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Production of a Doubled Haploid from a Haploid Pummelo Using Colchicine Treatment of Axillary Shoot Buds

Masaki Yahata
The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima-shi, Kagoshima 890-0065, Japan

Hisato Kunitake, Tsutomu Yabuya, and Kensuke Yamashita
Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadainishi, Miyazaki-shi, Miyazaki 889-2192, Japan

Yukiko Kashihara and Haruki Komatsu[1]
School of Agriculture, Kyushu Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869-1404, Japan

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Abstract. To produce the homozygous strain of a haploid plant derived from small seed-derived seedlings of 'Banpeiyu' pummelo (*Citrus grandis* Osbeck), we carried out colchicine treatment to axillary shoot buds of the haploid. Many shoots with cytochimeras (X+2X and 2X+4X) arose from the colchicine-treated axillary buds. When cytochimeric buds of 2X+4X were top-grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf.], a complete diploid shoot with 18 chromosomes was obtained from the cytochimera. This diploid strain showed vigorous growth compared with the original haploid. The leaf weight per unit area and the stomata size in this diploid were significantly larger than those of the original haploid plant, and were almost equal to those of 'Banpeiyu' pummelo. The diploid strain was confirmed to be a doubled haploid of a haploid from 'Banpeiyu' pummelo, based on random amplified polymorphic DNA (RAPD) analysis and chromosome composition analysis by chromomycin A_3 (CMA) staining.

The possibility of genetic fixation through doubling of the chromosome complement is an attractive feature of breeding programs using haploid plants (Lespinasse et al., 1999). In fruit crops, haploids have been obtained from some species such as *Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa*, *Mulus pumila* Mill., *Musa acuminata* Colla, *Prunus avium* L., *Prunus persica* (L.) Batsch., and *Pyrus pyrifolia* Nakai; and doubled haploids have also been induced by treating shoots and leaves with antimitotic agents such as colchicine and oryzalin (Assani et al., 2003; Bouvier et al., 2002; Chalak and Legave, 1996; Hesse, 1971; Horfer and Grafe, 2003; Ochatt and Zhang, 1996; Zhang and Lespinasse, 1991).

In *Citrus* L. species and related species, some haploid plants have been produced (Germana and Chiancone, 2001, 2003; Hidaka et al., 1979; Oiyama and Kobayashi, 1993). These haploids were very weak, and grew slower than original diploid plants did. Additionally, doubled haploid plants were seldom obtained (Germana and Chiancone, 2003). To date, in the haploid plants obtained from clementine (*C. clementina* Hort. ex Tanaka), doubled haploids have only been induced by Germana and Chiancone (2003) using anther culture.

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[1]Corresponding author; e-mail: hkomatsu@ktmail.ktokai-u.ac.jp

Toolapong et al. (1996) selected a haploid progeny among small seed-derived seedlings obtained from a cross between 'Banpeiyu' pummelo and 'Ruby Red' grapefruit (*C. paradisi* Macf.). When this haploid was grafted onto trifoliate orange, it showed vigorous growth and flowered for the first time 7 years after germination. Yahata et al. (2005a) reported that this haploid produced fertile pollen, and some diploid progenies were obtained following pollination of four diploid plants with pollen from this haploid (Yahata et al., 2005b). However, it was very difficult to use this haploid for genetic analysis and for planned breeding because it had no fertile female gamete (1.6% stainable and 0.4% viable) and slightly fertile pollen grains.

In the present study, we carried out colchicine treatment to the axillary shoot buds in order to produce the homozygous strain of a haploid plant derived from a seedling of 'Banpeiyu' pummelo.

Materials and Methods

Plant materials. A haploid plant obtained from the cross between 'Banpeiyu' pummelo and 'Ruby Red' grapefruit (Toolapong et al., 1996) was used in the present study. The haploid was grafted onto trifoliate orange and maintained for about 10 years in the greenhouse of the School of Agriculture, Kyushu Tokai University.

Colchicine treatment of axillary shoot buds. The axillary shoot buds of the haploid were treated with 0.05%, 0.1%, and 0.2% colchicine dissolved in 1% DMSO (dimethyl sulfoxide) for 10 d. Each axillary bud was bound with cotton saturated with colchicine solution, then covered with parafilm (Pechiney Plastic Packaging, Chicago) to prevent the cotton from drying out. Two months

after colchicine treatment, the ploidy level of shoots arisen from colchicine-treated axillary shoot buds was determined by flow cytometry and chromosome counting. Furthermore, cytochimeric shoots obtained by colchicine treatment were bud-grafted onto trifoliate orange. After 2 months, the ploidy level of the newly produced part of the cytochimeric shoot was also examined by flow cytometry and chromosome counting.

Confirmation of ploidy level. Flow cytometry. Young leaves of about 1-cm^2 segments were collected from the new shoots obtained from colchicine-treated axillary shoot buds of the haploid and chopped with a razor blade. These samples were treated for 5 min in 1 mL buffer solution containing 1.0% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan), 140 mM mercaptoethanol, 50 mM Na_2SO_3, and 50 mM Tris-HCl at pH 7.5, according to the preparation method of Yahata et al. (2005a). Crude samples were filtered through Miracloth (Merck KGaA, Darmstadt, Germany) and stained with 25 $\mu g \cdot L^{-1}$ propidium iodide (PI). The relative fluorescence of total DNA was measured for each nucleus with a flow cytometry system (EPICS XL; Beckman Coulter, Fullerton, Calif.) equipped with an argon laser (488 nm, 15 mW).

Chromosome observation. Young leaves (≈3–5 mm long) were excised from new shoots obtained from colchicine-treated axillary shoot buds of the haploid, immersed in 2 mM 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 the method of 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 Industry Co., Tokyo), 1.0% (w/v) Macerozyme R-200 (Yakult Pharmaceutical Industry Co.), 0.3% (w/v) Pectolyase (Kyowa Chemical Products Co., Osaka, Japan), and 200 mM EDTA at 37 °C for 40 min. After rinsing with distilled water, the macerated samples were treated with fixative solution. The mixtures were then transferred to a glass slide. After air drying of the slide, the chromosomes were stained with 2.0% Giemsa solution (Merck KGaA) in 1/30 phosphate buffer (pH 6.8) for 30 min, rinsed with distilled water, air dried, and observed under an optical microscope.

Morphological characteristics of leaves. Leaf weight per unit and the size of the stomata of fully expanded leaves in 'Banpeiyu' pummelo, the haploid, the doubled haploid, and cytochimeras (X+2X and 2X+4X) obtained from colchicine-treated current shoots of the haploid were measured using 10 samples.

Confirmation of homozygosity. RAPD analysis. Total DNA was extracted from young leaves of the original 'Banpeiyu' pummelo, the haploid, and the doubled haploid obtained from colchicine-treated axillary shoot buds of the haploid according to the method of Doyle and Doyle (1987). RAPD analysis was performed by the modified method of Williams et al. (1990). The reaction mixture (25 μL) contained 10 mM Tris-HCl, pH 8.9, 80 mM KCl, 1.5 mM $MgCl_2$, 100 μM each dNTP, 0.3 μM primer, 2.5 U Tth *Taq* DNA polymerase (TaKaRa Bio, Otsu, Japan), and 10 ng of genomic DNA. Reactions were carried out by repeating 45 cycles of the following thermal treatments: 94 °C for 30 s, 37 °C for 2 min, and 72 °C for 3 min in ASTEC Program Control System PC-700 (ASTEC Co., Fukuoka, Japan). The primers used were OPA11, OPA18, OPB5, OPB7, OPB11, and OPB15 in Operon random 10-mer primers (Operon Technologies, Huntsville, Ala.). Reaction products were electrophoresed on 1.5% agarose gels containing 0.5 $\mu g \cdot mL^{-1}$ 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.

Chromomycin A_3 (CMA) analysis. After the chromosomes were stained with Giemsa and each position was confirmed on the slide, the chromosomes were de-stained with 70% methanol. Chromosomes were re-stained with 0.1 $mg \cdot L^{-1}$ CMA (Sigma-Aldrich Co., Steinhein, Germany) according to Befu et al. (2000) with some modifications, and observed under a fluorescence microscope with a BV filter cassette. Chromosomes were classified into the following five types based on the number and position of CMA bands according to Befu et al. (2000) and Yamamoto and Tominaga (2003): A = two telomeric bands and one proximal band, B = one telomeric and one proximal band, C = two telomeric bands, D = one telomeric band, and E = no band.

Results and Discussion

Two months after colchicine treatment of axillary shoot buds of the haploid, sprouting was observed under all the respective treatment conditions. New shoots arising from colchicine-treated axillary shoot buds often showed abnormal morphology (i.e., short nodes and dark green round and/or thick leaves). Ploidy level analysis of these new shoots by flow cytometry and chromosome counting showed that haploid and cytochimeras of haploid and diploid (X+2X) and diploid and tetraploid (2X+4X) were all produced (Fig. 1). The cytochimera of X+2X appeared at a high frequency under all treatment conditions: 40.0% at 0.05%, 25.0% at 0.1%, and 37.5% at 0.2% colchicine treatment, respectively. The cytochimera of 2X+4X was obtained from 20.0% and 12.5% of new shoots arising from axillary shoot buds treated with colchicine at 0.05% and 0.1%, respectively. A complete diploid was not obtained under any treatment conditions.

For some of these cytochimeras, each bud of their shoots was topgrafted onto trifoliate orange. After 2 months, shoots arising from topgrafted buds were also examined to determine their ploidy levels by flow cytometry and chromosome observation. Although most cytochimeras maintained the original cytochimeric conditions, the reversion to complete haploid was observed in some cytochimeras of X+2X. In cytochimeras of 2X+4X, a similar phenomenon was observed. One of two new shoots (B-1-2) obtained from a cytochimera of 2X+4X (strain B-1) showed two peaks, corresponding to diploid and tetraploid, whereas another shoot (strain B-1-1) had only one peak, which corresponded to diploid (Fig. 2A). Furthermore, chromosome counting of young leaf cells revealed that the chromosome number of this shoot was 18 (Fig. 2B). This diploid showed vigorous growth compared with the haploid (Fig. 2C), and also produced thorns (Fig. 2D).

It has been reported that when axillary buds, seeds, and callus have been treated with antimitotic agents, including colchicine and oryzalin, many cytochimeras appeared in fruit crops such as *Actinidia deliciosa*, *Citrus* spp., *Fortunella crassifolia* Swingle, *Musa acuminata*, *Pyrus pyrifolia*, and *Vitis vinifera* L. (Chalak and Legave, 1996; Kadota and Niimi, 2002; Notsuka et al., 2000; Van Duren et al., 1996; Wu and Mooney, 2002; Yahata et al., 2004). Notsuka et al. (2000) reported that complete tetraploids were obtained from cytochimeras of 2X+4X by stem cutting in *Vitis vinifera*. In the present study, it was possible to obtain a complete diploid (strain B-1-1) from a cytochimera of 2X+4X by bud grafting onto trifoliate orange. Presumably, this cytochimera might be a sectorial chimera.

'Banpeiyu' pummelo, the haploid, B-1-1, A-1-1 (cytochimera of X+2X), and B-1-2 (cytochimera of 2X+4X) were investigated

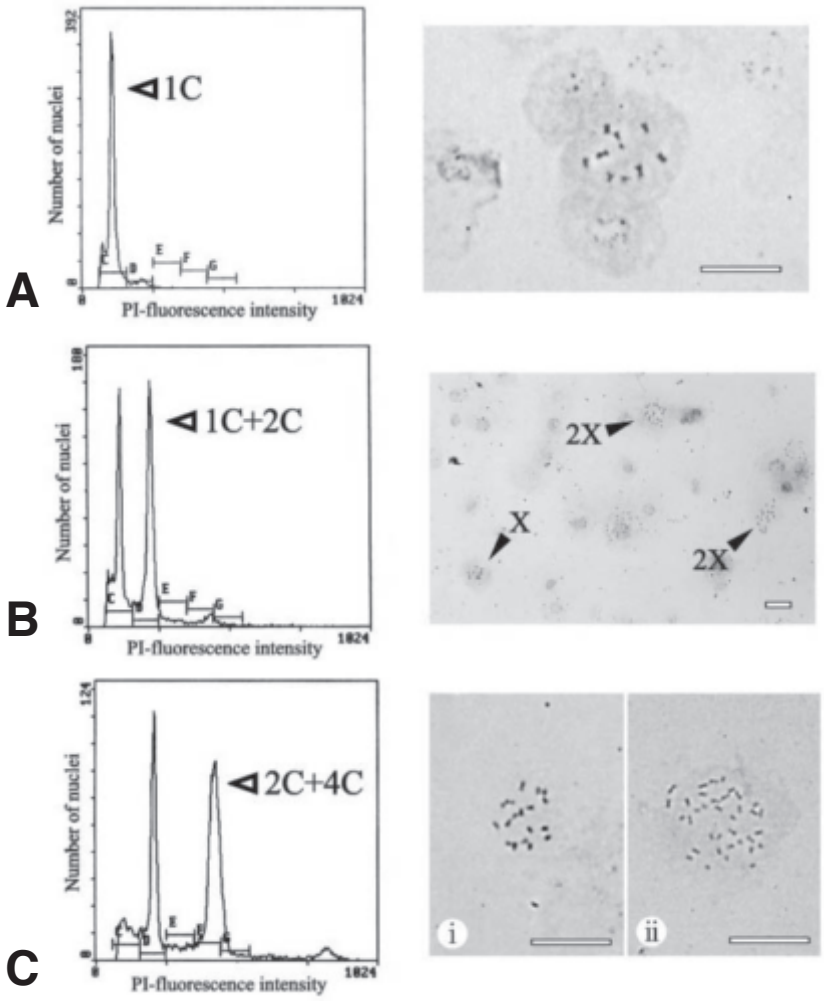

Fig. 1 (*above*). Flow cytometric analysis and chromosome number analysis (bar = 20 μm) of new sprouting shoots obtained from colchicine treatment to axillary shoot buds of the haploid; A = haploid (2n = X = 9), B = cytochimera [haploid (2n = X = 9) and diploid (2n = 2X = 18)], C = cytochimera [diploid (i = 2n = 2X = 18) and tetraploid (ii = 2n = 4X = 36)].

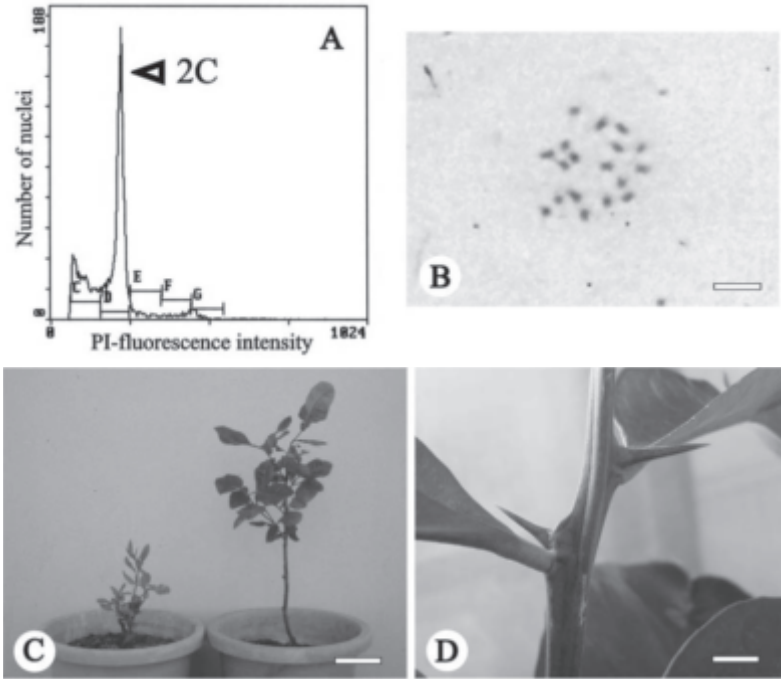

Fig. 2 (*left*). The doubled haploid obtained from bud grafting of 2X+4X cytochimera; A = flow cytometric analysis, B = chromosomes of young leaf cells (2n = 2X = 18, bar = 10 μm), C = comparison of initial growth between the haploid (left) and the doubled haploid (right), 1 year after grafting (bar = 10 cm), D = occurrence of thorns on the shoot of the doubled haploid (bar = 1 cm).

to determine the morphological characteristics of the leaf weight per unit area and the stomata size (Table 1). The leaf weights per unit area of 'Banpeiyu' pummelo, the haploid, B-1-1, A-1-1, and B-1-2 were 37.3, 21.4, 31.4, 36.9, and 51.6 mg·cm^{-2}, respectively. The leaf of B-1-1 was significantly thicker than that of the haploid, and was almost equal to that of 'Banpeiyu' pummelo. No difference in stomata size was observed among 'Banpeiyu' pummelo, B-1-1, A-1-1, and B-1-2. The stomata size of strain B-1-1 was significantly bigger than that of the haploid.

To confirm the homozygosity of B-1-1 obtained from the cytochimera (2X+4X), we employed RAPD analysis for 'Banpeiyu' pummelo, the haploid, and B-1-1. As shown in Fig. 3, the RAPD analysis revealed that B-1-1 had a portion of the specific bands obtained from 'Banpeiyu' pummelo, and the same banding patterns as the haploid. Furthermore, the haploid and B-1-1 were subjected to analysis for chromosome composition by CMA staining. The haploid was composed of 1A+1B+1C+2D+4E (Fig. 4A). On the other hand, B-1-1 had twice the number of chromosomes of the haploid and a chromosome composition of 2A+2B+2C+4D+8E (Fig. 4B). Thus, RAPD analysis and CMA staining confirmed that B-1-1 was a doubled haploid. To our knowledge, this is the first report on the production of a doubled haploid from a haploid plant in *Citrus*.

In previous studies (Germana and Chiancone, 2003; Germana et al., 1994, 2000), isozyme, RAPD, and microsatellite analysis were used for the identification of doubled haploids in *Citrus*. Recently, a combination of 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 has been applied for biotechnological studies such as genome analysis, somatic hybridization, and ploidy manipulation (Befu et al., 2000; Guerra, 1993; Miranda et al., 1997a, 1997b; Yahata et al., 2005b; Yamamoto and Tominaga, 2004; Yang et al., 2002). By CMA staining of the chromosomes, Yamamoto and Tominaga (2004) demonstrated that diploid cells in a haploid–diploid periclinal chimera were doubled haploid cells arisen from a haploid plant obtained from clementine. In the present study, we could also easily identify that the diploid strain was the complete doubled haploid of a haploid from 'Banpeiyu' pummelo by using CMA staining.

The shoot apical meristem of angiosperms consists of a stratified structure with three histogenic layers, which, from outside to inside, are

Table 1. Comparison of leaf weight and stomata size in 'Banpeiyu' pummelo, the haploid, doubled haploid, and cytochimera (X+2X and 2X+4X).

Strains	Ploidy level	Leaf wt ($mg \cdot cm^{-2}$)	Stomata size (μm) Length	Width
'Banpeiyu' pummelo	2X	37.3 b[z]	22.1 a	20.3 a
Haploid	X	21.4 d	17.0 b	15.1 b
A-1-1	X+2X	31.4 c	22.6 a	20.2 a
B-1-1	2X	36.9 b	22.4 a	20.4 a
B-1-2	2X+4X	51.6 a	22.8 a	20.4 a

[z]Different letters represent significant differences in Tukey's multiple range test, 1% level.

designated L-1, L-2, and L-3. Each tissue of the plant develops from the specific cells of one or two of the histogenic layers. In the leaves, it is known that L-1 cells of an apical meristem give rise to the epidermis, and the cells of L-2 and L-3 give rise to the mesophyll (Frost and Krug, 1942; Sugawara et al., 2002). In the present study, B-1-2, which is the cytochimera of 2X+4X, had stomata size similar to and leaves thicker than those of 'Banpeiyu' pummelo. Consequently, it is probable that B-1-2 is a complete periclinal chimera, which consists of 2X L-1 cells and 4X or 2X+4X in the cells of L-2 and L-3. Frost and Krug (1942) reported that reproductive organs such as anthers and embryos originate from the cells of L-2. If the cells of L-2 of B-1-2 were tetraploid, it is possible that B-1-2 could be used as an important parent for triploid breeding. Further study is needed to reveal the ploidy level of apical histogenic layers in this cytochimera.

In conclusion, although a number of cytochimeras (X+2X and 2X+4X) were obtained from colchicine-treated axillary shoot buds of a haploid plant from 'Banpeiyu' pummelo, a complete diploid was induced from the cytochimera of 2X+4X only by bud grafting. This diploid had thorns and showed vigorous growth as compared with the haploid. Furthermore, this diploid was almost equal in stomata size and leaf thickness to 'Banpeiyu' pummelo. The homozygosity of this diploid was confirmed by RAPD analysis and chromosome composition by CMA staining. The doubled haploid obtained in the present study will be valuable for genetic analysis and possibly for planned breeding.

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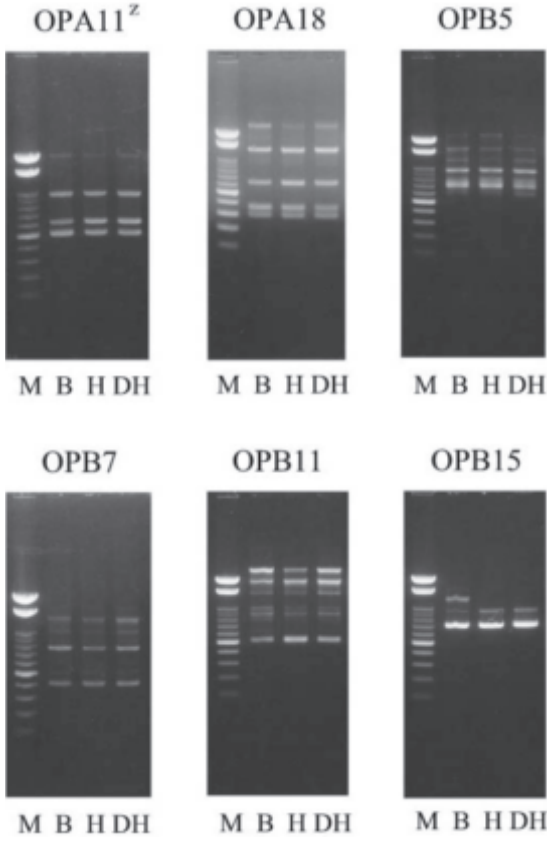

Fig. 3. RAPD analysis on 'Banpeiyu' pummelo, the haploid, and the doubled haploid; M = 100 bp ladder marker, B = 'Banpeiyu' pummelo, H = haploid, DH = doubled haploid. [z]Kit and number of Operon random 10-mer primer (Operon Technologies, Huntsville, Ala.).

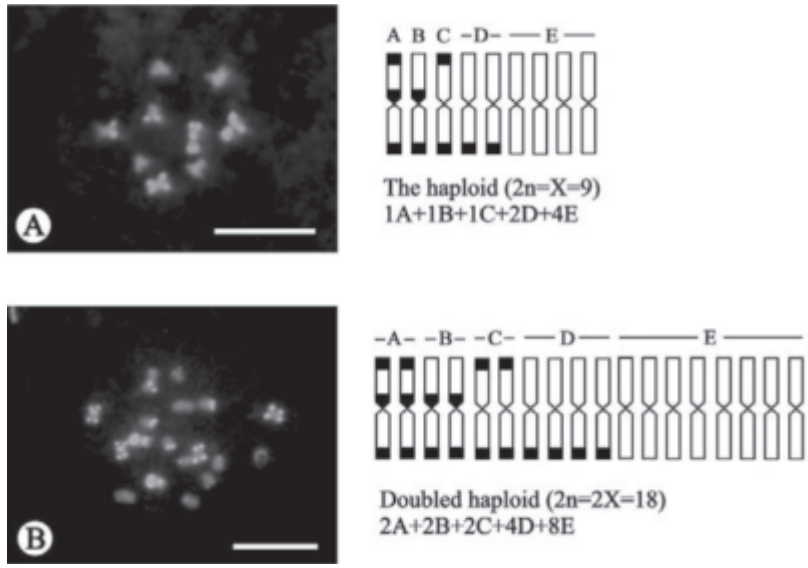

Fig. 4. Photographs and idiograms of the somatic chromosomes with chromomycin A_3 (CMA) banding patterns of the haploid and the doubled haploid (bar = 10 μm); A = haploid, B = doubled haploid. The black regions shown in the idiograms indicate CMA positive bands; type A = two telomeric bands and one proximal band, type B = one telomeric and one proximal band, type C = two telomeric bands, type D = one telomeric band, type E = no band.

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