Induction and Genetic Verification of Haploid-Derived Plants Using Anther culture of *Citrus Aurantium* L. (Sour Orange)

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Abstract

This study aimed to genetically verify whether *Citrus aurantium* L. (sour orange) plants grown using anther culture were obtained from somatic embryos or haploids. The ploidy of the individuals was assessed using ploidy measuring instruments and simple sequence repeat (SSR) molecular markers. The anthers of sour oranges were cultured onto a medium supplemented with 0.44 mg/L thidiazuron (TDZ), 0.8 mg/L 6-benzylaminopurine (BA), 0.43 mg/L Zeatin, 0.44 mg/L Kinetin, 0.2 mg/L a-naphthalene acetic acid (NAA), 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 500 mg/L malt extract. Somatic embryos and callus were obtained using dentin in N6 medium supplemented with malt extract (500 mg/L) and N6 liquid medium supplemented with spermidine (200 μM), and gibberellic acid (GA_3, 1 mg/L) was added to the anther surface. Regenerated plants were obtained from Murashige and Skoog medium supplemented with 0.5 mg/L TDZ, 1.0 mg/L GA_3 (or single use 0.5 mg/L) and 500 mg/L malt extract. For the genetic verification of the obtained plants, haploid-derived plants were investigated by polymerase chain reaction using two SSR markers. In addition, verification with a ploidy meter confirmed that approximately 80–90% of the regenerated plants were triploids, and two plants among the investigated plants were haploids. This study was the first to report these results among haploid and triploid studies obtained from an anther culture of sour oranges. The study findings suggested that the anther culture technique can produce seedless triploid cultivars of domestic citrus varieties.

Key Message

This is the first study to obtain haploid and triploid sour orange plants using the anther culture technique and genetically verify that this technique can develop triploid, seedless varieties.

1. Introduction

Citrus fruits (Rutaceae family) are one of the most economically important fruit crops (Cardoso et al. 2014; Huh et al. 2011; Jung et al. 2005). They have high supply and demand and have the third largest area under cultivation worldwide, after apple and banana (Aboshama, 2011). However, citrus fruit cultivation has a disadvantage, as it requires long time and intense labor to cultivate superior pure lines using conventional breeding methods, such as crosses, because of characteristics such as high heterozygosity, long juvenility, self-incompatibility, and polyploidy (Benelli et al. 2010; Cardoso et al. 2014; Chiancone et al. 2015; Chiancone and Germanà 2016; Germanà 2007, 2009). Furthermore, selecting a purebred line requires an extensive amount of breeding time (Lee et al. 2016).

The breeding time required to select purebred lines can be shortened by using haploid plants obtained from anther culture, which produces completely homozygous lines from heterozygous parents in one step (Chiancone and Germanà 2016; Karjee et al. 2020). Using this technique, the phenotype of recessive traits can be confirmed immediately (Burbulis et al. 2005; Lee et al. 2016; Niroula and Bimb 2009). However, haploid plants are smaller and have vitality lower than their parents, and they cannot undergo normal meiosis, which makes generational progress impossible (Karjee et al. 2020; Lee et al. 2016). In contrast, doubled haploids (homozygous) that are obtained from natural or artificial treatments can enable

generational progress (Lee et al. 2016). Consequently, individuals of homozygous lines obtained from these haploids can be used for important breeding applications, such as mutagenesis, transformation, genetic analysis, and gene sequencing (Chiancone and Germanà 2016; Cimò et al. 2016; Germanà et al. 2013).

The anther culture has a high potential for plant breeding and crop improvement (Das et al. 2018; Mishra and Goswami 2014) and has been widely studied for many plant species (Aboshama 2011; Hidaka 1984). A few cultivars of citrus plant obtained using anther culture include *Poncirus trifoliata* (L.) Raf., *C. clementina* Hort. ex Tan, *C. sinensis* (L.) Osbeck (sweet orange), *C. sinensis* (L.) Osbeck (Valencia sweet orange), and *C. limon* (L.) Burm. f. (lemon), Clausena excavated the Burm. (Cao et al. 2011; Cardoso et al. 2014; Froelicher and Ollitrault 2000; Germanà et al. 2005; Germanà and Chiancone 2003; Wang et al. 2015). In addition, haploid citrus plants have been obtained by irradiation (Aleza et al. 2009; Froelicher et al. 2007; Kundu et al. 2017; Wang et al. 2016; Yahata and Kunitake 2018). However, until now, systematic breeding of citrus varieties using a combination of various pure lineages has been insufficient. Previously, citrus varieties were produced using traditional crossbreeding methods, such as tetraploid and diploid crossbreeding, to obtain seedless triploid-free citrus fruits (Germanà et al. 2005; Grosser et al. 2000; Grosser and Gmitter 2011). Recent studies have shown that a triploid plant can also be obtained using anther culture (Chiancone et al. 2006; Germanà et al. 2005; Germanà 2007, 2009, 2011).

In this study, we aimed to identify the composition of anther culture medium that is most suitable for developing citrus embryos and investigate the anther culture efficiency of “sour orange,” which is commercialized as an anther tissue culture material. We verified the ploidy and investigated the genetic change in the plant obtained from a haploid.

2. Materials And Methods

2.1 Plant materials and pretreatment

In this study, approximately 100 flower buds of “sour orange” (a variety in which seeds are formed) were harvested from citrus trees (approximately 15 years old) in the Citrus Research Center greenhouse in April 2020 (Figs. 1A and B). Flower buds with a diameter of 3–5 mm were selected for the study. The harvested sour orange flower buds were stored in the dark at 4 ℃ for 24 h and sterilized by immersion in 70% ethanol for 30–60 s. Thereafter, the flower buds were dried in a sterilized filter for approximately 2–3 h to completely dry the alcohol in the aseptic workbench. Petals of the buds were aseptically removed using a small forceps and scalpel, and the anthers were collected in a 60-mm diameter Petri dish containing solid medium (the medium composition is explained in the next section). Subsequently, based on the efficiency of the anther culture, liquid N6 liquid medium—supplemented with GA_3 (1 mg/L) and spermidine (200 μM)—was added or not on two types of medicinal culture media (Dunwell 2010) (N6 and Murashige and Skoog (MS) basic media supplemented with plant growth hormone) (Fig. 2C).

2.2 Anther medium composition and culture condition

Induction of callus and embryo from an anther culture was performed on an N6 medium (Chu 1978) supplemented with Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969) and MS medium (Murashige and

Skoog 1962) containing MS vitamin, supplemented with sucrose (50 g/L) and malt extract (500 mg/L). Moreover, the following growth regulators were added to the two media: 0.2 mg/L 2,4-dichlorophenossiacetic acid (2,4-D), 0.2 mg/L a-naphthalene acetic acid (NAA), 1.0 mg/L kinetin (KI), 0.8 mg/L 6-benzyladenine (6-BA), 0.43 mg/L Zeatin (ZI), and 0.44 mg/L thidiazuron (TDZ). The pH was adjusted to 5.8 using 1 N KOH and 0.1 N HCl, and 0.8% (w/v) agar was added. Anthers were cultured in the dark at 4 ℃ for the first 14 d and then transferred to a 16-h photoperiod at 25 ± 2 °C.

2.3 Plant regeneration

The callus cells induced in the anther culture medium were transferred to a somatic cell induction medium (MS medium supplemented with 500 mg/L of malt extract and 164 mM of lactose) according to Jin et al. (2007) and cultured for six weeks. Somatic embryos were transferred to MS medium supplemented with TDZ (0.5 mg/L), GA_3 (1 mg/L), malt extract (500 mg/L), sucrose (50 g/L), and gelrite (0.2%) to induce germination. The embryos in which the shoots formed were transferred and cultured in a magenta box containing GA_3 (0.5 mg/L), malt extract (500 mg/L), sucrose (50 g/L), and agar (8 g/L) in MS medium. After six weeks of culture, roots were induced. Each of the newly formed shoots was grafted onto the cultivated citron root. Subsequently, these plants were transferred from the greenhouse to the external natural environment for acclimatization.

2.4 Genetic analysis of regenerants

To verify whether the plants obtained using anther culture were haploid-derived, a simple sequence repeat (SSR) marker primer specific to the heterozygote was manufactured by Biomedic (Korea) (Table. 1), and polymerase chain reaction (PCR) was performed. Total genomic DNA was extracted using automatic nuclear extraction (MX 16, Promega, Madison, WI, USA) from approximately 0.2 g of plant material (leaves) and stored at -20 °C until further use. The PCR reaction solution comprised genomic DNA 15- in AccuPower® Multiplex PCR PreMix (Bioneer, Corp., Daejeon, Korea) [250 μM dNTP, 1.5 mM $MgCl_2$, 1.0 unit Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), and 40 mM KCl]. Next, 20 ng and 0.5 μM of primer were added to adjust the volume to 20 μL. The PCR reaction was amplified 35 times at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s after DNA denaturation at 94 °C for 5 min, followed by elongation at 72 °C for 30 min. The PCR amplification products were confirmed using the QiAxcel Advanced System (Qiagen, Hilden, Germany) electrophoresis apparatus.

2.5 Polyploidy analysis

To verify the ploidy of the plants obtained through anther culture, the leaves of each plant were collected using *in vitro* grafting and an analysis sample was prepared according to Chiancone et al. (2006). The ploidy of the prepared samples was determined using flow cytometry (CyStain UV Precise PAm Flugplatz 13 02828; Sysmex Partec GmbH, Görlitz, Germany). Approximately 0.1 g of the sample (plantlet leaves obtained from anther culture) and heterozygous donor plant leaves of the sour oranges were chopped using a sharp razor blade and added to a plastic Petri dish with 0.5 mL nuclei ectration buffer 2 ml staining buffer. The sample was filtered through 30-μm Partec CellTrics (Sysmex Partec GmbH Am Flugplatz 13

02828 Görlitz, Germany) directly into the sample tube, and the test tube was loaded into the machine for analysis. In total, 109 regenerant lines were analyzed.

2.6 Phylogenetic analysis

Analysis of the genetic relationship between the plant and donor plant obtained using anther culture was performed according to previously reported methods (Jin et al. 2016; Jin et al. 2018), and the entire internal transcribed spacer (ITS) region (ITS15.8S-ITS2) in the nuclear ribosomal DNA was analyzed. PCR was performed for the entire ITS region of nuclear ribosomal DNA using a primer combination of ITS1F1 primer (5′-GAAGGATCATTGTCGACCTGCCAGCAGACG-3′) and ITS2R2 primer (5′-GACCTGGGGTCGCAATGCGAGCGCCGCTT-3′) (Jin et al. 2016, 2018). The amplified PCR product was identified as a band by electrophoresis on a 1.2% agarose gel at 100 V for 30 min. The amplified product confirmed on agarose gel was obtained using the GeneAll® Gel purification kit (GeneAll Biotechnology, Co., Seoul, Korea), and the purified amplification product was cloned using the pLUG-Prime® TA-Cloning Vector kit (iNtRON, Korea) (Jin et al. 2018). The cloned PCR product was sent to Solgent (Solgent Co., Daejeon, Korea) to determine the nucleotide sequence. The nucleotide sequence of the determined nuclear ribosomal DNA ITS region was analyzed by Jin et al. (2018) and edited with Bioedit (Hall 1999), and phylogenetic relationships were analyzed using MEGA 5.2 software.

3. Results

3.1 Establishment of plant regeneration

The anthers swelled within one or two weeks, and some observed that callus cells and embryos were formed again within three or four weeks (Fig. 1D). Furthermore, when the N6 liquid medium supplemented with 1 mg/L GA_3 and 200 μM spermidine was added to the anther culture medium, the induction rate of callus and embryo was approximately 10% higher than the medium without these supplements (data not shown). The obtained callus cells were transferred to a somatic embryo induction medium to induce embryo formation after six weeks of culture (Fig. 1E). The formed somatic embryos were transferred to a plant regeneration medium, and approximately 50–60% of normal plants were obtained after four to six weeks of culture (Fig. 1F). Furthermore, normal plants, which had roots and shoots until this stage, were transferred to MS medium without adding hormones, and the remaining abnormal embryos were transferred to MS containing 500 mg/L of malt extract, 50 g/L sucrose, and 0.5 mg/L of GA_3, which can induce continuous plant growth from non-regenerated callus and embryos, to induce approximately 70–80% of normal plants. Normal plants were rapidly induced to proliferate and grow through grafting *in vitro* and *in vivo* (Fig. 1G and H). Over 1000 regenerated plants were obtained using the anther culture method.

3.2 Genetic verification

To confirm whether the plant obtained by citrus anther culture is a haploid-derived plant, two CiSSR-P1,-P2) SSR markers were used out of five combinations of SSR markers, which were heterozygous to sour oranges (Table 1). Among the total plants obtained using anther culture, 271 plants (*in vivo* or *in vitro* grafting plants) were investigated. As shown in Fig. 2A, the SSR marker CiSSR-P1 showed the same amplification

pattern (double band) and double amplification peak as the control in four plants (Lanes 2–5) that were regenerated using anther culture (Fig. 2A top). The regenerated plants were presumed to be haploid-derived plants obtained a single amplification product and a single amplification peak (Fig. 2A mid., bottom). Furthermore, the SSR marker CiSSR-P2 showed three amplification bands and peaks in the control, unlike the SSR marker CiSSR-P1 (Fig. 2B, top). Plants assumed to be heterozygous (Lanes 2–5) also showed the same amplification band and peak pattern as the control (Fig. 2B, top). However, haploid-derived putative plants showed a single band (Fig. 2B (Lanes 6–12), and a peak pattern (Fig. 2B, middle bottom). Because of examining the PCR amplification products and peak patterns with three markers other than these two SSR markers, three or four amplification products were obtained from the control that were not suitable for selecting haploid-derived plants (Online Resource 1). In contrast, regenerated plants were presumed to be haploid-derived plants obtained a single amplification product and a single amplification pattern, unlike the control. Thus, haploid-derived plants were obtained in approximately 16% of cases (43 plants selected from 271 plants).

3.3 Polyploidy verification and morphological selection

Among the plants obtained using the anther culture, polyploidy was investigated in 109 plants, including 39 of 41 genetically presumably selected plants and 66 plants identified as heterozygous (Fig. 3). Consequently, eight individuals (approximately 7%) identified as diploid were obtained (same as control) (Fig. 3A). Two specimens (approximately 2%) were obtained from 109 individuals identified as haploids (Fig. 3B and C), and the remaining individuals (approximately 91%) were identified as triploid plants (Fig. 3D). Approximately 97% of the 39 individuals identified in the genetic analysis were triploid plants, whereas one diploid plant was also identified. Approximately 12% of the heterozygous plants were diploids and the remaining 88% were identified as triploids.

Moreover, anther-derived triploid plants were morphologically different from the control; in the hetero cultivars, there were differences in leaf width size and growth (Fig. 4A, left: hetero diploid, right: putative homo tri haploid plant). A morphological difference in leaf width, size, and growth among plants identified as triploids was observed (Fig. 4B, left: hetero diploid, middle and right: putative homo triploid). In addition, the haploid plants showed a shape in which, unlike the control group and the triploid plants, only the thin thread roots proliferated without shoot formation, or only the thin thread roots proliferated long without shoot elongation (not shown Figure: Sample loss by contamination).

3.4 Phylogenetic analysis

To investigate whether the plant obtained using citrus drug culture was a somatically mutated plant or a haploid-derived plant, the genetic relationship of the ITS region, which is a chromosomal genetic region, was analyzed. Consequently, a genetic difference in the chromosome ITS region was confirmed between plants identified as SSR markers and control plants (Fig. 5).

4. Discussion

In this study, we investigated the embryo formation and callus induction in sour orange varieties using anther culture based on medium composition. We obtained haploid and triploid plants using this culture. *Citrus* cultivars have difficulty in securing haploid-derived plants using anther culture compared to herbaceous plants (Cardoso et al. 2014) because they are characterized by long juvenility (Chiancone et al. 2015). Therefore, successful cases of securing haploid plants using anther culture have mainly been reported for grain species (mainly Cruciferae and some Solanaceae) (Parra-Vega et al. 2013; Prem et al. 2012; Seguí-Simarro et al. 2011; Wedzony et al. 2009). However, recent studies have reported embryo induction and plant regeneration using anther citrus cultures (Cardoso et al. 2014; Germanà 2006, 2007, 2009). In addition, haploid (homozygous diploid) and triploid plants have been reported (Chiancone et al. 2006; Germanà 2005, 2007, 2009). Among citrus fruits, sour orange varieties are known for industrial (mainly used as rootstock) and tissue culture and are a model for anther culture (Castle et al. 1992; Hidaka 1984). However, there have been no studies on obtaining haploid and triploid plants, and it has been reported that most are diploid plants (Hidaka et al. 1982).

According to the results of previous studies (Cardoso et al. 2014; Hidaka et al. 1982; Hidaka 1984) on sour orange and anther culture, N6 medium was more effective than MS medium (both media were supplemented with growth hormone) in embryo and callus formation, but the results were not significant. However, when the N6 liquid medium was supplemented with spermidin and GA_3 was added to the anther-cultured solid medium, the anthers swelled and the pear and callus formation effects increased by approximately 34–50%. The addition of a liquid medium to a solid medium (Dunwell 2010) was effective in the cultivation of anthers of several plant varieties, such as barley (Ziauddin et al. 1990), wheat (Touraev et al. 1996), tobacco (Dunwell and Thurling 1985), and black pepper (Supena et al. 2006). This result was like this study. In addition, the composition of plant growth hormones during anther culture differed from that in previous studies (Cardoso et al. 2014; Germanà and Chiancone 2003; Hidaka et al. 1982; Hidaka 1984). Satisfactory results were obtained only when a combination of plant growth hormones was used, similar to the hormone composition used by Chiancone et al. (2006).

For genetic verification of the plants obtained using anther culture, five combinations of SSR markers specific to heterozygotes for sour orange variety were purchased from Biomedic (Korea) and 271 redifferentiated plants were investigated. Consequently, the SSR markers CiSSR-P1 and CiSSR-P2 could confirm whether there were haploid-derived plants. However, PCR with the remaining CiSSR-P3, CiSSR-P4, and CiSSR-P5 (Table 1) confirmed that 3–4 bands and peaks were formed in the control plant; therefore, it was impossible to confirm whether there was a haploid or homozygous diploid (Online Resource 1). As shown in Online Resource 1, the control and heterozygous plants confirmed the same amplification pattern, but the amplification patterns of the plants presumed to be haploid and homozygous differed from those of the control. In addition, amplification products not found in the control were identified. Germanà et al. (2005) suggested this result is presumed to occur during meiosis. The plants obtained in this study could be estimated as triploid plants rather than haploid-derived plants (Germanà et al. 2005).

Because of ploidy verification, based on the above genetic verification results, most plants were identified as triploids, as in previous studies (Germanà et al. 2005 or Chiancone et al. 2006). The above results could be attributed to the treatment with spermidin, which stimulates embryogenesis during anther culture

(Chiancone et al. 2006). In this study, the triploid plants had two types of morphological characteristics. Chiancone et al. (2006) identified individuals with a thin leaf width and a leaf width larger than diploid individuals, similar to the results of this study. To confirm whether these individuals were formed by somatic mutations or anther walls during culture (Germanà 2011), the chromosomes were determined through the investigation of the ITS region (Jin et al. 2016, 2018; Kim et al. 2021) related to parental inheritance. These types differed from the control group and both could be judged as the same plants (Fig. 5). Thus, it was possible to establish these two plant types were not generated from somatic cell mutations or the anther wall. Therefore, the triploid plants obtained in this study were prepared according to Germanà et al. (2005) and were presumed to have a mutation that occurred during meiosis. This study identified the haploid and homo diploid plants. According to Hidaka et al. (1982), haploid plants could not be obtained from sour orange. In Clementine (Chiancone et al. 2006; Germanà et al. 2005) anther cultures, most plants were triploid and few or no haploids were obtained. In this study, we obtained two haploid plants (although sample loss was due to contamination), which is a similar to the results of Germanà et al. (2005). The stems of these two plants did not grow, and only small shoots proliferated as if they were stacked in layers. Some observed that the roots grew extremely thin compared to other plants. Different haploid results were obtained from the photographs (plant types similar to this result were all identified as triploids) reported by Chiancone et al. (2006). Similar to the results of Germanà et al. (2005), the leaves were small and dense, and there was no node elongation. In addition, it was possible to observe embryos in which only thin roots were grown without the formation of new shoots. Therefore, more studies are needed to clearly confirm whether the plants obtained from this study are triploid or tri-haploid.

Furthermore, the vacuole size of microspores in anthers in anther culture is an important factor responsible for differences in regeneration efficiency (Cardoso et al. 2014; Chiancone et al. 2006).

Moreover, among citrus fruits, *Citrus unshiu* cultivars have a low regeneration rate (Jin et al. 2007; Yun et al. 2006). To solve this problem, a method for selecting cells capable of forming somatic cells through a Percoll concentration gradient according to the size of callus cells has been proposed (Jin et al. 2007). Therefore, in microspore culture, if the Percoll concentration gradient method and the liquid medium culture method are grafted, the microspore culture is considered successful.

5. Conclusions

In this study, we performed sour orange anther culture method and obtained haploid and triploid citrus. Except for some citrus fruits (such as oranges and mandarins), most citrus fruits capable of self-pollination and cross-pollination develop from the seed. As seed formation is alienated from consumers, the production of seedless citrus fruits is preferred. The production of seedless citrus of varieties has previously been achieved via the crossing between diploid and tetraploid plants, but this method is time-consuming. In contrast, the production efficiency of triploid citrus fruits obtained using anther culture was approximately 80% higher than that achieved using crosses. Hence, the findings of this study can be applied for effective production of seedless non-nuclear citrus fruits. This may be especially useful for the *C. junos* cultivar with problems with seed formation. Future studies should focus on investigating to obtain fruit from sour orange triploid plants and verify whether the fruit is seedless.

Declarations

Author contributions

JSB and KMJ performed the genetic analysis and system establishment of anther culture, and CCW and YSH assisted in performing the experiments. JSB wrote the manuscript with inputs from all other authors. JSB, KMJ, CCW, and YSH reviewed and revised the manuscript. All the authors have read and approved the final manuscript.

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Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

Data availability

The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Tables

Table 1
Nucleotide sequence and repeat motifs of the SSR markers used in the study.

Product name	Primer sequence (5'->3')		Repeat motif	Homozygous cultivars
	Forward	Reverse		
BM-CiSSR-P1	CCCCCTCTTCTTTCACACAA	GGTGAGCAGCCATCTTCTTC	(TA)6	*C. clementina* 'Fina Sodea', *C. erythrosa* 'Dingjeongkyul'
BM-CiSSR-P2	GAATTGGGAGGACGAACTGA	CGAGCCCTAGACAGAGATGG	AGA)7	*C. pseudogulgul*, Citrus hybrid 'Haruka'
BM-CiSSR-P3	GCCTGAGTTTCTTTGTTATG	CATTCCATCGTCTCCTATTGT	(TATG)4	*C. maxima* 'Mato Buntan', *C. grandis* 'Dangyooja'
BM-CiSSR-P4	GTTTTCAGCTGGATTCGAGG	CACGTGTCCTCCTGGAACTT	(GCC)5	*C. maxima* 'Banbeiyu', *C. maxima* 'Mato Buntan'
BM-CiSSR-P5	GCAACGTGTACTGACGCTTG	GCTCGTATCTGAAGCTCGCC	(TAT)7	*C. maxima* 'Mato Buntan, *C. pseudogulgul* 'Sadoogam'

Figures

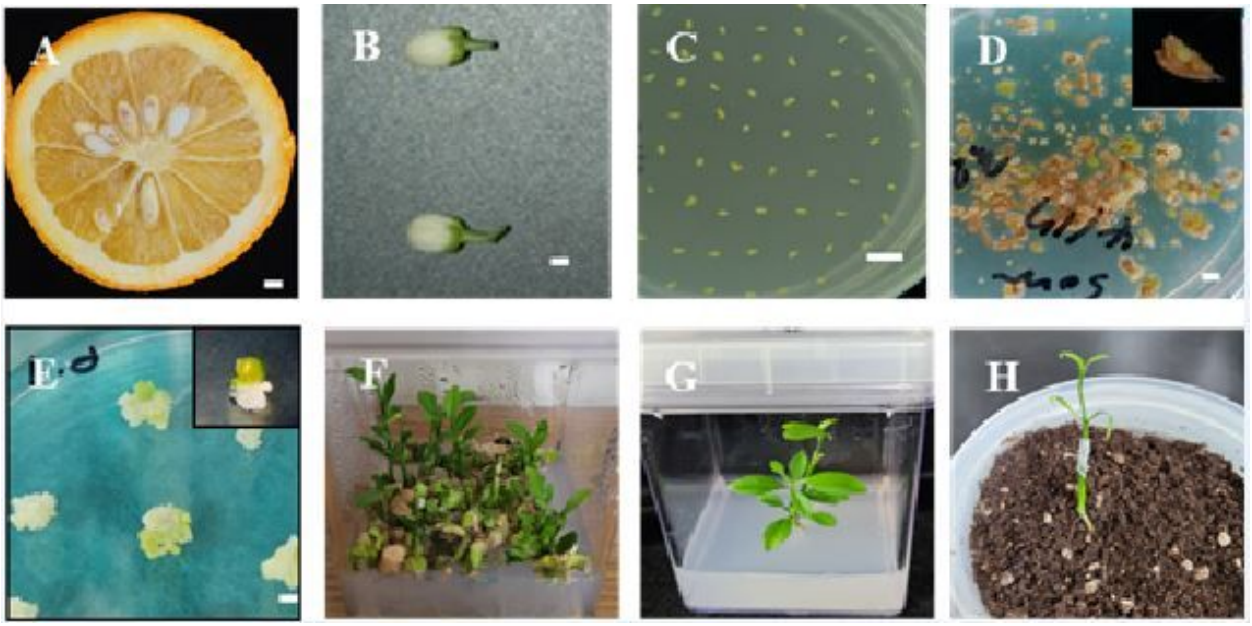

Figure 1

Citrus aurantium L. (sour orang) anther culture. (A); cut plane of sour onge fruit (B); Flower bud of-*aurantium* L. (sour orang) (C); anther culture in Petri dish (D) callus or direct embryo formation (E) somatic embryo formation from callus (F) plant regeneration (G) *in vitro* grafting (H) *in vivo* grafting. Scare bar is 0.1 mm

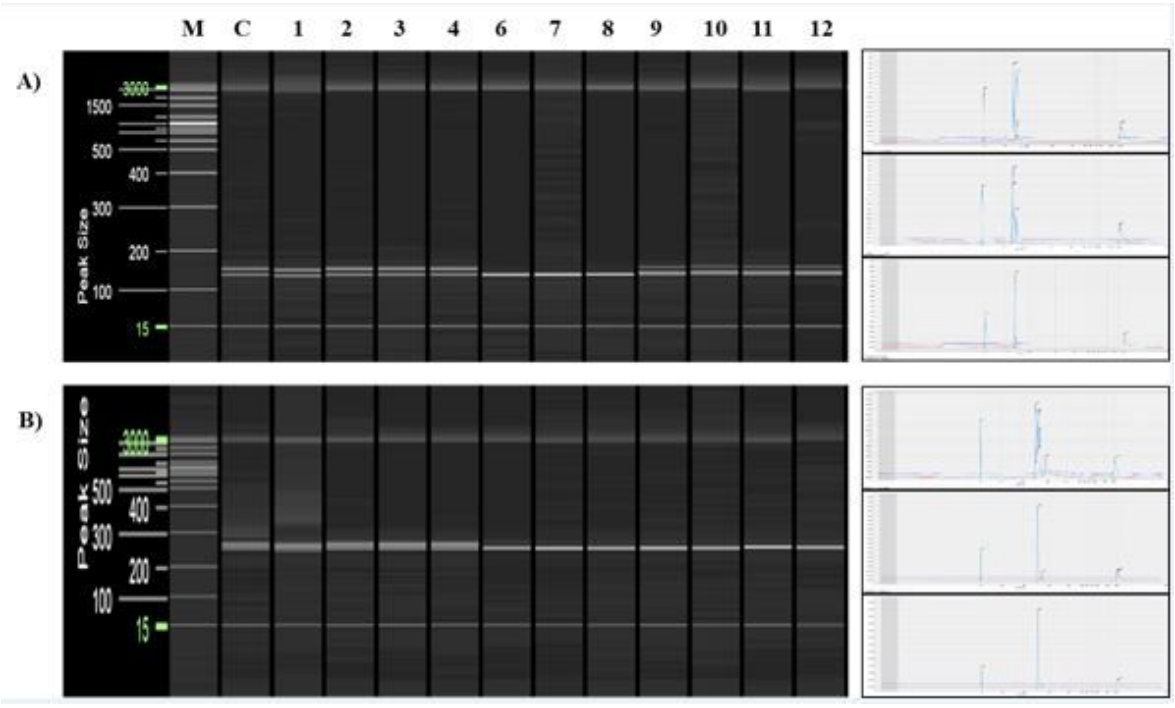

Figure 2

Simple Sequence Repeat (SSR) molecular analysis of control plant and regenerated plants obtained from anther culture. A-left PCR analysis using primer SSR CiSSR-P1 sets. Polyacrylamide gel electrophoresis

using the QiAxcel Advanced System (Qiagen); M: molecular marker (20 and 100 bp DNA ladder, Qiagen); C: control plant; lanes 1–12: regenerated plants obtained from anther culture. A-right PCR analysis amplified peak using primer SSR CiSSR-P1 sets. Polyacrylamide gel electrophoresis using the QiAxcel Advanced System (Qiagen). Up: control plant and lanes 1–4; middle: lanes 6–8; down: lanes 9–12. B-left PCR analysis using primer SSR CiSSR-P2 sets. Polyacrylamide gel electrophoresis using the QiAxcel Advanced System (Qiagen); M: molecular marker (20 and 100 bp DNA ladder, Qiagen); C: control plant; lanes 1–12: regenerated plants obtained from anther culture. A-right PCR analysis amplified peak using primer SSR CiSSR-P2 sets. Polyacrylamide gel electrophoresis using the QiAxcel Advanced System (Qiagen). Up: control plant and lanes 1–4; middle: lanes 6–8; down: lanes 9–12

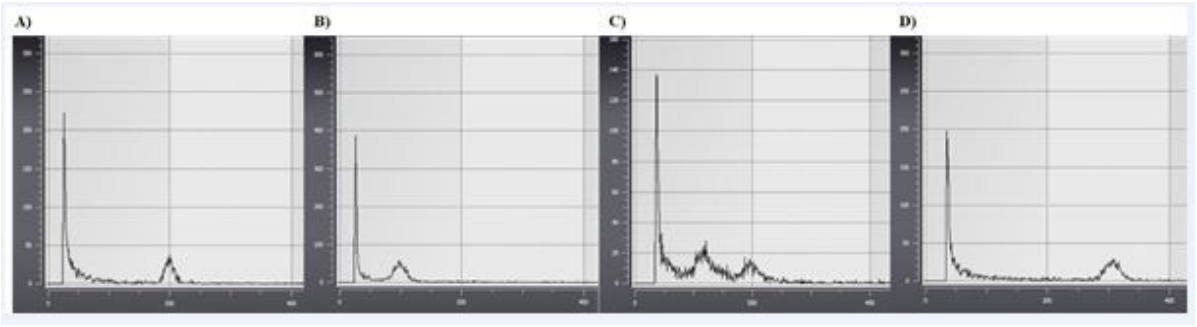

Figure 3

Ploidy analysis using flow cytometry. Donor plant used as control (A), haploid obtained from anther culture (B) analysis result after mixing haploid and diploid obtained from anther culture (C) triploid obtained from anther culture (D)

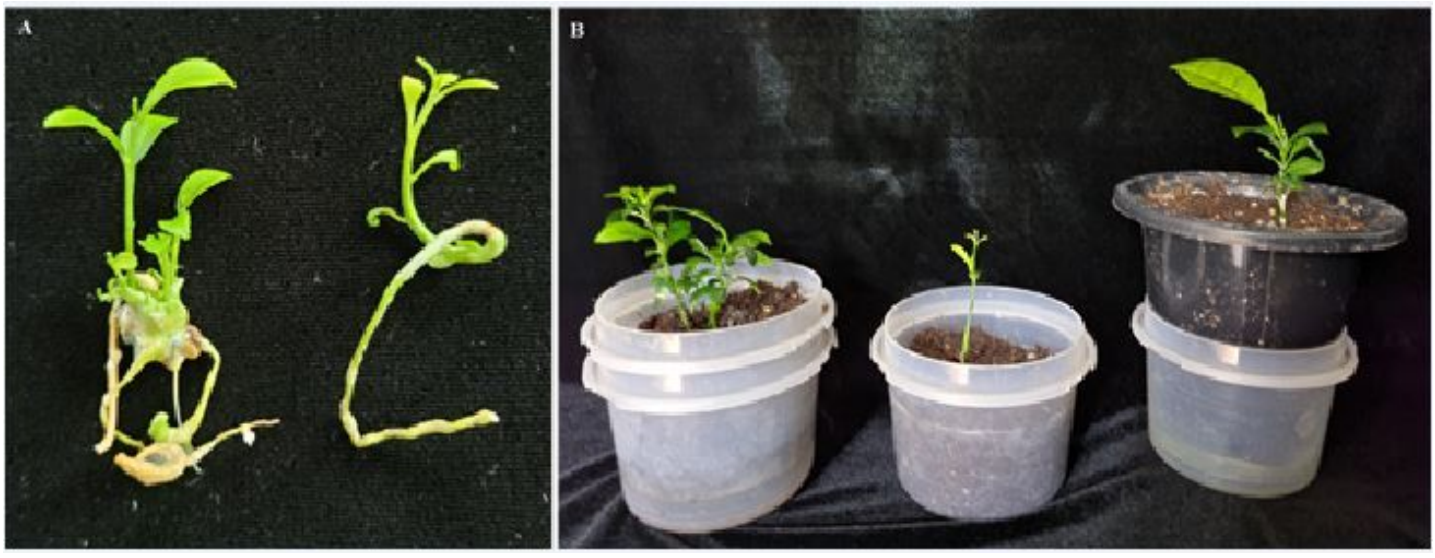

Figure 4

Morphological characteristics of plants secured through anther culture. (A) Left; Hetero diploid plants, right; putative homo Tri haploid plant, (B) left; hetero diploid, right: middle, and right: putative homo tri haploid plant

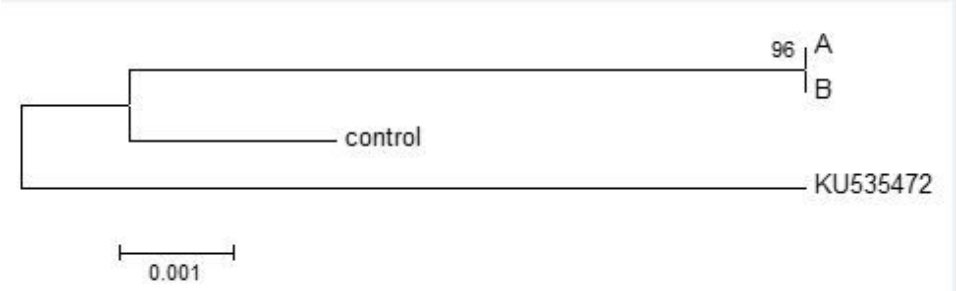

Figure 5

Maximum-likelihood phylogenetic tree of the regenerated plants obtained from anther culture based on total internal transcribed spacer (ITS; ITS1+5.8S rDNA+ ITS2) region sequences. Regenerated A and B plants obtained from anther culture; control is donor plant and hetero plant; Ku535472 is the accession number of 'Sour orange' registered at GenBank

Supplementary Files

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