

REVIEW

Morphological Characterization and Evaluation of Reproductive Function in a Haploid Pummelo [*Citrus maxima* (Burm.) Merr.]

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Abstract

To obtain basic information about haploid plants in *Citrus*, the morphological characteristics and reproductive potential of a haploid plant obtained from the 'Banpeiyu' pummelo [*C. maxima* (Burm.) Merr.] were examined. The leaves, flowers and fruits of the haploid were significantly smaller than those of the 'Banpeiyu' pummelo. Although the haploid showed remarkably reduced pollen fertility compared with the 'Banpeiyu' pummelo, it produced slightly fertile pollen grains. In crosses with diploid cultivars, when the haploid was the seed parent, there was no seed. But when diploid cultivars were pollinated with pollen of the haploid, diploid progenies were obtained. During female gamete formation, no embryo-sac mother cell was formed in the ovules, and consequently no normal embryo sac was observed at anthesis, owing to female sterility caused by the lack of embryo-sac mother cell (EMC) formation in the haploid. During male gamete formation, however, univalents were not distributed to opposite poles at anaphase I in some dividing cells; instead, they remained near the equatorial plate. The nine univalents then performed mitosis, and the univalents were equally distributed to opposite poles. And at the tetrad stage, dyads were also produced in the haploid at a ratio of 24.7%. These results suggest that fertile pollen grains in the haploid would be dyad derivation, produced by abnormalities in the first meiotic division such as first division restitution (FDR).

Discipline: Horticulture

Additional keywords: female sterility, male fertility, unreduced gamete

Introduction

Haploid and doubled haploid (DH) plants are of great value for genetic analysis and premeditated breeding (Germanà 2006, Ollitrault et al. 2012, Xu et al. 2013, Wang et al. 2015). This is particularly the case for woody species, which are generally characterized by a long reproductive cycle, high degree of heterozygosity, large plant size, and self-incompatibility. And in *Citrus* and related genera, new forms of interspecific and intergeneric triploid hybrids can be obtained through the fusion of haploid protoplasts (Kobayashi et al. 1997).

Several haploid induction methods such as *in vitro* androgenesis, induced by anther culture and *in vitro* gynogenesis and *in situ* parthenogenesis, induced by pollination with irradiated pollen and followed by

the application of new anti-microtubule herbicides for chromosome doubling, have been described in literature (Dunwell 2010, Germanà 2006, Germanà 2011).

In *Citrus* and related genera, Karasawa (1971) first reported haploid production from the seeds of *C. natsudaidai* Hayata that had been exposed to γ -rays irradiation. Since then, haploids have been produced by anther culture (Germanà 2011, Hidaka et al. 1979, Wang et al. 2015), interploid hybridization (Oiyama & Kobayashi 1993), and pollination of irradiated pollen (Froelicher et al. 2007, Yahata et al. 2010). However, these haploids were very weak and grew more slowly than the original diploid plants. Therefore, these haploids have hardly been used for genetic analysis and premeditated breeding. Furthermore, the flowering and fruiting of haploids have rarely been reported, as well as information on the

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reproduction in *Citrus* and related genera (Aleza et al. 2009).

Toolapong et al. (1996) selected a haploid progeny from among small seed-derived seedlings obtained from a cross between the ‘Banpeiyu’ pummelo and ‘Ruby Red’ grapefruit (*C. paradisi* Macfad.). This haploid derived from the female gamete of ‘Banpeiyu’ pummelo showed dwarf growth behavior and rosette morphology, similar to that of haploids thus far obtained from other cross combinations or methods. However, it grew very well when grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf.]. Seven years after germination, this haploid fortunately had many flowers (Fig. 1A). And three years after achieving reproductive growth, the haploid bore fruits. In the present review, we summarize our recent studies on the morphological characteristics and reproductive potential of this haploid pummelo.

Morphological characterization

The haploid had very small and narrow leaves, and small guard cells, as compared to those of the ‘Banpeiyu’ pummelo (Fig. 1B) (Yahata et al. 2005a). The flowers of the haploid were approximately half the size of those of the ‘Banpeiyu’ pummelo (Fig. 1C) (Yahata et al. 2005a). In addition, the haploid had a significantly reduced number of stamens and ovules compared with the ‘Banpeiyu’ pummelo. In flowers of the haploid, such abnormalities as the adhesion of pistils and stamens were observed. The fruit weight of the ‘Banpeiyu’ pummelo

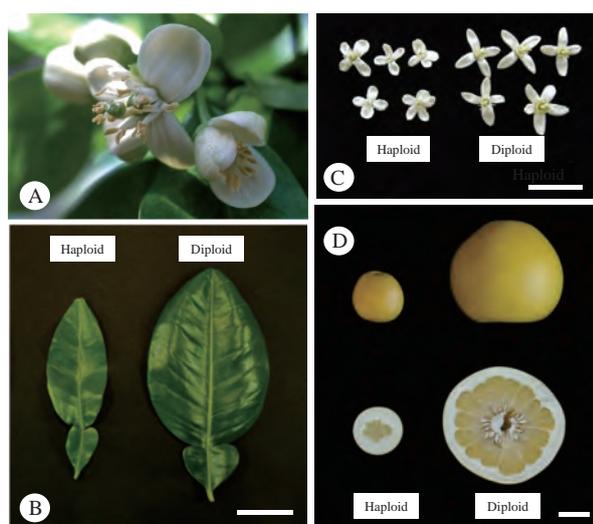


Fig. 1. Flowers of haploid pummelo (A) and the morphological characteristics of leaves (B), flowers (C) and fruits (D) in the haploid and the ‘Banpeiyu’ pummelo. Bars: 5 cm.

was approximately 1800 g, whereas that of the haploid was approximately 200 g, much smaller than that of the ‘Banpeiyu’ pummelo (Fig. 1D) (Yahata et al. 2011b). Approximately 100 seeds per fruit were obtained from the ‘Banpeiyu’ pummelo, whereas there were no seeds at all in the fruit of the haploid. There was no significant difference between the haploid and the ‘Banpeiyu’ pummelo in terms of Brix and titratable acidity of the fruit juice.

The haploid had a remarkably reduced number of pollen grains per anther (ca. 9,000) compared with the ‘Banpeiyu’ pummelo (ca. 63,000). Furthermore, the fertility of the pollen grains was evaluated by stainability with acetocarmine (Fig. 2) (Yahata et al. 2005a). Consequently, the ‘Banpeiyu’ pummelo showed a 97.5% stainability rate, whereas that of the haploid was only 14.1%. However, a few fertile pollen grains from the haploid were observed.

Evaluation of reproductive potential of male and female gametes in the haploid by cross pollination

The reproductive potential of the female and male gametes of the haploid crossed with several diploid cultivars revealed that when the haploid was the seed parent, no fruit was set in any of the cross combinations (Yahata et al. 2005b). However, when monoembryonic diploid cultivars were pollinated with pollen of the haploid, fruits were set and many developed seeds were obtained. These seeds germinated almost normally (Fig. 3A), and their seedlings grew vigorously and developed large wing leaves, typical of the haploid (Fig. 3B). The ploidy level of these seedlings was confirmed by flow cytometry analysis and chromosome observation, and they were diploid. This result reveals that fertilization occurred between the normal eggs of diploid cultivars and pollen grains with nine chromosomes from the haploid pummelo.

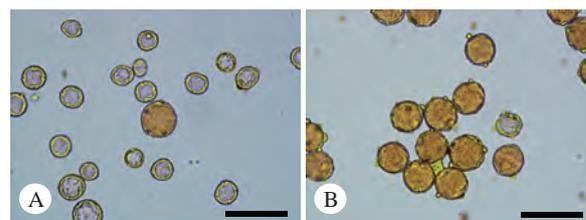


Fig. 2. Pollen grains stained by 1% acetocarmine in the haploid (A) and the ‘Banpeiyu’ pummelo (B). Bars: 50 μ m.

Microscopic observation of female and male gamete formation

1. Meiotic behavior during megasporogenesis

The process of female gamete formation was observed using the paraffin-sectioning method. The ‘Banpeiyu’ pummelo had normal morphologies in the inner and outer integuments (Fig. 4C), whereas the haploid showed abnormalities in its ovules, such as detached growth of the integuments from the nucellar tissue, and the formation of a void between the inner and outer integuments (Fig. 4A) (Yahata et al. 2011a).

The formation of EMCs was first recognized in the ovules at 1/4 the size of flower buds (SOFB) of ‘Banpeiyu’ pummelo (Yahata et al. 2011a). Subsequently, the initiation of meiosis and tetrad formation were observed at 1/3 SOFB and 2/5 SOFB, respectively. Approximately 20% of the ovules contained EMC or further developed embryo sacs. Afterwards, embryo sacs developed rapidly at the flowering stage, 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 days after flowering (DAF) (Fig. 4D), at a frequency of approximately 25%. In the haploid, on the other hand, no EMCs were formed throughout flower bud development, and no embryo sac was formed in the flowers at 2 DAF (Fig. 4B).

2. Meiotic behavior during microsporogenesis

The process of male gamete formation was observed using the squash method. The male meiosis of the

‘Banpeiyu’ pummelo occurred normally (Fig. 5J-Q). In the first meiotic division at prophase I, duplicated chromatin condensed (Fig. 5J), and the condensed chromosomes were visible (Yahata et al. 2011a). At metaphase I, homologous chromosomes aligned at the equatorial plate, and nine bivalents were observed (Fig. 5K). The bivalents separated into univalents and migrated towards each pole at anaphase I (Fig. 5L & M). In the second division, the chromosomes aligned at the equatorial plate at metaphase II (Fig. 5N), and the chromatids migrated towards each pole separated at anaphase II (Fig. 5O & P). Consequently, the ‘Banpeiyu’ pummelo produced a tetrad (99.2%) having four microspores of equal size (Figs. 5Q & 7).

In the haploid pummelo, meiotic division also occurred twice in the pollen mother cell (PMC), but abnormalities were observed in most dividing cells (Fig. 5A-I) (Yahata et al. 2011a). Although nine univalents aligned on the equatorial plate at metaphase I (Fig. 5A & B), they migrated unequally to each pole (Fig. 5C & D). In the second division, their chromatids also migrated separately to each pole (Fig. 5E-G). Another type of abnormal division was also observed in some meiocytes (Fig. 6), in which all the univalent chromosomes

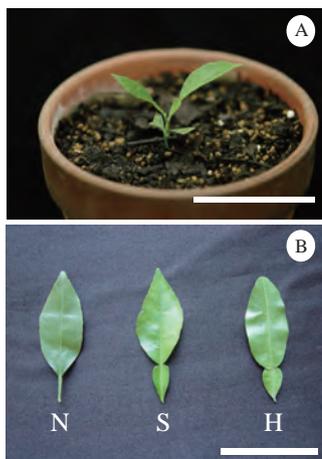


Fig. 3. Seedling obtained from the cross between ‘Nanpu’ tangor and the haploid (A) and a comparison of its leaf morphology with the parents (B). Bars: 5 cm. N: ‘Nanpu’ tangor, S: Seedling, H: Haploid.

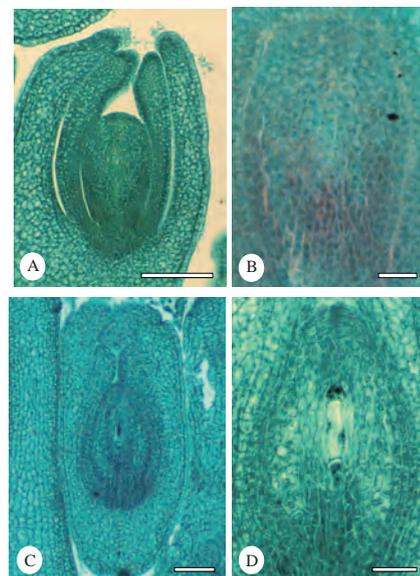


Fig. 4. Comparison of ovule morphology and embryo sac development in the haploid (A & B) and the ‘Banpeiyu’ pummelo (C & D).

A: Ovule morphology of the haploid at 3/4 the size of flower bud. Bars: 100 μ m. B: No embryo sac in the haploid at 2 days after flowering. Bars: 20 μ m. C: Ovule morphology of the ‘Banpeiyu’ pummelo at 2 DAF. Bars: 100 μ m. D: Eight-nucleate embryo sac in the ‘Banpeiyu’ pummelo at 2 DAF. Bars: 20 μ m.

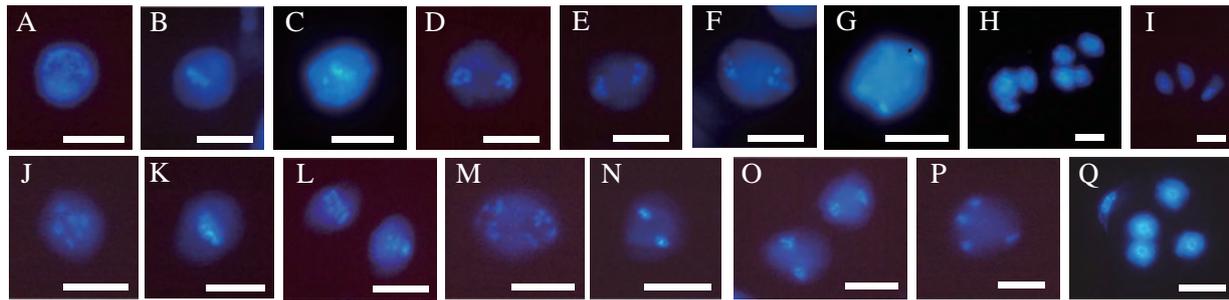


Fig. 5. Meiotic stages in the haploid (A - I) and the 'Banpeiyu' pummelo (J - Q).

A & J: Prophase I, B & K: Metaphase I, C & L: Anaphase I, D & M: Telophase I and prophase II, E & N: Metaphase II, F & O: Anaphase II, G & P: Telophase II, H, I & Q: Tetrad stage (H: Triad and tetrad, I: Dyad, & Q: tatrads). Bars: 10 μ m.

remained near the equatorial plate without distributing to either pole at anaphase I (Fig. 6A). In addition, nine univalents that remained on the equatorial plate showed mitotic division to segregate each set of chromosomes in the direction of opposite poles during the second meiosis (Fig. 6C). Consequently, microspore types from monads to hexads were observed in the tetrad stage of the haploid (Fig. 5H & I). The sizes of their microspores were unequal except for the dyads (Fig. 5I). Moreover, the haploid had a high frequency of dyads (24.7%) as compared with the 'Banpeiyu' pummelo (Fig. 7).

Discussion

In the present review, we summarize our recent studies on the morphological characteristics and reproductive potential of the haploid pummelo (Yahata et al. 2005a, 2005b, 2011a, 2011b).

It has been reported that haploids were obtained in some fruit crops such as apple, peach and pear (Dunwell 2010, Germanà 2006). These haploids generally show poor growth, and their leaves, flowers and fruits tend to

be smaller than those of diploid plants (Aleza et al. 2009, Hesse 1971, Höfer & Lespinasse 1996, Pooler & Scorza 1995, Toyama 1974). The haploid pummelo also showed similar morphology to that of the haploids of other fruit crops.

In the reciprocal crosses between the haploid and some diploid cultivars, no fruit set followed the pollination of the haploid with the pollen of 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 DAF (Bacchi 1943). The 'Banpeiyu' pummelo showed normal meiosis and produced mature embryo sacs at 2 DAF, although at a low frequency of formation, whereas in the haploid, female gamete formation was abnormal and no EMCs were formed in the ovules. Furthermore, the morphology of the inner and outer integuments of the ovules in the haploid was similar to that in the haploid plant of clementine mandarin (Aleza et al. 2009). We revealed that the lack of EMC formation was responsible for complete sterility in the haploid pummelo.

However, only a few fertile pollen grains from the haploid were observed. In the crosses with some diploid cultivars, when the haploid was used as the pollen parent, diploid progenies were obtained. And some species could form fertile gametes in the haploid plants (Hesse 1971, Pooler & Scorza 1995, Toyama 1974, Veilleux 1985, Yan et al. 2000). For fertile gamete formation to occur in the 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 such an event occurring in the pummelo haploid is theoretically $(1/2)^9 = 0.2\%$. However, the pollen fertility of the haploid was 14.1%, and much higher than the expected fertility rate. Meiotic nuclear restitution has been identified as a causal factor of this phenomenon (Ramanna & Jacobsen 2003). In the haploid plant of

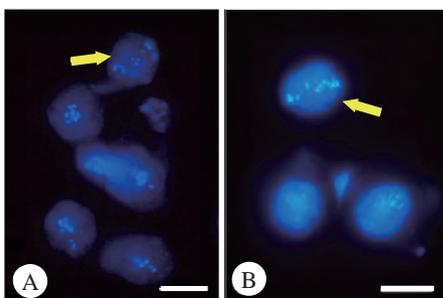


Fig. 6. Abnormalities of the meiotic stage of the haploid.

Arrows indicate the abnormal cell, A: Chromosomes remaining in the equatorial plane, B: Nine bivalents aligned in the equatorial plane. Bars: 10 μ m.

Capsicum annuum L., Yan et al. (2000) found laggards in many meocytes of the first division at meiosis of the PMC, which resulted in 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 in normal meiosis, the following abnormalities were observed in some meocytes: all the univalent chromosomes remained 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. At the tetrad stage, many dyads were also formed. This observation indicates that fertile pollen grains in the haploid pummelo were of dyad derivation, as previously reported in the haploid plant of *C. annuum*. Since the dyads were formed through the arrest of first meiotic division, it can be considered that meiotic nuclear restitution such as FDR took place in the haploid pummelo.

Conclusion and prospects

Our recent studies on the morphological characteristics and reproductive potential of the haploid pummelo were summarized in the present review. This haploid pummelo showed similar morphology to that of the haploids of other fruit crops. When the haploid was the seed parent, 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 found that the lack of EMC formation was responsible for complete sterility of the female gamete and that unreduced gamete formation by FDR caused partial fertility of the male gamete.

In *Citrus*, seedlessness is an essential trait for any new cultivar intended for fresh fruit and for the manufacturing market. Therefore, the method of producing seedless cultivars entails the use of triploids (Cuenca et al. 2013). In the haploid of *Citrus* and related genera, triploid somatic hybrids can be produced through the fusion of haploid protoplasts (Kobayashi et al. 1997). In particular, superior triploid pummelo-grapefruit hybrids such as ‘Oroblanco’ (Soost & Cameron 1980) may be produced by somatic hybridization between this haploid pummelo and diploid grapefruit cultivars.

Haploid and DH plants also have contributed to various fields of research such as mutant isolation, transformation, cytogenetic analysis, linkage maps, and genome sequencing (Dunwell 2010). Haploid and DH plants provide beneficial information on the location of major genes and quantitative trait loci (QTLs) for agronomically important traits, and were used for genome sequencing in some fruit crops such as peach, pear, and apple (Dunwell 2010, Germanà 2011). In *Citrus*, a rough draft of the genome was recently completed using the haploid clementine mandarin and the DH sweet orange (Ollitrault et al. 2012, Xu et al. 2013). This genomic information has been applied in the development of DNA markers, genetic analysis, and the production of new cultivars (Ahmad et al. 2013, Cuenca et al. 2013, Garcia-Lor et al. 2013).

Our research group has also succeeded in producing

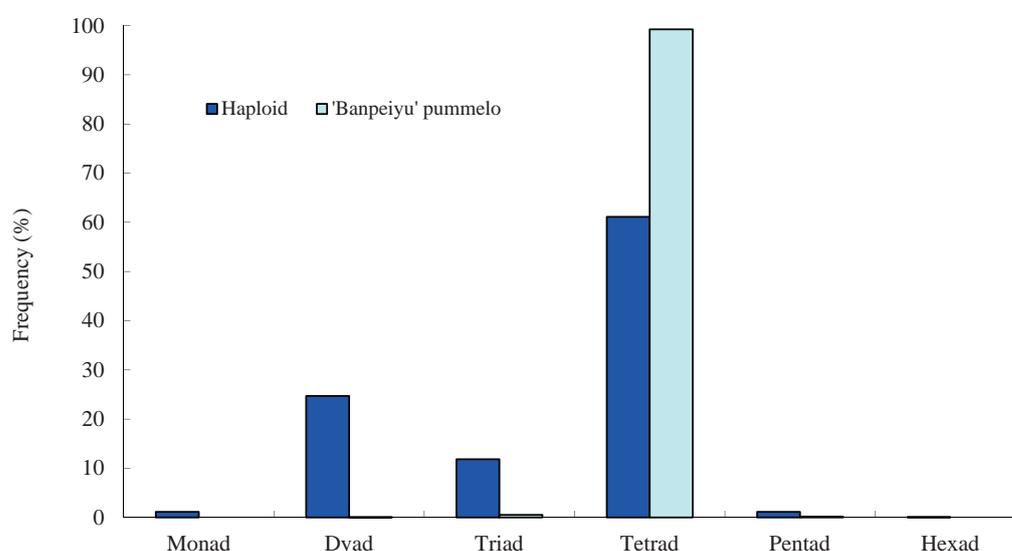


Fig. 7. Frequency of microspore type appearance in the tetrad stage of the haploid and the ‘Banpeiyu’ pummelo.

the DH plant of this haploid pummelo using colchicine-treated axillary shoot buds (Yahata et al. 2005c). Five years after being top-grafted onto trifoliolate oranges, this DH fortunately showed vigorous growth, and had many flowers and fruits for the first time. The DH pummelo had fertile pollen grains and developed seeds (Yahata et al. 2015). Moreover, we obtained some haploid plants by means of ovule culture (Yahata et al. 2010) and interploid hybridization in ‘Banpeiyu’ pummelo. Our haploid and DH pummelos will also be utilized as alternative materials for related genetic and breeding studies in the future.

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