

Evaluation of Reproductive Functions in a Haploid Pummelo by Crossing with Several Diploid Citrus Cultivars

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Summary

To evaluate the reproductive potential of female and male gametes in a haploid plant derived from seedlings of 'Banpeiyu' pummelo, we performed crosses between the haploid and several diploid citrus cultivars. In the crosses with the diploid cultivars as pollen parents, no fruit set on the haploid in all cross combinations. However, developed seeds were obtained in four cross combinations, when some monoembryonic diploid cultivars were pollinated with pollen of the haploid. These seeds germinated normally and developed into diploid, vigorous seedlings with large wing leaves, typical of 'Banpeiyu' pummelo. Furthermore, the hybridity of a seedling obtained from the cross between 'Kiyomi' tangor and the haploid was confirmed by random amplified polymorphic DNA (RAPD) analysis and chromosome composition by chromomycin A₃ (CMA) staining. These results suggest that the haploid produced fertile pollen grains (n=9).

Key Words: chromomycin A₃, *Citrus grandis* Osbeck, flow cytometry, RAPD, un-reduced pollen.

Introduction

Haploid plants are of great value in plant breeding because by doubling of their chromosomes, homozygous plants can be obtained that are very useful for genetic analysis and premeditated breeding. It has been reported that haploids have been obtained in fruit crops such as peach, apple, pear, and banana (Assani et al., 2003; Bouvier et al., 2002; Hesse, 1971; Ochatt and Zhang, 1996; Zhang and Lespinasse, 1991). Haploid plants generally show poor growth compared with their mother diploid parents. Flowering of haploids is rare but it was observed in one peach plant among fruit crops (Hesse, 1971; Pooler and Scorza, 1995; Toyama, 1974). Hesse (1971) reported that some pollen grains of the haploid peach germinated and set fruit following the pollination of diploid plants. Pooler and Scorza (1995) showed that two open-pollinated genotypes of haploid peach formed fertile seeds. Thus, it has been recognized that some haploid peaches can produce a few fertile female and male gametes.

In *Citrus* and related species, haploid production has been reported in *C. natsudaidai* Hayata, trifoliata orange (*Poncirus trifoliata* (L.) Raf.), calamondin (*C. madurensis* Lour.), clementine (*C. clementina* Hort. ex Tanaka), and 'Lee' (Chen et al., 1980; Germana and Chiancone, 2001, 2003; Hidaka et al., 1979; Karasawa, 1971; Oiyama and Kobayashi, 1993). These haploids are also very weak, and grow slower than the diploid plants. Therefore, no flowering of haploids in *Citrus* species has ever been reported and consequently, the reproductive potential of these haploids has not been examined. On the other hand, Toolapong et al. (1996) selected a haploid progeny among small seed-derived seedlings that were obtained from the cross between 'Banpeiyu' pummelo (*C. grandis* Osbeck) and 'Ruby Red' grapefruit (*C. paradisi* Macf.). When this haploid was grafted onto trifoliata orange, it showed vigorous growth and flowered for the first time seven years after germination. Yahata et al. (2005) confirmed that this haploid was derived from the female gamete of 'Banpeiyu' pummelo, and reported that the haploid produced a few fertile pollen grains that were 1.6% stainable and 0.4% viable. However, the haploid had small flowers and produced a few pollen grains, compared with that of the diploid.

In this study, we performed crosses between the haploid from 'Banpeiyu' pummelo and several citrus diploid cultivars to evaluate the reproductive potential of

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female and male gametes in the haploid. Furthermore, we investigated the hybridity of the seedlings obtained from these crosses by using RAPD and chromosome identification by chromomycin A₃ (CMA) staining techniques.

Materials and Methods

Plant materials

A haploid plant, obtained from the cross between 'Banpeiyu' pummelo and 'Ruby Red' grapefruit (Toolapong et al., 1996) in this study was grafted onto trifoliolate orange, and maintained for approximately 10 years in the greenhouse in School of Agriculture, Kyushu Tokai University. To evaluate the reproductive potential of male and female gametes of the haploid, 8 diploid species and cultivars, 'Banpeiyu' pummelo, 'Kiyomi' tangor (*C. unshiu* Marc. × *C. sinensis* Osbeck), 'Nanpu' tangor ('Kiyomi' tangor × 'Fairchild' mandarin), 'Miyauchi-Iyokan' (*C. iyo* hort. ex Tanaka), 'Tosa-Buntan' pummelo (*C. grandis* Osbeck), 'Kitou-Yuzu' (*C. junos* hort. ex Tanaka), 'Kawano-Natsudaidai' (*C. natsudaidai* Hayata), and Hyuga-natsu (*C. tamurana* hort. ex Tanaka) were used. The cross combinations conducted between the haploid and diploids are shown in Table 1.

The flowers were pollinated immediately after emasculation and covered with paraffin paper bags. Seeds were collected from each fruit of all crosses at maturity and were classified into two groups, developed and undeveloped seeds, according to their size and shape. Developed seeds were placed on moistened filter paper and kept at 25°C. Undeveloped seeds were cultured on Murashige and Tucker (MT) medium (1969) containing 500 mg · L⁻¹, malt extract, 30 g · L⁻¹ sucrose, and 2 g · L⁻¹ gellan gum at 25°C under continuous illumination (38 μmol · m⁻² · s⁻¹). After germination, the seedlings were transplanted into pots containing vermiculite and transferred to a greenhouse.

Confirmation of ploidy level

1. Flow cytometry

Young leaves of approximately 1cm² segments were collected from all seedlings obtained from the crosses between the above diploid citrus cultivars and the haploid. The samples were chopped with a razor blade and blended for 5 min in 2 mL buffer solution containing 1.0% (v/v) Triton X-100, 140 mM mercaptoethanol, 50 mM Na₂SO₃, and 50 mM Tris-HCl at pH 7.5, according to the preparation method of Harusaki et al. (2000). An aliquot (550 μL) of each sample was filtered through Miracloth (Merck KGaA, Darmstadt, Germany) and the filtrate stained with 50 μL 0.5 g · L⁻¹ propidium iodide (PI). The relative fluorescence of total DNA was measured for each nucleus with a Flow Cytometry System (EPICS XL; Beckman Coulter, Inc., CA, USA) equipped with an argon laser (488 nm, 15

mW).

2. Chromosome observation

Young leaves (approximately 3–5 mm long) were excised from the above seedlings, immersed in 2mM 8-hydroxyquinoline for 10 h at 4°C, and fixed in a mixed solution of ethanol and acetic acid (3:1) for 12 h at 4°C. Enzymatic maceration and air drying were performed according to Fukui (1996) with some modifications. The young leaves were washed in distilled water to remove the fixative and then macerated in an enzyme mixture containing 2.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo), 1.0% (w/v) Macerozyme R-200 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo), 0.3% Pectolyase (w/v) (Kyowa Chemical Products Co., Ltd., Osaka), and 200 mM EDTA at 37°C for 40 min. The macerated samples were rinsed with distilled water and added to a fixative solution. The mixtures were transferred to a slide glass. After air drying the slide glass, the chromosomes were stained with 2.0% Giemsa solution (Merck KGaA, Darmstadt, Germany) in 1/30 phosphate buffer (pH 6.8) for 30 min. Then, they were rinsed with distilled water to remove excess stain, air dried, and observed under an optical microscope.

Confirmation of hybridity

1. RAPD analysis

Total DNA was extracted from young leaves of a seedling obtained from the cross between 'Kiyomi' tangor and the haploid, according to Doyle and Doyle (1987). RAPD analysis was performed using a modified method of Williams et al. (1990), in which the reaction mixture (25 μL) contained 10 mM Tris-HCl, pH 8.9, 80 mM KCl, 1.5 mM MgCl₂, 100 μM each dNTPs, 0.3 μM primer, 2.5 U Tth *Taq* DNA polymerase, and 10 ng of genomic DNA. Reactions were carried out by repeating 45 cycles of the following thermal treatments; 94°C for 30 sec, 37°C for 2 min, and 72°C for 3 min, in ASTEC Program Control System PC-700 (ASTEC Co., Ltd., Fukuoka). The primers used were OPA-7, OPA-11, OPA-12, and OPB-18 in Operon random 10-mer primers (Operon Technology Inc., CA, USA). Reaction products were electrophoresed on 1.5% agarose gels containing 0.5 mg · L⁻¹ ethidium bromide and subsequently photographed under ultraviolet light (360 nm). For each combination of samples and primers, PCR was carried out twice and only stable polymorphisms were taken into account.

2. Analysis of chromosome composition by chromomycin A₃ (CMA)

In a seedling obtained from the cross between 'Kiyomi' tangor and the haploid, after the chromosomes were stained with Giemsa and each position was confirmed on the slide glass, the chromosomes were de-stained with 70% methanol. Chromosomes were also stained with 0.1 mg · L⁻¹ CMA according to Befu et al. (2000) with some modifications and observed under a

fluorescence microscope with the BV filter cassette. The chromosomes were classified into five types based on the number and position of CMA bands according to Befe et al. (2000) and Yamamoto and Tominaga (2003): A, telomeric band in both arms and proximal band in one arm, B, telomeric band in one arm and proximal band in the other arm, C, telomeric band in both arms, D, telomeric band in one arm, and E, no band.

Results and Discussion

The reproductive potentials of female and male gametes of the haploid crossed with several diploid cultivars (Table 1) revealed that when the haploid was the seed parent, no fruit set in any of the cross combinations. But, when five monoembryonic diploid cultivars were pollinated with pollen of the haploid, fruits were set in all of the cross combinations. Many developed seeds and some undeveloped seeds were obtained from four cross combinations. The numbers of developed seeds per fruit, obtained from crosses with ‘Kiyomi’ tangor, ‘Nanpu’ tangor, ‘Miyuchi-Iyokan’, and ‘Tosa-Buntan’ pummelo were 4.7, 5.1, 0.2, and 15.0, respectively. The frequency of developed seeds in these cross combinations was similar to that of open pollination. In the cross between ‘Banpeiyu’ pummelo and the haploid, fruit set was 35.7%, but no fruit contained seeds.

No fruit set following the pollination of the haploid with the pollen of diploid cultivars because all flowers

dropped within two weeks after pollination in spite of crossing to the inflorescence with leaves. It seems that no fertilization occurred between the eggs of this haploid and the pollen grains from the diploid parents. Further study is needed to examine the formation of the female gamete and its fertilization process in the haploid.

In the cross between the ‘Banpeiyu’ pummelo and the haploid, seeds were not completely obtained. ‘Banpeiyu’ pummelo is known to show strong self-incompatibility (Isobe et al., 1982; Iwamasa, 1976). Isobe et al. (1982) reported that the fruits obtained from the self-pollination of ‘Banpeiyu’ pummelo were smaller than those pollinated with pollen from diploid citrus cultivars, such as ‘Kawano-Natsudaidai’, and seeds were rarely obtained from their fruits. In this study, the fruits obtained by pollinating with the pollen of the haploid were significantly smaller than those from open-pollinated fruits. Self-pollination of ‘Banpeiyu’ pummelo yielded results similar to those of cross between ‘Banpeiyu’ pummelo and the haploid, indicating that self-incompatibility is expressed by both partners.

Both developed and undeveloped seeds obtained from the crosses between diploid cultivars and the haploid were germinated on moistened filter paper and MT medium, respectively. Although no seedlings were obtained from undeveloped seeds cultured, developed seeds germinated almost normally (Table 2). Consequently, 14, 45, 1, and 13 seedlings were obtained from

Table 1. Fruit set and seed contents in the crosses between the haploid and several diploid citrus cultivars.

Cross combination		No. of flowers Pollinated	No. of fruits set	% of fruits set	Av. fruit wt. (g)	No. of seed		No. of developed seeds per fruit	% of developed seeds ^z
Seed parent	Pollen parent					Developed	Undeveloped		
Haploid	Open pollination	-	0	-	-	-	-	-	-
	Haploid	10	0	0	-	-	-	-	-
	‘Banpeiyu’	10	0	0	-	-	-	-	-
	‘Kitō-Yuzu’	10	0	0	-	-	-	-	-
	‘Kawano-Natudaidai’	10	0	0	-	-	-	-	-
	Hyuga-natsu	10	0	0	-	-	-	-	-
‘Banpeiyu’	Open pollination	-	3	-	1484.0 a ^y	279	15	93.0	94.9
	Haploid	14	5	35.7	807.4 b	0	0	0	0
	‘Banpeiyu’	5	4	80.0	905.0 b	0	0	0	0
‘Kiyomi’	Open pollination	-	5	-	275.2	68	3	13.6	95.8
	Haploid	5	3	60.0	212.3	14	0	4.7	100
‘Nanpu’	Open pollination	-	5	-	272.4	26	5	5.2	83.9
	Haploid	33	10	30.3	280.3	51	18	5.1	73.9
‘Miyuchi-Iyokan’	Open pollination	-	3	-	496.3	26	8	8.7	76.5
	Haploid	11	6	54.5	340.0	1	0	0.2	100
‘Tosa-Buntan’	Open pollination	-	3	-	593.7	140	17	46.7	89.2
	Haploid	10	1	10.0	388.0	15	2	15.0	88.2

^z (Developed seed / total seed) × 100.

^y Different letters represent significant differences in Tukey’s multiple range test, 5% level.

cross combinations with 'Kiyomi' tangor, 'Nanpu' tangor, 'Miyachi-Iyokan', and 'Tosa-Buntan' pummelo, respectively. After being transplanted to soil, these seedlings grew vigorously and developed large wing leaves, typical of 'Banpei-yu' pummelo (Fig. 1).

All seedlings were analyzed to determine the ploidy level by flow cytometry analysis and chromosome observation (Table 2). In flow cytometric analysis, most of the seedlings showed the fluorescence intensity equal to that of the diploid control, but one seedling obtained

Table 2. Ploidy level of seedlings obtained from the crosses between four diploid citrus cultivars and the haploid.

Seed parent	No. of seeds examined	No. of germinated seeds	% of germination	No. of seedlings examined	Ploidy level		
					2X	3X	Other
'Kiyomi'	14	11	78.6	11	0	0	
'Nanpu'	51	45	88.2	45	44	0	
'Miyachi-Iyokan'	1	1	100	1	0	0	
'Tosa-Buntan'	15	13	86.7	13	13	0	

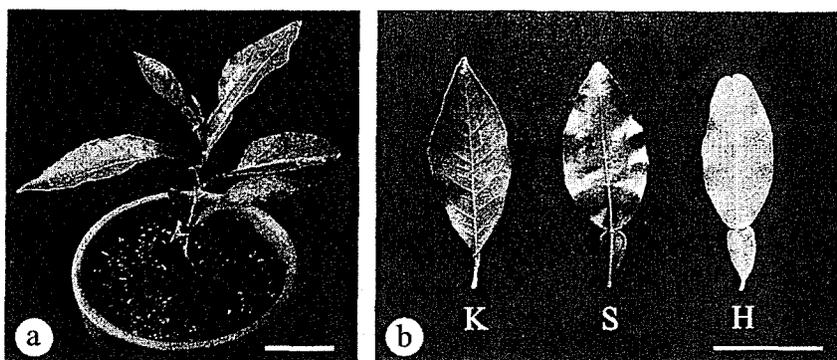


Fig. 1. The seedling obtained from the cross between 'Kiyomi' tangor and the haploid (a) and the comparison of its morphology of leaf with the parents (b). K: 'Kiyomi' tangor, S: Seedling, H: The haploid. Bars=5 cm.

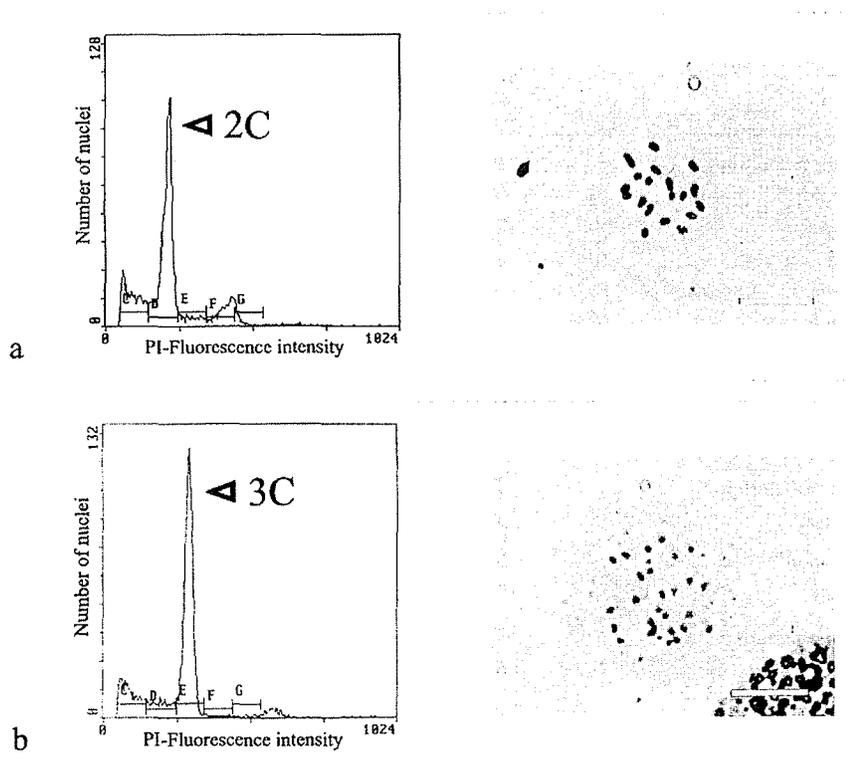


Fig. 2. Flow cytometric analysis and chromosome observation of seedlings obtained from the cross between 'Nanpu' tangor and the haploid. a: Diploid ($2n=2X=18$), b: Triploid ($2n=3X=27$). Bars=10 μ m.

from the cross between 'Nanpu' tangor and the haploid showed a triploid DNA value. Chromosome observation on immature leaflets revealed that the former seedlings had 18 and the latter 27 chromosomes (Fig. 2). Moreover, the appearance of an aneuploid was not observed among any seedling.

To confirm the hybridity of diploid seedlings obtained from the cross between 'Kiyomi' tangor and the haploid,

we employed RAPD analysis for one seedling and its parents that yielded bands specific to both parents (Fig. 3). Furthermore, when the seedling and its parents were subjected to analysis for chromosome composition by CMA staining, 'Kiyomi' tangor was composed of 1B+2C+7D+8E (Fig. 4a), whereas the chromosome composition of the haploid was 1A+1B+1C+2D+4E (Fig. 4b). However, the seedling with a chromosome

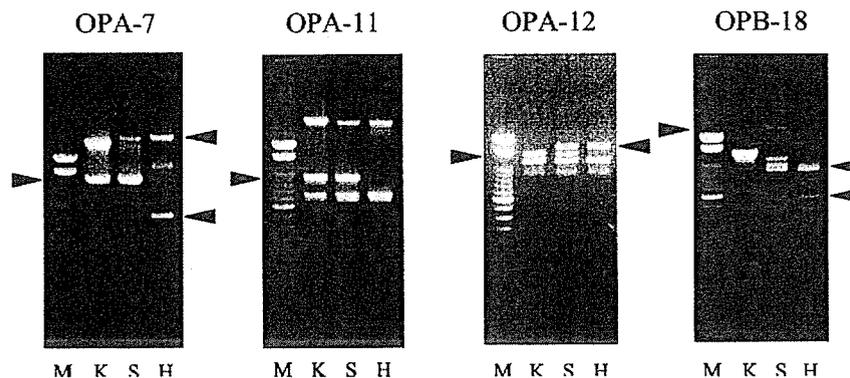


Fig. 3. Gel plates for RAPD analysis of the seedling obtained from the cross between 'Kiyomi' tangor and the haploid. Four primers (OPA-7, OPA-11, OPA-12, and OPB-18) were used for the analysis. Arrows indicate the bands specific to each parent. M: 100 bp ladder marker, K: 'Kiyomi' tangor, S: Seedling, H: The haploid.

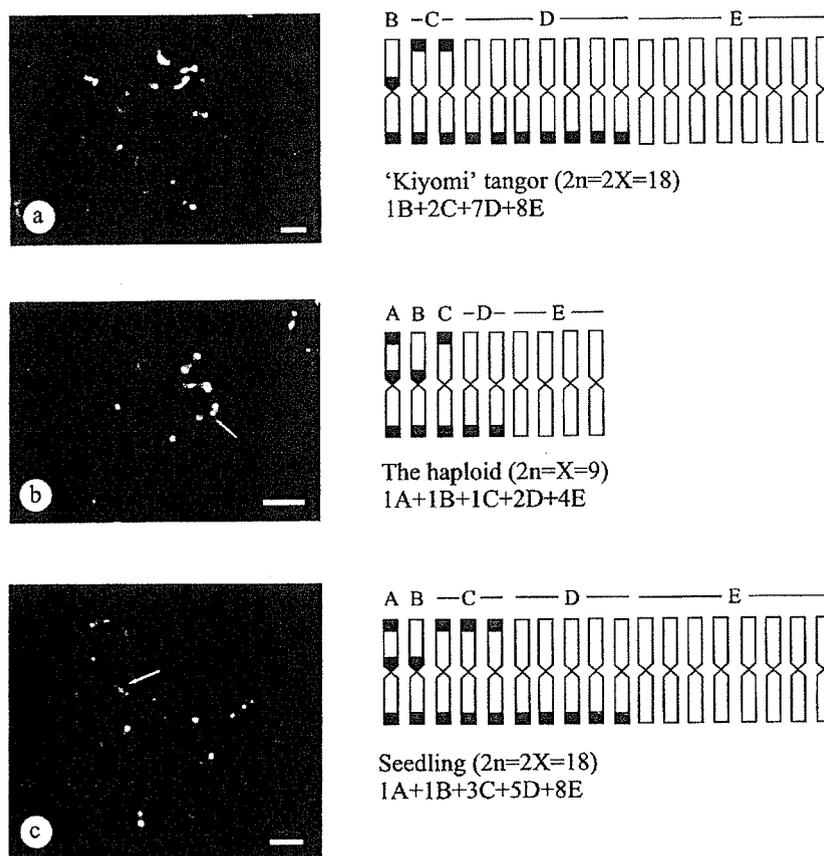


Fig. 4. Photographs and idiograms of CMA banding patterns in the somatic chromosome of 'Kiyomi' tangor (a), the haploid (b), and the seedling obtained from the cross between 'Kiyomi' tangor and the haploid (c). The black regions shown in the idiograms indicate CMA positive bands. Arrows indicate type A chromosome. A: Telomeric band in both arms and proximal band in one arm. Bars=5 μ m.

composition of 1A+1B+3C+5D+8E (Fig. 4c) possessed a type A chromosome, specific to 'Banpeiyu' pummelo. Thus, RAPD analysis and CMA staining confirmed that the seedling was a diploid hybrid between 'Kiyomi' tangor and the haploid. This result reveals that fertilization occurred between the normal eggs of 'Kiyomi' tangor and pollen grains with 9 chromosomes from the haploid.

To date, it has been reported that small seeds obtained from the crosses among several citrus diploids produced triploids at high frequencies (Esen and Soost, 1971; Toolapong et al., 1996; Wakana et al., 1981). In this study, one triploid progeny was obtained from the cross between 'Nanpu' tangor and the haploid. It is presumed that this triploid was produced by the fertilization between 2n unreduced egg of 'Nanpu' tangor and pollen grains with 9 chromosomes from the haploid. However, this triploid was produced from a seed having a similar weight (0.27 g) as normally developed seeds (0.24 g). Yahata et al. (2005) reported that this haploid produced bigger fertile pollen grains (>40 μm) than other normal sized ones (35 μm), although they constituted only 4% of fertile pollen grains. Consequently, it is also possible that the triploid was produced by the fertilization between a normal egg of 'Nanpu' tangor and chromosome doubled 2n pollen grains of the haploid.

It has been reported that some species such as *Brassica napus*, *Prunus persica*, *Lycopersicon esculentum*, and *Capsicum annuum* could form fertile gametes in haploid plants (Hesse, 1971; Pooler and Scorza, 1995; Toyama, 1974; Veilleux, 1985; Yan et al., 2000). Regarding the mechanism of fertility restitution in the haploid plant of the peach, Pooler and Scorza (1995) noted that all chromosomes in the meiocyte migrated to the same pole by chance during meiosis I that resulted in the formation of fertile unreduced gametes, whereas Hesse (1971) suggested that parallel spindle formation during meiosis resulted in a second division restitution. In the haploid plant of *C. annuum*, Yan et al. (2000) found laggards in many dividing cells of the first division at meiosis, which resulted in the first division restitution (FDR) at meiosis that led to the restitution of fertility in the haploid. In the haploid, it is also necessary to clarify the mechanism involved in the production of normal pollen grains.

Recently, a combination of the enzymatic maceration and fluorescent staining such as CMA and/or 4'-6-diamidino-2-phenyl-indole (DAPI) has been used for identifying *Citrus* chromosomes and applied for biotechnological studies such as genome analysis, somatic hybridization, and ploidy manipulation (Befu et al., 2000; Guerra, 1993; Miranda et al., 1997a, b; Pedrosa et al., 2000; Yamamoto and Tominaga, 2003; Yang et al., 2002). In this study, type A chromosome could be distinguished as a specific marker for the haploid by using the CMA staining (Fig. 4). By using this marker chromosome, the hybridity of the seedling obtained

from the cross between diploids and the haploid was confirmed. Moreover, the inheritance of the marker chromosome was also confirmed in the progenies. Therefore, CMA staining is considered to be an efficient and useful method for analysing hybridity by finding species-specific markers, such as the type A chromosome.

In conclusion, numerous diploid seedlings with large wing leaves were obtained from crosses between several diploid cultivars and the haploid. Furthermore, the hybridity of one seedling obtained from the cross between 'Kiyomi' tangor and the haploid was confirmed by RAPD analysis and chromosome composition by CMA staining. These results suggest that the haploid has the ability to produce viable pollen grains ($n=9$). In the future, we plan to carry out detailed studies on the formation of female and male gametes in the haploid.

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二倍体カンキツ品種との交雑による半数体ブンタンの生殖機能の評価

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摘 要

‘晩白柚’の実生から得られた半数体における雌性および雄性配偶子の生殖機能の評価するために、半数体と種々の二倍体カンキツ品種との交雑を行った。半数体に二倍体の花粉を授粉した場合には全く着果しなかったが、半数体を花粉親とした場合、4つの交雑組合せにおいて完全種子が得られた。これらの完全種子は正常に発芽し、多くの二倍体実生が

得られた。これらの実生は旺盛に成長し、‘晩白柚’の形態的特徴である翼葉を有していた。さらに、‘清見’と半数体との交雑から得られた1個体の実生について、RAPD分析およびCMA染色による染色体構成を解析したところ、雑種であることが確認された。これらの結果から、本半数体では正常な花粉(n=9)が形成されていることが示唆された。