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A tetraploid-dominated cytochimera developed from a natural bud mutant of the nonapomictic mandarin variety 'Orah'

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Abstract

Nonapomictic citrus tetraploids are desirable in citrus breeding for the production of triploid, seedless varieties, and polyploid rootstocks. However, only a few lines have been reported, and they were all generated using chemical methods. A 2x + 4× cytochimera of the nonapomictic citrus variety 'Orah' mandarin, which developed from a bud mutant, was found due to its morphology differing from that of diploid plants and characterised via ploidy analysis combining flow cytometry and chromosome observation. The chimaera was stable, and there were 1.86–1.90 times as tetraploid cells as diploid cells. Anatomical structure observation revealed that the 'Orah' chimaera may be a periclinal chimaera with diploid cells in the L1 layer and tetraploid cells in the L2 and L3 layers. The chimaera showed some typical traits of polyploid plants, including thicker shoots, wider and thicker leaves, larger flowers and fruits, and fewer but larger seeds in fruits than in diploid plants. Almost all the seeds of the chimaera were monoembryonic. Most of the self-pollinated progenies of the chimaera were identified as tetraploids, and some triploid, pentaploid, and hexaploid plants were found. As a female, the chimaera produced allotriploids when crossed with Australian finger lime. In addition, 6 plants developed from polyembryonic seeds of the chimaera were identified as sexual tetraploid progenies with low-level recombinant genomes. Therefore, the 'Orah' 2x + 4× chimaera can be used as a female parent to produce hybrid triploid and tetraploid citrus plants with high efficiency. Identification of the chimaera demonstrated that tetraploid citrus plants, especially nonapomictic varieties, can be generated from shoot bud mutants.

Keywords Nonapomixis · 'Orah' mandarin · Tetraploid · Cytochimera · Triploid · Seedlessness

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Introduction

Citrus is among the fruit types that contribute the most to fruit commerce worldwide. Seedlessness is a quality of superior citrus varieties (Cuenca et al. 2018). Triploids play a major role in producing seedless crops. Tetraploids can be used to efficiently breed triploids (Ollitrault et al. 2020). Therefore, tetraploids are important materials for breeding seedless fruit via the triploid pathway.

Apomixis is widely found in various citrus plants and negatively affects sexual reproduction (Zhang et al. 2014; Wang et al. 2017). As a result, when polyembryonic materials are used as female parents, hybrids can be obtained only at very low frequencies (Cao et al. 2011) or at higher frequencies by means of complicated measures, such as embryo rescue (Tan et al. 2007). Although polyembryonic tetraploid citrus plants can be easily obtained via nucellar seedling screening (Zhou et al. 2020), the use of polyembryonic tetraploid citrus lines as female parents to produce triploid citrus lines for nucellar embryo interference is labour intensive. Tetraploids can be used as male parents to produce triploid citrus plants, but normal seeds were at low proportions, and the proportion of triploids is generally less than 50% among plants developed from normal seeds (Aleza et al. 2012a). Although the proportion of triploids reached 100% among plants developed from undeveloped seeds, in vitro culture of embryos is necessary, and much labour is needed. In contrast, triploid plants were more efficiently generated when nonapomictic tetraploids were crossed with diploids and used as female parents (Aleza et al. 2009, 2012b). Therefore, monoembryonic tetraploids are desirable for triploid citrus production because of their high efficiency. However, only a few existing nonapomictic tetraploid citrus species can be used in triploid breeding (Dang et al. 2021).

Monoembryonic tetraploids had been obtained according to chemical induction assisted by micrografting (Oiyama and Okudai 1986; Aleza et al. 2009) and selected from progeny of monoembryonic diploid varieties (Xiang et al. 2008; Cui et al. 2021). Bud mutation plays major roles in citrus breeding (Mendel 1981; Wang et al. 2021). Tetraploid cells had been observed in somatic tissue, but most of the bud variations were diploid (Raghuvanshi 1962). And very few tetraploid bud sports have been identified (Ollitrault et al. 2020). Until now, only a few (maybe one case) was reported, and it was from a polyembryonic variety 'Ponkan' (a 2x + 4× chimaera) (Chen and Wan 1996). Therefore, nonapomictic tertaploid developed from bud mutant is rare, and remarkable for citrus polyploid breeding.

The 'Orah' mandarin is a well-known excellent nonapomictic citrus variety bred by Spiegel-Roy and Vardi (1992) with 'Temple' tangor (♀) (*Citrus reticulata* × *C. sinensis*) × 'Dancy' mandarin (♂) (*C. reticulata*) or 'Kinnow' mandarin ('King' mandarin [*C. nobilis*] × 'Willowleaf' mandarin [*C. deliciosa*]) (Barry et al. 2015). It was introduced in China in 2004 (Jiang and Cao 2011). 'Orah' mandarin is seedy variety, and seedlessness is a main breeding objective. Maybe, tetraploid 'Orah' can be efficient to produce triploid for seedlessness breeding. In the present paper, a diploid and tetraploid chimaera of 'Orah' is reported. The

chimaera developed from a natural bud mutant. This chimaera provides a new approach for producing triploids by using tetraploids as female parents and demonstrates a feasible pathway for breeding tetraploid citrus plants, especially nonapomictic varieties.

Materials and methods

Materials

The main material (named DL-1) was a 5-year-old tree found in 2018 in an 'Orah' mandarin orchard in Dongxing district, Neijiang, in Sichuan, China. The tree grew from an 'Orah' bud grafted on 1-year-old rootstock of *Citrus junos* cv. Ziyang xiangcheng and planted in 2015 when it was 1 year old. It fruited for the first time in 2019. Ten plants were cultivated by grafting scions of DL-1 on stumps of 'Kiyomi' tangor in the same orchard in 2020. These 'Kiyomi' tangor trees were grafted onto *Poncirus trifoliata* rootstocks and were 8 years old. 'Orah' mandarin plants from the same orchard ('Orah' 2) and germplasm resource nursery of the Fruit Research Institute at the Chongqing Academy of Agricultural Sciences (Jiangjin, Choongqing, China) ('Orah'1–1, 'Orah'1–2) were used as contrasts.

'Daoxianyeju' mandarin, 'Nianju' mandarin, 'Washington' navel orange, 'Jincheng' sweet orange, and 'Taroko' blood orange plants were obtained from the Germplasm Resource Nursery of the Fruit Research Institute at the Chongqing Academy of Agricultural Sciences (Jiulongpo district in Chongqing, China). 'Wanzhouhongju' mandarin was obtained from Wanzhou Ancient Red Mandarin Park (Wanzhou district in Chongqing, China).

Chromosome observation

Tender stem tips (DL-1) or root tips (some selfing and hybrid progenies of DL-1) were used as materials for preparing chromosome samples. The method described by Dang et al. (2019) was used with some modifications. First, stem tips or root tips were pretreated in 0.002 mol/L 8-hydroxyquinoline solution for 3 h and fixed overnight using Carnoy's fluid. After rinsing with deionized water 3 times, the fixed materials were dissociated in mixed enzyme solution (3% cellulose + 1% macerozyme) for 1 h. The mixed enzyme solution was removed, and the materials were gently rinsed with deionized water once. The materials were subsequently immersed in Carnoy's fluid. Finally, the materials were spread on glass slides by using tweezers and torrefied using the flame of an alcohol lamp. The slides were stained with 5% Giemsa dye. Chromosomes were observed using a microscope (×1000) (BX63, Olympus, Tokyo, Japan) and photographed by a charge-coupled device camera. All chromosomes on the slides at metaphase were observed, and the dispersed chromosomes were photographed.

Flow cytometry

The method reported by Dang et al. (2023) was used with some modifications. Fresh fully expanded leaves were used as materials. Approximately 1 cm^2 of leaf tissue was used to prepare the samples. Leaf tissues were placed in polystyrene dishes, immersed in 100 μL of buffer solution (Chinese patent: ZL 201610578478.1), and cut rapidly using a razor blade. Another 850 μL of buffer solution was added, and the samples were filtered through a 30-μm cell strainer after slight oscillation. Then, 50 μL of 5 μg/mL DAPI (4′,6-diamidino-2-phenylindole) was added to the filtrate, and the mixture was incubated at 4 °C for approximately 10 min. Then, the samples were detected by a CyFlow® Ploidy Analyser (Sysmex Partec GmbH, Goerlitz, Germany). Diploid 'Orah' plants were used for comparison. More than 200 cells per sample were collected.

Cell size observation

Cross-sections of leaves of DL-1 and diploid 'Orah' ('Orah'-2) plants were prepared according to the paraffin sectioning method and stained with saffron and solid green (Wang 2008). The cell sizes of different tissues (upper and lower epidermis and palisade tissue) were measured. For every tissue, 50 cells were measured. Five replicates were used.

Phenotypic observation

The diameter of the branch (mature spring shoot) and the length, width, and thickness of the leaf (on mature spring shoot) were measured in October. The length and diameter of the flowers were measured in April. The fruit (open-pollinated) weight, fruit vertical diameter, fruit transverse diameter, soluble solid content, and seed number were investigated. Fruit shape indices were calculated (fruit transverse diameter/fruit vertical diameter). Three replicates were performed, and every replicate included 10 fruits.

One hundred randomly selected seeds were weighed when the episperm were dry and grey−white, and their average weight was calculated. Three replicates were performed.

The diploid 'Orah' was used for comparison. All the data were analysed by using Microsoft Excel 2013, and *t* tests were used to examine the differences.

Pollination and progeny ploidy investigation

For self-pollination, DL-1 plants were enveloped using a colourless plastic film sheet before any flowers bloomed in late March. When approximately 1/3 of the flowers bloomed, a hair brush was used for pollination, first, to gently brush the dehiscent anthers and then to gently brush the stigmas. When most of the flowers fell, the late flowers were removed. Approximately 1 month later, the plastic film sheet was also removed.

Flowers of DL-1 were pollinated by the pollen of DL-1 and Australian finger lime (*Microcitrus australasica*) (2n = 2x = 18; Fig. S1) on sunny days in early April. Flowers of Australian finger lime were collected before they bloomed. The anthers were peeled from the stamens and dried to collect the pollen. The stamens and petals of the unopened DL-1 flowers were removed. In addition, Australian finger lime pollen was gently spread on the stigmas of DL-1. The pollinated flowers were enveloped by white wax paper bags. Approximately 1 month later, the paper bags were removed. The fruits were harvested in January of the next year. Seeds were collected to breed seedlings. All plants developed from self- and cross-pollinated seeds were analysed via flow cytometry. The presence of pentaploids and hexaploids was confirmed by chromosome observation.

Seed germination

The episperm and endopleura of normal and small DL-1 seeds (from self-pollinated fruits) were removed. The seeds were embedded in moist peat soil, with the embryos facing down. Approximately 0.5 cm thick moist peat soil was used to cover all the seeds. One week later, the seed germination rates were recorded. If the epicotyl was elongated, the seed was recorded as germinating. Three replicates were established for each type of seed, and 100 seeds were included in each replicate.

Genome resequencing

DL-1, 3 'Orah' mandarin plants ('Orah' 1–1, 'Orah'1–2, and 'Orah' 2), 'Daoxianyeju' mandarin, 'Nianju' mandarin, 'Wanzhouhongju' mandarin, 'Washington' navel orange, 'Jincheng' sweet orange, 'Taroko' blood orange, and 6 plants developed from polyembryonic seeds of DL-1 were used as materials for resequencing. High-quality DNA was extracted by the CTAB method (Cheng et al 2003). DNA was sequenced on the Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA) by PersonalBio (Shanghai, China). FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to obtain high-quality reads. High-quality reads from all the samples were separately aligned to the 'Valencia' V3 (*Citrus sinensis* v3.0, http://citrus.hzau.edu.cn/download.php) assembly sequence reads (Wang et al. 2021) using BWA (0.7.12-r1039) (Li and Durbin 2009). All the materials were individually sequenced at depths of 10×–20×. GATK 3.8 (McKenna et al. 2010) was used to call single nucleotide polymorphisms (SNPs). All the materials were genotyped according to the SNP loci.

Genetic relationship analysis

DL-1, 3 'Orah' mandarin plants ('Orah' 1–1, 'Orah'1–2, and 'Orah' 2), 'Daoxianyeju' mandarin, 'Nianju' mandarin, 'Wanzhouhongju' mandarin, 'Washington' navel orange, 'Jincheng' sweet orange, and 'Taroko' blood orange plants were used for genetic relationship analysis. Genetic distance was estimated using genome-wide

SNPs (Zhang et al. 2018). All the analyses were carried out by using Microsoft Excel 2013.

Analysis of triploid progeny from hybridization between DL-1 and Australian finger lime

To investigate the genomic constitution of triploid progeny from hybridization between DL-1 and Australian finger lime, reduced-representation sequencing was employed to identify variations in the parents and progeny (Dang et al. 2023). The sequencing depths of all SNP loci were investigated, and parent-specific InDel allelic loci frequencies in the genomes of the progeny were calculated by using the methods reported by Dang et al. (2023). The total depth of every exonic InDel locus was calculated, and scatter diagrams at all depths were generated. All the data points were plotted according to their relative location on the chromosome. The relative depths of all chromosomes were calculated as average depth of the corresponding chromosomes/average depth of the whole genome. All parents were genotyped for exonic InDel loci. Parent-specific homozygous loci at depths greater than 3/4 the average depth and less than 5/4 the average depth were selected. The corresponding alleles in the hybrids were considered parent-specific alleles. The frequencies of the parent-specific InDel alleles in the hybrid genome were calculated. Scatter diagrams were generated according to the methods described above. All steps were carried out by using Microsoft Excel 2013.

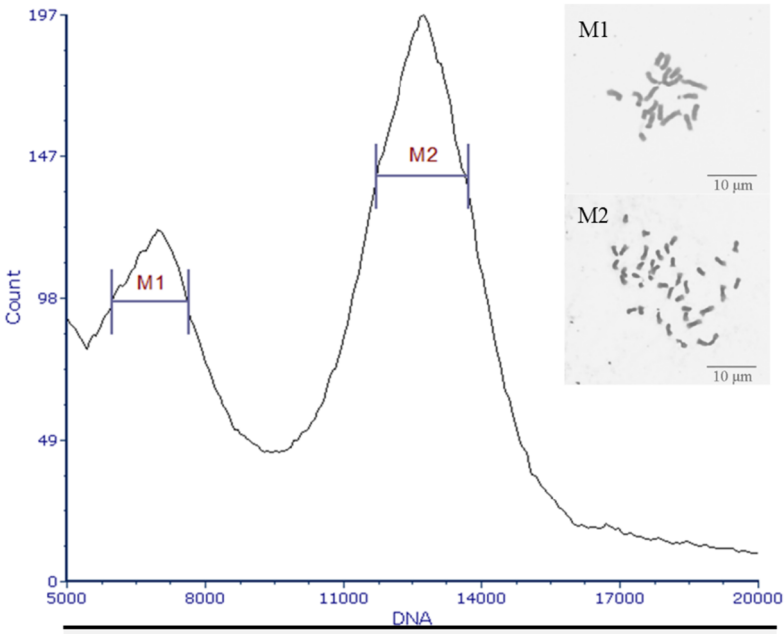
Genotyping plants developed from DL-1 self-pollinated polyembryonic seeds

Sequence information from 6 plants developed from DL-1 polyembryonic seeds was analysed, using DL-1, 'Orah' 1–1, 'Orah'1–2, and 'Orah' 2 for comparison. Heterozygous SNP loci (those whose depth was more than 1/2 of the average depth of the whole genome and whose depth of reference allelic loci and alternative allelic loci was near 1:1) of DL-1 were selected. The frequencies of homozygosity at these loci were investigated in the genomes of plants developed from DL-1 self-pollinated polyembryonic seeds.

Results

DL-1 was a 2x + 4× cytochimera

All twenty-four shoot tips from 8 directions (3 from each direction) surrounding DL-1 were used as materials for preparing the chromosome samples. Tetraploid (2n = 4x = 36) cells were found in all the shoot tips (Fig. 1). Diploid cells were occasionally found in a few tips. This result confirmed that the DL-1 was a 2x + 4× cytochimera. Some branches may have been chimaeras, and some branches were tetraploid.



Parameter	Merker	# of Events	Median	Geometric Mean	Cv
DNA	M1	2813	6763.00	2559.61	6.76
DNA	M2	5248	12617.00	7571.93	4.35

Fig. 1 Column diagram of the leaf cell DNA fluorescence strength of the chimaera detected by flow cytometry. M1 indicates the DNA fluorescence peak and chromosomes of 2×cells; M2 indicates the DNA fluorescence peak and chromosomes of 4×cells

To verify these queries, flow cytometry was used to analyse leaves from 8 directions surrounding DL-1 and randomly selected leaves on the grafted trees. Both diploid and tetraploid cells were detected on all the leaves, and the ratio of tetraploid to diploid cells ranged from 1.86–1.90 (Fig. 1). Therefore, DL-1 was truly a cytochimera with diploids and tetraploids, and tetraploids were dominated. The chimaera was essentially stable.

The cell sizes in the inner layers of DL-1 leaves were larger than those of diploid

The cell sizes of the leaves were observed via paraffin sectioning (Fig. 2A). The cell sizes of the upper epidermis and lower epidermis of the leaves of DL-1 and diploid 'Orah' plants were not significantly different ($p < 0.01$) (Fig. 2B, C). The average horizontal lengths of the upper epidermal cells were 14.72 ± 0.31 μm (diploid) and 14.52 ± 0.49 μm (DL-1), and the average horizontal lengths of the lower epidermal cells were 12.28 ± 0.60 μm (diploid) and 12.66 ± 0.77 μm (DL-1). The average

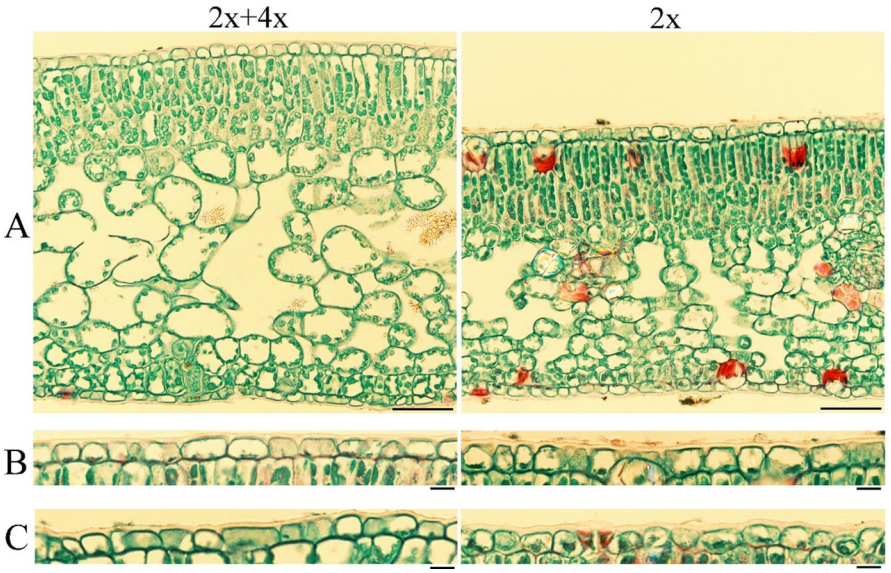


Fig. 2 Leaf vertical sections of the chimaera (2x + 4x) and the diploid 'Orah' (2x). **A** Overview of whole vertical sections; bar = 50 μm; **B** upper epidermis; bar = 20 μm; **C** lower epidermis; bar = 20 μm

horizontal length of palisade cells was also measured. It was 6.97 ± 0.47 μm for diploids and 10.55 ± 0.23 μm for DL-1, and these two sizes were significantly different ($p < 0.01$). The cells in the spongy mesophyll were found to be of various sizes in paraffin sections (Fig. 2A). Alternatively, cells in spongy mesophyll were possibly too large, and paraffin sections showed various profiles of cells with different diameters in paraffin sections of 8 μm thickness. However, the cells in the spongy mesophyll of DL-1 were clearly larger than those in the diploid mesophyll (Fig. 2A).

DL-1 had larger leaves and flowers but fewer seeds and larger fruits than diploid plants

Most of the traits of the chimaera were different from those of the diploid (Table S1, Table S2, Table S3, and Fig. 3). First, the tree canopy of the chimaera was obviously more compact than that of the diploid plants. Second, the diameter of the spring shoot (6 months old) of the chimaera was 4.20 ± 0.64 mm, whereas that of the diploid was 2.68 ± 0.40 mm, which was a significant difference ($p < 0.01$). Third, the leaves (from spring shoots) of the chimaera were wider and thicker than those of the diploid. Fourth, the flowers of the chimaera were larger than those of the diploid. Fifth, the fruits of the chimaera were somewhat larger than those of the diploid plants ($p < 0.05$), but the shapes of the two groups were similar. Although the peel of the chimaera fruits was thicker than that of the diploid fruits, the total soluble solid content (TSSC) of the chimaera fruits was greater than that of the diploid fruits. As in polyploids, the number of seeds (developed seeds) in the fruit of the chimaera

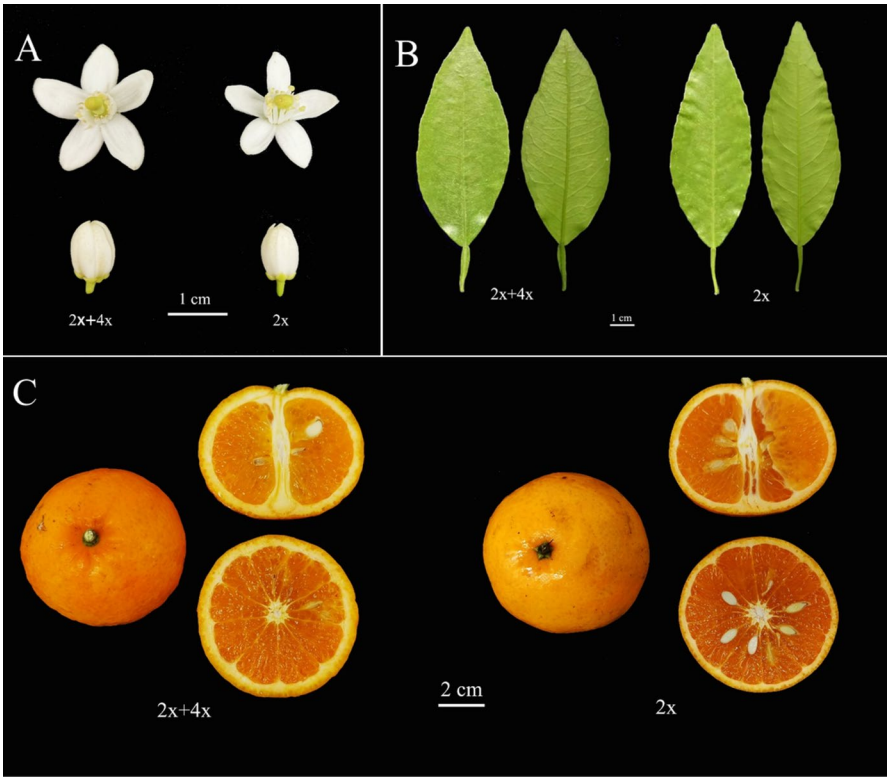


Fig. 3 Flower (**A**), leaf (from spring shoots) (**B**) and fruit (**C**) of the 'Orah' mandarin chimaera and diploid plants

(9.10 ± 2.13 per fruit) was far less than that in the fruit of the diploid (15.30 ± 5.12 per fruit). Sixth, the most developed seeds of the chimaera were monoembryonic, and they were larger than those of the diploid (the average seed weight of the chimaera was 101.68 ± 9.30 mg, and the average seed weight of the diploid was 77.26 ± 4.42 mg) ($p < 0.01$) (Fig. 4A).

Some small developed seeds (1/3–1/5 the weight of normal seeds) (Fig. 4A) were found in the fruits of DL-1, i.e. 22.19% (178/802) in 2021 and 24.59% (105/724) in 2023. Most of the seeds germinated, but the data were not recorded in 2020 and 2021. We carried out a test this year; the germination rate of the normal seeds was 96.33 ± 2.05%, and that of the small seeds was 95.33 ± 1.24%, with no significant difference ($p > 0.05$).

Most self-pollinated progeny were tetraploids, and triploid, pentaploid and hexaploid plants were found

To determine the ploidy of the self-pollinated progeny, plants developed from developed seeds were investigated via flow cytometry. In 2020, all 100 randomly

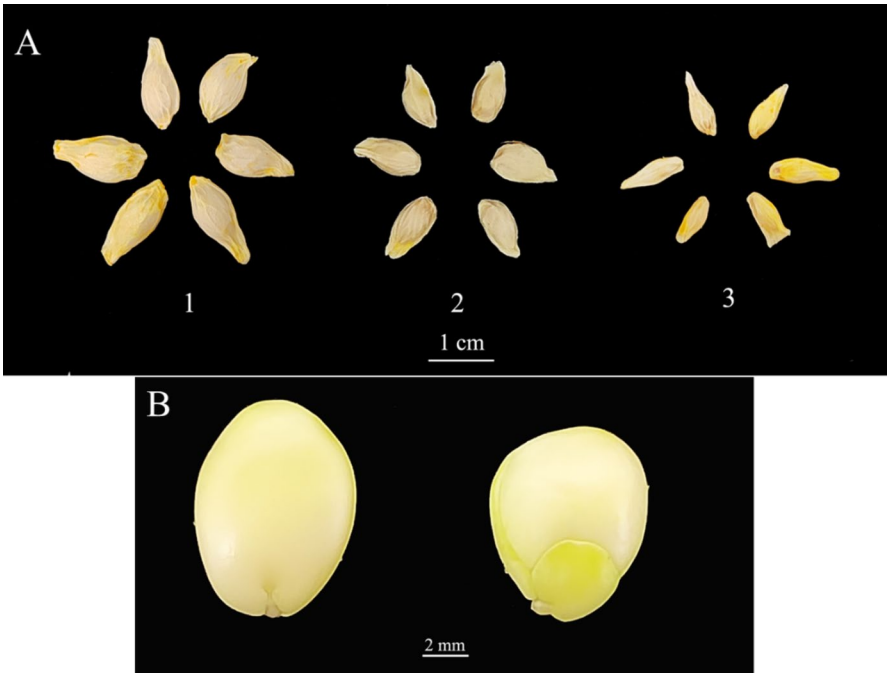


Fig. 4 Seeds of the 'Orah' mandarin chimaera. **A** Seeds of the chimaera (1) and diploid (2). 3 indicates small seeds of the chimaera. **B** Monoembryonic seed (right) and polyembryonic seed (left) of the chimaera without seed coats

selected unassorted seeds were used, and only 93 plants produced leaves. In addition, 120 small seeds were used, and 115 produced leaves. And all plants with leaves were used for ploidy identification.

The results of flow cytometry in 2020 showed that triploid (18.28%), tetraploid (73.12%), pentaploid (2.15%) (Fig. S2), and hexaploid (6.45%) plants developed from self-pollinated seeds of the chimaera (parent tree) (Table 1). However, among the plants that developed from small seeds, the proportion of triploids was much greater (69.57%), the proportion of tetraploids was much lower (11.30%), the proportion of pentaploids was only 2.61%, and the proportion of hexaploids (Fig. S3) was greater (15.65%). In addition, diploid were found among the plants that developed from small seeds, but the proportion was low (0.87%). That was to say, triploid and hexaploid might be enriched in small seeds.

To examine the enrichment of triploid and hexaploid in plants developed from small seeds, in 2021, only plants developed from small self-pollinated seeds were investigated. All 100 seeds were used, but there were respectively 99 and 95 produced leaves and were used for ploidy identification. Diploids, triploids, tetraploids, and hexaploids were found among plants developed from small seeds obtained from the parent tree and grafted trees (Table 1). The proportions of diploid and triploid plants were much greater than those of plants developed from unassorted seeds.

Table 1 Ploidy of plants developed from self-pollinated seeds of DL-1

Year 2020					
Unassorted seeds			Small seeds		
Ploidy	Plants number	Proportion (%)	Ploidy	Plants number	Proportion (%)
2x	0	0.00	2x	1	0.87
3x	17	18.28	3x	80	69.57
4x	68	73.12	4x	13	11.30
5x	2	2.15	5x	3	2.61
6x	6	6.45	6x	18	15.65
Year 2021 (small seeds)					
Parent tree			Grafted trees		
2x	10	10.10	2x	20	23.26
3x	84	84.85	3x	58	67.44
4x	1	1.01	4x	1	1.16
5x	0	0.00	5x	0	0.00
6x	4	4.04	6x	7	8.14

That is, most plants developed from self-pollinated seeds of the chimaera were 4x. Furthermore, 3×plants mostly developed from small seeds, 2×and 5×plants were not consistently found, and 6×plants were found at low frequencies.

DL-1 was identified as an asexual mutant of 'Orah'

The genetic distances of 10 citrus materials were calculated based on SNPs (Table S4). According to the genetic distances, these 10 materials can be divided into 3 groups. The first group is the 'Orah' group and includes 'Orah' 1–1, 'Orah' 1–2, 'Orah' 2, and DL-1. The second group is the mandarin group, which includes the three mandarin varieties, and the third group is the orange group, which includes the three orange varieties. In the 'Orah' group, the distance between any two materials was 0.022536 (DL-1 and 'Orah' 2)–0.024251 ('Orah' 1–1 and 'Orah' 1–2). That is, the genetic distance between DL-1 and any one of the materials was less than 0.024251. 'Orah' 1–1 and 'Orah' 1–2 were both developed from 'Orah' buds. Therefore, DL-1 was concluded to be an asexual mutant of 'Orah' mandarin.

Triploid hybrids between DL-1 and Australian finger lime were obtained

In 2020, hybridization between DL-1 and Australian finger lime was carried out. In total, 40 DL-1 flowers were pollinated with finger lime pollen, 3 ripe fruits were harvested, 7 developed seeds were obtained, and all the seeds developed into plants. Among the 7 plants, 5 grew normally (Fig. S4A). The 5 normally growing plants were investigated via chromosome observation, and they were all identified as triploid plants. Resequencing depths indicated that there was no aneuploidy (Fig. 5A). To confirm the genomic constitution of the 5 triploids, parent-specific InDel allelic loci

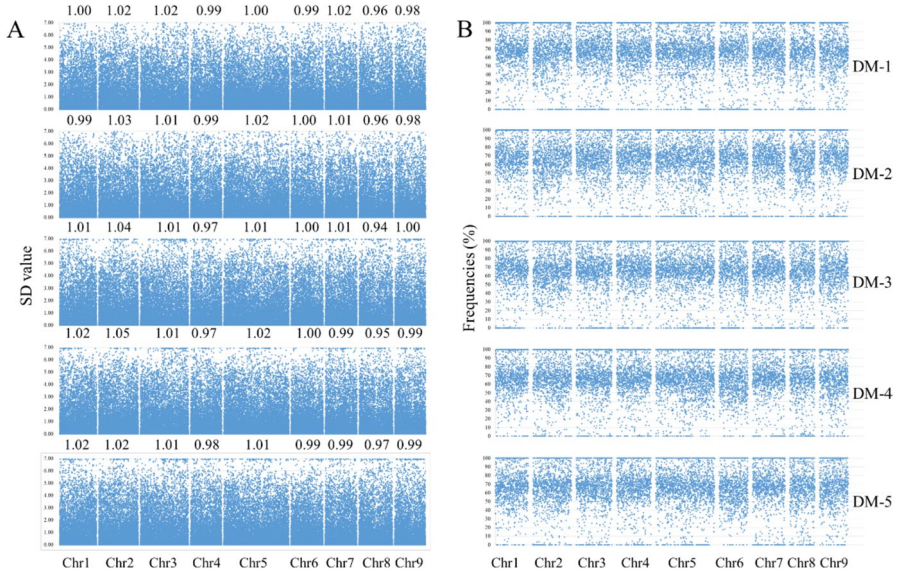


Fig. 5 InDel locus sequencing depths of the hybrids and frequencies of DL-1-specific InDel allelic loci on 9 chromosomes of 5 triploids. Each hollow dot indicates one InDel locus. The numbers above every scatter diagram indicate the relative depths (**A**). The chromosome no. are listed at the bottom (**A**, **B**). The IDs of the hybrids are listed on the left side (**A**, **B**)

frequencies were calculated. The results showed that the frequencies of DL-1-specific InDel allelic loci in 9 chromosomes of 5 triploids were approximately 70% (Fig. 5B). Therefore, the triploids were identified as allotriploids, and DL-1 contributed 2/3 of the genome. All 5 triploids grew into shrubby trees similar to those of the female parent but with longer thorns than the parents and larger leaves than the female parent.

Plants developed from polyembryonic seeds of DL-1 were identified as sexual progeny

Several polyembryonic seeds (5 were found among all 4032 seeds) (Fig. 4B) were found among the self-pollenated seeds of DL-1. Six plants (P1, P2, P3, P4, P5, and P5-1) (Fig. S4B) that developed from 5 polyembryonic seeds were identified as tetraploids using chromosome observation and flow cytometry. P5 and P5-1 were developed from the same seed. Perhaps all 6 plants were developed by the path of apomixes. To test our hypothesis, the genomic DNA of the 6 plants was subjected to genome resequencing, and all the plants were genotyped based on whole-genome InDel loci (depths greater than 10×). The homozygous ratio of DL-1 heterozygous loci in the genomes of these plants was calculated. 'Orah' 1–1, 'Orah' 1–2, and 'Orah' 2 were used as contrasts. Among the 6 plants, 7.15% (P1), 6.54% (P2), 13.96% (P3), 4.48% (P4), 13.00% (P5), and 12.73% (P5-1) were homozygous. The homozygous ratios of 'Orah' 1–1, 'Orah'1–2, and 'Orah' 2 were only 1.25%, 1.36%, and 1.21%, respectively. If the heterozygous loci of the parent were homozygous in self-pollenated progeny, the

genome of the progeny was surely recombined and developed from the zygote. The homozygous ratios of the 6 plants were much greater than those of the 3 asexual relatives of DL-1 ('Orah' 1–1, 'Orah'1–2, and 'Orah' 2). This finding indicated that all 6 plants developed from the zygote but not the nucellar tissue of DL-1.

Discussion

DL-1 may be a periclinal chimaera

Using flow cytometry, we determined that DL-1 was a stable cytochimera. Yasuda et al. (2008) confirmed the periclinal ploidy profile of meiwa kumquat (*Fortunella crassifolia*) by using flow cytometry to analyse the ploidy of various organs and tissues. And then Nukaya et al. (2019) associated ploidy with cell size to confirm another periclinal ploidy chimaera of meiwa kumquat. Their results showed that the outermost layer (L1) of the chimaera was diploid, and tetraploid cells were found in the inner layers (L2 and L3). The leaf epidermis derived from the L1 of the chimaeras exhibited the same cell size result as that of the diploid kumquat, and the cell sizes of the inner tissue of the chimaeras were significantly greater than those of the diploid.

In our study, the leaf epidermal cell sizes of the DL-1 and diploid 'Orah' plants were not significantly different, but the cell sizes in the inner tissue of the DL-1 leaves were significantly greater than those in the diploid 'Orah'. Thus, Dl-1 may be a chimaera with diploid cells in the L1 layer and tetraploid cells in the L2 and L3 layers.

Bud mutation screening is a pragmatic pathway for acquiring autotetraploid nonapomictic citrus plants

It is easier to obtain polyembryonic tetraploids; many tetraploids have been selected from nucellar seedlings of various citrus varieties (Ollitrault et al. 2020; Zhou et al. 2020), and some tetraploids have developed from nucellar embryos induced by colchicine and oryzalin (Yahata et al. 2004). Regeneration of citrus adult tissue has been difficult, and very few tetraploid bud mutants have been identified, probably due to unfavourable competition between diploid and tetraploid cells (Ollitrault et al. 2020). As a result, tetraploids could be induced by using adult tissue as a material. Early in 1986, Oiyama and Okudai obtained tetraploid plants of the nonapomictic varieties 'Clementine', 'Hassaku', and 'Hyuganatsu' using colchicine/oryzalin induction by micrografting (Oiyama and Okudai 1986). Aleza et al. (2009) obtained tetraploid 'Clemenules', 'Fina', 'Marisol', and 'Moncada' plants. All of these were nonapomictic citrus varieties. In addition, some tetraploids can be found among the progeny of nonapomictic varieties (Xiang et al. 2008; Cui et al. 2021). These tetraploids were very likely to be formed by 2n gametes via sexual reproduction, and the genotypes were different from those of both the female and male parents and cannot be predicted. Therefore, these tetraploids cannot be used as parents to produce triploids directly before necessary evaluations are carried out. To date, only a few natural polyploid citrus bud mutant plants and even ploidy chimaeras have been reported (Chen and Wan 1996). Perhaps the 'Orah' ploidy

chimaera is the first case in nonapomictic varieties, confirming a pragmatic pathway for obtaining polyploids (or chimaeras) of citrus plants, especially nonapomictic varieties. Although the frequency is very low, morphological observation may enhance efficiency. And the frequency may be higher in high latitudes and high altitude localities for ploypoid (Oustric et al. 2017; Rice et al. 2019).

Segregating chimaeras to recover tetraploid plants

The chimaera DL-1 is stable and represents a periclinal ploidy chimaera. The segregation schema described by Aleza et al. (2009) is ineffective for DL-1. Other methods (Wang et al. 2016; He et al. 2023) can be used to segregate tetraploids from chimaeras if the inner layers of cells regenerate into plants. However, it is difficult for plants to recover from adult citrus tissues. In the present study, the genotypes of plants developed from polyembryonic seeds were analysed, but no asexual plants were found. This was similar to the results reported by Aleza et al. (2010). However, these plants all had low proportions of recombined InDel loci, and the proportion of one plant (P4) was only 4.48%. The phenotypes of these plants may be similar to that of DL-1 plants.

Possible origin of the diploid, triploid, pentaploid, and hexaploid plants

In addition to tetraploids, diploids, triploids, pentaploids, and hexaploids were produced from the chimaera at different low frequencies. It was reported that diploids were obtained from 2x×4x (Aleza et al. 2012a) and 4x×2x (Aleza et al. 2012b) hybridizations. Diploid can produce gynogenesis (Aleza et al. 2012b). But the possibility that diploids develop from zygotes formed by haploid gametes cannot be excluded. Additionally, triploids develop from zygotes formed by diploid and haploid gametes. Pentaploid and hexaploid plants have been reported as progeny of tetraploids (Aleza et al. 2012a, 2012b). It is possible that pentaploids develop from zygotes formed from diploid and triploid gametes or haploid and tetraploid gametes (unreduced gametes of tetraploid). Hexaploidy may develop from zygotes formed by two triploid gametes or diploid and tetraploid gametes (unreduced gametes of tetraploid). It was difficult to determine the origin of these various ploidies because there was only one parent.

Triploids are enriched in small seeds and were mostly found in small developed seeds (Aleza et al. 2012b). This phenomenon can be explained by the endosperm balance number (EBN) (Carputo et al 2003). Pentaploid and hexaploid were not consistently enriched in small seeds. It is possible that other unknown mechanisms regulate the formation of pentaploid and hexaploid plants, and we may research these mechanisms in the future.

Possible application of DL-1 in citrus production and breeding

DL-1 can be used as a female parent to produce triploid citrus plants for seedlessness, and triploid plants should be generated in a high proportion when the chimaera is crossed with diploids. Only a few progenies were obtained from hybridization between DL-1 and finger lime. This difference might be related to the large genetic distance between the two

parents (Wu et al. 2018). Australian finger lime is tolerant of Huanglongbing (HLB; citrus greening) (Ramadugu et al. 2016; Alves et al. 2021). The triploid hybrids obtained in the present study can be used in the breeding of Huanglongbing-tolerant citrus plants. In addition, DL-1 produced pentaploids and hexaploids. Some plants still survive as of this writing and can be used to research the responses of citrus plants to high ploidy.

Furthermore, 'Orah' is a fertile mandarin. And there were approximately 9 developed seeds per fruit of the chimaera. Almost all the seeds were monoembryonic. This strain may be used as a male and female parent to produce triploids without embryo rescue, saving a large amount of labour. It is very likely that some delicious citrus plants can be bred by using DL-1 as a female parent because of the excellent quality of 'Orah'. More tetraploids can be produced via self-crossing of the chimaera. Research by Tan et al. (2019) showed that autotetraploid (4x) Ponkan fruit had higher TSSC and total acid, ascorbic acid, and total phenolic compound contents than diploid (2x) fruit. The TSSC of the mixed-ploidy 'Orah' fruit was also greater than that of the diploid fruit. It may be possible to develop a scion line with higher sugar content and fewer seeds than 'Orah'.

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Author contribution All the authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Jiangbo Dang and Cai Li. The first draft of the manuscript was written by Jiangbo Dang. Danni Sun carried out most supplemental experiments during the revision. Qigao Guo reviewed the manuscript. Guolu Liang supervised the project and reviewed the manuscript. All the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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