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RESEARCH ARTICLE

UNREDUCED MEGAGAMETOPHYTE FORMATION VIA SECOND DIVISION RESTITUTION CONTRIBUTES TO TETRAPLOID PRODUCTION IN INTERPLOIDY CROSSES WITH 'ORAH' MANDARIN (*CITRUS RETICULATA*)

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KEYWORDS

Citrus, 2n gamete, interploidy hybridization, pericentromeric SNP marker, second division restitution

HIGHLIGHTS

- In addition to triploid progeny, tetraploid hybrids derived from the fertilization of 2n megagametophytes are frequently regenerated from 2x × 4x crosses that utilize 'Orah' mandarin as the female parent.
- Data here indicate that 'Orah' mandarin is a cultivar that readily produces 2n megagametophytes.
- Second division restitution is the mechanism underlying 2n megagametophyte formation in 'Orah' mandarin.

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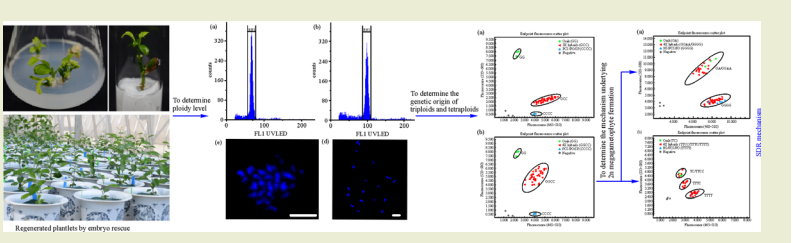
GRAPHICAL ABSTRACT



ABSTRACT

Seedless fruits are desirable in the citrus fresh fruit market. Triploid production via diploid × tetraploid interploidy crosses is thought to be the most efficient and widely-used strategy for the breeding of seedless citrus. Although 'Orah' mandarin has desirable organoleptic qualities, seeds in the fruits weaken its market competitiveness. To produce new seedless cultivars that are similar to 'Orah' mandarin, we performed three 2x × 4x crosses using 'Orah' mandarin as the seed parent to regenerate triploid plantlets. A total of 182 triploid and 36 tetraploid plantlets were obtained. By analyzing their genetic origins using nine novel single nucleotide polymorphism (SNP) markers, all of the triploids and tetraploids derived from these three crosses were proven to be hybrids. Also, we demonstrated that 2n megagametophyte formation in 'Orah' mandarin result in tetraploid production in these three interploidy crosses. These tetraploid plantlets were genotyped using eight pericentromeric SNP markers and nine centromere distal SNP markers. Based on the genotypes of the 2n megagametophytes, the parental heterozygosity rates in 16 SNP loci and all 2n megagametophytes were less than 50%, indicating that second division restitution was the mechanism underlying 2n megagametophyte formation at both the population and individual levels. These triploid hybrids enrich the

germplasm available for seedless breeding. Moreover, the tetraploid hybrids are valuable as parents for ploidy breeding for the production of seedless citrus fruits.

1 INTRODUCTION

Polyploidy, which contains more than two sets of chromosomes in one somatic cell, is believed to be a major force in plant evolution and a valuable trait for the improvement of woody plants, especially *Citrus*[1]. *Citrus* and related genera are mostly diploid (2n = 2x = 18)[2]. Although self-incompatibility and parthenocarpy have led to the production of seedless citrus fruit, most citrus cultivars still produce fruits with seeds because of cross-pollination[3]. Thus, ploidy manipulation for developing triploid progeny has become an important component of citrus breeding programs because triploid seedless fruits are more competitive in the citrus fresh fruit market[4]. Diploid × tetraploid hybridization is the most common strategy used for breeding seedless citrus[5]. Using this approach, several citrus breeding programs have recently produced numerous triploid hybrids[6–10]. In citrus, 2x × 4x crosses also produce tetraploids, which mostly regenerate from developed seeds[6–10]. Based on analyses of their genetic origins, chromosome doubling of nucellar cells and the fertilization of unreduced (2n) megagametophytes are thought to be the main sources of almost all spontaneous polyploidy in citrus[6–10].

Although the frequency of 2n gamete formation depends on the genotype in citrus, 2n gamete formation seems to be an intrinsic trait[11,12]. Some citrus genotypes, such as 'Nadorcott' tangor, 'Fina' clementine and 'Fortune' mandarin, have been reported to frequently produce 2n megagametophytes[9–11,13]. Various mechanisms are responsible for 2n gamete formation in citrus. Second division restitution (SDR) appears to be the predominant mechanism for 2n megagametophyte formation in mandarin cultivars[13–15]. In contrast, first division restitution (FDR) has been reported to be the major mechanism for 2n pollen formation in a clementine × sweet orange hybrid[16]. Also, although post meiotic chromosome doubling (PMD) has been reported in lemon, it is not the predominant mechanism[17,18]. The mechanism affects the transmission of parental heterozygosity restitution (PHR) to the progeny. The 2n gametes produced by FDR and SDR transmit about 80% and 40% PHR to the progeny, respectively. The 2n gametes derived from PMD possess full homozygosity due to an extra round of genome duplication that occurs after the formation of the haploid gametes[4]. The differences in the transmission of PHR in the different types of 2n gametes can greatly impact the gametic structures and thus the efficiency of a particular breeding strategy. Ascertaining the genetic origin of the 2n gamete can therefore facilitate their use in the breeding of polyploids. Although determining the mechanism of 2n gamete formation requires a large number of randomly selected molecular markers[15], it can be easily achieved using a few pericentromeric markers[14].

'Orah' mandarin (*Citrus reticulata* Blanco) is an excellent monoembryonic genotype cultivated widely due to its desirable organoleptic qualities and late maturing trait[19]. However, many seeds in each fruit weaken its market competitiveness. In our previous work, we produced triploids from crosses that utilized 'Orah' mandarin as the female parent and two distinct tetraploids[10]. In addition to triploid hybrids, these crosses also yielded tetraploid hybrids, indicating that 'Orah' mandarin is predisposed to produce 2n megagametophytes[10]. Here, we describe three additional 2x × 4x interploidy crosses using 'Orah' mandarin as the female parent to (1) produce more triploid hybrids from the 'Orah' mandarin lineage to breed new cultivars with fewer seeds in each fruit, (2) test whether 'Orah' mandarin is a cultivar that readily produces 2n megagametophytes, and (3) obtain insight into the mechanism of 2n megagametophyte formation in 'Orah' mandarin by analyzing the heterozygosity restitution for pericentromeric single nucleotide polymorphism (SNP) markers mined using a whole genome resequencing technique.

2 MATERIALS AND METHODS

2.1 Plant materials

'Orah' mandarin was pollinated with pollen from three allotetraploid somatic hybrids, PCS ['Page' tangelo + (clementine × satsuma orange)[20], PO ['Page' tangelo + 'Ortanique' tangor][20] and SP ['Succari' sweet orange + 'Page' tangelo][21] to produce triploids plantlets. The progeny from the three crosses are designated as OPCS, OPO and OSP, respectively. 'Orah' mandarin is cultivated at the Guangxi Citrus Research Institute located in Guilin city, Guangxi province, China.

2.2 Pollination, embryo rescue, plant regeneration and *in vitro* grafting

Hand pollination was conducted as described by Xie et al.[8]. Young fruits were collected 85 d after pollination. Embryo rescue was conducted as described by Xie et al.[10]. Immature seeds were classified as either developed or undeveloped and cultured on MT (Murashige and Tucker) medium supplemented with 1 mg·L^{-1} gibberellic acid. After germination the embryoids/shoots were transferred to MT medium supplemented with 0.5 mg·L^{-1} 6-benzyl aminopurine, 0.5 mg·L^{-1} kinetin and 0.1 mg·L^{-1} α-naphthalene acetic acid to promote the regeneration of shoots. The shoots were then grafted onto trifoliate orange (*Poncirus trifoliata*) rootstock *in vitro* to avoid a rooting phase. The grafted plantlets were transferred to plastic pots in a greenhouse when their growth appeared robust.

2.3 Ploidy analysis

The ploidy of each regenerated plantlet was determined using flow cytometry (CyFlow Space, Münster, Germany) as described by Guo et al.[22] with minor modifications. Young leaves from 'Orah' mandarin were used as a control. Histograms form each regenerated plantlet were generated automatically from an analysis of at least 3,000 nuclei. Chromosome counting analysis was performed with root-tips from randomly selected polyploid progeny to determine their ploidy level as described by Wang et al.[23]. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole, mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), and examined with a Zeiss Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany).

2.4 DNA extraction and SNP marker development based on whole genome resequencing

Whole genome resequencing was conducted to obtain polymorphic SNP markers to verify the genetic origin of progeny and determine the mechanism of 2n megagametophyte formation in 'Orah' mandarin. Qualified genomic DNA from 'Orah' mandarin, PO and PCS were extracted as described by Cheng et al.[24] and used to strictly construct DNA-seq libraries as described by Xia et al.[25] that were sequenced using the Illumina Hiseq2500 (PE250) at Beijing Novogene Bioinformatics Technology Co., Ltd. The raw data from the three parental accessions are available from the NCBI Sequence Read Archive (SRA) under accession number PRJNA678816. The DNA resequencing data for SP were downloaded from NCBI (SRA PRJNA613394). The clean DNA-seq read (i.e., sequences with adapters and reads with>10% of the bases called as N removed) from 'Orah' mandarin, PCS, PO and SP were aligned to the sweet orange reference genome[26] using BWA (v0.7.4-r385)[27] with default parameters. Variants (SNP and indel) were called using SAMtools mpileup[28] and annotated with SnpEff[29].

To verify the genetic origin of progeny, SNPs were selected from alleles that were homozygous and different in 'Orah' mandarin and the three male parents, which were defined as aa × bbbb type. To determine the mechanism of 2n megagametophyte formation in 'Orah' mandarin, SNPs were selected from alleles that were heterozygous in 'Orah' mandarin and homozygous in each of the three male parents, which were defined as the ab × aaaa/bbbb type. Based on the physical location[25] of citrus centromeres, we chose SNPs that were located both proximal and distal to the centromeres. Also, there are no additional SNPs known within 50 bp of each SNP. The primers used to score these SNPs were designed from flanking sequences using Primer 5 (www.premierbiosoft.com) and have lengths ranging from 18 to 22 bp, GC contents ranging from 45% to 55%, and Tm values ranging from 50°C to 60°C.

2.5 Determining the genetic origin and mechanism of 2n gamete formation using KASP genotyping

The competitive allele specific PCR (KASP) genotyping method was used to determine the genetic origin of polyploid progeny and the mechanism of 2n megagametophyte formation in 'Orah' mandarin as described by Cuenca et al.[30]. The samples with clusters between parents were considered to be hybrids when using the SNP markers (the aa × bbbb type) to identify the genetic origin of progeny.

Once we demonstrated that the 2n gametes in the tetraploid plantlets were derived from female parents, SNP markers (the ab × aaaa/bbbb type) were used to analyze the mechanism of 2n megagametophyte formation. The allelic configurations of 2n megagametophytes were deduced from the genotypes of pertinent tetraploids as described by Cuenca et al.[30]. The percentage of parental heterozygosity restitution (PHR) for each SNP locus was calculated as recommended by Xie et al.[15] using the formula PHR = N_{he} / (N_{he} + N_{ho}) × 100, where N_{he} is the number of heterozygous genotypes and N_{ho} is the number of homozygous genotypes. The tetraploid plantlets were genotyped using pericentromeric SNP markers to distinguish between the FDR and SDR (or PMD) hypotheses. If the PHR approaches 0%, SDR and/or PMD may be responsible for 2n megagametophyte formation. If the PHR approaches 100%, FDR is responsible for 2n megagametophyte formation[14]. Additionally, a set of centromere distal SNP markers distributed along Chr5 were used to differentiate between PMD and SDR. Full homozygosity

for these loci is expected if PMD is responsible for the formation of the 2n gametes. Heterozygosity at these loci indicates that SDR is responsible for 2n megagametophyte formation[17].

3 RESULTS

3.1 Regeneration of triploids and tetraploids from the three 2x × 4x crosses

From the three interploidy crosses conducted with 'Orah' mandarin as the seed parent (Table 1), 711 flowers were pollinated, yielding 287 harvested fruits with an average fruit-set rate of 40.4%. When the male parents were PCS, PO and SP, a total of 79, 128 and 80 fruits were harvested, respectively. Using an embryo rescue procedure (Fig. 1(a–c),) 1672 seeds that were developed and 2347 seeds that were undeveloped were cultured *in vitro*. The seed numbers per fruit crossed with the PCS, PO and SP male parents were 15.9, 13.8 and 12.5, respectively. Approximately one month later, the 315 developed seeds and the 466 undeveloped seeds were germinated (Fig. 1(d), Table 1). A total of 4.9, 1.6 and 2.4 seeds germinated per fruit that were derived from crosses with the PCS, PO and SP male parents, respectively. Moreover, among the three crosses, the seeds from the 'Orah mandarin × PCS' cross had the highest overall germination rate (30.8%), followed by 'Orah mandarin × SP' (18.8%) and 'Orah mandarin × PO' (11.7%). These rates are consistent with the ranking of germination rates from the developed and undeveloped seeds. In total, 365 progeny were regenerated from these three crosses. The overall average of regenerated plants per fruit was 1.3.

The ploidy of these plantlets was determined using flow cytometry (Fig. 2(a–c)) and chromosome counting (Fig. 2(d–f)). A certain proportion of diploid progeny appeared due to the missing of

Table 1 The fruit set and numbers of seeds and polyploids recovered from the 2x × 4x crosses

Cross	No. pollinated flowers	No. fruits set	No. seeds obtained		No. seeds germinated		No. plantlets obtained	No. diploids	No. triploids	No. tetraploids
			Dev.	Undev.	Dev.	Undev.				
Orah × PCS	210	79	323	930	115	271	145	37	90	18
Orah × PO	238	128	859	906	99	108	132	35	81	16
Orah × SP	263	80	490	511	101	87	88	75	11	2
Total	711	287	1672	2347	315	466	365	147	182	36

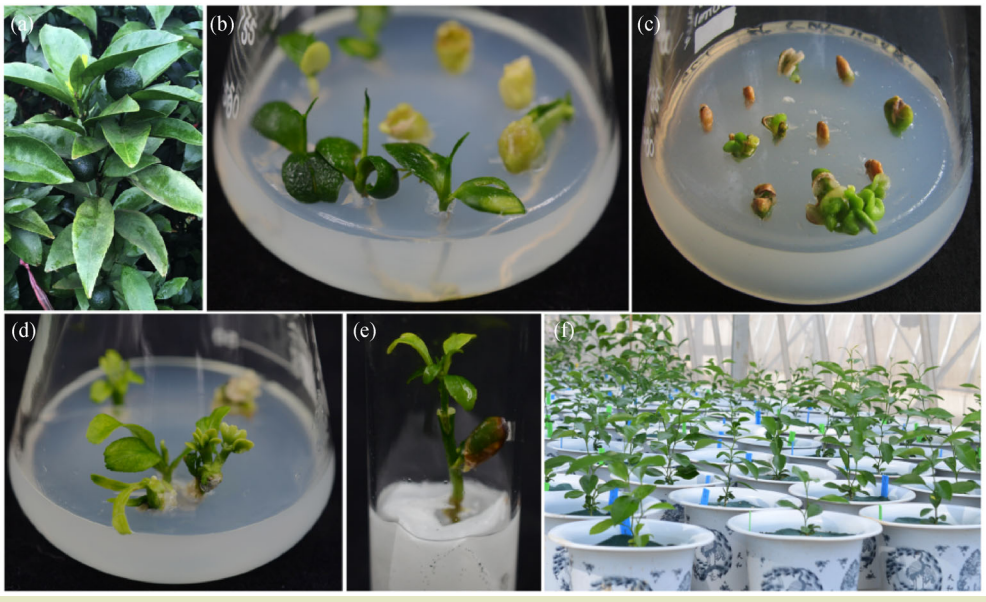


Fig. 1 Embryo rescue, plant regeneration and transplantation for citrus triploid production. (a) Young fruits 85 d after pollination. (b) Germination of developed seeds after approximately two weeks of culturing *in vitro* on germination medium. (c) Germination of undeveloped seeds after about four weeks of culturing *in vitro* on germination medium. (d) Regeneration of shoots from embryoids after their transfer to the shoot-induction medium. (e) A shoot grafted *in vitro* to the rootstock (*Poncirus trifoliata*). (f) Transplanted seedlings in a greenhouse.

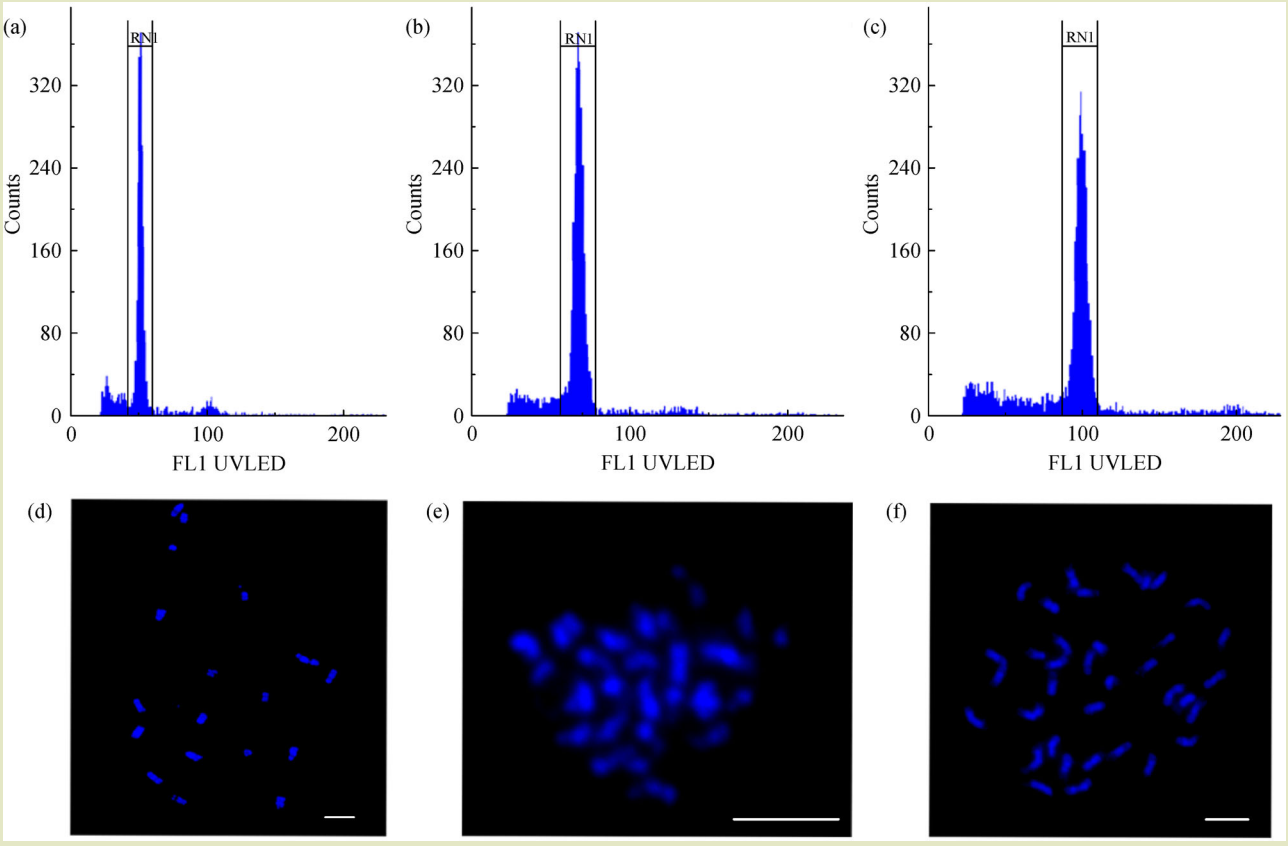


Fig. 2 Ploidy determination for regenerated citrus plantlets using flow cytometry and chromosome counting. (a–c) Histograms of diploid progeny (peak = 50), triploid progeny (peak = 75) and tetraploid progeny (peak = 100). (d–f) Chromosome counting for diploid (2n = 2x = 18), triploid (2n = 3x = 27) and tetraploid (2n = 4x = 36) plantlets. Scale bars = 5 μm.

bagging after pollination and the contamination of 2x pollen. In total, 182 triploids and 36 tetraploids were obtained from all of the regenerated plantlets (Table 1). The numbers of triploid plantlets obtained per fruit crossed with the SP, PCS and PO male parents were 0.1, 1.1 and 0.6, respectively. The numbers of tetraploid plantlets obtained per fruit were 0, 0.2 and 0.1, respectively (Table 1). They were grafted onto the etiolated seedlings of trifoliate orange *in vitro* to shorten the rooting phase of these polyploids (Fig. 1(e)). The grafted plantlets were then transferred to plastic pots in the greenhouse (Fig. 1(f)).

3.2 Mining of polymorphic SNP markers

Whole genome resequencing of the four parents was conducted to mine SNP markers that are useful for determining the genetic origin of progeny and for revealing the mechanism underlying 2n megagametophyte formation. By mapping the clean data to the reference genome of sweet orange, the average coverage depth was distributed in 20.7–30.7 X and mapping rate of the four samples ranged from 82.7% to 96.6%. A total of 4,167,442 variants covering the nine chromosomes were mined and annotated. Of these, 3,786,875 variants were in regions upstream of genes, 3,731,100 variants were in regions downstream of genes, 492,804 variants were in exons and 1,261,019 variants were in introns (Table S1). From these variants, 3,369,386 SNPs were determined. To obtain enough markers for further analysis, 11,004 SNPs (aa × bbbb type) and 80,079 SNPs (ab × aaaa/bbbb type) were selected *in silico* using a custom Python script.

3.3 Determination of hybrid origin of selected triploid and tetraploid progeny

From the 11,004 SNP markers (aa × bbbb type), 26 SNPs that are scattered across the nine chromosomes were selected to identify the genetic origins of 43 randomly selected triploids (22, 15 and 6 derived from crosses that used PCS, PO and SP as male parents, respectively) and all of the 36 tetraploids. Nine SNPs (Table S2; Fig. S1) were polymorphic and useful in determining the genotypes of the polyploid progeny. All of the selected triploids and tetraploids were clustered between the male and

female parents (Fig. 3; Table 2), indicating their hybrid origin. Furthermore, based on our analysis of all the tetraploid hybrids, we conclude that they were derived from the fertilization of 2n megagametophytes of 'Orah' mandarin with diploid pollen (Fig. 3(b); Table 2).

3.4 Determination of SDR mechanism for 2n megagametophyte formation in 'Orah' mandarin

Because these 36 tetraploid hybrids were derived from 2n megagametophytes, we determined the mechanism responsible for their formation at the population and individual levels using pericentromeric SNP markers and centromere distal SNP markers. We selected 20 pericentromeric SNPs and 20 centromere distal SNPs from the 80,079 SNPs that are heterozygous in 'Orah' mandarin and homozygous in the three male parents (ab × aaaa/bbbb type) (Table S1) to genotype these tetraploids. Eight pericentromeric SNP markers (Chr1-12169985, Chr3-7913461, Chr5-17395118, Chr5-18735709, Chr6-5337355, Chr7-20190172, Chr8-7202641 and Chr9-8606082) and nine centromere distal SNP markers (Chr5-1014992, Chr5-1051787, Chr5-1103777, Chr5-1323430, Chr5-1580076, Chr5-22348846, Chr5-24661722, Chr5-24798525 and Chr5-26120337) (Table S2) were polymorphic and were used to determine the mechanism underlying 2n megagametophyte formation.

To distinguish between FDR and SDR (or PMD), 36 tetraploid

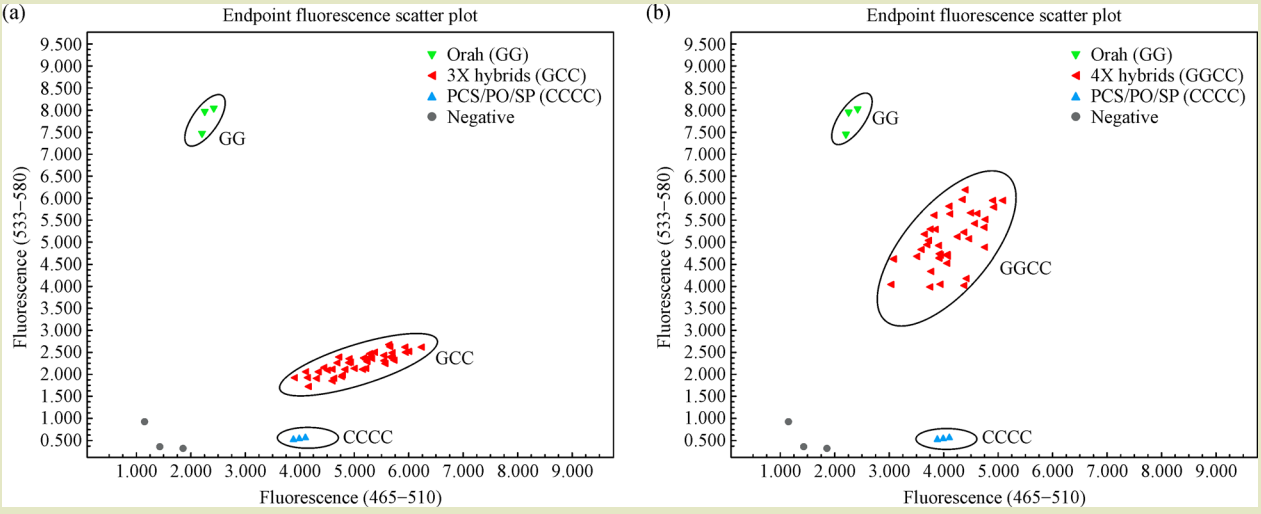


Fig. 3 Determining the genetic origin of triploids and tetraploids using KASP genotyping and aa × bbbb type SNP markers. Genotyping plots of (a) 43 randomly selected triploid progeny and (b) 36 tetraploid progeny with SNP marker Chr2-25841537 demonstrating their hybrid origins. Green, blue, red and gray represent the genotypes of maternal parents, paternal parents, triploid or tetraploid progeny and negative controls, respectively.

Table 2 Genotypic analysis of nine SNP markers (aa × bbbb type) in the triploid and tetraploid hybrid populations

SNP marker	Orah (aa)	Male parents (bbbb)	NI	abb	aabb
Chr2-24850985	CC	AAAA	79	43	36
Chr2-25841537	GG	CCCC	79	43	36
Chr3-18395328	AA	GGGG	78	43	35
Chr3-24832283	CC	TTTT	79	43	36
Chr4-8664085	GG	CCCC	77	42	35
Chr4-8689111	GG	TTTT	79	43	36
Chr5-12876197	CC	TTTT	79	43	36
Chr6-1932038	TT	CCCC	79	43	36
Chr9-815315	AA	GGGG	76	43	33

Note: NI, number of individuals genotyped; abb and aabb, number of individuals of each genotype.

hybrids obtained in the three interploidy crosses were genotyped using eight pericentromeric SNP markers. These tetraploid hybrids clustered with either the maternal or paternal parents (Fig. 4(a)), showing that the allelic configurations in all 2n megagametophytes were homozygous at these eight SNP pericentromeric loci (Table 3; Table 4), thus allowing us to discard the FDR hypothesis. The 36 tetraploid hybrids were further analyzed using nine centromere distal SNP markers. We found that particular hybrids clustered between the parents using two markers (Chr5-1103777 and Chr5-24798525) (Fig. 4(b)). At least one SNP locus was heterozygous in all of the 2n megagametophytes (Table 3; Table 4), allowing us to reject the PMD hypothesis. These data show that, at an individual level, SDR was the mechanism responsible for the formation of all 2n megagametophytes. At the population level, except for Chr5-1103777, the PHR of the 2n megagametophytes at the remaining 16 SNP loci was less than 50%, with an average PHR of 2.26%, confirming the predominance of the SDR mechanism.

4 DISCUSSION

4.1 Rapidly distinguishing between FDR and SDR (or PMD) using pericentromeric markers

The centromere is the primary constriction on the chromosome and is also a prominent feature in the genetic maps of plants. The inhibition of recombination in centromeric or pericentromeric regions has been reported in many plant species[31,32]. In general, crossover interference occurs most frequently in the chromosomal region that is most distal from the centromere, and the pericentromeric region has the lowest frequency of crossover interference. Although determining the mechanism of 2n gamete formation requires a large number of randomly selected molecular markers[15], it can be easily achieved using a few pericentromeric markers for genotyping the individual 2n gametes or a population derived from these gametes. When the 2n gametes are totally heterozygous for these markers, an FDR mechanism is indicated. When the 2n gametes are homozygous, an SDR (or PMD) mechanism is indicated[14].

Therefore, the application of pericentromeric markers is useful for rapidly distinguishing an FDR from SDR (or PMD) mechanisms at the population and individual levels in citrus and *Populus*[16–18,33,34]. In citrus, by genotyping with pericentromeric SSR and SNP markers, FDR has been shown to be the predominant mechanism driving 2n pollen formation in diploid 'CSO' tangor[16], and SDR for 2n megagametophyte formation in lemon (*Citrus limon*)[17,18]. Here, we screened eight pericentromeric SNP markers from centromeric regions, which located previously by half-tetrad analysis and chromatin immunoprecipitation technique[25] and determined that SDR was the

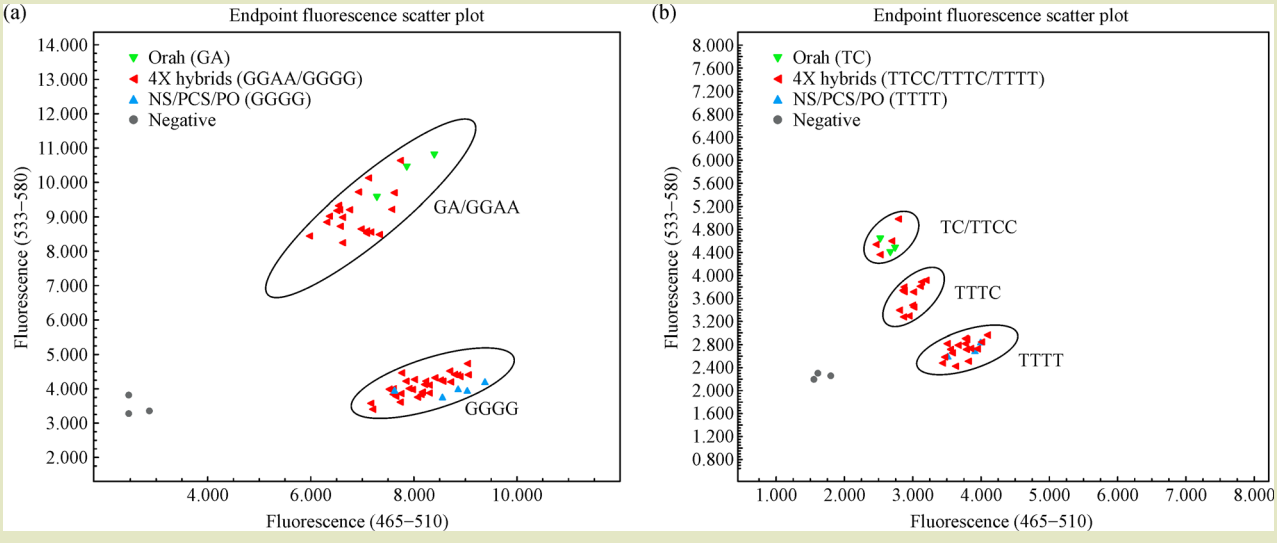


Fig. 4 Determining the mechanism of 2n megagametophyte formation in the 36 tetraploids using KASP genotyping and ab × aaaa/bbbb type SNP markers. (a) Under pericenteomeric locus Chr5-17395118, the maternal genotype (green) is GA, the paternal genotype (blue) is GGGG, and the tetraploid plantlets (red) clustered with their parents; the genotypes of the tetraploids are GGAA and GGGG with a GG contribution from the paternal parent and therefore homozygous AA and GG for the 2n megagametophyte. (b) Under the centromere distal locus Chr5-24798525, the maternal genotype (green) is TC, the paternal genotype (blue) is TTTT, and the tetraploid plantlets (red) clustered into three groups; the genotypes of the tetraploids are TTCC, TTTT and TTTC with a TT contribution from the paternal parent and therefore homozygous CC, TT and TC for the 2n megagametophyte.

Table 3 Genotypes of 18 tetraploids from 'Orah × PCS' hybridization generated using eight pericentromeric SNP markers and nine centromere distal SNP markers

SNP markers	Orah	Male parents	OPCS 1	OPCS 2	OPCS 3	OPCS 4	OPCS 5	OPCS 6	OPCS 7	OPCS 8	OPCS 9	OPCS 10	OPCS 11	OPCS 12	OPCS 13	OPCS 14	OPCS 15	OPCS 16	OPCS 17	OPCS 18	Het	PHR
Chr1-12169985	GA	GGGG	GG	AA	GG	GG	AA	AA	GG	GG	GG	AA	AA	GG	GG	AA	GG	AA	AA	GG	0	0
Chr3-7913461	GA	GGGG	GG	AA	GG	GG	AA	AA	GG	AA	AA	AA	AA	GG	AA	GG	AA	GG	GG	GG	0	0
Chr5-17395118	GA	GGGG	GG	AA	AA	GG	AA	GG	AA	AA	GG	AA	AA	GG	AA	GG	AA	GG	GG	AA	0	0
Chr5-18735709	TA	TTTT	TT	TT	AA	TT	TT	AA	TT	TT	TT	TT	TT	AA	TT	AA	TT	TT	TT	TT	0	0
Chr6-5337355	TG	TTTT	TT	TT	GG	GG	GG	TT	GG	TT	TT	TT	GG	GG	TT	TT	GG	GG	GG	TT	0	0
Chr7-20190172	CA	CCCC	CC	AA	CC	AA	AA	CC	AA	AA	CC	CC	CC	CC	CC	AA	CC	AA	AA	AA	0	0
Chr8-7202641	AG	AAAA	AA	AA	GG	AA	GG	AA	AA	AA	GG	GG	AA	GG	AA	AA	GG	GG	GG	GG	0	0
Chr9-8606082	GT	GGGG	TT	GG	GG	GG	TT	TT	GG	GG	GG	TT	GG	TT	TT	GG	TT	GG	TT	GG	0	0
Chr5-1014992	CA	CCCC	AA	CC	AA	AA	CC	AA	AA	AA	AA	AA	AA	AA	AA	CC	AA	CC	AA	AA	0	0
Chr5-1051787	AT	AAAA	TT	AA	AA	AA	AA	TT	TT	TT	AA	TT	TT	AA	AA	AA	AA	AA	AA	AA	0	0
Chr5-1103777	TC	TTTT	TC	TC	TC	TC	TT	TC	TC	TC	TC	TC	TC	CC	TC	TT	TC	TC	TC	TC	15	83.33
Chr5-1323430	CT	CCCC	TT	CC	CC	CC	CC	TT	TT	TT	TT	TT	TT	CC	CC	CC	CC	CC	CC	TT	0	0
Chr5-1580076	TC	TTTT	CC	TT	CC	CC	TT	CC	CC	CC	CC	CC	CC	CC	CC	TT	CC	TT	CC	TT	0	0
Chr5-22348846	GC	GGGG	GG	GG	GG	CC	CC	GG	CC	CC	CC	CC	GG	GG	GG	CC	GG	CC	GG	CC	0	0
Chr5-24661722	AG	AAAA	GG	AA	GG	GG	GG	GG	GG	GG	AA	GG	GG	GG	GG	AA	GG	AA	GG	AA	0	0
Chr5-24798525	TC	TTTT	TT	TT	TT	TC	TC	TC	TC	CC	TT	TT	TC	TC	TT	TC	TC	CC	TT	TT	8	44.44
Chr5-26120337	CT	CCCC	CC	CC	TT	TT	CC	TT	TT	TT	TT	CC	TT	TT	TT	CC	TT	CC	TT	CC	0	0
Het			1	1	1	2	1	2	2	1	1	1	2	1	1	1	2	1	1	1		
PHR				5.88	5.88	5.88	11.76	5.88	11.76	11.76	5.88	5.88	5.88	11.76	5.88	5.88	5.88	11.76	5.88	5.88	5.88	

Table 4 Genotypes of 18 tetraploids from 'Orah × PO' and 'Orah × SP' hybridizations generated using eight pericentromeric SNP markers and nine centromere distal SNP markers

SNP markers	Orah	Male parents	OPO1	OPO2	OPO3	OPO4	OPO5	OPO6	OPO7	OPO8	OPO9	OPO10	OPO11	OPO12	OPO13	OPO14	OPO15	OPO16	OSP1	OSP2	Het	PHR
Chr1-12169985	GA	GGGG	GG	GG	AA	GG	AA	AA	AA	AA	GG	GG	AA	AA	GG	GG	AA	GG	AA	AA	0	0
Chr3-7913461	GA	GGGG	AA	AA	GG	AA	GG	GG	GG	AA	GG	GG	GG	AA	AA	GG	GG	AA	GG	GG	0	0
Chr5-17395118	GA	GGGG	AA	AA	GG	AA	GG	AA	GG	AA	AA	GG	GG	AA	GG	AA	AA	GG	AA	AA	0	0
Chr5-18735709	TA	TTTT	AA	AA	TT	AA	TT	AA	AA	AA	TT	TT	TT	AA	TT	AA	TT	TT	TT	TT	0	0
Chr6-5337355	TG	TTTT	TT	TT	TT	GG	TT	GG	GG	TT	TT	GG	TT	GG	GG	GG	TT	GG	GG	GG	0	0
Chr7-20190172	CA	CCCC	CC	CC	CC	AA	CC	CC	AA	CC	CC	AA	CC	CC	CC	AA	AA	AA	CC	CC	0	0
Chr8-7202641	AG	AAAA	AA	AA	AA	AA	GG	AA	GG	AA	AA	AA	AA	GG	GG	AA	AA	GG	GG	GG	0	0
Chr9-8606082	GT	GGGG	GG	GG	GG	TT	TT	GG	GG	GG	TT	TT	TT	GG	GG	GG	TT	GG	GG	GG	0	0
Chr5-1014992	CA	CCCC	CC	AA	AA	CC	CC	CC	CC	CC	CC	AA	AA	CC	AA	CC	AA	CC	CC	AA	0	0
Chr5-1051787	AT	AAAA	AA	AA	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	AA	AA	AA	AA	AA	AA	0	0
Chr5-1103777	TC	TTTT	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC	TT	TT	TC	16	88.89
Chr5-1323430	CT	CCCC	CC	CC	CC	CC	CC	CC	CC	CC	CC	TT	CC	CC	CC	CC	CC	CC	CC	CC	0	0
Chr5-1580076	TC	TTTT	TT	CC	CC	TT	TT	TT	TT	TT	TT	CC	CC	TT	CC	TT	CC	TT	TT	CC	0	0
Chr5-22348846	GC	GGGG	GG	CC	CC	GG	GG	GG	GG	GG	GG	CC	GG	GG	GG	GG	CC	CC	CC	GG	0	0
Chr5-24661722	AG	AAAA	AA	GG	AA	AA	AA	AA	AA	AA	AA	AA	GG	AA	GG	AA	GG	GG	GG	GG	0	0
Chr5-24798525	TC	TTTT	TT	TC	CC	TT	TT	TT	TT	TT	TT	CC	TT	TT	TT	TT	TC	TC	TC	TC	5	27.78
Chr5-26120337	CT	CCCC	CC	TT	CC	CC	CC	CC	CC	CC	CC	TT	TT	CC	TT	CC	CC	TT	CC	CC	0	0
Het			1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2		
PHR			5.88	11.76	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	11.76	5.88	5.88	11.76		

predominant mechanism underlying 2n megagametophyte formation in 'Orah' mandarin.

The parental heterozygosity restitution transferred by FDR and SDR to 2n gametes varies greatly. Rapidly determining the mechanism underlying 2n gamete formation using pericentromeric markers can provide guidance for selecting suitable parents depending on the purpose of the breeding and thus, improves breeding efficiency. In addition, the mechanisms underlying 2n gamete formation affects the breeding efficiency for particular traits and is related to both the genetic distance between centromeres and the major locus controlling the particular trait[35]. The 2n gametes produced by the SDR mechanism transmits about 40% PHR to the progeny[4]. The genotypes of progeny produced by interploidy crosses that utilize 'Orah' mandarin as the female parent may show great variation and be expected to be useful for breeding new elite cultivars.

4.2 Implications for breeding new cultivars of triploids

Triploid production using ploidy manipulation is one of the most important strategies for breeding new seedless cultivars[4]. In addition to the exploitation of 2n gametes in 2x × 2x hybridization, the use of allotetraploid parents in interploidy crosses (2x × 4x and 4x × 2x) is popular in triploid plantlet production because when the allotetraploids are used as parents, there is a greater probability that triploid progeny will harbor genomes from three elite diploid parents. The enhanced genotypic variation of these triploid hybrids is extremely useful for selecting new cultivars. For example, the first commercially available seedless triploid mandarin hybrid in the USA was C4-15-19, which was derived from a 2x × 4x hybridization with an allotetraploid as the male parent. A total of 34 high-quality seedless triploid mandarin hybrids with commercial potential were selected from hybridizations between diploids and allotetraploids (2x × 4x)[4]. In the present study, 182 triploid and 36 tetraploid hybrids were recovered from the three 2x × 4x crosses conducted with allotetraploid somatic hybrids as male parents. PCS, PO and SP are allotetraploids with different maturation periods that produce fruits with acceptable flavor[20,21]. Although 'Orah' mandarin has elite fruit quality and late maturation trait[19], it has not been used as a female parent in ploidy hybridizations. These triploid hybrids provide promising germplasm for breeding seedless cultivars with high Brix to acid ratios and staggered maturation dates.

Additionally, the production of tetraploid hybrids shows that 'Orah' mandarin seems to be a cultivar that readily produces 2n megagametophytes at a frequency of about 10%. Triploid hybrids can be recovered from 2x × 2x crosses using 'Orah' mandarin as the female parent. Despite an increased contribution to the gene pool from male parents, these triploid hybrids will be genetically more similar to the female parent due to the presence of 2n megagametophyte and thus may be useful for breeding new 'Orah' mandarin-like seedless cultivars. Furthermore, these hybrids were made from 'Orah' mandarin, a typical monoembryonic citrus cultivar, and the three male parents that are each polyembryonic. There is also a chance of screening monoembryonic tetraploid hybrids from these 36 tetraploid progeny. Using a marker associated with polyembryony in citrus as reported by Wang et al.[36], we can select monoembryonic tetraploid hybrids at an early stage. When the monoembryonic tetraploid hybrid is pollinated with diploid pollen, the triploid hybrids can be obtained directly by germinating mature seeds without performing embryo rescue, and this will greatly improve the efficiency of recovering citrus triploid hybrids.

5 CONCLUSIONS

In total, 182 triploid and 36 tetraploid hybrids were regenerated from three interploidy crosses that utilized 'Orah' mandarin as the female parent and three allotetraploid somatic hybrids as the male parent. Also, the production of tetraploid hybrids at a high frequency indicates that 'Orah' mandarin is a cultivar that readily produces 2n megagametophytes. Using pericentromere and centromere distal SNP markers, SDR was demonstrated to be the mechanism of 2n megagametophyte formation in 'Orah' mandarin at both the population and individual levels.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2021385 contains supplementary materials (Tables S1–S2; Fig. S1).

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Compliance with ethics guidelines

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