Chapter 6

Flowering and Fruiting Haploid and Doubled Haploid Pummelos

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Additional information is available at the end of the chapter

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Abstract

Haploid and doubled haploid (DH) plants are of great value for genetic analyses and premeditated breeding. This is especially true for woody species, which are generally characterized by a long reproductive cycle, a high degree of heterozygosity, a large plant size, and self-incompatibility. In *Citrus* and related genera, some haploid and DH plants have been produced by techniques such as anther culture, interploid hybridization, and the pollination of irradiated pollen. However, there are few reports of the characteristics of haploid and DH plants' flowers, fruits, or reproductive potential. We selected a haploid progeny among small seed-derived seedlings obtained from 'Banpeiyu' pummelo [*C. maxima* (Burm.) Merr.], and we produced the DH plant of this haploid using colchicine-treated axillary shoot buds. Both this haploid pummelo and the DH pummelo showed normal growth and produced many flowers and fruit. In this chapter, we describe about the morphological characteristics and the reproductive potential of the haploid pummelo and the DH pummelo.

Keywords: first division restitution (FDR), homozygosity, reproductive function, unreduced gamete

1. Introduction

Haploid and doubled haploid (DH) plants are of great value for genetic analyses and developmental studies, as well as for premeditated plant breeding [1–5]. Technologies using DH plants also enhance the effectiveness of the selection of desired recombinants, especially when quantitative traits are evaluated [6]. This is the case for fruits, which are generally characterized by a long reproductive cycle, a high degree of heterozygosity, a large plant size, and self-incompatibility. In *Citrus* and related genera, triploid somatic hybrids can be obtained

through the fusion of haploid protoplasts [7, 8] although one of the method for producing seedless cultivars is the use of triploids [9–12].

Several haploid induction methods such as *in vitro* androgenesis induced by anther culture, *in vitro* and *in situ* gynogenesis induced by pollination with irradiated pollen, and followed by the application of new anti-microtubule herbicides for chromosome doubling, have been described in the literature [1, 13, 14].

In *Citrus* and related genera, haploid seedlings were first obtained by the application of γ-rays in natsudaidai (*C. natsudaidai* Hayata) [15]. Esen and Soost [16] described a haploid embryo obtained from an immature seed of clementine mandarin (*C. clementina* hort. ex Tanaka). Since then, haploid plants have been produced by anther culture [17–20], interploid hybridization [21–23] and the pollination of irradiated pollen [24–28]. However, these haploids were very weak and grew more slowly than the original diploid plants. To date, the flowering haploids are only a haploid of clementine mandarin by gynogenesis *in situ*, induced by irradiated pollen [26], and the flowering and fruiting of haploids have rarely been reported. The available information on the reproduction of haploids is also quite limited.

Reports regarding DH plants' production are very few in number. DH plants have been induced only by anther culture in clementine mandarin [19] and sweet orange [*C. sinensis* (L.) Osbeck] [20, 29], and by the pollination of irradiated pollen in Clementine mandarin [26]. Detailed information regarding the morphological characteristics and the reproductive potential of the DH plants in *Citrus* and related genera have not yet been reported.

Our research group selected a haploid progeny among small seed-derived seedlings obtained from the 'Banpeiyu' pummelo, and we produced the DH plant by using colchicine-treated axillary shoot buds of the haploid pummelo. Both the haploid and DH plants continue to grow normally, and they flowered and fruited. In this chapter, we present the morphological characteristics and the reproductive potential in the haploid pummelo [30–33] and the DH pummelo [34, 35].

2. Production of the haploid pummelo and DH pummelos

We selected a haploid (2n = x = 9) from among small seed-derived seedlings obtained from the cross between 'Banpeiyu' pummelo and 'Ruby Red' grapefruit (*C. paradisi* Macfad.) (**Figure 1A**–**C**) [22]. The haploid was confirmed to be derived from female gamete of 'Banpeiyu' pummelo by molecular biological techniques: isozyme (**Figure 1D**), random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) analyses. This haploid pummelo also showed dwarf growth behavior and rosette morphology, similar to that of the haploids obtained from other methods [17, 21, 25]. However, it grew very well while maintaining the haploidy when it was grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf.]. The tree growth habit of the haploid pummelo showed intermediate between upright and spreading (**Figure 2A**). Seven years after germination, the haploid pummelo had many flowers for the first time (**Figure 2B**) [30]. Three years after achieving reproductive growth, the haploid pummelo bore fruits for the first time (**Figure 2C**) [33].

Chromosome doubling of the haploid pummelo was achieved with colchicine treatment of axillary shoot buds of the haploid [34]. Many shoots with cytochimeras (X + 2X and 2X + 4X)

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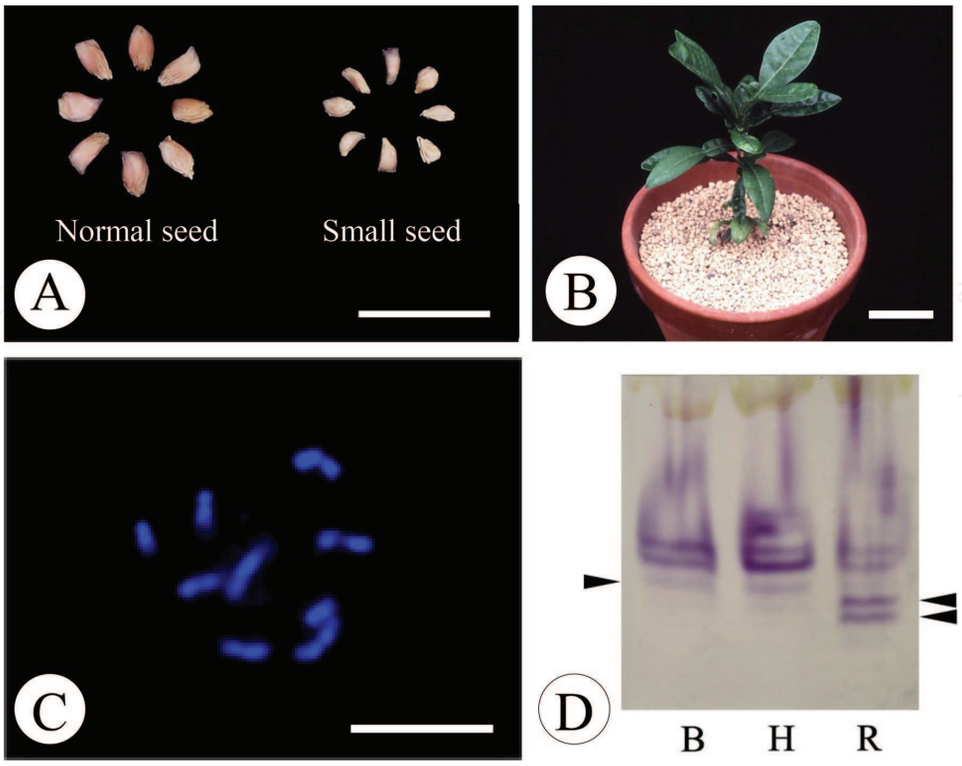

Figure 1. Production of the haploid pummelo [22, 30]. A: Normal (left) and small (right) seeds obtained from the cross between 'Banpeiyu' pummelo and 'Ruby Red' grapefruit. Bar = 5 cm. B: Initial growth of haploid plant, one year after grafting. Bar = 3 cm. C: The chromosomes of young leaf cells (2n = x = 9) Bar = 10 μm. D: Zymogram patterns of shikimate dehydrogenase (SADH) in 'Banpeiyu' pummelo (B), the haploid (H), and 'Ruby Red' grapefruit (R).

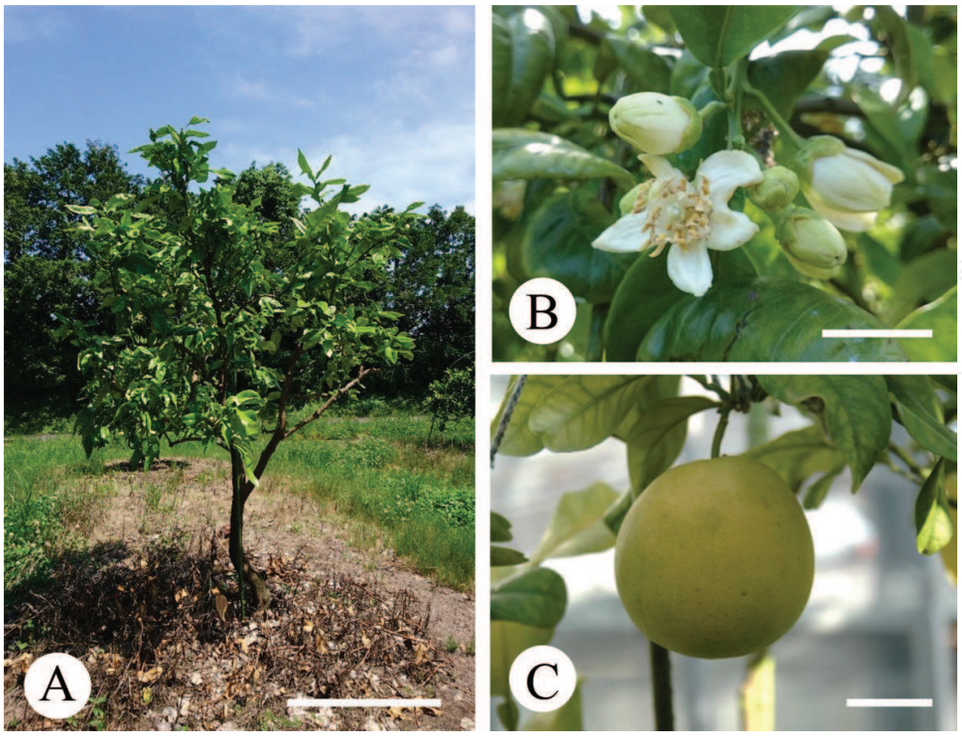

Figure 2. The haploid from among small seed-derived seedlings obtained from the cross between 'Banpeiyu' pummelo and 'ruby red' grapefruit [22, 33]. A: 10-year-old haploid tree. Bar = 30 cm. B: Flowers. Bar = 3 cm. C: Fruit. Bar = 5 cm.

arose from the colchicine-treated axillary buds. When cytochimeric buds of 2X + 4X were top-grafted onto trifoliate orange, a complete diploid shoot with 18 chromosomes was obtained from the cytochimera (**Figure 3A**, **B**). This DH pummelo produced thorns, and it showed vigorous growth compared to the original haploid pummelo. The tree growth habit of the DH pummelo showed spreading similar to that of 'Banpeiyu' pummelo (**Figure 4A**). The DH pummelo also produced many flowers and fruit for the first time at 5 years after the top-grafting onto trifoliate orange (**Figure 4B**, **C**) [35]. Moreover, thorns of the DH pummelo disappeared with advancing age.

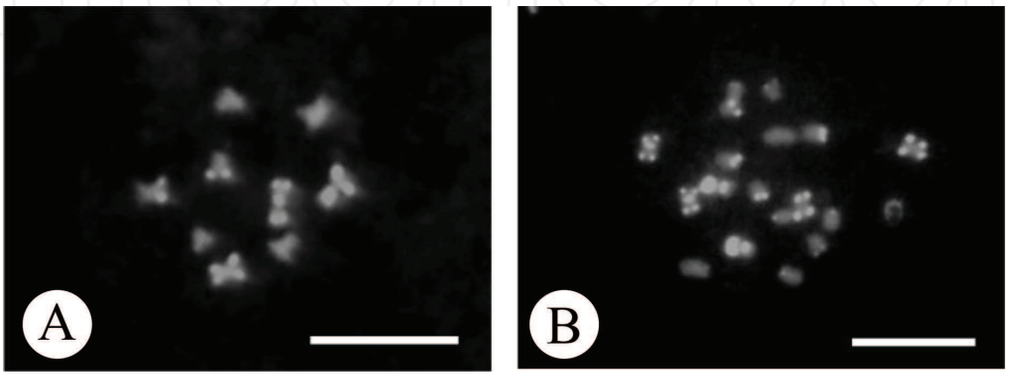

Figure 3. Photographs of the chromosomes with chromomycin A_3 banding patterns of the haploid (1A + 1B + 1C + 2D + 4E) and the DH pummelo (2A + 2B + 2C + 4D + 8E) [34, 52]. A = two telomeric bands and one proximal band, B = one telomeric and one proximal band, C = two telomeric bands, D = one telomeric band, E = no band. Bars = 10 μm.

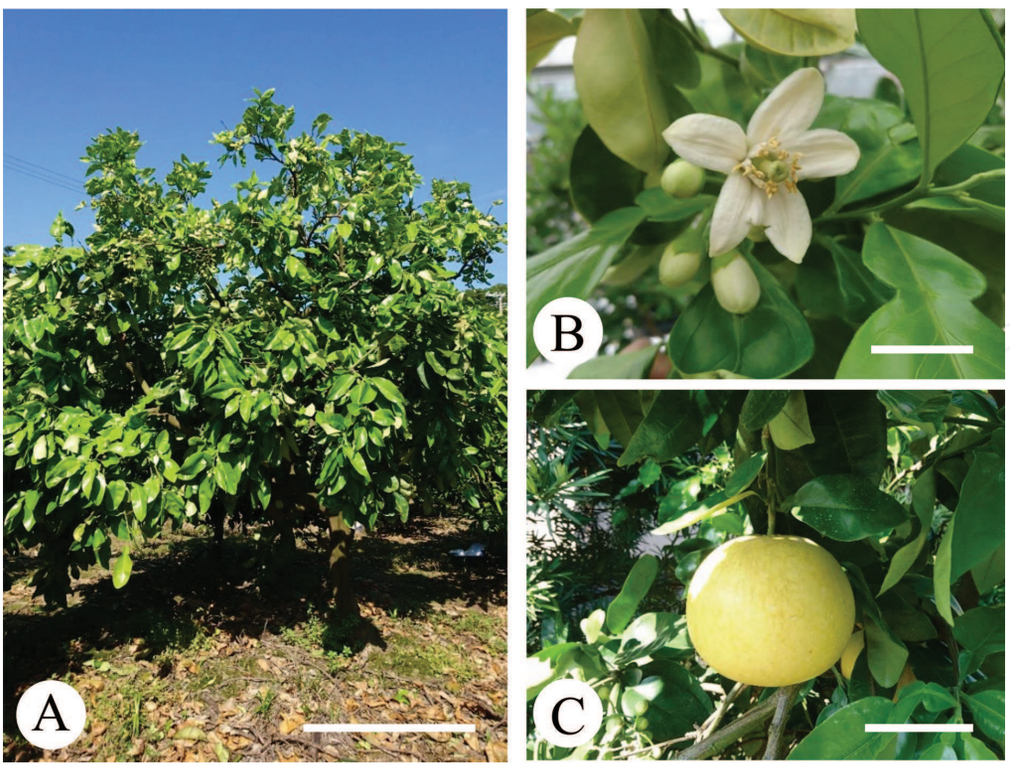

Figure 4. The DH induced by colchicine-treated axillary shoot buds of a haploid plant from 'Banpeiyu' pummelo [35]. A: 15-year-old DH tree. Bar = 100 cm. B: Flowers. Bar = 3 cm. C: Fruit. Bar = 10 cm.

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3. Morphological characterization of the haploid and DH pummelos

The leaves of haploids of fruit crops tend to be smaller than those of diploid plants [2, 36, 37]. Haploids of trifoliate orange, mandarin, tangor and tangelo also show rosette morphology with small leaves in *Citrus* and related genera [17, 21, 25]. Although the flowering of haploids has rarely been reported for fruit crops, the morphology of haploid flowers has been reported in peach (*Prunus persica* Batsch) and clementine mandarin. These haploids had smaller flowers than the original diploids, and they shed very few pollen grains [26, 36–38]. In peach haploids, fertile pollen grains were observed [37, 38]. Among fruit crops, the fruiting of haploids have been observed only in peaches [37, 38]. Hesse [38] reported that two genotypes of haploid peaches showed very small fruit compared to the original diploid plants. Pooler and Scorza [37] found that five out of seven genotypes of haploid peach had fruits that were smaller than those of the original diploid cultivar, whereas the other two genotypes produced large fruits with fertile seeds.

Our group [30, 32, 33, 35] observed that the haploid pummelo had small, narrow, and lightness leaves compared to those of 'Banpeiyu' pummelo (**Figure 5A**). The guard cell size of the haploid was also significantly smaller than that of 'Banpeiyu' pummelo. The haploid formed raceme inflorescence (**Figure 2B**). The flowers of the haploid were approximately half the size of those of 'Banpeiyu' pummelo (**Figure 5B**). In addition, the haploid had a significantly reduced number of stamens and ovules compared to those of 'Banpeiyu' pummelo. In the flowers of the haploid, moreover, abnormalities such as the adhesion of pistils and stamens were rarely observed. Regarding the morphology of the pollen grains, most of pollen grains of 'Banpeiyu' pummelo were elliptical in shape (**Figure 6A**), whereas the shape of the pollen grains of the haploid showed severely depressed morphology; these pollen grains were thus presumed to be sterile, although a few normally shaped pollen grains from the haploid were also observed (**Figure 6B**). The average size of the pollen grains from the haploid was smaller than that of the grains from 'Banpeiyu' pummelo. While the 'Banpeiyu' pummelo showed a 97.5% acetocarmine-stainability rate, the haploid rate was only 14.1% (**Figure 6D**, **E**), and the haploid had slightly fertile pollen grains. The fruit weight of the 'Banpeiyu' pummelo was approx. 1800 g, whereas that of the haploid pummelo was only approx. 200 g, or about 11% that of 'Banpeiyu' pummelo (**Figure 5C**). The number of seeds per fruit obtained from 'Banpeiyu' pummelo was approx. 100, whereas the haploid had no seeds. Whereas the 'Banpeiyu' pummelo showed low parthenocarpy and rarely produced seedless fruits, the development of the haploid's fruit might be caused by parthenocarpy. We are planning detailed studies of the expression of parthenocarpy in the haploid pummelo.

Details of the morphology of DH plants have rarely been reported for fruit crops, although DH plants of several species have been produced, e.g., kiwifruit, apple, banana, sweet cherry, peach, and Japanese pear [1, 2]. Several DH plants of apple were produced by *in vitro* androgenesis and *in situ* parthenogenesis, and their morphology and reproductive potential have been reported [39–41]. Those studies showed that most of the DH apple lines had smaller leaves, flowers and fruit than the original diploid cultivars, and some of these DH lines also showed aberrant morphology of flowers.

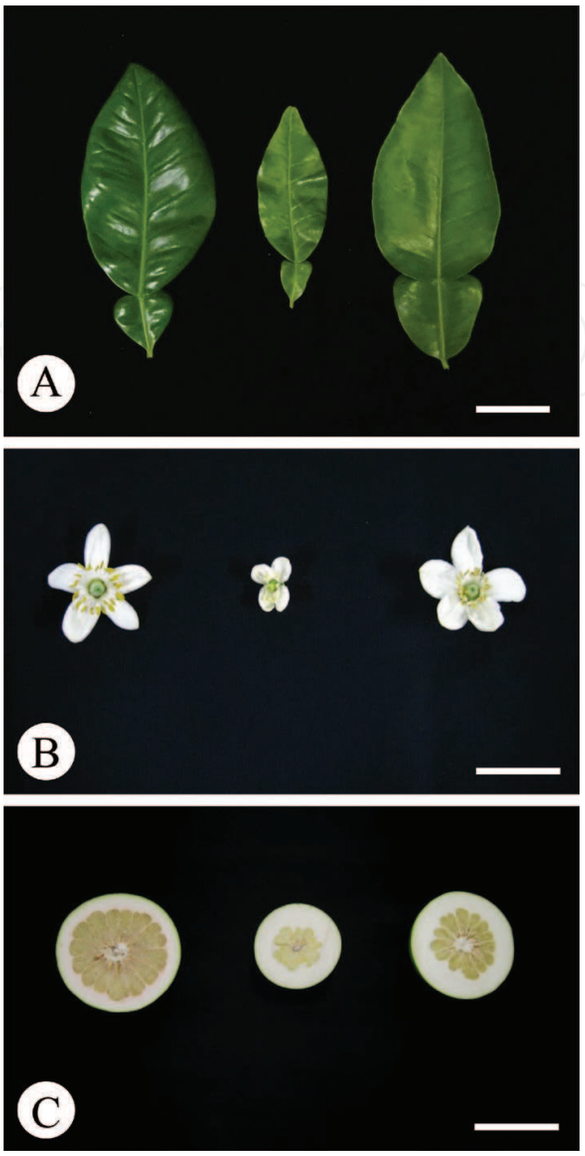

Figure 5. The morphological characteristics of leaves (A, Bar = 10 cm), flowers (B, Bar = 3 cm) and fruit (C, Bar = 10 cm) in 'Banpeiyu' (left), the haploid (center) and the DH (right) pummelo [35].

The sizes of the leaves and guard cells of the DH pummelo were almost equal to those of the 'Banpeiyu' pummelo (**Figure 5A**). The inflorescence of the DH plant was also raceme (**Figure 4B**). The flower organs of the DH showed normal morphology. The DH plant' flowers were larger than those of the haploid, and no difference in flower size was observed compared to those of the 'Banpeiyu' pummelo (**Figure 5B**). However, the DH had a reduced number of locules and ovules per ovary (approx. half) compared to that of the 'Banpeiyu' pummelo. The pollen fertility of the DH (an acetocarmine-stainability rate of ca. 85.0%) was a bit lower than that of 'Banpeiyu' pummelo (**Figure 6C**, **F**). The fruit size of the DH was approx. 900 g, which was approx. Half that of 'Banpeiyu' pummelo (**Figure 5C**). The number of seeds per fruit obtained from the DH plant was significantly less than that of the 'Banpeiyu' pummelo at approx. 60. Moreover, there was no difference among the haploid, the DH and the 'Banpeiyu' pummelos in terms of Brix and the titratable acidity of the fruit juice [35].

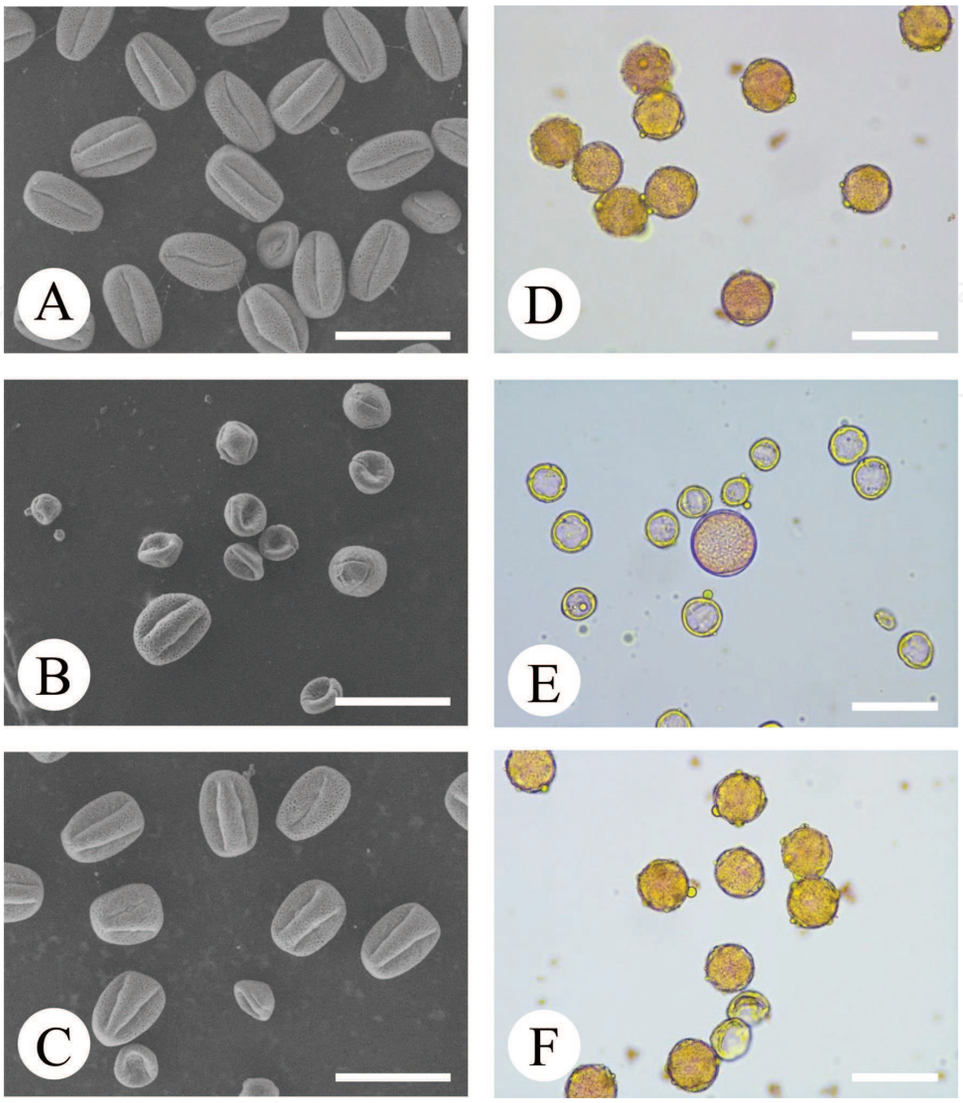

Figure 6. Micrographs of scanning electron (A-C) and stainability by 1% acetocarmine (D-F) in pollen grains of 'Banpeiyu' (A, D), the haploid (B, E) and the DH (C, F) pummelo. Bars = 30 μm.

4. Evaluation of the reproductive potential of male and female gametes in the haploid and DH pummelos by cross pollination

We carried out crosses with some diploid cultivars in order to evaluate the reproductive potential of the haploid and DH pummelos [31, 35]. When the haploid was the seed parent, no fruit set followed the pollination of the haploid with the pollen of diploid cultivars, because all flowers dropped within a month after pollination despite the crossing to the inflorescence with leaves. In the crosses with the haploid as pollen parents, conversely, fruits were set and some developed seeds were obtained. The developed seeds obtained from these crosses germinated almost normally, and their seedlings grew vigorously and developed large wing leaves, which is typical of the haploid (**Figure 7A**). The ploidy level of these seedlings was diploid with 18 chromosomes (**Figure 7B**). This result reveals that fertilization occurred between the normal eggs of diploid cultivars and pollen grains with nine chromosomes from the haploid.

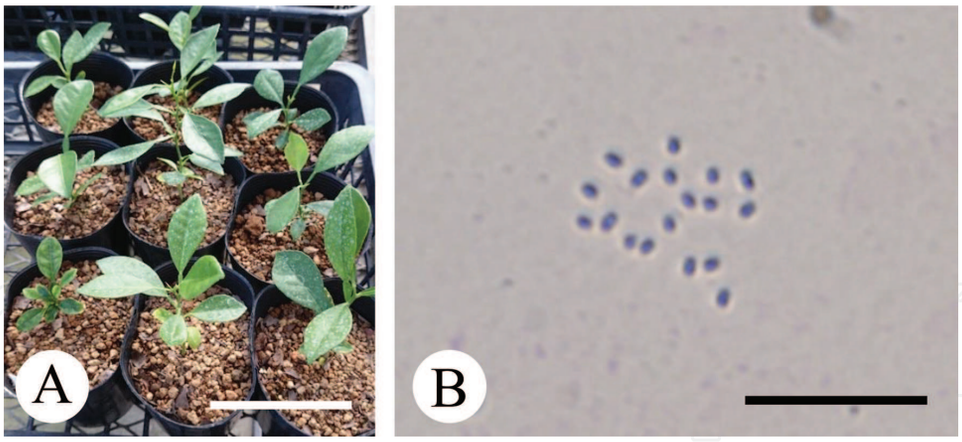

Figure 7. The seedlings obtained from the crosses between the diploid cultivars and the haploid (A, Bar = 10 cm) and the metaphase chromosomes in a root tip cell in one of the seedlings (B, 2n = 2x = 18, Bar = 20 μm).

In the reciprocal crosses between the DH and diploid cultivars, in contrast, when the DH was used as the seed and/or pollen parent, fruit and developed seeds were obtained compared to those of the haploid. Most of these developed seeds showed normal germination, and all of the seedlings examined were diploid [35]. In apple, it was difficult to use the DH lines as breeding materials because most of them had low and/or no reproductive potential, and no or only a few progeny were obtained in their cross combinations [39–41]. Our DH pummelo has no problem in term of the reproductive potential of female and male gametes.

4.1. Cause of the sterility of female gametes in the haploid pummelo

No fruit set followed the pollination of the haploid with the pollen of diploid cultivars in the reciprocal crosses between the haploid and some diploid cultivars. In *Citrus* species, the formation of embryo sacs is incomplete at the flowering stage, and the sacs remain at the two- or four-nucleate stage until the mature embryo sacs are formed at 3 or 4 days after flowering (DAF) [42]. We used the paraffin-sectioning method to observe the process of female gamete formation [32]. The formation of the embryo-sac mother cell (EMC) was detailed in the ovules at 1/4 of the size of flower buds (SOFB) of the 'Banpeiyu' pummelo (**Figure 8A**, **B**). Subsequently, the initiation of meiosis and tetrad formation were observed at 1/3 SOFB and 2/5 SOFB, respectively (**Figure 8A**, **B**). Approx. 20% of the ovules contained EMCs or further developed embryo sacs. The, embryo sacs then developed rapidly at the flowering stage (**Figure 8A**, **B**), and embryo sacs at the two-nucleate stage were observed at 3/4 SOFB. Eight-nucleate mature embryo sacs were formed in the flowers at 2 DAF (**Figure 8A**, **B**), at a frequency of approx. 25%.

In the haploid pummelo, in contrast, no EMCs were formed throughout flower bud development, and no embryo sac was formed in the flowers at 2 DAF (**Figure 9A**, **B**). We concluded that the lack of EMC formation was responsible for the complete sterility in the haploid pummelo. Regarding the morphology of the inner and outer integuments of the ovules, moreover, that of the haploid showed abnormalities such as detached growth of the integuments from the nucellar tissue, and the formation of a void between the inner and outer integuments (**Figure 10A**–**C**) [32]. These morphological abnormalities of the ovules has also been observed in the haploid plant of the clementine mandarin [26].

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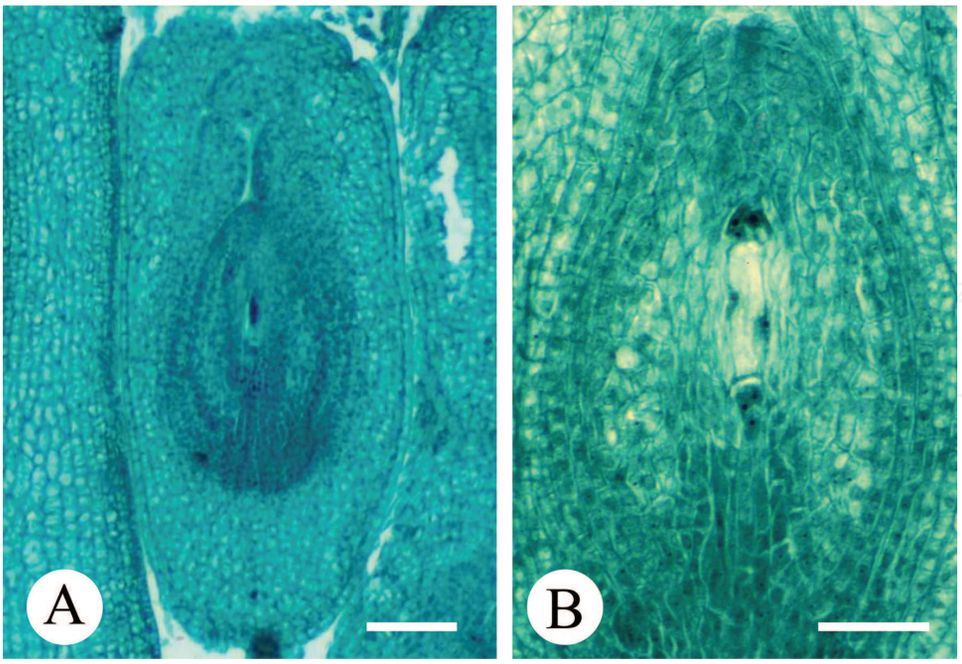

Figure 8. Ovule morphology and embryo sac development in 'Banpeiyu' pummelo [32]. A: Ovule morphology of 'Banpeiyu' pummelo at 2 days after flowering. Bar = 100 μm. B: Eight-nucleate embryo sac in at 2 days after flowering. Bar = 50 μm.

4.2. Formative mechanism of fertile pollen grains in the haploid pummelo

We observed the process of male gamete formation by the squash method [32]. The male meiosis of the 'Banpeiyu' pummelo occurred normally (**Figure 11**). In the first meiotic division at prophase I, duplicated chromatin condensed (**Figure 11A**), and condensed chromosomes were

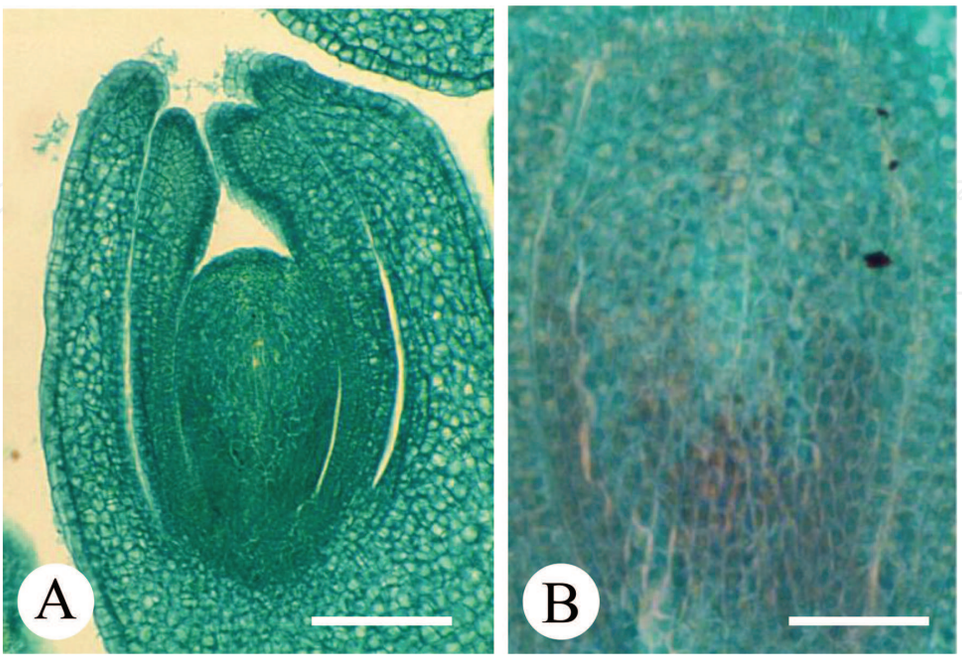

Figure 9. Ovule morphology and embryo sac development in the haploid pummelo [32]. A: Ovule morphology at 3/4 size of flower bud. Bar = 100 μm. B: No embryo sac at 2 days after flowering. Bar = 50 μm.

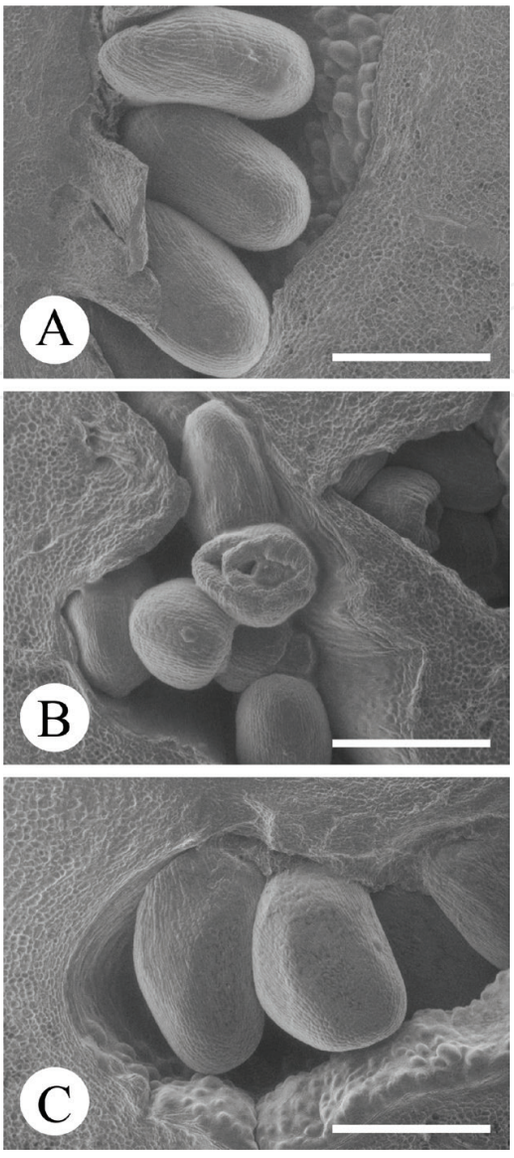

Figure 10. Scanning electron micrographs of ovule morphology in 'Banpeiyu' (A), the haploid (B) and the DH (C) pummelo. Bars = 500 μm.

visible. At metaphase I, homologous chromosomes aligned at the equatorial plate, and nine bivalents were observed (**Figure 11B**). The bivalents separated into univalents and migrated towards each pole at anaphase I (**Figure 11C**, **D**). In the second division, the chromosomes aligned at the equatorial plate at metaphase II (**Figure 11E**), and the chromatids migrated towards each pole separated at anaphase II (**Figure 11F**, **G**). Consequently, the 'Banpeiyu' pummelo predominantly produced normal tetrads (99.2%) with four microspores of equal size (**Figure 11H**).

In the haploid pummelo, meiotic division also occurred twice in the pollen mother cell (PMC), but abnormalities were observed in most dividing cells (**Figure 12**). Although nine univalents aligned on the equatorial plate at metaphase I (**Figure 12A**, **B**), they migrated unequally to each pole (**Figure 12C**, **D**). In the second division, their chromatids also migrated separately to each pole (**Figure 12E–G**). Another type of abnormal division was also observed in some meiocytes (**Figure 13**), in which all of the univalent chromosomes remained near the equatorial plate without distributing to either pole at anaphase I (**Figure 13A**, **B**). In addition, the nine

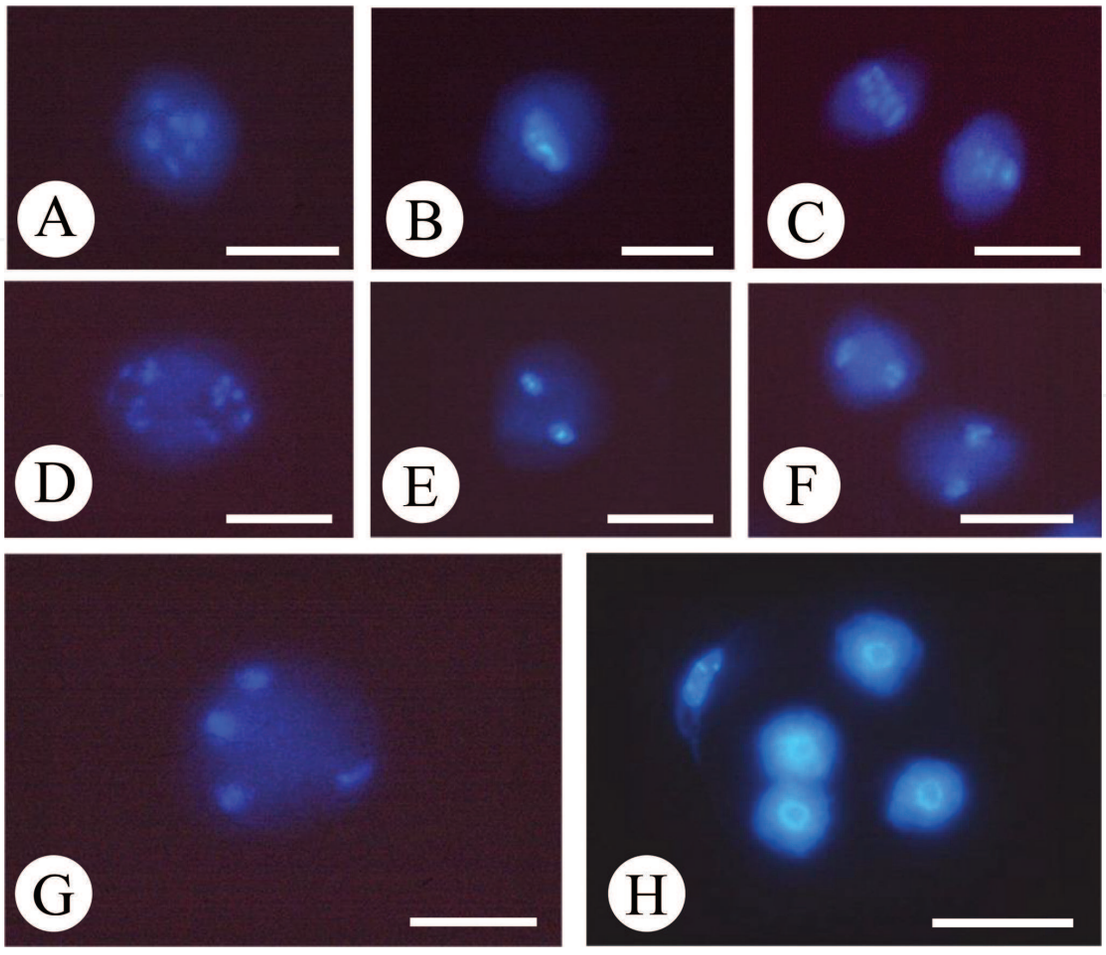

Figure 11. Meiotic stages in 'Banpeiyu' pummelo [32]. A: Prophase I, B: Metaphase I, C: Anaphase I, D: Telophase I and prophase II, E: Metaphase II, F: Anaphase II, G: Telophase II, H: Tetrad stage. Bars = 10 μm.

univalents that remained on the equatorial plate showed mitotic division to segregate each set of chromosomes in the directions of opposite poles during the second meiosis (**Figure 13C**). Consequently, microspore types from monads to hexads were observed in the tetrad stage of the haploid (**Figure 12H**, **I**). Notably, the dyads appeared at a high frequency (24.7%) and produced two microspores of equal size (**Figure 12I**).

Some species can form fertile gametes in haploid plants [36–38, 43, 44]. For fertile gamete formation to occur in a haploid plant, the complete set of the haploid genome (i.e., all chromosomes in the meiocyte) should migrate to the same pole during meiosis I. The probability of the occurrence of such an event in the pummelo haploid is theoretically $(1/2)^9 = 0.2\%$. However, the pollen fertility of the haploid was 14.1%, which was higher than the expected fertility rate. Meiotic nuclear restitution has been identified as a causal factor of this phenomenon [45].

In the haploid plant of *Capsicum annuum* L., Yan et al. [44] found laggards in many meiocytes of the first division at meiosis of the PMC, which resulted in first division restitution (FDR) at meiosis that led to the restitution of pollen fertility in the haploid. They also reported that the microspores formed by FDR were dyads. In the haploid pummelo, although two successive divisions occurred in the PMC (as occurs in normal meiosis), we observed the following abnormalities in some meiocytes: all of the univalent chromosomes remained

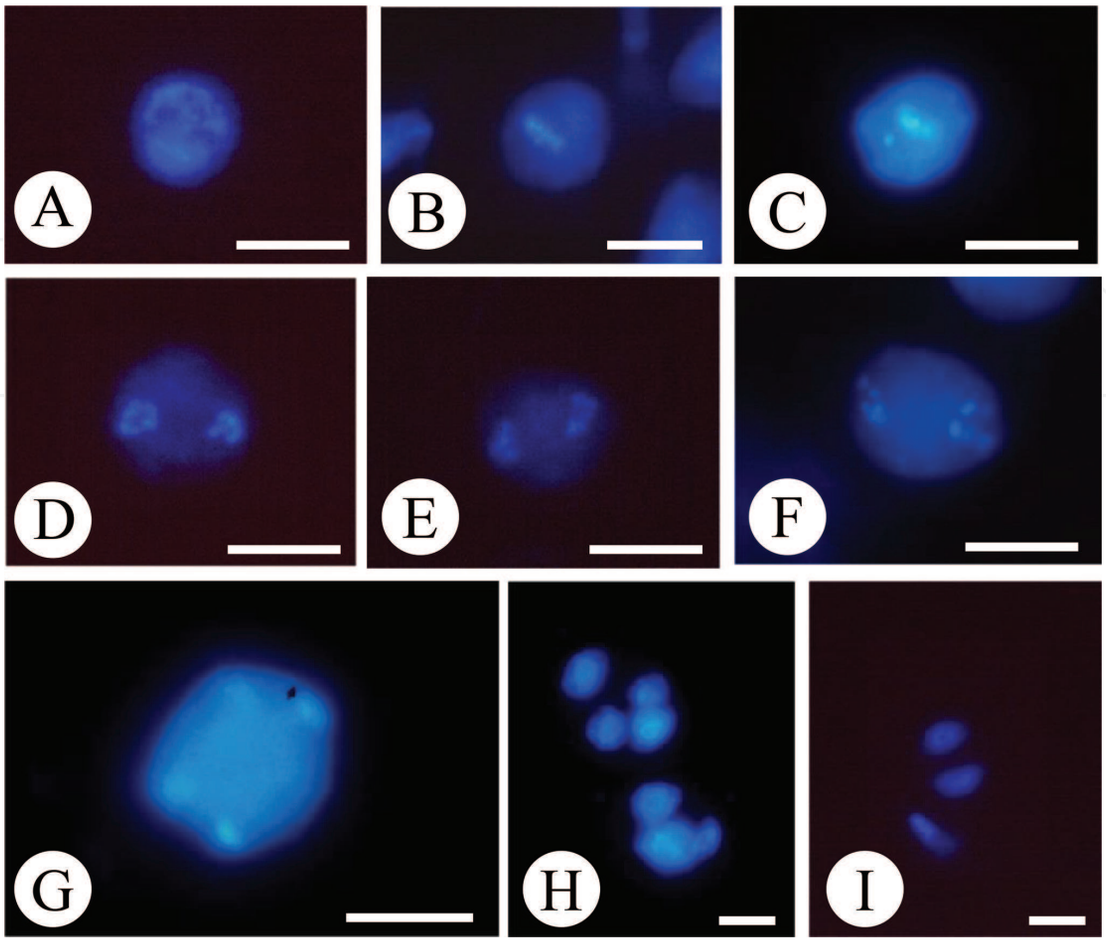

Figure 12. Meiotic stages in the haploid pummelo [32]. A: Prophase I, B: Metaphase I, C: Anaphase I, D: Telophase I and prophase II, E: Metaphase II, F: Anaphase II, G: Telophase II, H, I: Tetrad stage [H: Tetrad (upper) and triad (lower), I: Dyad]. Bars = 10 μm.

near the equatorial plate without distributing towards either pole at anaphase I, and nine univalents on the equatorial plate showed normal mitotic division to segregate each set of chromosomes in the direction of opposite poles during the second meiosis. Moreover, many dyads were formed at the tetrad stage. This observation indicates that the fertile pollen grains in the haploid pummelo were of dyad derivation, as was reported in the haploid plant of *C. annuum*.

Since the dyads were formed through the arrest of the first meiotic division, it can be speculated that meiotic nuclear restitution such as FDR took place in the haploid pummelo. By using single pollen genotyping, Honsho et al. [46] demonstrated that unreduced 2n pollen grains of 'Nishiuchi Konatsu' hyuganatsu (*Citrus tamurana* hort. ex Tanaka) had heterozygosity transmission exceeding 50% in all six alleles, and fitness tests indicated that the FDR map function better fitted the heterozygosity transmission observed rather than the second division restitution (SDR) function. We concluded that the formation of fertile pollen grains in the haploid pummelo was due to abnormalities in the first meiotic division such as FDR.

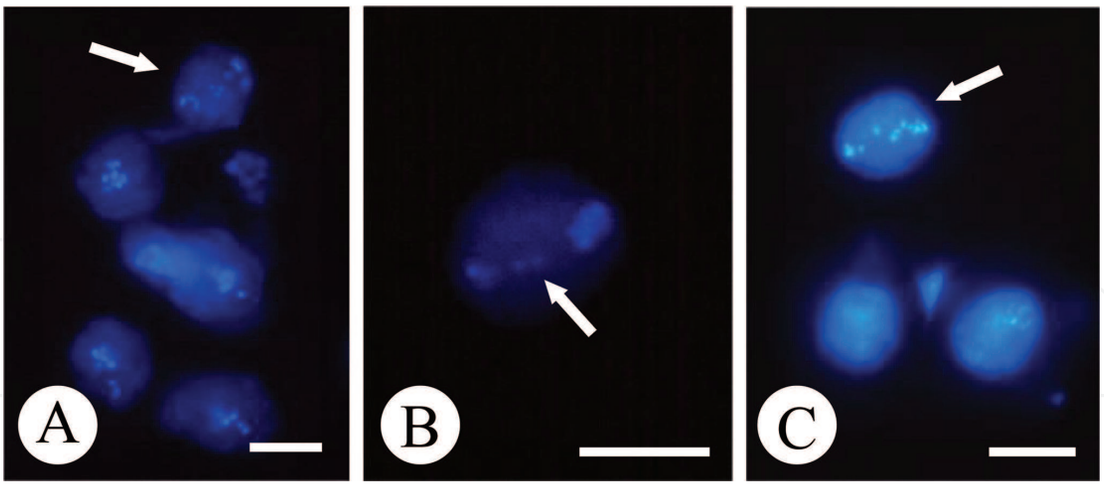

Figure 13. Abnormalities of the meiotic stage of the haploid pummelo [32]. A, B: Chromosomes remained in the equatorial plane, C: Nine bivalents aligned in the equatorial plane. Bars = 10 μm. Arrows indicate the abnormal cell.

5. Conclusion and prospects

Our studies of the morphological characteristics and reproductive potential of haploid [30–33] and DH pummelos [34, 35] are summarized in this chapter. The haploid pummelo showed morphology similar to that of the haploids of other fruit crops. When the haploid was the seed parent, there was no fruit set in any of the cross-combinations. However, when diploid cultivars were pollinated with pollen of the haploid, fruits were set and many developed seeds were obtained. We examined the process of meiosis in both gametes in the haploid pummelo, and our findings revealed that the lack of EMC formation was responsible for the complete sterility of the female gamete and that unreduced gamete formation by FDR caused partial fertility of the male gamete. The DH pummelo showed morphology similar to that of 'Banpeiyu' pummelo, and it had significantly large leaves, flowers and fruit compared to those of the original haploid pummelo. The DH pummelo also showed higher pollen fertility and a larger number of seeds than the haploid. In the reciprocal crosses with some diploid cultivars, the DH plant produced many developed seeds as both seed and pollen parents. These seeds germinated normally and developed into diploid plants.

Haploid and DH plants provide beneficial information regarding the location of major genes and quantitative trait loci (QTLs) for agronomically important traits, and they have been used for genome sequencing in some fruit crops such as apple, peach and pear [13, 14]. In *Citrus*, a rough draft of the genome was completed using the haploid clementine mandarin and the DH sweet orange [3–5]. This genomic information has been applied in the development of DNA markers, genetic analyses, and the production of new cultivars [47–49]. Chang et al. [50] reported that they constructed the detailed genetic linkage maps based on RAPD and SSR markers for 'Fina Sodea' clementine and Byungkyul (*C. platymamma*), using the information of whole-genome sequencing.

Figure 14. Variant shoot with spindly and variegated leaves arose from the haploid plant of pummelo. Arrows indicate the variant shoot. Bar = 5 cm.

Our research group also obtained some haploid plants by means of interploid hybridization and the pollination of irradiated pollen [51] in 'Banpeiyu' pummelo. These haploid pummelos showed vigorous growth (like the haploid pummelo introduced in this chapter), and flowering and fruiting lines among them were also observed. Bud mutation with spindly and variegated leaves arose from one of these haploids (**Figure 14**). We are now conducting studies on self-incompatibility, mutagenesis by ion-beam irradiation, and genetic analyses of mutants using these haploid and DH pummelos and their mutants. Our haploid and DH plants can also be used in various research fields such as plant breeding, mutant isolation, transformation, cytogenetic analyses, linkage maps, and the genome sequencing of *Citrus* and related genera.

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