther-derived plantlets was diploid. Simple sequence repeat (SSR) analysis determined the origin and homozygous status of the anther-derived plantlets. The results of *S-RNase* polymerase chain reaction (PCR)-digestion analysis reinforced the homozygous state. The morphological characteristics and reproductive potential of the doubled haploids were investigated. It is especially interesting that one of the doubled haploids derived from anther culture had fertile pollen grains (rate of in vitro germination was 20.6%–33.0%), and several progenies were obtained from the cross between ‘Prima’ apple and the doubled haploid. The hybridism of these seedlings was confirmed by SSR analysis. Furthermore, the female gametes of the doubled haploid showed slight fertility. Thus, this doubled haploid will be valuable for breeding and genetic studies.

The obstacles to breeding programs and genetic analysis in apple are the long reproductive cycle with a juvenile phase that lasts several years, the highly heterozygous nature of the genome, the large canopy size, and self-incompatibility that generally prevents inbreeding and promotes out-crossing. As homozygosity can reduce the number of genetic recombinations occurring in the progeny of a cross, a better understanding of the genetic control of characteristics would be possible by using homozygous genotypes as the parents. Therefore, homozygous genotypes would offer new possibilities for breeding and genetic studies in apple and would facilitate the search for molecular markers related to important agronomic characteristics. In the 1970s, the first approaches were conducted to produce doubled haploids in apple by in vitro anther culture (Nakayama et al., 1972). Since then, various homozygous genotypes induction methods such as in vitro anther culture and in situ parthenogenesis in apple have been reported (De Witte, 2000; Hidano, 1982; Höfer, 2004; Höfer and Hanke,

1990; Kadota et al., 2002; Lespinasse et al., 1999; Zhang and Lespinasse, 1991). Although the number of technical methods for inducing homozygous genotypes has increased, information on the morphological characterization of doubled apple haploids is rather limited.

In recent years, several homozygous apple plants have flowered (De Witte, 2000; Höfer et al., 2004, 2008; Lespinasse et al., 1999; Okada et al., 2006; Vanwynsberghe et al., 2005). In other perennial species [e.g., a haploid plant from a female gamete of ‘Banpeiyu’ pumelo (*Citrus grandis* Osbeck)], fertile pollen grains have been reported (Yahata et al., 2005). However, homozygous apple plants have had extremely low pollen fertility, with the exception of the results from Höfer et al. (2008) obtained by in situ parthenogenesis.

In our previous study (Matsuno et al., 1996), several homozygous genotypes were obtained by in vitro anther culture from ‘Senshu’ apple. These genotypes were grafted onto an apple rootstock in the field, and 2 to 4 years after grafting, three of the genotypes produced several flowers for the first time.

The objectives of the present study were to confirm the ploidy level of the androgenic genotypes by flow cytometry; to confirm the zygoty state of the androgenic genotypes by SSR markers; to analyze S-alleles of the androgenic genotypes by the specific polymerase chain reaction (PCR)-digestion method; and to compare the morphological characteristics and pollen

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MORPHOLOGICAL CHARACTERISTICS. For evaluation of several morphological characteristics of the androgenic genotypes and ‘Senshu’ apple, leaf length, leaf width, diameter of flower, and number of stamens and petals per flower were measured using 10 samples. For investigation of fruit quality, depending on yield, 8

to 10 apples per androgenic genotype were used. The source of the pollen used to produce the fruit was open pollinated. After measuring the diameter and weight of the fruit, the firmness of peeled fruit was tested by Magness-Taylor pressure tester (Fujiwara, Tokyo). Thereafter, soluble solids were measured using a digital refractometer (PR-101; Atago, Tokyo). Acidity was measured by titration with NaOH and was calculated as malic acid. In addition, seeds were extracted from the fruit and their size was measured. Data were subjected to analysis of variance by STATISTICA (version 03J; StatSoft, Tulsa, OK). Mean comparisons, except for soluble solids and acidity, were conducted using Tukey’s multiple range test at $P = 0.05$.

For the determination of seed viability, seeds were wrapped with moistened paper, placed in a polyethylene pack, and maintained at 5.5 °C until sowing time.

CROSSING FOR EVALUATION OF REPRODUCTIVE POTENTIAL. ‘Prima’ apple was used as the seed parent and androgenic homozygous genotype 95P6 was used as the pollen parent for the investigation of reproductive potential. The flowers were pollinated immediately after emasculation and were covered with paraffin paper bags. The fruit were collected at maturity and were maintained for 3 months at 4 °C. The seeds were then extracted from the fruit and immediately planted in a 45-mL cell tray (72 cells per tray), which were placed in a greenhouse. After germination, preliminary examination of the hybridism of about 10 of the seedlings was carried out by SSR analysis using CH03d10, MS14h03, CH02h11a, CH05c07, MS06g03, CH04d10, CH05d11, and CH02c09. These eight SSR markers were used

Fig. 1. Flower morphology of three androgenic apple genotypes (92P3, 95P5, and 95P6) and their donor genotype, ‘Senshu’. (A) ‘Senshu’, (B) 92P3, (C) 95P5, (D) 95P6; bar = 20 mm.

Fig. 2. Flow cytometry analysis of leaves in three androgenic apple genotypes (92P3, 95P5, and 95P6) and their donor genotype, ‘Senshu’. (A) ‘Senshu’, (B) 92P3, (C) 95P5, (D) 95P6. Peak 1 = ‘Senshu’ or the androgenic genotype; Peak 2 = ‘Jonagold’.

to evaluate homozygous genotypes of anther culture-derived genotype 95P6, showed heterozygous genotypes for ‘Prima’ apple and were distributed in different linkage groups (LGs).

Results and Discussion

ANDROGENIC PLANTLETS DERIVED FROM ANTHR CULTURE IN APPLE. Anther culture experiments that were carried out between 1992 and 2008 resulted in 17 androgenic genotypes. Although seven genotypes were dropped from the experiment during subculture and two genotypes were maintained in vitro, the other eight androgenic genotypes survived in the experimental field after planting and, moreover, only three of the eight androgenic genotypes flowered between 2002 and 2008, namely, genotypes 92P3, 95P5, and 95P6 (Fig. 1). These genotypes continued to flower each year after the first flowering.

EVALUATION OF PLOIDY LEVEL. Ploidy analysis by flow cytometry revealed that the fluorescence intensity of the flowered androgenic genotypes was two-thirds that of the triploid genotype ‘Jonagold’ apple (Fig. 2). This result showed that the flowered androgenic genotypes were diploid.

RESULTS OF SSR ANALYSIS. In apples, SSR markers are used for molecular characterization and identification and for mapping (Gianfranceschi et al., 1998; Guilford et al., 1997; Kitahara et al., 2005; Silfverberg-Dilworth et al., 2006). SSR is also used for analyzing the genetic origin of haploid/diploid regenerants (Höfer et al., 2002, 2004, 2008; Kenis and Keulemans, 2000; Vanwynsberghe et al., 2005). In the present study, about 100 SSR markers (Gianfranceschi et al., 1998; Liebhard et al., 2002), whose positions were identified in apple genetic linkage maps, were tested for evaluation of anther culture-derived genotypes. Among them, 35 SSR markers showing heterozygous genotype for the donor genotype ‘Senshu’ were used for characterization of three anther culture-derived genotypes 92P3, 95P5, and 95P6 (Table 1). Thirty-five SSR markers used in this study were distributed in almost all LGs except for LGs 1,7,13, and 17. All alleles of the donor genotype were also found in the anther culture-derived genotypes, but only one

Fig. 3. *S-RNase* PCR-digestion analysis for identification of the homozygosity state and the *S*-genotypes of three androgenic apple genotypes (92P3, 95P5, and 95P6) and their donor genotype, ‘Senshu’. The *S-RNase* fragments were amplified by PCR using ASPF3 and ASPR3 primers (A) and were digested with *S*-allele-specific restriction enzymes: *Mva*I (*S*¹) (B) and *Hind*III (*S*⁷) (C). Line 1 = ‘Senshu’; line 2 = 92P3, line 3 = 95P5, line 4 = 95P6.

allele per locus was amplified in each sample. This indicates that all of the anther culture-derived genotypes have a haploid origin, although they are now diploid.

Table 2. Comparison of leaf morphological characteristics in three doubled haploids of apple (92P3, 95P5, and 95P6) and their donor genotype, ‘Senshu’.

Genotype	Leaf length [mean ± SD (cm)]	Leaf width [mean ± SD (cm)]	Length/width ratio [mean ± SD]	Petiole length [mean ± SD (cm)]	Petiole diam [mean ± SD (mm)]
‘Senshu’	9.1 ± 1.0 a ^z	5.0 ± 0.5 a	1.8 ± 0.1 b	2.4 ± 0.2 a	1.9 ± 0.2 a
95P6	8.2 ± 0.7 b	3.9 ± 0.4 bc	2.1 ± 0.1 a	2.5 ± 0.3 a	1.5 ± 0.1 b
95P5	7.1 ± 0.9 c	3.9 ± 0.3 c	1.8 ± 0.2 b	1.7 ± 0.3 b	2.0 ± 0.2 a
92P3	8.0 ± 0.5 b	4.4 ± 0.4 b	1.8 ± 0.2 b	1.9 ± 0.2 b	1.6 ± 0.1 b

^zMeans in columns with different letters are significantly different via Tukey’s test at *P* = 0.05.

Table 3. Comparison of flower morphological characteristics in three doubled haploids of apple (92P3, 95P5, and 95P6) and their donor genotype, ‘Senshu’.

Genotype	Full bloom date ^z	Flowers [mean ± SD (no./cluster)]	Flower diam [mean ± SD (cm)]	Petals [mean ± SD (no.)]	Stamens [mean ± SD (no.)]	Pistils [mean ± SD (no.)]
‘Senshu’	30 Apr.	5.1 ± 0.7 ab ^y	4.3 ± 0.4 a	5.0 ± 0.0 a	19.4 ± 1.1 NS	5.3 ± 0.5 NS
95P6	2 May	5.6 ± 0.8 a	3.9 ± 0.2 ab	5.0 ± 0.0 a	19.4 ± 0.8	5.0 ± 0.0
95P5	2 May	4.6 ± 0.8 ab	3.6 ± 0.2 b	5.0 ± 0.0 a	17.3 ± 2.8	5.3 ± 0.5
92P3	4 May	4.2 ± 1.0 b	3.6 ± 0.5 b	3.9 ± 1.0 b	17.2 ± 2.5	5.3 ± 0.5

^zAverage date of ‘Senshu’ and 92P3 was investigated between 2003 and 2009; that of 95P6 and 95P5 was investigated between 2008 and 2009.

^yMeans in columns with different letters are significantly different via Tukey’s test at *P* = 0.05; NS means not significantly different within a column.

Table 4. Comparison of fruit morphological characteristics in two doubled haploids of apple (95P5 and 95P6) and their donor genotype, 'Senshu'.

Genotype	Harvest date	Fruit wt		Fruit length		Fruit diam		Basin depth		Basin width		Carvity depth		Carvity width		Stalk length		Stalk diam		Soluble solids		Acidity ^y		Fruit firmness		Seeds		Seed length		Seed diam	
		[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	[mean ± sd]	
'Senshu'	14 Sept.	252.4 ± 17.9 a ^x	76.6 ± 5.2 a	83.4 ± 1.8 a	5.5 ± 1.1 a	28.7 ± 3.6 a	11.5 ± 0.8 a	39.2 ± 2.2 a	2.0 ± 0.4 a	3.3 ± 0.4 ab	14.6	0.51	66.4 ± 4.1 b	10.0 ± 3.1 a	8.2 ± 0.4 a	4.5 ± 0.4 ab															
	5 Sept.	112.0 ± 15.0 b	60.6 ± 5.3 b	59.6 ± 3.0 c	4.8 ± 1.4 a	23.1 ± 2.5 b	6.9 ± 1.1 b	27.9 ± 2.1 b	1.3 ± 0.3 b	3.0 ± 0.4 b	14.3	0.21	71.4 ± 3.0 b	2.6 ± 2.0 b	8.4 ± 0.5 a	4.7 ± 0.4 a															
	8 Oct.	114.9 ± 22.9 b	51.9 ± 4.1 c	63.1 ± 3.9 b	2.2 ± 0.5 b	23.4 ± 1.9 b	7.7 ± 1.9 b	30.8 ± 3.1 b	1.6 ± 0.6 ab	4.4 ± 2.1 a	17.1	0.10	93.9 ± 8.0 a	1.3 ± 1.2 b	7.4 ± 0.5 b	4.1 ± 0.5 b															

^aMeans in columns with different letters are significantly different via Tukey's test at $P = 0.05$.

^ySoluble solids and acidity were analyzed using mixed juice from 8 to 10 fruit.

RESULTS OF S-ALLELE ANALYSIS. Generally, diploid apple cultivars contain two different alleles of the *S*-gene and therefore the androgenic genotype induced by in vitro anther culture from the diploid cultivars should have only one of the alleles of the parent. Consequently, analysis of the *S*-alleles was a valuable approach for confirming their haploid origin (Verdoodt et al., 1998). In addition, the knowledge of the *S*-alleles was useful for cross-pollinating and genetic studies. In the previous study, the parental cultivar used was 'Senshu', which contained *S*¹*S*⁷ alleles (Kitahara et al., 1997; Komori et al., 2000; Matsumoto et al., 1999; Sakurai et al., 1997). Using the *S*-*RNase*-specific primers (ASPF3 and ASPR3), the results of PCR analysis of 'Senshu' and the three flowered androgenic genotypes (92P3, 95P5, and 95P6) are shown in Fig. 3A. The PCR products 844 bp (*S*¹) and 618 bp (*S*⁷) were amplified from

Fig. 4. Fruit of three androgenic apple genotypes (92P3, 95P5, and 95P6) and their donor genotype, 'Senshu'. (A) 'Senshu', (B) 92P3, (C) 95P5, (D) 95P6; bar = 50 mm.

Table 5. Rate of anther dehiscence and in vitro pollen germination at different developmental flower stage in three doubled haploids of apple (92P3, 95P5, and 95P6) and their donor genotype, 'Senshu'.

Genotype	Developmental flower stage ^z	Anther dehiscence (%) ^y		In vitro pollen germination (%) ^x	
'Senshu'	Balloon	78.3		82.9	
	Early bloom	97.8		85.3	
	Middle bloom	99.4		85.3	
95P6	Balloon	0.9		1.3	
	Early bloom	20.6		20.6	
	Middle bloom	68.9		33.0	
95P5	Balloon	0.0		0.0	
	Early bloom	16.2		0.0	
	Middle bloom	88.1		0.0	
92P3	Balloon	0.0		0.0	
	Early bloom	0.0		0.0	
	Middle bloom	94.1		0.0	

^zEarly bloom = before dehiscence of anthers in the field, middle bloom = after dehiscence of anthers in the field.

^yAverage number of anthers evaluated for 'Senshu', 95P6, 95P5, and 92P3 was 185, 232, 156, and 164, respectively.

^xAverage number of pollen grains evaluated for 'Senshu', 95P6, 95P5, and 92P3 was 383, 472, 117, and 137, respectively.

'Senshu'. However, 92P3 and 95P5 obtained only the S^7 allele, and 95P6 obtained only the S^1 allele, respectively. The S -*RNase* PCR fragments were digested with the S^1 - and S^7 -allele-specific restriction enzymes, *Mva*I (S^1) (Fig. 3B) and *Hind*III (S^7) (Fig. 3C) to confirm the homozygosity state and S genotypes of the three flowered androgenic genotypes. The 844-bp PCR fragments of 'Senshu' and 95P6 were cut with *Mva*I into two fragments of 712 and 132 bp (S^1 -allele) and the 618-bp PCR fragments of 'Senshu', 92P3 and 95P5, were cut with *Hind*III into 343 and 275 bp (S^7 -allele). The S^1 -specific enzyme *Mva*I was found to also digest the S^7 -allele, yielding 505-, 82-, and 31-bp fragments from 'Senshu', 92P3, and 95P5. Therefore, the S genotypes of 92P3, 95P5, and 95P6 were determined as S^7 , S^7 , and S^1 , respectively, by the PCR-digestion analysis, suggesting that the three flowered androgenic genotypes (92P3, 95P5, and 95P6) were homozygous genotypes.

The previously described results confirmed that the flowered androgenic genotypes were probably doubled haploids. In general, polyploidization easily occurs during anther culture (Hidaka, 1984; Hidaka et al., 1982; Höfer et al., 2008; Kadota et al., 2002; Vanwynsberghe et al., 2005; Verdoodt et al., 1998). In the present study, doubled haploids were also induced without antimitotic agents such as colchicines. This phenomenon was described by Höfer et al. (2008). It is probable that the vigor of the doubled haploids' secondary embryos or cotyledons was higher than haploid tissues. Therefore, their aptitude for shoot regeneration and micropropagation is higher, and haploid structures were likely eliminated during the development.

MORPHOLOGICAL CHARACTERISTICS. The morphological characteristics of the flowered doubled haploids were compared with those of 'Senshu'. The leaf length and width of doubled haploids were significantly smaller, whereas their shape, except for 95P6, was similar to that of 'Senshu' (Table 2). The shape of 95P6 seemed slight compared with the others. The petiole length of 95P5 and 92P3 was significantly shorter than that of 'Senshu' and 95P6. The petiole diameter of 95P6 and 92P3 was significantly thinner than that of 'Senshu' and 95P5.

The doubled haploids bloomed 2 to 4 d later than 'Senshu' (Table 3). 'Senshu' is one of the late-flowering commercial cultivars in Japan; therefore, the doubled haploids were regarded as the latest flowering and, when using them for examination of cross-pollination, attention must be paid to the season. The number of flowers per cluster of 95P6 and 95P5 did not differ significantly from that of 'Senshu', but 95P6 was significantly more than that of 92P3. 95P5 and 92P3 had significantly smaller flowers compared with 'Senshu'. 92P3 had a significantly reduced number of petals compared with 95P6, 95P5, and 'Senshu'. The number of stamens and pistils did not differ significantly. Aberrant flower morphology of androgenic genotypes obtained from in vitro culture of anthers of apple was described by Vanwynsberghe et al., (2005). On the other hand, seemingly normal flower aspects were reported by Höfer et al. (2008). In the present study, flower morphol-

ogy of the flowered doubled haploids was separated between normal and aberrant. 95P6 was similar to that of the donor genotype 'Senshu' (Fig. 1D). 95P5 differed from 'Senshu' only in flower diameter (Fig. 1C). 92P3 had aberrant flower morphology, except the number of stamens and pistils (Fig. 1B).

CHARACTERISTICS OF FRUIT. Table 4 show the results of observation on the fruit characteristics of doubled haploids, with the exception of 92P3, which had too small a yield for comparison with the others. Ripening time of 95P6 was earlier than 'Senshu', and that of 95P5 was later than 'Senshu'. The fruit color of 'Senshu' was a striped red type (Fig. 4A), whereas that of 92P3 and 95P6 was green-yellow (Fig. 4, B and D) and that of 95P5 was solid red (Fig. 4C). Thus, 'Senshu' was heterozygous for anthocyanin production. Although the study of all aspects of fruit color is complex (Janick et al., 1996), it is interesting that solid red occurred from striped red via anther culture. The fruit size of doubled haploids was significantly smaller than that of the donor genotype 'Senshu'. A similar

Fig. 5. In vitro pollen germination of three androgenic apple genotypes (92P3, 95P5, and 95P6) and their donor genotype 'Senshu'. (A) 'Senshu', (B) 92P3, (C) 95P5, (D) 95P6.

Fig. 6. The progenies obtained from cross between 95P6 and 'Prima' apple: (A) seedlings 7 months after germination (bar = 100 cm), (B) nursery stock 3 months after softwood grafting on 'JM7' apple rootstock (bar = 10 cm).

Table 6. Molecular biological examination of apple seedlings from ‘Prima’ × 95P6 and the parental genotypes.

Genotype	SSR markers							
	CH03d10	MS14h03	CH02h11a	CH05c07	MS06g03	CH04d10	CH05d11	CH02c09
	LG2 ^z	LG3	LG4	LG9	LG10	LG11	LG12	LG15
	[SSR band size (bp)]							
‘Prima’	168/170	117/121	120/126	137/145	169/194	168/208	173/177	228/239
95P6	184/184	121/121	126/126	145/145	184/184	164/164	201/201	228/228
1 ^y	170/184	121/121	120/126	137/145	184/194	164/208	173/201	228/239
2	168/184	121/121	126/126	137/145	184/194	164/208	173/201	228/228
3	168/184	121/121	126/126	145/145	169/184	164/168	177/201	228/239
4	168/184	121/121	126/126	145/145	184/194	164/208	177/201	228/239
5	170/184	121/121	126/126	137/145	184/194	164/208	177/201	228/228
6	168/184	117/121	126/126	137/145	169/184	164/208	173/201	228/239
7	168/184	121/121	126/126	145/145	184/194	164/208	177/201	228/239
8	170/184	117/121	120/126	145/145	184/194	164/168	177/201	228/228
9	170/184	121/121	126/126	137/145	184/194	164/208	173/201	228/239
10	170/184	121/121	120/126	145/145	184/194	164/168	173/201	228/239

^zLG = linkage group.^yNumber of the seedlings obtained from the cross ‘Prima’ × 95P6.

tendency of small fruit size in doubled haploids obtained by in situ parthenogenesis was demonstrated by Höfer et al. (2008). Although a statistical comparison of soluble solids and acidity was not conducted, it appears that 95P5 had high soluble solids and low acidity compared with the others.

EVALUATION OF MALE AND FEMALE FERTILITY. The doubled haploids had a significantly reduced number of seeds per fruit compared with that of ‘Senshu’, but the seed size for 95P6 was similar to that of ‘Senshu’ (Table 4). The open-pollinated seeds of 95P6 were viable and the rate of seed germination was 95.0%.

The rate of anther dehiscence of the flowered doubled haploids was dependent on the developmental stage of the flower (Table 5). A later developmental stage tended to increase the rate of anther dehiscence. The fertility of the pollen grains was evaluated by in vitro germination. ‘Senshu’ showed a pollen germination rate of more than 80% (Table 5, Fig. 5A), whereas the rate for 95P5 and 92P3 was 0% (Fig. 5, B and C). On the other hand, the pollen germination rate of 95P6 also depended on the developmental stage of the flower. The pollen germination rate at the balloon stage was only 1.3%, but that at the early to middle bloom stage was 20.6% to 33.0% (Fig. 5D). These results were rather better than those obtained by Vanwynsberghe et al. (2005) and Höfer et al. (2008) in androgenesis.

To evaluate the reproductive potential of male gametes of 95P6, crosses with ‘Prima’ apple were carried out. As described above, 95P6 showed considerably later flowering compared with ‘Prima’; therefore, hand-pollination was carried out at Morioka, located about 250 km north of Fukushima. The number of crossing flowers was 150 and after pollination, 24 fruit were set. The efficiency of this result was low at 16.0% and may have been caused by the small amount of pollen grains of 95P6. In total, 122 seeds were obtained from the fruit and the average number of seeds per fruit was 5.1 (SD = 2.7). When these seeds were planted in a cell tray with compost, the germination rate was 77.9%, and 81.1% of the germinated seedlings survived. The seedlings were transferred to an 18-cm polyethylene basin and maintained outside a greenhouse (Fig. 6A). Moreover, parts of these seedlings were grafted onto ‘JM7’ apple rootstock in the greenhouse (Fig. 6B).

For verification of hybridism of those seedlings, SSR analysis was carried out using eight SSR markers. All of the seedlings showed, with each primer combination, one allele from the maternal parent, and a second allele from the paternal parent (Table 6). This result confirmed that the seedlings were indeed a hybrid between ‘Prima’ apple and 95P6. All of the other surviving seedlings also had one allele from the maternal parent and a second allele from the paternal parent (data not shown).

In conclusion, the use of homozygous genotypes in apple breeding programs is currently not a realistic approach (Höfer et al., 2008). Homozygous genotypes in apple generally have low vigor, aberrant tree architecture, abnormal flower morphology, and infertility by inbreeding depression (Vanwynsberghe et al., 2005). In the present study, the flowered homozygous genotypes obtained from in vitro culture of anthers of ‘Senshu’ were confirmed as doubled haploids. One of them, 92P3, showed definitely low vigor, bark morphology comparable with a very old apple tree (similar result reported by Höfer et al., 2008), aberrant flower morphology and pollen infertility, whereas the other genotype, 95P6, showed similar morphological characteristics, except for fruit color, and shape and size of the leaf and fruit compared with the donor genotype ‘Senshu’, and also produced fertile pollen grains. Moreover, several progenies were obtained from the cross between ‘Prima’ and 95P6. This progenies grafted onto ‘JM7’ rootstock are planting in our experimental field. Furthermore, although pollination was open, the female gametes of 95P6 showed slight fertility. Even though several problems arose (e.g., small amount of pollen grains), 95P6 will be used in future studies for breeding and analysis of favorable genes. For the present, we will practice genetic mapping by using the progenies obtained from the cross between ‘Prima’ and 95P6.

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