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Original Articles

Relationship between Weight of Seeds and the Ploidy Level of the Seedlings Obtained from the Cross Between 'Banpeiyu' Pummelo and Somatic Hybrid 'Citrus parental line No. 4'

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Summary : The possibility of using somatic hybrid 'Citrus parental line No.4' (Yuzu, *Citrus junos* Siebold. ex Tanaka+ navel orange, *C. sinensis* (L.) Osbeck var. *brasiliensis* Tanaka) for triploid production was investigated by crossing it with diploid 'Banpeiyu' pummelo. 'Citrus parental line No.4' showed high pollen fertility as assessed by stainability (88.2 %) and germination rate (21.2 %), although one of the parents of this somatic hybrid was a male sterile navel orange. When the 'Banpeiyu' pummelo (*C. maxima* (Burm.) Merr.) was pollinated with pollen of 'Citrus parental line No.4', triploid seedlings were predominantly obtained from developed seeds at a high frequency (83.3 %), i.e., 105 triploid, 20 tetraploid, and 1 hexaploid seedlings. No clear correlation was observed between the weight of the seeds and the ploidy level of the seedlings, and triploids, tetraploids, and hexaploid were obtained from seeds with weights of 0.2-0.8 g, 0.4-0.6 g, and 0.2 g, respectively. In contrast, all of the seedlings obtained from undeveloped seeds were triploids. These results indicate that 'Citrus parental line No.4' can be useful as a parent for triploid breeding.

Key words : 'Citrus parental line No.4', Flow cytometry, Triploid breeding, Unreduced gamete

Introduction

Seedlessness is a trait that is essential for any new citrus cultivar intended for fresh fruit and for the manufacturing market. The most important *Citrus* cultivars for fresh fruit in the world, such as the navel oranges (*Citrus sinensis* (L.) Osbeck var. *brasiliensis* Tanaka), satsuma mandarins (*C. unshiu* Marcow.) and some cultivars of grapefruits (*C. paradisi* Macfad.), are virtually seedless. For the breeding of seedless cultivars, crossing with male sterile cultivars (Nishiura *et al.*, 1983 ; Matsumoto *et al.*, 1991 ; Okudai *et al.*, 1991a, 1991b) and the creation of mutants by irradiation (Hearne, 1986) have mainly been used. Another method for producing seedless cultivars is the use of triploids (Soost & Cameron, 1980, 1985). In general, triploids have efficiently been obtained by 2X × 4X and 4X × 2X crosses (Longley, 1926 ; Esen & Soost, 1972 ; Kaneyoshi *et al.*, 1997 ; Yahata *et al.*, 2003) and by selecting the seedlings derived from small seeds in 2X × 2X crosses (Esen & Soost, 1971 ; Wakana *et al.*, 1981 ; Toolapong *et al.*, 1996). Besides triploids, however, unexpected tetraploids resulted from fertilization between diploid unreduced female gametes and diploid male gametes as well as undeveloped seeds degenerated by an unbalanced of ploidy ratio between the embryo and endosperm were ob-

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tained in great numbers in 2X × 4X crosses (Tachikawa *et al.*, 1961 ; Yamashita, 1976 ; Oiyama *et al.*, 1982 ; Takahara *et al.*, 1982 ; Yahata *et al.*, 2003). Furthermore, no tetraploids are available in commercial cultivars for the production of triploids. These problems are now considered to be major barriers in the triploid breeding of *Citrus*.

Since the first success in the production of somatic hybrids in *Citrus* and its related genera by Ohgawara *et al.* (1985), somatic hybridization techniques have become well been developed and employed to facilitate improvement of *Citrus* scion and rootstock (Grosser & Chandler, 2000 ; Grosser *et al.*, 2000). To date, more than 150 citrus somatic hybrids have been produced in the world, including intergeneric combinations (Moreira *et al.*, 2000 ; Guo *et al.*, 2002 ; Liu *et al.*, 2002). In Japan, 'Citrus parental line No.1' (Satsuma mandarin + naval orange), 'Citrus parental line No.2' (grapefruit + naval orange), 'Citrus parental line No.3' ('Murcott' tangor + naval orange) and 'Citrus parental line No.4' (Yuzu + navel orange) have been registered as cultivars (Kobayashi *et al.*, 1988 ; Ohgawara *et al.*, 1989 ; Kobayashi *et al.*, 1991 ; Kobayashi *et al.*, 1995). Although these somatic hybrids are of great interest as breeding materials, they have not been cultivated commercially because they were amphidiploids (2n=4x=36) and showed undesirable characteristics such as a thick rind and a high number of seeds (Kobayashi *et al.*, 1995).

In the present study, we estimated pollen fertility in the somatic hybrid cultivar 'Citrus parental line No. 4' produced between diploid Yuzu and diploid 'Ohmishima' navel orange. Furthermore, we investigated the relationship between the weight of seeds and the ploidy level of the seed-derived plantlets obtained from the cross between diploid 'Banpeiyu' pummelo (*C. maxima* (Burm.) Merr.) and tetraploid 'Citrus parental line No.4'.

Materials and Methods

Morphological Characteristics and Pollen Fertility of 'Citrus Parental Line No.4'

Tetraploid somatic hybrid cultivar 'Citrus parental line No.4' and its parents, Yuzu and 'Ohmishima' navel orange were used in the observation of morphological characteristics and of fertility in pollen. The morphological characteristics of pollen (e.g., overall size and shape index of pollen grains) were measured at the full bloom stage using one hundred samples for each plant material. Dried pollen grains were sputter-coated with gold using an ion-coater (IB-3, EIKO Engineering Co., Ltd., Tokyo, Japan) and observed using scanning electron microscopy (SEM, ALPHA-30A, ABT Co., Ltd., Tokyo, Japan). Pollen fertility was investigated by testing the stainability and *in vitro* germination of the pollen grains. Pollen stainability was estimated by staining the samples with 1 % acetocarmine after squashing mature anthers just before dehiscence on a slide glass. *In vitro* germination of the pollen grains was performed on a slide glass covered with a 2-mm layer of 1 % (w/v) agar medium containing 10 % sucrose. Five stamens, each from different flowers, were rubbed on the agar medium, and the slides were then incubated for 10 h in a moistened chamber at 25℃ in the dark. One thousand pollen grains were observed for each sample.

Reproductive Potential as Breeding Material

In early May, 2002, emasculated flowers of monoembryonic diploid cultivar 'Banpeiyu' pummelo were hand-pollinated with the pollen of somatic hybrid cultivar 'Citrus parental line No.4'. The flowers were then enclosed using paraffin paper bags to prevent open pollination. The pollinated flowers were harvested at maturity in early November, 2002 and seeds were collected from each fruit. The seeds were classified into two groups : developed and undeveloped seeds, according to their size and shape. After being numbered and weighed, the developed seeds were placed on moistened filter paper and kept at 25 ℃ in the dark. The undeveloped seeds were cultured on Murashige and Tucker (MT) medium (1969) containing 500 mg/l malt extract, 30 g/l sucrose and 2 g/l gellan gum at 25℃ under continuous illumination (40 μmol m^{-2} s^{-1}). After germination, the seedlings were transplanted onto vermiculite in pots and grown in a greenhouse.

Confirmation of Ploidy Level

Flow Cytometry

Segments of young leaves approximately 1 cm^2 in size were collected from the seedlings obtained from the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4' and chopped with a razor blade. The samples were treated for 5 min in 1 ml buffer solution containing 1 % (v/v) Triton X-100, 140 mM mercaptoethanol, 50 mM Na_2SO_3 and 50 mM Tris-HCl at pH7.5, according to the preparation method of Harusaki *et al.* (2000). Crude samples were filtered through Miracloth (Merck KGaA, Drarmstadt, Germany) and stained with 25 μg/l 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).

Chromosome Observation

Young leaves (approximately 3-5 mm long) were excised from the seedlings obtained from the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4', immersed in 2 mM 8-hydroxyquinoline for 6 h at 4℃, and fixed in a mixed solution of ethanol and acetic acid (3 : 1) for 12 h at 4℃. Enzymatic maceration and air drying were performed according to the method of Fukui (1996) with some modifications. The young leaves were washed in distilled water to remove the fixative and then were macerated in an enzyme mixture containing 2 % (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 1 % (w/v) Macerozyme R-200 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 0.3 % Pectolyase (w/v) (Kyowa Chemical Products Co., Ltd., Osaka, Japan), and 200 mM EDTA at 37℃ for 40 min. The macerated samples were rinsed with distilled water, and were added with fixative solution. The mixtures were transferred to a slide glass. After air drying of the slide glass, the chromosomes were stained with 2 % Giemsa solution (Merck KGaA, Drarmstadt, Germany) in 1/30 phosphate buffer (pH6.8) for 30 min, rinsed with distilled water, air dried, and observed under an optical microscope.

After confirmination of ploidy level, the relationship between weight of the seeds and the ploidy level of the seed-derived plantlets obtained by the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4' was investigated.

Results and Discussion

The morphological characteristics and fertility of pollen grains in 'Citrus parental line No.4' and the parents of this somatic hybrid are shown in Table 1. Although the 'Ohmishima' navel orange, one of the parents, had no pollen grains, 'Citrus parental line No.4' showed a high pollen stainability (88.2 %) and germination rate (21.2 %) that were relatively high, but which were slightly lower than those of another parent, Yuzu. However, the average size of the pollen grains of 'Citrus parental line No.4' was larger than

Table 1. Comparison of morphological characteristics of pollen grains in Yuzu, somatic hybrid 'Citrus parental line No. 4' and 'Ohmishima' navel orange.

Strains	Ploidy level	Pollen grain (μm)		Shape index of pollen grain[z]	No. of germ pore	Pollen fertility (%)	
		Length	Width			Stainability	*In vitro* germination
Yuzu	2X	31.2	17.3	1.8	4.2	92.1	44.9
'Citrus parental line No. 4'	4X	36.2	32	1.1	5	88.2	21.2
'Ohmishima' navel orange	2X	-	-	-	-	-	-
t-test[y]		*	*	*	*	NS	*

[z] Length of pollen grain/Width of pollen grain.
[y] NS and * indicate non-significant and significant at 1% levels, respectively.

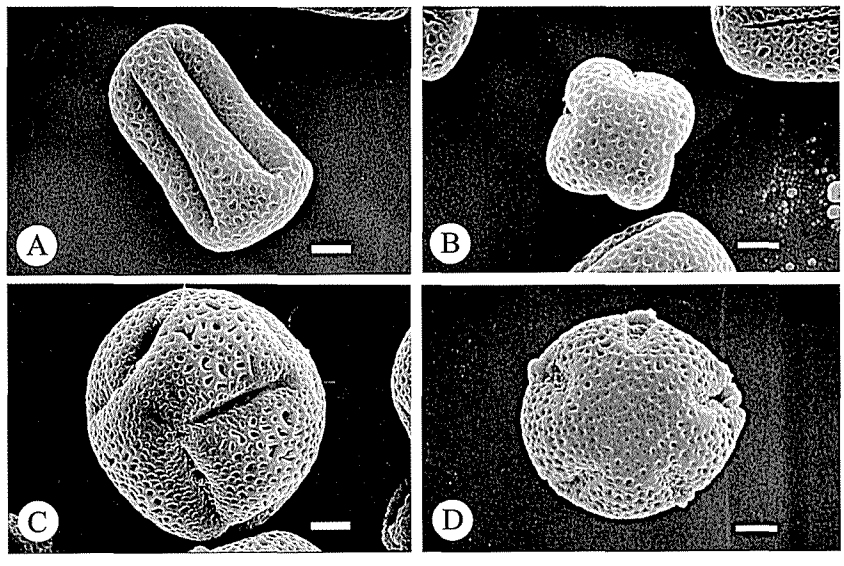

Fig. 1. Morphological characteristics of pollen grains in Yuzu (A and B) and somatic hybrid 'Citrus parental line No. 4' (C and D) (Bar = 5 μm).

that of the pollen grains of Yuzu. SEM observation revealed that most pollen grains of Yuzu had an elliptical shape (Fig. 1A), whereas those of 'Citrus parental line No.4' had a round shape (Fig. 1C). Furthermore, 'Citrus parental line No.4' had a significantly higher number of germ pores than Yuzu (Figs. 1B, 1D).

Previous studies confirmed that somatic hybrids of *Citrus* produced fertile pollen grains (Ohgawara *et al.*, 1991 ; Oiyama *et al.*, 1991 ; Kobayashi *et al.*, 1995). Ohgawara *et al.* (1991) reported that the pollen grains of the somatic hybrid between 'Bahia' navel orange and 'Troyer' citrange (*C. sinensis* × *Poncirus*) had high stainability and sufficient germinability. Kobayashi *et al.* (1995) also reported that four somatic hybrid cultivars including 'Citrus parental line No.4', which were produced by using a male sterile navel orange as one of the parents, produced fertile pollen grains. In the present study, we reconfirmed that 'Citrus parental line No.4' had high pollen fertility.

To evaluate the reproductive potential of the male gamete of 'Citrus parental line No.4', crossing with 'Banpeiyu' pummelo was carried out (Table 2). The frequency of developed seeds in 'Banpeiyu' pummelo was 40.8 % when crossed with pollen from 'Citrus parental line No.4', whereas that in open-pollinated fruit was 94.9 %. Compared with open pollination, a lot of undeveloped seeds were obtained when pollinated with 'Citrus parental line No.4'. However, the developed seeds obtained in the cross with pollen of 'Citrus parental line No.4' were slightly larger than those obtained by open-pollination, i. e., ca. 0.5 g in the former and ca. 0.4 g in the latter (Fig. 2).

In a 2X × 4X cross of *Citrus*, it was reported that the percentage of undeveloped seeds was high, and that the average weight of developed seeds was small compared with that of 2X × 2X (Tachikawa *et al.*, 1961 ; Esen & Soost, 1972 ; Yamashita, 1976 ; Oiyama *et al.*, 1991 ; Kobayashi *et al.*, 1995 ; Yahata *et al.*, 2003). Yahata *et al.* (2003) reported that when crosses between 'Banpeiyu' pummelo and 4X Yuzu or Natsudaidai (*C. natsudaidai* Hayata) were carried out, many undeveloped seeds were obtained, and the developed seeds were small compared with those of open-pollinated 'Banpeiyu' pummelo. In particular, Yamashita (1976) suggested the use of 4X *Citrus* as a pollinizer for producing seedless fruits in Hyuganatsu

Table 2. Fruits set and development of seeds in the cross between 'Banpeiyu' pummelo and somatic hybrid 'Citrus parental line No. 4'.

Cross combination		No. of flowers pollinated	No. of fruits set	% of fruits set	Av. fruit wt. (g)	No. of seeds		No. of developed seeds per fruit	Average seed weight (g)		% of developed seeds[y]
Seed	Pollen					Developed	Undeveloped		Total[z]	Developed	
'Banpeiyu'	Open-pollinated	—	3	—	1484.0	279	15	93	0.37	0.39	94.9
	'Citrus parental line No. 4'	6	4	66.7	1991.0	154	223	38.5	0.35	0.47	40.8
t-test[x]					**				NS	*	

[z] Developed seeds + Undeveloped seeds.
[y] (Developed seeds/Total seeds) × 100.
[x] NS, * and ** indicate non-significant, significant at 5% and 1% levels, respectively.

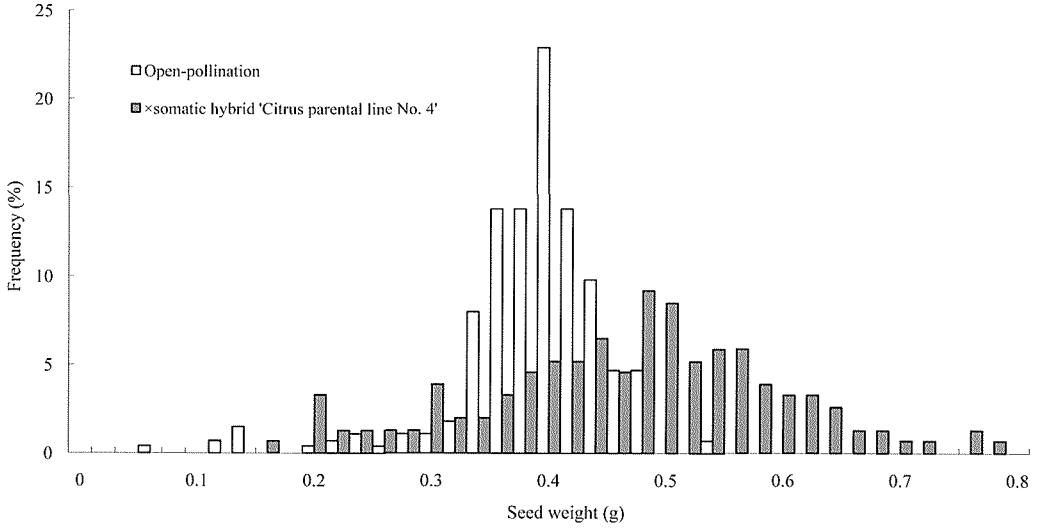

Fig. 2. Comparision of distribution frequency of seed weight obtained from open-pollination and crossing with somatic hybrid 'Citrus parental line No. 4' in 'Banpeiyu' pummelo.

(*C. tamurana* hort. ex Tanaka) because undeveloped seeds were mostly obtained when tetraploids were used as the pollen parent.

One hundred twenty-six seedlings were obtained from developed seeds in the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4'. The ploidy level of these seedlings was confirmed by flow cytometry analysis and chromosome observation (Fig. 3). One hundred five triploids (2n=3X=27), 20 tetraploids (2n=4X=36), and 1 hexaploid (2n=6X=54) were determined (Table 3). Oiyama *et al.* (1991) reported that when clementin mandarin (*C. clementina* hort. ex Tanaka) was pollinated with pollen from the tetraploid somatic hybrid between 'Trovita' orange (*C. sinensis* (L.) Osbeck) and trifoliate orange (*Poncirus trifoliata* (L.) Raf.), all of the seedlings obtained from developed seeds were tetraploid. Kobayashi *et al.* (1995) also reported that the developed seeds obtained from the crosses between 'Southen Red' mandarin and three somatic hybrids, 'Citrus parental line No.1', 'Citrus parental line No.2' and 'Citrus parental line No.3', were all tetraploids. In the present study, however, it was possible to obtain triploid seedlings from developed seeds at a high frequency of 83.3 % in the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4'. In a 2X × 4X cross of *Citrus*, therefore, the ploidy level of the progeny might be influenced by the genotype of the male parent used or by the combination of both parents.

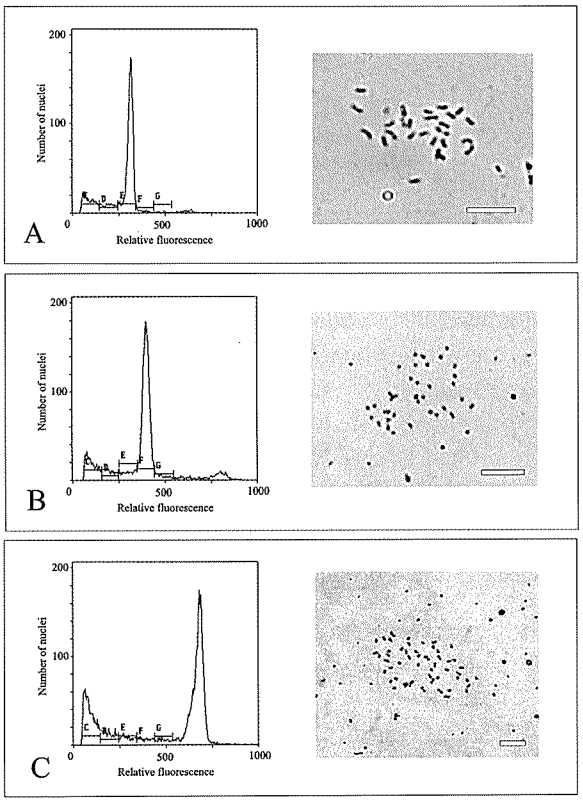

Fig. 3. Flow cytometric analysis and chromosome observation of the seedlings obtained from the cross between 'Banpeiyu' pummelo and somatic hybrid 'Citrus parental line No.4' (Bar = 10 μm). (A) Triploid (2n = 3X = 27). (B) Tetraploid (2n = 4X = 36). (C) Hexaploid (2n = 6X = 54).

Furthermore, one hexaploid was obtained from the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4'. The appearance of a hexaploid in a 2X × 4X cross was reported by Tachikawa *et al.* (1961), Esen & Soost (1972), Oiyama *et al.* (1982), and Yahata *et al.* (2003). Esen & Soost (1972) presumed that the hexaploid originated from the fertilization between the tetraploid female gamete produced by chromosome doubling of the unreduced gamete and the diploid reduced male gamete. However, Yang *et al.* (2002) investigated the chromosome composition of triploids and tetraploids obtained from the cross between 2 diploid cultivars, 'Tosa-Buntan' and 'Suisyo-Buntan' pummelo, by chromomycin A_3 (CMA) staining, and revealed that these polyploid were originated from the fertilization with not only unreduced female gamete but also unreduced male gamete. Therefore, it is also possible that one hexaploid obtained in the present study was produced by the fertilization between unreduced diploid female gamete and unreduced tetraploid male gamete or between tetraploid female gamete produced by chromosome doubling of unreduced gamete and diploid reduced male gamete. Further study is needed to reveal the

Table 3. Ploidy level of the seedlings obtained from the cross between 'Banpeiyu' pummelo and somatic hybrid 'Citrus parental line No. 4' and comparision of the seed weight of their seedlings.

Cross combination		Ploidy level	No. of seedlings obtained	Frequency (%)[z]	Average developed seed weight (g)	Developed seed weight (g)	
Seed	Pollen					Maximum	Minimum
'Banpeiyu'	'Citrus parental line No. 4'	3X	105	83.3	0.46	0.78	0.16
		4X	20	15.9	0.51	0.6	0.42
		6X	1	0.8	0.2	0.2	—

[z] (No. of seedlings / No. of total seedlings) × 100.

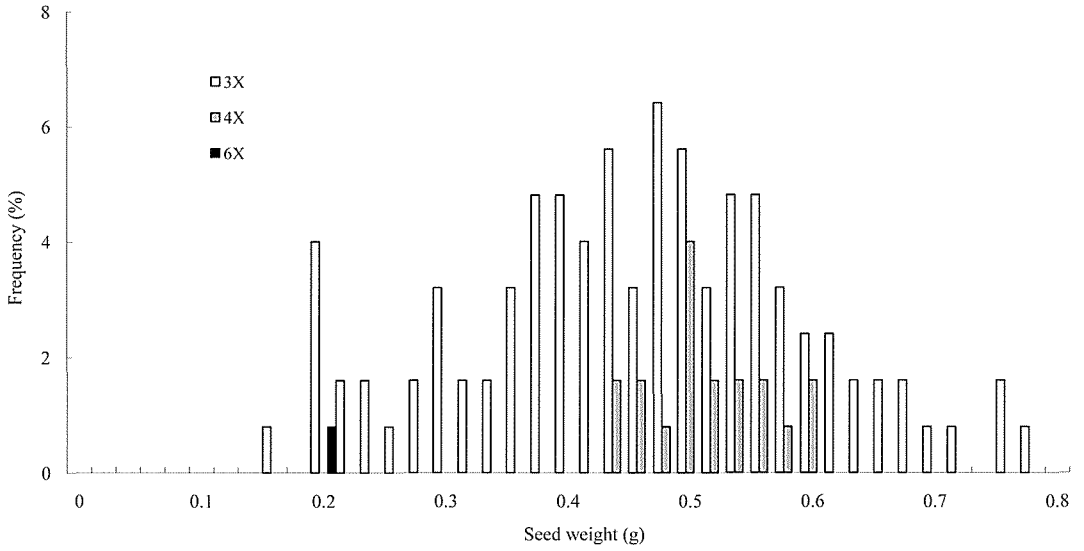

Fig. 4. Relationship between weight of seeds and ploidy level of seedlings obtained from the cross between 'Banpeiyu' pummelo and somatic hybrid 'Citrus parental line No. 4'.

origin of the hexaploid using cytogenetic analysis.

The relationship between seed weight and the ploidy level of the seedlings obtained from the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4' was investigated (Fig. 4). Differences in the distributions of the seed weight were recognized between triploids and tetraploids. Triploids were obtained from seeds of the varying weights, ranging from 0.2-0.8 g, while tetraploids were obtained from 0.4-0.6 g seeds. Moreover, one hexaploid was obtained from a small seed of 0.2 g.

Yahata *et al.* (2003) previously reported that the average weight of triploid seeds obtained from the crosses between 'Banpeiyu' pummelo and 4X Yuzu and/or 4X natsudaidai was significantly lower than that of tetraploid seeds. In particular, a clear difference was observed in the distribution of seed weight for triploids and tetraploids in the cross between 'Banpeiyu' pummelo and 4X natsudaidai, i. e., ca. 0.25 g in triploid seeds and ca. 0.5 g in tetraploid seeds, respectively. Therefore, the distribution of the seed weight of triploids and tetraploids in 2X×4X crosses is considered to be greatly influenced by the cross combinations.

When all undeveloped seeds obtained from the cross between 'Banpeiyu' pummelo and 'Citrus parental line No.4' were cultured, all of the 29 seedlings obtained were determined to be triploid. It has also been reported that undeveloped seeds obtained from crosses between diploids and autotetraploids and/or somatic hybrids (allotetraploid) produced almost triploid progenies (Starrantino & Recupero, 1981 ; Oiyama *et al.*, 1991 ; Kobayashi *et al.*, 1995 ; Yahata *et al.*, 2003). These results show that the undeveloped seeds might be useful for obtaining triploids, certainly even in the case where triploids are difficult to obtain from developed seeds.

In the present study, 'Citrus parental line No.4' was confirmed to show high pollen fertility. When it was crossed with 'Banpeiyu' pummelo, many triploid seedlings were obtained from seeds of varying weight. Furthermore, all seedlings obtained from undeveloped seeds were triploids. These results clearly indicate that 'Citrus parental line No.4' could be used as the pollen parent in the triploid breeding of *Citrus*.

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'晩白柚'と体細胞雑種'カンキツ中間母本農4号'との交雑から得られた種子の重さと倍数性との関係

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要　約

ユズ（*Citrus junos* Siebold ex Tanaka）とネーブルオレンジ（*C. sinensis* (L.) Osbeck var. *brasiliensis* Tanaka）との体細胞雑種である'カンキツ中間母本農4号'を三倍体育種に利用するために，'晩白柚'と交雑を行い，得られた実生の倍数性を調査した．'カンキツ中間母本農4号'の花粉稔性は，片親のネーブルオレンジに花粉が全くないにも関わらず，染色稔性と発芽稔性がそれぞれ88.2と21.2%であり，高い稔性を示した．そこで，'カンキツ中間母本農4号'を'晩白柚'に交雑した結果，完全種子からは三倍体（105個体），四倍体（20個体）および六倍体（1個体）の実生が得られ，三倍体（83.3%）が多く出現した．種子の重さと倍数性との間に相関は認められなかったが，やや大きい種子（0.4～0.6 g）から四倍体が，小さい種子（0.2 g）から六倍体が出現していたのに対し，三倍体は種子の重さに関係なくいずれの重さの種子（0.2～0.8 g）からも発生していた．なお，不完全種子から得られた実生の倍数性は全て三倍体であった．本研究の結果より，'カンキツ中間母本農4号'は三倍体育種の親として利用できることが示唆された．

キーワード：フローサイトメトリー，非還元配偶子，'カンキツ中間母本農4号'，三倍体育種