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ORIGINAL ARTICLE

Recovery and characterization of homozygous lines from two sweet orange cultivars via anther culture

Shu-Ming Wang[1] · Hong Lan[1] · Hong-Bo Cao[1] · Qiang Xu[1] · Chun-Li Chen[1] · Xiu-Xin Deng[1] · Wen-Wu Guo[1]

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Abstract Homozygous genotypes are valuable for breeding and genomic studies in higher plants. The production of haploids and DHs through gametic embryogenesis allows a single-step development of complete homozygous lines from heterozygous parents, requiring much less time than the conventional selfing method. Here, we report the regeneration of haploid and double haploid lines of citrus species through anther culture. The anthers of seven citrus cultivars at the uninucleate stage were cultured and induced using four previously reported mediums. Ten haploid lines (2n = x = 9), six DH lines (2n = 2x = 18), two tetraploid lines (2n = 4x = 36) of 'Early Gold' sweet orange, and one haploid line of 'Rohde Red' Valencia sweet orange were obtained, as identified by ploidy, karyotype and simple sequence repeats (SSRs) analysis. All of them were confirmed to be fully homozygous by SSR analysis using 31 primer pairs that are distributed evenly on each of the chromosomes. Among them, plants regenerated from two DH lines of 'Early Gold' sweet orange grew vigorously in the greenhouse. To our knowledge, this is the first report on sweet orange anther culture with successful DH plant regeneration. The haploid, DH and tetraploid lines reported here hold great potential for future citrus genome resequencing in genetic studies and seedless breeding via somatic fusion.

Keywords Citrus · Anther culture · Haploid · Doubled haploid · Karyotype analysis · Molecular characterization

Abbreviations

DH	Doubled haploid
MT	Murashige and Tucker (1969) medium
N_6	Germanà (2005) medium
6-BA	6-benzyaminopurine
NAA	Naphthalene acetic acid
IAA	Indoi-3-acetic acid
KT	Kinetin
2,4-D	2, 4-dichlorophenoxyacetic acid
GA	Gibberellic acid
TDZ	Thidiazuron
DAPI	4′,6-diamidino-2-phenylindole

Introduction

Haploids are plants with a gametophytic chromosome number while doubled haploids are haploids that have undergone chromosome duplication (Germanà 2011a). Haploid plants and their derivatives e.g. DH, tri-haploid or tetra-haploid, which are collectively referred to as homozygous genotypes, have great potential for germplasm creation like triploid breeding via somatic fusion (Kobayashi et al. 1997; Ollitrault et al. 2000), dwarfing breeding (Dunwell 2010), QTLs (Lu et al. 1996; Xiao et al 2014a). Especially in recent years, the development of structural genomics and genome sequencing has led to a growing interest in generating haploid and DH plants, due to their significant advantage in fragment assembly (Forster et al. 2007; Aleza et al. 2009; Germanà et al. 2013). With a

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✉ Wen-Wu Guo
guoww@mail.hzau.edu.cn

[1] Key Laboratory of Horticultural Plant Biology (Ministry of Education), Huazhong Agricultural University, Wuhan 430070, China

highly heterozygous genome, the homozygous genome sequencing has been performed for several woody species, such as peach, apple, cocoa and citrus (Dunwell 2010; Argout et al. 2011; The International Peach Genome Initiative 2013; Xu et al. 2013; Wu et al. 2014).

Despite the number is growing, the frequency of spontaneous haploids is still too low for practical application in breeding (Germanà 2011b). Conventional breeding method that employs several generations of selfing is unpredictable and time-consuming (Srivastava and Chaturvedi 2008). Gametic embryogenesis, serves as an effective single-step approach to produce homozygous lines, have been applied to regenerate the majority of plant haploids and DHs. Gametic embryogenesis is divided into two ways as androgenesis which includes anther culture, microspore culture and microspore suspension culture, and gynogenesis which includes in vitro pollination with triploid pollen or irradiated pollen, in vivo parthenogenesis and cross. The first haploid was regenerated in vitro from immature anthers in *Datura innoxia* (Guha and Maheshwari 1964). Since then, homozygous lines have been regenerated from more than 250 plant species by gametic embryogenesis (Dunwell 2010). It is reported that haploids could also be obtained through transgenics in *Arabidopsis thaliana* (Ravi and Chan 2010; Wijnker et al. 2012). However, whether such an approach works on other species is still unknown.

Citrus is one of the most important fruit trees of a great economic and health value worldwide (Gmitter et al. 2012). Citrus homozygous lines can hardly be developed through conventional methods due to the high heterozygosity, sexual incompatibility, nucellar embryony, severe inbreeding depression, large size, and long juvenility in most citrus species (Germanà and Chiancone 2001). However, these problems could be solved by gametic embryogenesis, in which anther culture is considered as the most efficient method due to its simplicity in manipulation and applicability in a wide range of genotypes (Benelli et al. 2010; Germanà 2011a, b).

In citrus, the first case of haploid derivation from anther culture was reported in 1975 (Drira and Benbadis 1975), which greatly stimulated the motivation of inducing haploid plants in *Citrus* genus. To date, through citrus gametic embryogenesis, homozygous lines have been obtained from several major cultivated species, including trifoliate orange (Hidaka et al. 1979; Hidaka 1984a, b; Deng et al. 1992), mandarin (Germanà et al 1994, 2005a, b; Germanà and Chiancone 2003; Chiancone et al. 2006; Froelicher et al. 2007; Aleza et al. 2009; Cardoso et al. 2014), pummelo (Toolapong et al. 1996; Yahata et al. 2010, 2015) and lemon (Germanà et al. 1991). However, the majority of the generated homozygotes of mandarin have the genetic background of clementine tangerine (Germanà 2006; Srivastava and Chaturvedi 2008). The genotypes of sweet orange have shown high level of recalcitrance for gametic embryogenesis except for two reports of Cao et al. (2011) (only DH callus lines of Valencia sweet orange were maintained) and Cardoso et al. (2014) (only tri-haploid callus lines of hybrid sweet orange crossed with clementine tangerine were produced). The recovery of sweet orange homozygous individual plants was not yet reported. Thus the induction of homozygous lines of sweet orange particularly the recovery of sweet orange homozygous individual plant will be of great importance.

In the present study, we report the recovery of ten haploid lines, six DH lines, two tetraploid lines of 'Early Gold' sweet orange, and one DH callus line of Valencia sweet orange cv. Rohde Red, by inducing seven citrus cultivars with four referenced mediums through anther culture. The most influencing medium was determined by comparing embryogenesis rates among mediums. We also characterized the homozygous lines by ploidy, karyotype and simple sequence repeats (SSRs) analysis, and found that all the homozygous lines were fully homozygous and the two tetraploid lines were doubled heterozygous diploids.

Materials and methods

Plant materials

Immature citrus floral buds were collected from the National Center of Citrus Breeding (NCCB) in the Institute of Citriculture, Huazhong Agricultural University (HZAU), Wuhan, China. The citrus species are 'Early Gold' sweet orange (*Citrus sinensis* [L.] Osbeck), 'Rohde Red' Valencia sweet orange (*C. sinensis* [L.] Osbeck), Red tangerine (*C. reticulata* Blanco), Ponkan mandarin (*C. reticulata* Blanco cv. Egan No. 1), Huanong Bendizao tangerine (*C. reticulata* Blanco), HB pummelo (*C. grandis* [L.] Osbeck 'Hirado Buntan') and Huanong red pummelo (*C. grandis*). The developmental stage of the microspore collected from floral buds of different size was checked by acetic-carmine staining (Germanà 2005). The floral buds containing anthers with high percentage of microspores at the uninucleate stage were chosen (Fig. 1a, b), followed by pretreatment under 4 °C for 3–7 day in darkness.

Anther culture

Collected floral bud were surface sterilized by immersion in 1 mol/L HCl for 30 s, followed by decontamination in sodium hypochlorite (active Cl^- more than 3 %), and rinsed 3–5 times for 3 min in sterile distilled water. Anthers were excised without petals and filaments, and then placed on the surface of 50 mL medium in 100 mL conical flasks (Deng et al. 1992). Four culturing mediums

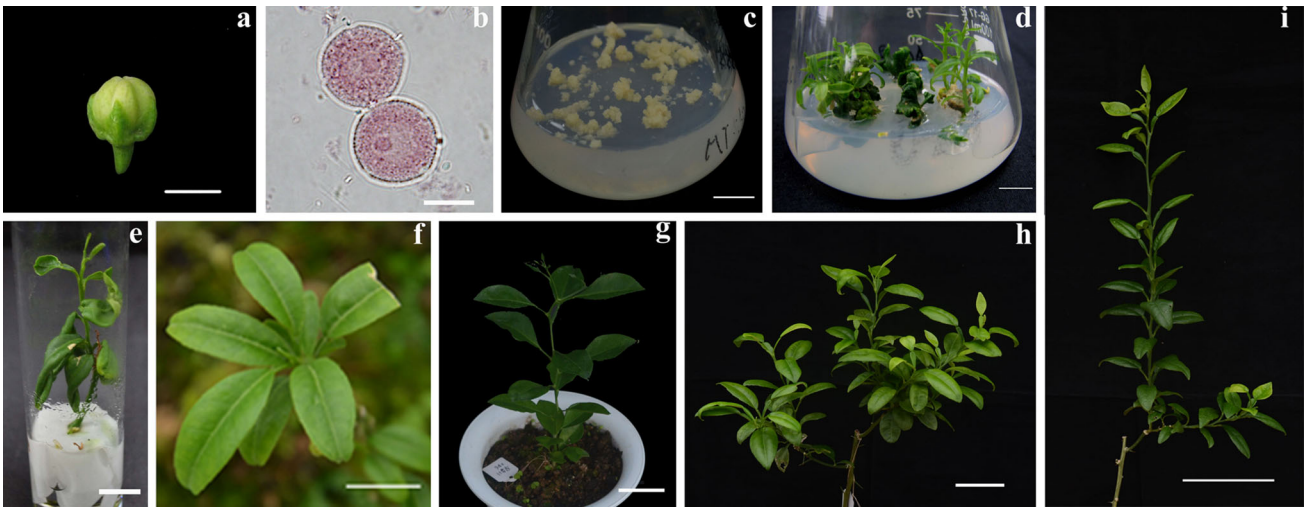

Fig. 1 Anther culture and plant regeneration of 'Early Gold' sweet orange. **a** The morphology of flower at uninucleate stage, **b** Microspores at uninucleate stage, **c** The callus of homozygous line, **d** Embryoid germination in the shoot induction medium, **e** In vitro grafting of shoot, **f** Line A haploid plant grafted on trifoliate orange in vitro, **g** Plant of tetraploid line, **h** Line A DH plant grafted on trifoliate orange, and **i** Line B Plant grafted on trifoliate orange. *Bars* in **a** = 0.5 cm, **b** = 10 μm, **c**, **d**, **e**, **f** = 1 cm, and **g**, **h**, **i** = 5 cm

Table 1 Four mediums used for citrus anther culture

Medias	Basal media	Carbon source (g/L)	Hormone (mg/L)	Other	References
M1	1/2MT	Sucrose 25	NAA 0.1	0.5 g/L active carbon	Deng et al. (1992)
M2	MT	Sucrose 50	IAA 0.2; KT 0.2		Hidaka et al. (1979)
M3	N_6	Lactose 18; galactose 9	NAA 0.02; 2,4-D 0.02; KT 1.0; 6-BA 1.0; GA 0.5; TDZ 0.1; Zeatin 0.5	0.5 g/L casein	Germanà and Chiancone (2003)
M4	N_6	Sucrose 35	NAA 0.2; KT 1.0; 6-BA 0.5; GA 0.5; Zeatin 0.5		Germanà (2005)

were used and named as M1, M2, M3, and M4 (Table 1). For each genotype, more than 1000 anthers were induced on each of the mediums, making a total of 64,000 (Table S1). The anther-derived embryoids and calluses emerged 3 months after culture at 25 °C in the dark without any subcultures. Callus and embryoid were counted and recorded. The inducing rate is calculated as the number of callus and embryoid lines divided by the number of cultured anthers.

Culture maintenance, embryoid germination and plant regeneration

The anther-derived callus lines were sub-cultured onto the MT medium (40 mL in 100 mL conical flasks) without any plant growth regulators at 25 °C in the dark to store experimental materials and in the light (40 μmol m^{-2} s^{-1} illumination for 16 h daily) to induce embryoids. The anther-derived embryoid lines and callus-derived embryoids were sub-cultured onto shoot-induction medium (MT basal media supplemented with 0.5 mg/L 6-BA, 0.2 mg/L KT, 0.5 mg/L NAA, 0.1 mg/L IBA, 40 g/L sucrose, 8 g/L agar, pH 5.8) at 25 °C under 40 μmol m^{-2} s^{-1} illumination for 16 h daily. Subsequently, the induced shoots were subcultured onto root-induction medium (1/2 MT basal medium supplemented with 0.5 mg/L NAA, 0.1 mg/L IBA, 25 g/sucrose, 8 g/L agar, pH 5.8). Finally, the induced rooting plantlets were transferred to 200 mL plastic pots containing steam sterilized artificial soil mix suitable for growing citrus (40 % black peat, 20 % washed sand, and 40 % soil). To obtain homozygous plants, the transplanted shoots in the root-induction medium that could hardly root but could elongate significantly with lignified stems were grafted onto citrange (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf.) in the greenhouse, whereas the weak shoots without elongated and lignified stems were micro-grafted onto citrange seedling rootstocks grown in the culture medium (MT basal medium supplemented with 20 g/L sucrose) in darkness.

Ploidy analysis and chromosomal cytogenetic analysis

Ploidy analysis was conducted using a 'CyFlow space' flow cytometer (Cyflow space, Munster, Germany) as described by Guo et al. (2007) and Xiao et al. (2014b). Approximately 0.5 cm^3 fresh leaf was chopped with 400 μL Partec HR-A buffer in a plastic Patri dish. The sample was then stained with 1.6 mL Partec HR-B buffer. After being filtered by using 30 μm micropore filter, the fluorescence of total DNA was measured, and for each sample at least 3000 cells were analyzed.

For chromosome analysis, root-tips were pretreated with saturated p-dichlorobenzene for 2 h at 20 °C, then with 0.075 mol KCl for 30 min at room temperature, finally fixed in 3:1 ethanol-acetic acid (v/v) for 24 h at room temperature and stored in 75 % ethanol at 4 °C. Sections were stained by DAPI and CMA according to Miranda et al. (1997) with minor modification. Images were captured by a fluorescent microscope (Olympus BX 61, Japan) with UV and BV filters.

Molecular characterization

The extraction of genomic DNA was conducted according to Cheng et al. (2003). The isolated DNA was diluted to 100 ng/ μL for SSR analysis. Out of 223 SSR primers mapping to the genome of sweet orange (http://citrus.hzau.edu.cn/orange), 31 (Table S2) were selected because of the allelic diversity of their loci represented in the genome and the polymorphism they revealed among the corresponding anther donor parents. PCR amplifications were performed in a MJ-PTC-200 thermal controller (MJ Research, Waltham, MA, USA) in a 20-μL final volume containing 1 U of *Taq* DNA polymerase, 100–200 ng/mL citrus DNA, 0.25 μM forward primer, 0.25 μM reverse primer, 0.2 mM of dNTP mix, 1×Taq PCR buffer and 1.75 mM $MgCl_2$. The PCR program was set as following: denaturation at 94 °C for 5 min, followed by 32 cycles of (1 min at 94 °C, 45 s at 57 °C, 1 min at 72 °C), and a final elongation step of 4 min at 72 °C. Polyacrylamide gel analysis was performed according to Cheng et al. (2003). DNA banding pattern was visualized with silver staining as described by Ruiz et al. (2000).

Results

Inducing medium M1 is most effective for sweet orange anther culture

In this study, to test the embryogenesis efficiency of the inducing medium, we adopted four previously reported mediums in this experiment and calculated the embryogenesis percentages for all the seven cultured cultivars. M1 was effective for 'Early Gold' sweet orange, Rohde Red orange and Egan No. 1 Ponkan, with inducing rate of 0.85, 0.09 and 0.08 %, M3 was effective for 'Early Gold' sweet orange and Red tangerine, with inducing rate of 0.07 and 0.05 %, whereas M4 was only effective for 'Early Gold' sweet orange with an inducing rate of 0.20 %. Taken together, M1 was the most effective medium for citrus anther culture particularly for sweet orange, with embryogenesis in three cultivars, whereas M2 was noneffective on all of the selected citrus cultivars (Table 2). The inducing effect of distinct medium was different among citrus cultivars. Calluses or embryoids were induced from only four cultivars, whereas the other three cultivars (Huanong Bendizao tangerine, HB pummelo and Huanong red pummelo) were recalcitrant to regenerate via anther culture. On M1 medium, 'Early Gold' sweet orange showed the highest embryogenic callus and embryoid inducing rate (0.85 %), which was much higher than the average inducing rate (0.35 %) of the other six tested cultivars (Table 2), suggesting that specific genotype might play an important role for regeneration via anther culture.

Callus/embryoid induction and regeneration from anther culture

After 3-month incubation in darkness, embryoids and calluses were observed to grow from yellowish and brownish anthers (Fig. 1c). We obtained 26 callus lines and 14 embryoid lines from 'Early Gold' sweet orange, two callus lines from Rohde Red orange, three embryoid lines from Egan No. 1 Ponkan, and one embryoid line from Red tangerine (Table 2). However, no callus line or embryoid line was obtained from the remaining three cultivars (not shown in Table 2). Fourteen 'Early Gold' sweet orange callus lines and one Rohde Red orange callus lines died before genetic analysis. One haploid callus line of 'Early Gold' sweet orange was too weak for DNA extraction.

The molecular genetic analysis was performed for all of the obtained embryoid lines and the 12 callus lines (one line of 'Rohde Red' sweet orange and 11 lines of 'Early Gold' sweet orange). The results indicated that five embryoid lines and 11 callus lines of 'Early Gold' sweet orange and one callus line of 'Rohde Red' sweet orange were homozygous. Two lines of 'Early Gold' sweet orange were heterozygous tetraploids (Fig. 1g).

By embryoid induction, eight anther-derived callus lines developed into embryoids (Fig. 1d). The pure embryoid lines including the anther-derived lines and callus-derived embryoid lines were induced to produce shoots and roots through several sub-cultures on shoot-induction medium and root-induction medium. However, the rooting

Table 2 Inducing rate of different citrus cultivars incubated with four culture mediums

Cultivar	Embryoids	Calluses	Inducing rate (%)	Total derived lines	Total inducing rate (%)	No. homozygous lines		The rate of homozygous lines (%)	Homozygous lines with regenerated plants
						Embryoids	Calluses		
'Early Gold' sweet orange									
M1	8	26	0.85	40	0.35	4	12	40	3 (2 DH lines and 1 tetraploid line)
M2	0	0	0						
M3	2	0	0.07						
M4	4	0	0.20						
'Rohde Red' sweet orange									
M1	0	2	0.09	2	0.03	0	1	100	0
M2	0	0	0						
M3	0	0	0						
M4	0	0	0						
'Egan No. 1' Ponkan mandarin									
M1	3	0	0.08	3	0.04	0	0	0	0
M2	0	0	0						
M3	0	0	0						
M4	0	0	0						
Red tangerine									
M1	0	0	0	1	0.01	0	0	0	0
M2	0	0	0						
M3	1	0	0.05						
M4	0	0	0						

The inducing rate was calculated as the number of callus and embryoid lines divided by the number of cultured anthers

The rate of homozygous lines was calculated as the number of homozygous lines divided by the number of callus and embryoid lines

percentage was low and most shoots could not root, except the two tetraploid lines showing strong rooting capability. Only three haploid (Fig. 1f) and three DH rooting-plants of a line (named line A) were obtained by inducing more than 100 shoots. Despite our efforts, all the obtained homozygous rooting-plants died after transplanting to the greenhouse. The regenerated shoots without rooting were grafted onto trifoliate orange to improve the survival rate (Fig. 1e). Currently, two grafted DH plants (an embryoid-derived line named line A and a callus-derived line named line B) of 'Early Gold' sweet orange that were transplanted to the greenhouse are growing vigorously (Fig. 1h, i), and more than 10 plants of each line were vegetatively produced and are normally growing. The homozygous lines without plant regeneration are in vitro preserved for potential basic research. The DH plants have visible morphological difference such as narrower leaves, shorter internodal segments and bigger wing cascades compared with their donor parent.

Ploidy and karyotype analysis of homozygous lines

The ploidy level analysis of all pure lines by flow cytometry demonstrated that, for 'Early Gold' sweet orange, eight callus lines and two embryoid lines were haploid (Fig. 2b), three embryoid lines and four callus lines were doubled haploids (Fig. 2c); the rest two embryoid lines were tetraploid (Fig. 2d) which proved to be doubled diploids by genetic analysis; for 'Rohde Red' Valencia sweet orange, the only one callus line was haploid. We also analyzed the two grafted DH plants of 'Early Gold' with vigorous growth in the greenhouse, and found that some of the plantlets of line A were doubled haploids resulted from spontaneous chromosome doubling derived from haploid embryoid, whereas the plantlets with weak growth were still haploid; however, all of the line B plantlets were doubled haploids derived from doubled haploid callus line.

Based on the chromosome CMA banding pattern and classification (Guerra 1993), chromosome karyotype analysis revealed that the two donor parents ('Early Gold' sweet orange and 'Rohde Red' Valencia sweet orange) had the same karyotype formula 1B + 1B′ + 2C + 6D + 1D′ + 7F (Fig. 3a) (Fig. 3b in Xu et al. 2013), indicating their close relationship. However, the two Bs were designated separately as B (with no fragile site) and B′ (with fragile site), and the two Ds as D (with no fragile site) and D′ (with fragile site). DH line A of 'Early Gold' sweet orange has the chromosome configurations as 2B′ + 4C + 6D + 6F

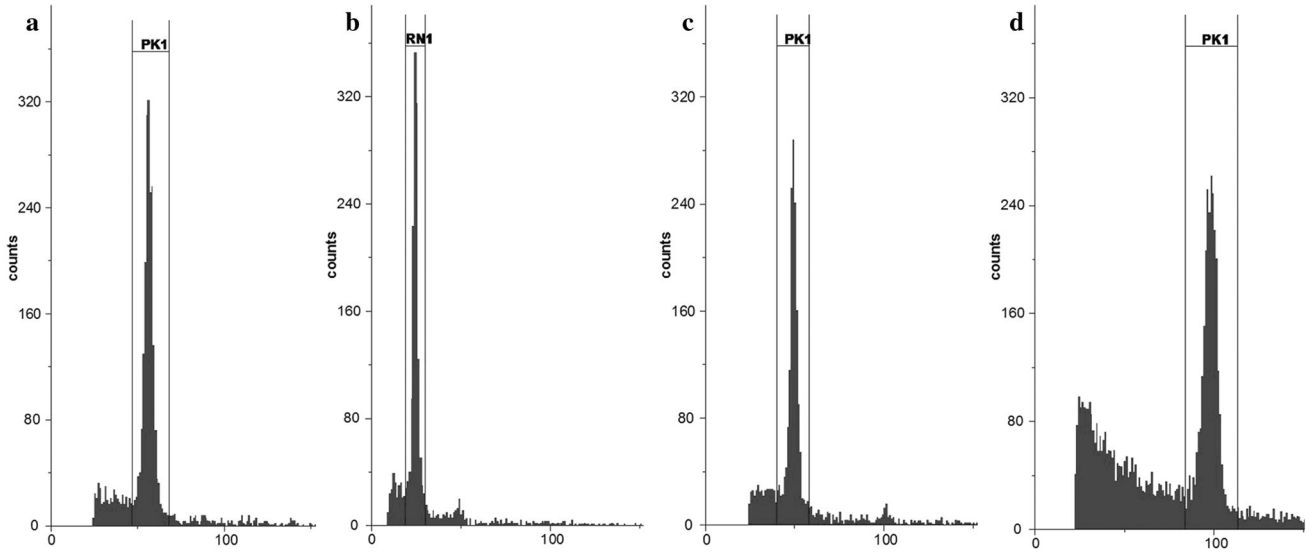

Fig. 2 Ploidy determination by flow cytometry analysis. **a** Diploid parent plant of 'Early Gold' sweet orange, **b** Anther-derived haploid line of 'Early Gold' sweet orange, **c** Anther-derived DH line of 'Early Gold' sweet orange, and **d** Anther-derived tetraploid line of 'Early Gold' sweet orange

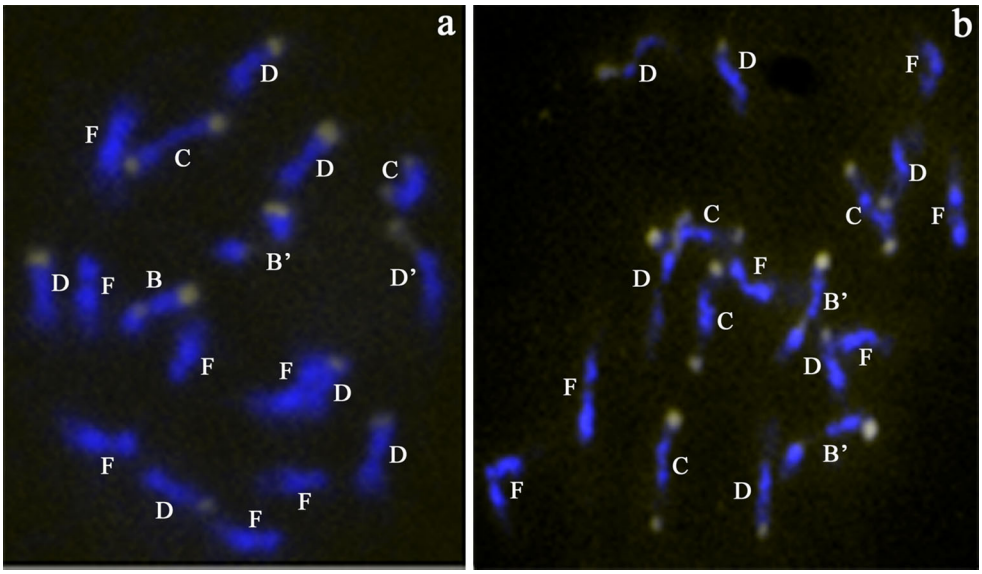

Fig. 3 Karyotype analysis of 'Early Gold' sweet orange and its DH line. **a** Karyotype analysis of 'Early Gold' sweet orange donor parent (1B + 1B′ + 2C + 6D + 1D′ + 7F), and **b** Karyotype analysis of 'Early Gold' sweet orange DH line A (2B′ + 4C + 6D + 6F)

(Fig. 3b), whereas the DH line of 'Rohde Red' Valencia sweet orange reported by Cao et al. (2011) as 2B + 2B′ + 2C + 4D + 2D′ + 6F (Fig. 3b in Xu et al. 2013). The results demonstrate that line A is homozygous with 18 chromosomes and has no homologous chromosome pair. The difference of karyotype formula between the two DH lines also suggests that they differed in chromosome combination. Meanwhile, the DH line A of 'Early Gold' sweet orange had only two fragile sites while the DH line of 'Rohde Red' Valencia sweet orange had four.

SSR analysis confirmed the homozygosity of the recovered lines

The allelic constitution of all regenerates derived from anther culture was compared with that of their donor

parents by using 10 polymorphic SSR markers with which two different alleles could be traced in the parent donors. The regenerated lines would be homozygous when exhibiting only one of the allele pairs at all loci, but heterozygous when exhibiting the same banding patterns with those of their parent donors.

All of the obtained homozygous lines, and the two tetraploid lines were analyzed using another set of 31 SSR markers to further confirm their homozygous degree with the aid of the two anther donor parents and another four cultivars as control, including Mangshanyegan (*C. nobolis* Lauriro), Nanfengmiju tangerine (*C. reticulata* Blanco), Guanximiyou pummelo (*C. grandis* Osbeck) and Shatian pummelo (*C. grandis* Osbeck) (Fig. 4a–c). The 31 SSR markers had been mapped to the genome of sweet orange and their location on chromosomes was randomly distributed with at least two primer pairs per chromosome (Table S2). The results showed that all of the regenerated lines had a single allele in each locus, indicating that all homozygous lines were fully homozygous without homologous chromosomes, except primer M1H2Si17674 and M3H2Si35174, where the regenerated lines had a single or two alleles but the donor parents had three alleles (Table 3), and the fact that SSR analysis by primer M9H2Si25123 produced two bands in haploid No. 6 might be due to chromosome exchange and translocation in the process of microspore formation. However, these exceptions could not negate the homozygosity of these lines. Thus, the results indicated that all homozygous lines were fully homozygous. In 16 out of 29 primers, the allele segregation conformed to the law of allele segregation (a 1:1 ratio of the two alleles) shown in Table 3. In addition, the homozygous lines of the same cultivar showed the different banding patterns among single alleles, indicating their different gametophyte derivation. The two tetraploid lines with the same banding pattern of their donor parent were confirmed to be doubled diploids of 'Early Gold' sweet orange. The haploid line C of 'Rohde Red' Valencia sweet orange obtained herein and those reported previously by Cao et al. (2011) displayed different banding patterns, indicating that they were genetically different (Table 3).

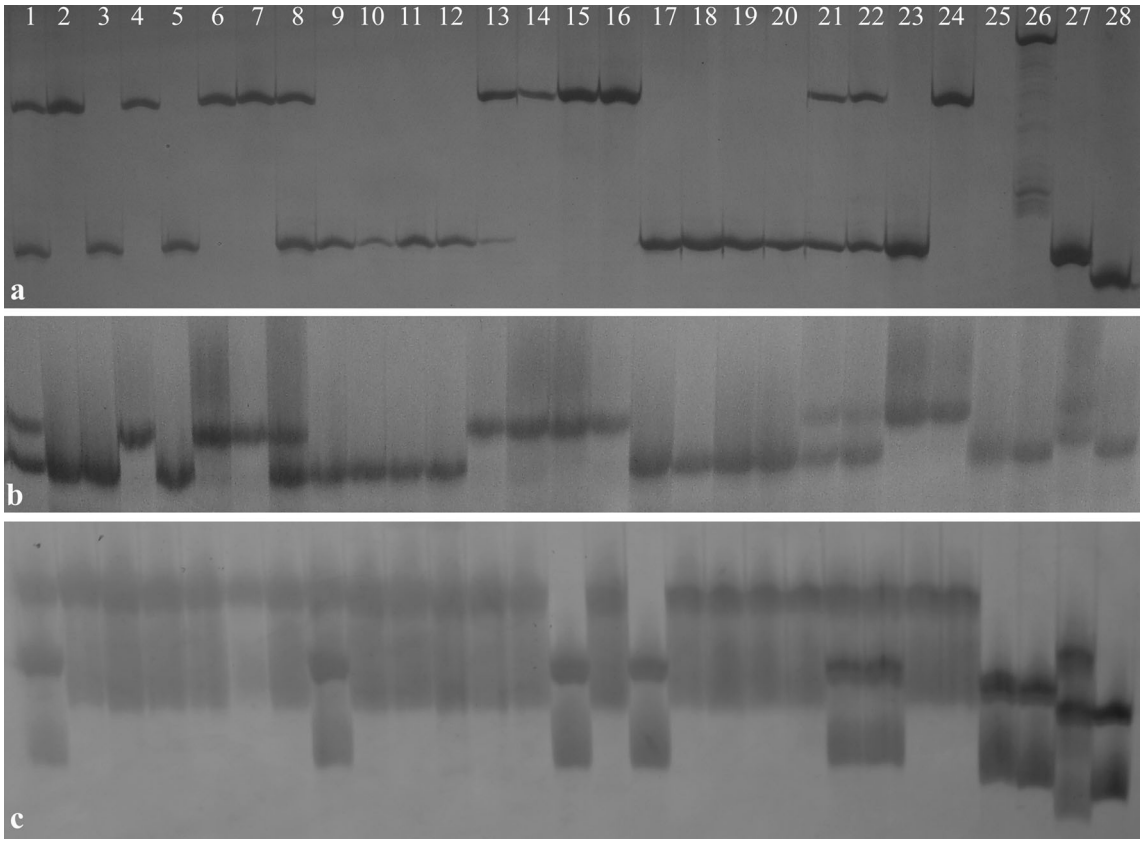

Fig. 4 Molecular analysis of homozygous lines by SSR markers. **a** Primer M3H2Si309, **b** Primer M4H3Si20399, and **c** Primer Mest 132. **1** 'Early Gold' sweet orange, **2–7** and **9–20** Pure lines of 'Early Gold' sweet orange, **8** and **21** Tetraploid line of 'Early gold' sweet orange, **22** 'Rohde Red' Valencia sweet orange, **23** Pure line of 'Rohde Red' Valencia sweet orange, **24** Pure line of 'Rohde Red' Valencia sweet orange reported by Cao et al. (2011), **25** Shatian pummelo, **26** Guanximiyou pummelo, **27** Nanfengmiju tangerine, and **28** Mangshanyegan mandarin

Table 3 SSR markers based Genetic analysis of donor parents, regenerated lines from anther culture of 'Early Gold' sweet orange and 'Rohde Red' Valencia sweet orange

Marker	Linkage group	EG	Haploid										DH						Tetraploid	RRV	Haploid	DH	Alleles a/b	χ
			A	1	2	3	4	5	6	7F	8	9	10	11	12	13	14	B	15		C	D		
M1H3Si6258	1	ab	a	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a	ab	ab	a	a	17/1	12.50
M1H2Si17674	1	abc	ab	a	a	a	a	a	bc	a	a	ab	ab	a	ab	bc	ab	ab	abc	abc	ab	ab	/	/
M1H2Si6452	1	ab	a	b	a	a	a	a	a	a	a	a	a	b	b	a	a	a	ab	ab	b	b	13/5	2.72 ★
M2H2Si20644	2	ab	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	ab	ab	a	a	18/0	7.56
mCrCIR04H06	2	ab	b	a	a	a	a	a	*	a	a	a	*	a	a	a	a	a	ab	ab	a	b	14/2	0.06 ★
mCrCIR03C08	2	ab	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	ab	ab	b	b	18/0	7.56
M3H2Si309	3	ab	b	a	b	b	b	b	a	b	b	a	a	b	a	a	a	a	ab	ab	b	a	9/9	0.06 ★
M3H3Si763	3	ab	b	a	b	b	b	b	a	b	b	b	a	b	b	a	b	b	ab	ab	b	a	4/14	4.50
M3H2Si35174	3	abc	c	c	c	c	c	c	ab	c	b	ab	b	c	c	ab	ab	ab	abc	abc	c	b	/	/
M3H3Si28170	3	ab	b	a	a	a	a	a	b	a	a	b	b	a	b	b	b	*	ab	ab	b	b	8/9	0.00 ★
M4H3Si5495	4	ab	b	a	b	b	b	b	b	b	b	b	b	a	a	b	b	b	ab	ab	a	b	4/14	4.50
M4H3Si20399	4	ab	b	b	b	b	b	b	a	b	b	a	a	b	a	a	a	a	ab	ab	a	a	9/9	0.06 ★
M4H2Si10740	4	ab	b	b	a	a	a	a	b	a	a	b	b	a	b	b	b	b	ab	ab	b	b	7/11	0.50 ★
M5H2Si33478	5	ab	a	a	a	a	a	a	b	a	a	b	b	a	b	b	b	b	ab	ab	b	b	9/9	0.06 ★
M5H10Si4489	5	ab	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	ab	ab	a	*	16/1	10.94
M6H2Si11486	6	ab	a	b	b	b	b	b	a	b	b	b	b	b	b	a	b	b	ab	ab	b	b	2/16	9.38
Mest 123	6	ab	a	a	b	b	b	b	b	b	b	a	a	a	a	b	a	a	ab	ab	b	a	9/9	0.06 ★
Mest 132	6	ab	a	a	a	a	a	a	b	a	a	a	a	a	a	b	a	a	ab	ab	a	a	16/2	9.39
M7H9Si2367	7	ab	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	ab	ab	b	a	17/1	12.50
M7H2Si19631	7	ab	a	b	a	a	a	a	b	a	a	a	b	b	b	b	a	a	ab	ab	a	a	12/6	1.39 ★
M7H2Si31270	7	ab	a	a	a	a	a	a	b	a	a	b	b	a	b	b	b	b	ab	ab	b	b	9/9	0.06 ★
Csin.0067	7	ab	a	b	a	a	a	a	a	a	a	a	a	b	b	a	a	a	ab	ab	a	a	15/3	6.72
M8H3Si29283	8	ab	b	a	a	a	a	a	b	a	a	b	a	a	b	b	b	b	ab	ab	b	a	10/8	0.06 ★
M8H2Si17550	8	ab	b	b	a	a	a	a	b	a	a	b	a	a	b	b	b	b	ab	ab	b	a	9/9	0.06 ★
M8H4Si9690	8	ab	b	b	a	a	*	a	b	a	a	a	b	a	b	b	a	a	ab	ab	b	b	9/8	0.00 ★
M8H3Si9989	8	ab	b	b	a	a	a	a	b	a	*	a	a	a	b	b	a	a	ab	ab	b	b	10/7	0.24 ★
Csin.0087	8	ab	a	a	b	b	b	b	a	b	b	a	b	b	a	a	a	a	ab	ab	a	b	9/9	0.06 ★
Csin.0080	8	ab	b	b	a	a	a	a	b	a	a	b	a	a	b	b	b	b	ab	ab	b	a	10/8	0.06 ★
M9H2Si25123	9	ab	a	b	a	a	a	a	ab	a	a	a	b	a	a	a	a	a	ab	ab	a	b	15/4	5.61
M9H2Si25847	9	ab	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	ab	ab	a	a	18/0	16.06

EG 'Early Gold' sweet orange; A, 1, 2, 3, 4, 5, 6, 7, 8, 9 represent the haploid lines of 'Early Gold' sweet orange, among which A and 1 were derived from embryoids, whereas 2–9 were derived from calluses; 10, 11, 12, 13, 14, B represent the DH lines of 'Early Gold' sweet orange, among which 10 and 11 were derived from embryoids, whereas 12–14 and B were derived from calluses; 15 represents the tetraploid line of 'Early gold' sweet orange; RRV represents 'Rohde Red' Valencia sweet orange; C represents the haploid line of 'Rohde Red' Valencia sweet orange; D represents the DH line of 'Rohde Red' Valencia sweet orange reported by Cao et al. (2011)

$\chi^2 = \sum \frac{(|O-E|-1/2)^2}{E}$, in this formula, O and E were observed and theoretical number of alleles a and b respectively. Symbol ★ represents the allele segregation fits a 1:1 ratio of the two alleles (χ^2 value (0.05, 1) <3.84)

* Represents no band was amplified by this primer in the experiment

Discussion

Culture medium composition is pivotal for haploid induction via anther culture (Germanà 2011a). The most commonly used basal mediums for anther culture are N_6 media (Germanà and Chiancone 2003), MS (Hidaka et al. 1979; Hidaka 1984b; Chen 1985) and MT (Deng et al. 1992; Cao et al. 2011). In our investigation, the recipe of M1 (basal medium is 1/2 MT medium) was found to be most suitable for microspore embryogenesis of sweet oranges, such as 'Early Gold' sweet orange and 'Rohde Red' Valencia sweet orange. In contrast, M3 (the basal medium is N_6 medium) was reported effective for the clementine and its relatives (Germanà et al. 1994; Germanà and Chiancone 2003; Cardoso et al. 2014). Thus, the medium seemed to be selective for the induced species. Additionally, as most researchers recognized, the genotype also played a major role. Factors affecting anther culture have been optimized by several experiments using different combinations (Hidaka 1984a; Germanà et al. 2005a, b; Chiancone et al. 2006). These included the endogenous factors like genotype, pollen developmental stage, physiological state and growth conditions of donor plants, as well as the exogenous factors like pretreatment, medium composition and culture conditions. However, the majority of citrus species remain recalcitrant for anther culture.

In addition to the challenge of low inducing rate and species recalcitrance, the regeneration of homozygous lines was also difficult. Cao et al. (2011) reported that two Valencia sweet orange lines derived from anther culture grew weakly and the leaves gradually etiolated and abscised even though the homozygous scions were grafted onto trifoliate orange rootstock. All haploid plants of clementine, obtained by androgenesis or gynogenesis, displayed a weak appearance and poor growth, and most of the regenerated plants typically died in test tubes or in the greenhouse (Aleza et al. 2009). The same problem is common in other woody plants (Peixe et al. 2000; Kadota et al. 2002; Li et al. 2013). The expression of recessive deleterious or lethal genes might be responsible for their weak growth and death.

Spontaneous chromosome doubling is quite common in the plant kingdom, and it also occurs during in vitro anther culture. The percentage of doubling in the anther culture process is affected by the genotype, developmental stage of the microspores, type of pretreatment and pathway of development, even if the average percentage is very high in some species such as rice, barley and wheat (Germanà 2011a). In citrus, DHs and even tri-ploid homozygous lines have been obtained in several cultivars (Deng et al. 1992; Germanà et al. 2005b; Cao et al. 2011; Cardoso et al. 2014). The origin of tri-haploids obtained from anther culture was explained by a spindle fusion mechanism (Germanà et al. 2005b): the endoreduplicate generative nucleus (n diplochromosomes) and the vegetative nucleus (n chromosome) divide on a common spindle, giving rise to 2 triploid daughter nuclei. In our study, three of the five homozygous embryoid lines and four of the twelve homozygous callus lines were DHs, demonstrating a high percentage of doubling. In addition to the DH lines, two tetraploid lines were also obtained, further revealing a common doubling in citrus. Chromosome doubling leads to increased cell size and genetic diversity, which could promote better adaptation to chronic injury or stress. Compared with the majority of haploid plants, the plantlets of DHs displayed more robust appearance and more vigorous growth, which could also be applied in crosses for breeding and in homozygous fruiting study after blossom.

Chromosomal identification was performed to reveal the relationship, origin and heterozygosity of citrus cultivars (Cornelio et al. 2003; Moraes et al. 2007). The chromosomal cytogenetic analysis is difficult to perform in the pure line, because of the low root inducing rate, less metaphase cells resulted from slow growth and undetermined enzymolysis condition. To our knowledge, the only reports available on the karyotype analysis of citrus homozygous lines are on a haploid clementine (Germanà and Chiancone 2003) and a haploid-DH variant clementine (Yamamoto and Tominaga 2004). In this investigation, we only obtained well-conditioned root tips from one DH line of 'Early Gold' for chromosomal karyotype analysis. The chromosomal cytogenetic analysis results not only confirmed the ploidy level but also proved the homozygosity status, which well supported the SSR results. The karyotype formula of the only DH 'Early Gold' line presented here was different from that of the DH line of Valencia sweet orange (Xu et al. 2013). The haploid and DH lines can be applied in the citrus resequencing and chromosomal variation study.

During embryogenic process of anther culture, the regenerated lines might originate from two different ways, i.e., haploid cell from microspores or somatic cell from anther tissues (Hofer et al. 2002). However, the study of microspore culture on an interspecific hybrid *Brassica napus* L. × *Brassica carinata* Braun showed that microspore culture preferentially selected unreduced (2n) gametes, with 26 of the 28 progenies (93 %) derived from unreduced gametes (Nelson et al. 2009), which posed the question of whether the heterozygous lines obtained in our study originated from 2n-male gametophytes. The 2n-male gametophytes would not be the same with their parent completely in genetic background because of chromatid exchange and translocation in the progress of chromosome synapsis. In this study, all markers were selected randomly

and evenly located at 9 linkage groups of citrus; some loci of the markers were closer to the centromere whereas the others closer to the telomere, so they could indicate whether the derived lines were the same to their donor parent. The banding patterns of the two regenerated tetraploid lines and all derived heterozygous diploid lines were the same as their donor parent, confirming that they all derived from somatic cell of anther tissues. So, to reduce the disturbance of anther somatic cells is a key to improve the homozygous rate via anther culture.

Sweet orange shows high levels of recalcitrance for gametic embryogenesis, with rare reports on homozygous induction, except the cases on a DH callus line (Cao et al. 2011) and a hybrid of sweet orange and clementina (Cardoso et al. 2014). In this study, using four mediums for anther culture, we recovered ten haploid lines, two tetraploid lines, six DH lines of 'Early Gold' sweet orange, and one haploid line of 'Rohde Red' Valencia sweet orange. The ploidy, karyotype and SSR analysis indicated that all the homozygous lines were completely homozygous and the two tetraploid lines were doubled heterozygous diploids. The identified haploid and tetraploid lines reported here hold great potential for future citrus seedless breeding by somatic fusion or sexual cross. The homozygous lines can be applied to elucidate the difference of morphology, chromosome, gene integrity, gene dosage, metabolism caused by haploidization, compared with their parents, as well as to genomic resequencing for explicating genomic rearrangements and citrus genetic relationship.

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Authors contribution SMW conducted most experiments, data analysis and wrote the manuscript. HL, HBC and CLC participated in chromosomal cytogenetic analysis. QX and XXD mined and provided some SSR markers. WWG conceived, supervised the research and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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