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Ploidy variation and genetic composition of open-pollinated triploid citrus progenies

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ABSTRACT. Triploid plants are the most important candidates for the selection of seedless cultivars. In an attempt to investigate whether a triploid could be used as a maternal parent in citrus breeding for germplasm creation, seeds from an open-pollinated triploid ($2n = 3x = 27$) citrus BHR (*Citrus reticulata* cv. Bendizao × tetraploid HR (*C. sinensis* cv. Hamlin + *C. jambhiri* cv. Rough lemon)) were cultured. There was significant difference in leaf morphology among the seedlings. Flow cytometry, simple sequence repeats (SSR), as well and chromosome counting were carried out to determine the ploidy variation and genetic composition of the progenies. Results showed that triploid and diploid ($2n = 2x = 18$) seedlings predominated in the 42 progenies at 52.3% and 35.7%, followed by aneuploids and tetraploids. SSR analysis indicated that triploids were derived from nucellar apomixis; diploids were most probably from sexual crosses of haploid female gametes and alien pollen grains. These results revealed that triploid plants could be used as maternal parents in sexual crossing to create novel citrus germplasm.

Keywords: Apomixis; Citrus; Flow cytometry; Simple sequence repeats; Sexual cross.

INTRODUCTION

Citrus is one of the most important fruit crops worldwide and plays a major role in the economic development of some areas. Seedlessness is an elite trait for fresh citrus (Deng, 2005). Triploid fruits are usually seedless due to abnormal meiosis and embryo abortion. Therefore, creation of a triploid via sexual cross between a diploid and a tetraploid has become one of the most important approaches for the selection of seedless cultivars. Since the first report on the production of a citrus triploid (Cameron and Burnett, 1978), a large number of triploid progenies have been obtained from diploid × tetraploid crosses (Starrantino and Recupero, 1981; Oiyama et al., 1991; Deng et al., 1996; Tusa et al., 1996; Yi and Deng, 1998; Chandler et al., 2000; Song et al., 2005; Viloria and Grosser, 2005), from which several agriculturally important triploids have been released (Soost and Cameron, 1980; 1985; Starrantino, 1992).

Triploids have been used as male parents for haploid plantlet regeneration (Oiyama and Kobayashi, 1993; Germana and Chiancone, 2001), which plays an important role in citrus breeding and genomic research. Although triploids have been used as female parents in several other plants, such as melon (Ezura et al., 1994), solanum (Carputo, 1999), lilium (Lim et al., 2003), and asparagus (Ozaki et al., 2004), so far, the use of citrus triploid as a maternal parent in sexual hybridization is far from understood. This is largely due to the unique reproductive nature of citrus, which is characterized by male or female sterility, polyembryony, sexual incompatibility, and long juvenility.

The triploid citrus BHR was obtained by Deng et al. (1996) from the interploidy crosses between diploid tangerine (*C. reticulata* cv. Bendizao) and allotetraploid somatic hybrid HR (*C. sinensis* cv. Hamlin + *C. jambhiri* cv. Rough lemon), and it has set fruits. An average of 2.3 seeds per fruit formed in the non-isolated block, and this was low enough to be considered commercially seedless. In an attempt to investigate whether triploid could be used as a maternal parent in citrus breeding to create a novel germplasm, seeds from the mature fruits of an open-pollinated allotriploid citrus plant BHR were cultured. Leafy morphology, ploidy variation, and genetic composition of the progenies were analyzed. The research revealed that an allotriploid could also be used as a female parent in citrus sexual breeding for novel germplasm creation.

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MATERIALS AND METHODS

Plant material

Mature fruits were harvested from an open-pollinated triploid citrus plant BHR grown at the Citrus Research Institute, Huazhong Agricultural University, Wuhan, China.

Seed culture

Both the well-developed and abortive seeds were collected from the fruits of BHR. They were sterilized and cultured on MT (Murashige and Tucker, 1969) basal medium supplemented with 500 mg l^{-1} ME (malt extract) and 1 mg l^{-1} GA_3 (Gibberellic acid), respectively and kept at 26 ± 1°C with 16-h photoperiod and a light intensity of 4 µmol $m^{-2}s^{-1}$. Seedlings at the five-leaf stage were transferred into plastic pots contained garden soil and kept in a greenhouse.

Ploidy determination and chromosome counting

To determine the ploidy, young leaves were chopped in 0.5 ml nuclear extraction buffer (Solution A of a Partec High Resolution Kit, Partec, Germany), incubated for 3 min, and then filtered in a nylon sieve with mesh diameter of 30 µm (CellTrics^{TM}, Partec, Germany), followed by addition of 2 ml of 4', 6-diamidino-2-phenylindole (DAPI, solution B of the kit). The samples were measured with a Flow CytoMetry (FCM, PA-I, Partec Corporation) 2 min later. Bendizao tangerine (*C. reticulata*) was used as a diploid control, and then the ploidy of seedlings in test tubes from both well-developed and abortive seeds were calculated. The detailed ploidy determination procedure was as described by Xu et al. (2007). Newly growing leaf apices of the progenies were used for chromosome counting with the protocol of Chen et al. (2004).

Genome DNA extraction and SSR analysis

Three grams of leaves were harvested from the triploid and seven surviving diploid progenies, the somatic hybrid HR and their three original parents. Genome DNA was extracted according to Cheng et al. (2003). PCR reactions were performed in a T1 thermocycler (Biometra, Germany) in 20 µl reaction mixture, containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µM forward and reverse primers, 1 U Taq polymerase (MBI Fermentas, Lithuania) and 50 ng of genome DNA. The amplification program consisted of an initial denaturing cycle at 94°C for 5 min, followed by 32 cycles of 1 min denaturing at 94°C, 30 sec at 55°C for primers TAA15 and 57°C for CAC19 (Kijas et al., 1997), and 1 min at 72°C, with a final extension at 72°C for 4 min (Kijas et al., 1995). Amplified products were detected by electrophoresis in 1% agar gel and TAE buffer. The confirmed products were then analyzed on 6.0% denatured polyacrylamide gels and visualized by silver-staining as described by Pang et al. (2003).

RESULTS

Plant regeneration, morphological observation and ploidy verification

A total of 42 seedlings were regenerated, including 25 from well-developed seeds and 17 from abortive ones. Leaf morphology among the seedlings differed significantly. As shown in Figure 1, plantlet No. 8 had the heart-shaped petiole wing, which is considered as a typical characteristic of the pummelo leaf; plantlet Nos. 4, 11, 32 (1) and 36(1) had sharp petiole wings, similar to those of orange while no apparent petiole wing appeared in plantlet Nos. 6 or 9. For leaf apex shape, plantlet Nos. 6 and 4 exhibited sharp-pointed apexes, similar to those of Hamlin sweet orange and Bendizao tangerine, while plantlet Nos. 8, 36(1), 11, 9, 32(1) and 24 were round-shaped, similar to that of Rough lemon. The leaf apex of plantlet No. 24 was not so sharp as that of the other plants shown in Figure 2. To determine the ploidy, flow cytometry and chromosome counting were performed. Results showed that seedlings from well-developed seeds included 13 triploids, 10 diploids, one tetraploid, and one aneuploid ($2n = 21 = 2x + 3$); but those from abortive seeds included 9 triploids, 5 diploids, and 3 aneuploids ($2n = 25 = 3x-2$, $2n = 24 = 3x-3$) (Table 1 and Figure 3, 4). The integrated results of leaf morphology and ploidy determination indicated

Figure 1. Comparison of leaf morphology between seven surviving diploid plants and their three original parents. Left to right: Rough lemon, Hamlin, Bendizao and diploid plant Nos. 8, 36(1), 6, 4, 11, 9, and 32(1). Note: The difference in size due to the difference in growth potential of seedlings. The three original parents grew in the field, and the rest in greenhouse.

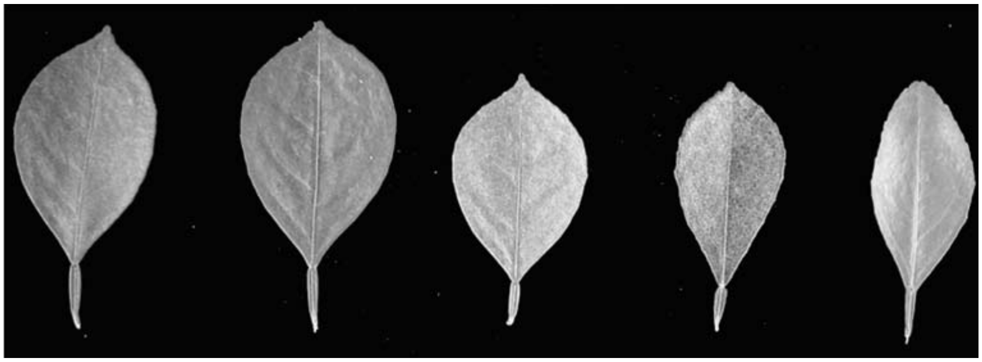

Figure 2. Comparison of leaf morphology among one triploid and four aneuploid citrus offspring. Left to right: No. 32(2) as triploid control and four aneuploids (Nos. 20, 15, 16 and 24).

significant differences in morphology among the diploid progenies (Figure 1), few differences among aneuploids (Figure 2), and no differences among the triploids (data not shown). After the seedlings were transferred to the pots in a greenhouse, 23 seedlings in total—including 12 triploids, 7 diploids, and 4 aneuploids—survived.

SSR analysis of the progeny

To identify the genetic composition of the triploid progeny and the diploid progenies with significant difference in leaf morphology, primers TAA15 and CAC19 screened from 14 primers in our laboratory were carried out for SSR analysis. As shown in Figure 5B, the triploid progeny and its maternal parent BHR had the same loci, which contained the specific locus of their three original parents revealed by CAC19. The seven surviving diploid progenies all had one specific locus of their original parent Bendizao tangerine. However, plants No. 4 (lane 8 in Figure 5B) and Nos. 6, 9, 8, 36 (1) (lane 7, 9, 10, 13 in Figure 5A) possessed additional loci (arrowed in Figure 5), which were absent in BHR, the somatic hybrid HR, and their three original parents.

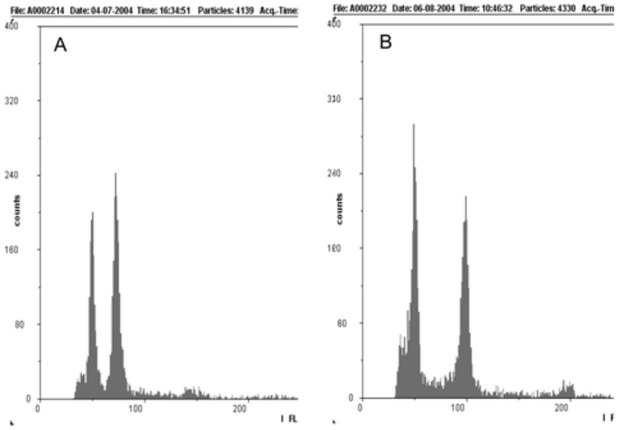

Figure 3. Flow cytometric analysis of triploid (A) and tetraploid (B) seedlings. Bendizao was used as a diploid control.

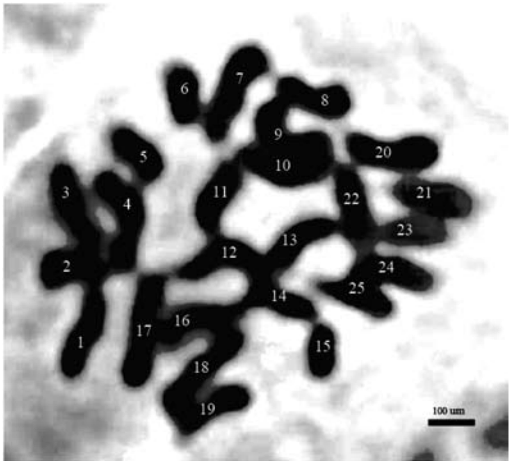

Figure 4. An aneuploid citrus seedling with 25 chromosomes.

DISCUSSION

Among the progeny population, triploid and diploid seedlings predominated with a ratio of 52.3% (22/42) and 35.7% (15/42), respectively. The high frequency of triploids presumably resulted from apomixis, a specific characteristic of citrus. Diploids were probably derived from sexual cross between haploid gametes from triploid citrus BHR and unknown alien 1x pollens. This could be interpreted by the hypothesis of Endosperm Balanced Number (EBN) (Johnston et al., 1980). Both the embryo

Table 1. Regeneration of plantlets with different ploidy from in- vitro cultured seeds from an open-pollinated triploid [*C. reticulata* cv. *Bendizao* × *tetraploid* HR (*C. sinensis* cv. Hamlin + *C. jambhir* cv. Rough lemon)].

Source	Triploid	Diploid	Tetraploid	Aneuploid	Total
Well-developed	13	10	1	1	25
Aborted	9	5	0	3	17
Total	22	15	1	4	42

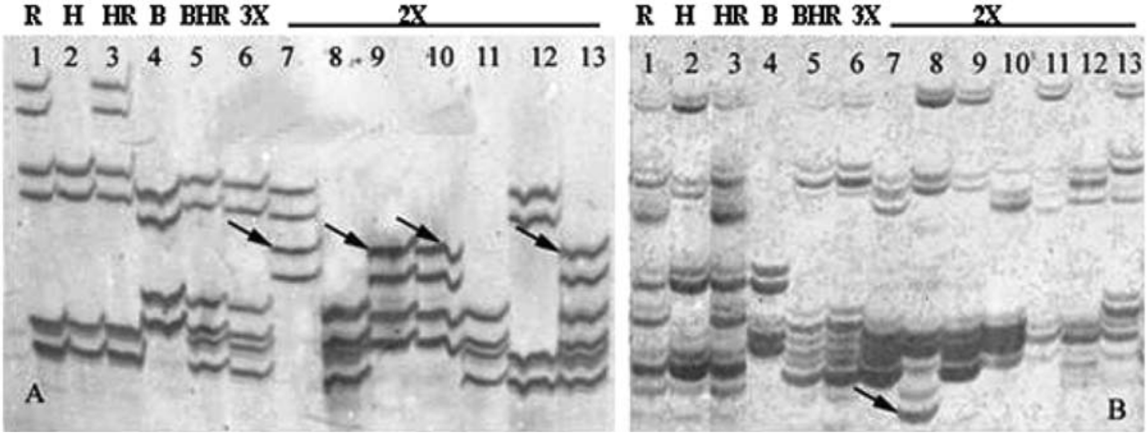

Figure 5. SSR alleles for citrus diploid progenies and their original parents on polyacylamide gel from primer TAA15 (A) and CAC19 (B). R: Rough lemon; H: Hamlin sweet orange; HR (Hamlin + Rough lemon); B: Bendizao tangerine; BHR: maternal parent; 3x: triploid progeny; 2x: diploid plantlet Nos. 6, 4, 9, 36(1), 8, 32(1), 11.

and seed development depend on functional endosperm (Brink and Cooper, 1947; Scott et al., 1998). However, during interploidy and interspecific crosses, endosperms often collapsed, resulting in abnormal seed development. Lin (1975) pointed out that a 2:1 ratio of maternal to paternal genome dosage was fundamental for normal growth of endosperm in maize. However, Lin (1984) found that a 3 maternal:1 paternal ratio in endosperm was subnormal but could still support fully viable 2x embryos. In the open-pollinated allotriploid citrus, we supposed that the maternal triploid plant BHR produced variable gametes ranging from haploid to triploid. Thus the maternal to paternal ratio in endosperm would be from 2:1 to 6:1 according to the theory of double–fertilization with normal 1x pollen. Excessive maternal nuclei could cause precocious cellulosing in seeds and stop endosperm growth early in development (Scott et al., 1998). Thus almost all seeds would abort, except for those diploids with a 2 maternal:1 paternal endosperm ratio. Based on the hypothesis mentioned above, the ploidy ratio of endosperm to embryo in the seeds of the open-pollinated triploid citrus was from 3:2 to 7:4. Only the diploid seeds with 3:2 were viable. This EBN hypothesis has been successfully used in citrus seedless production. After the diploid seedy *C. grandis* – Shatian pummelo was hand-pollinated with a tetraploid somatic hybrid, most seeds with 4x endosperm:3x embryo collapsed due to deviating from the 3x endosperm:2x embryo ratio. This technique had been widely applied under a patent (Patent No. ZL00109615.X).

Aneuploids were also obtained from the open-pollinated allotriploid citrus BHR. Unlike autotriploid, allotriploid BHR has three different sets of chromosomes, which originate from Bendizao tangerine, Hamlin sweet orange, and Rough lemon. Therefore, chromosome pairing would be more complex during meiosis, finally leading to higher percentages of univalents, bivalents, trivalents, and polyvalents. Lim et al. (2003) suggested that in triploid-diploid crosses of lilium the triploid parents produced aneuploid gametes ranging from near-haploid to triploid, and the progeny were predominantly near-diploid. However, within the four surviving citrus aneuploids, three were near-triploid ($2n = 25 = 3x$-2; $2n = 24 = 3x$-3) and one was near-diploid ($2n = 21 = 2x + 3$). This difference was probably due to the apomixis trait of citrus. However, the chromosome behavior of triploid citrus during meiosis needs further research, which could help in the future utilization of triploid in citrus breeding and citriculture.

The genetic composition analyzed by SSR indicated that the triploid progeny had the same band pattern as its triploid maternal parent BHR. It was thus verified that the triploid progenies originated from nucellar embryos of BHR. Some diploid progenies possessed an additional locus absent in their three original parents, HR and BHR, indicating that they probably derived from a sexual cross between a haploid gamete and an unknown alien pollen source. Chao et al. (2005) reported that the longest distance of pollen flow was more than 500 m or 92 rows. BHR grows in the citrus germplasm centre without isolation, where pollens from various citrus genera, species, and cultivars are numerous. Preventing cross-pollination between the BHR and compatible citrus by honey bees was impossible. However, the possibility that they came from the recombinated diploid gamete apomixis could not be excluded because genetic variation has been described in other plants like apomictic dandelions (Sorensen and Gudjonsson, 1946; Van der Hulst et al., 2000), just as chromosome pairing and chiasma formation during meiosis has been reported in triploid dandelions (Baarlen et al., 2000).

In this investigation, we have identified the ploidy variation and genetic composition, and verified the origin of the progenies from the open-pollinated triploid citrus BHR, derived from the crossing between monoembryognic tangerine and the somatic hybrids. During the culture, most seeds are monoembryonic, which makes it easier to obtain zygotic hybrids in a sexual cross. For the three original parents of BHR, Rough lemon and Hamlin sweet orange are tolerant to CTV (Citrus Tristeza Virus), and Bendizao tangerine is resistant (Grossor and Gmitter, 1990). Thus BHR is supposed to be a useful rootstock resistant to CTV. Meanwhile, Bendizao tangerine and Hamlin sweet orange are economically important cultivars. Thus, BHR is a potentially important maternal parent in a controlled sexual cross for selecting the cultivars with excellent traits in fruit quality and resistance to CTV from the segregated progenies. BHR has three sets of chromosomes, which are from three original parents contributing to different species and genus. Therefore, the behavior of chromosomes in the allotriploid citrus BHR is more complex than that in autotriploid during meiosis, which enables BHR to be used as a maternal parent in controlled sexual crossing for novel germplasm creation and also for meiosis research although we could not identify the origins of the diploid and aneuploid progenies from the open-pollinated triploid BHR. The results of this research have convinced us that triploid citrus could be used as a female parent in sexual breeding, a possibility which has been ignored by breeders given the unique reproductive characteristics of citrus—which include male or female sterility, polyembryony and sexual incompatibility—and the distinct characteristics of triploid plants like abnormal meiosis and embryo abortion.

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開放性授粉的三倍體後代的倍性變異和遺傳組成分析

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三倍體是無核品種選育重要的候選材料。我們對開放性授粉的異源三倍體 BHR (本地早橘 x（哈姆林甜橙 + 粗檸檬)) 的種子進行了離體培養和研究來證明三倍體能否在柑橘育種中作為母本進行種質的創造。通過觀察發現，後代個體在葉片形態上存在顯著的差異。流式細胞儀、SSR 分子標記和染色體計數等方法對後代的倍性變異和遺傳組成進行了分析。研究發現，三倍體和二倍體在所獲得的 42 株後代群體中所占的比例分別為 52.3% 和 35.7%，其次分別是非整倍體和四倍體。SSR 分析結果表明三倍體後代來源於柑橘珠心細胞的無融合生殖，而二倍體個體很可能是單倍體的雌配子與外界花粉雜交後代。本研究結果表明，三倍體在柑橘的有性雜交育種中可用作母本進行新種質的創造。

關鍵詞：無融合生殖；流式細胞儀；簡單序列重複；有性雜交。