Characterization of Allotetraploids Derived from Protoplasts Fusion between Navel Orange and Kumquat

Min Ju Kim

(Supervised by Professor Kwan Jeong Song, PhD)

A thesis submited in partial fulfillment of the requirement for the degree of Master of Science in Agriculture

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Min Ju Kim, a candidate for the degree of Master of Science in Agriculture is approved by the committee members

Chairman : _____
Hoon KANG, PhD

Member : _____
Kwan Jeong SONG, PhD

Member : _____
Soo Kuk PARK, PhD

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ABSTRACT

The study aimed to investigate the plant and fruit characteristic of the allotetraploids derived from protoplasts fusion between navel orange and kumquat. Giljun navel orange having large flesh with high soluble content and Jangsil kumquat having edible peel were used as an embryogenic callus line and a mesophyll line, respectively. Protoplast fusion was conducted using polyethylen glycol (PEG) method. The regenerated plants were analyzed by flow cytometry to select tetraploids, which were further evaluated by PCR analysis with simple sequence repeat (SSR) markers specific to the origin of necleus and cytoplasmic organelles to confirm allotetraploids and cybrids. Sixteen allotetraploids were finally produced and phenotypic characterization for leaf morphology and fruit quality was conducted. All allotertaploids contained mitochondria originated from Giljun navel orange. Twelve of them were identified to contain chloroplasts originated navel orange and 4 of them, from kumquat. Leaf size of allotetraploids is intermediate between both parents, but leaf shape is obovate different to spindle of both parents. Petiole wings were absent as following the kumquat trait. Flowering time is mid-May and pollen fertility is sterile which are similar to navel orange. Fruit size, external shape, soluble solids content, and acidity are intermediate between both parents, but the peel thickness and the number of segments were similar to kumquat and flesh weight is similar to navel orange. The results indicated that intergeneric allotetraploids with good traits incorporated from both parents, navel orange and kumquat might be produced and selected despite an existence of a little wide variation.

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INTRODUCTION

Citrus is among the most important crops in the world due to their economic and nutritional value, and has played a significant role in the economy of Jeju Island, Korea. As of 2018, the planting area of citrus accounts for more than 82% of the island's total area (MAFRA, 2015). However, the majority of the citrus cultivars currently cultivated in Jeju Island have been introduced from Japan, and hence there may be issues relating to the payment of significant amounts of royalties. Therefore, there has been a growing invest in developing new citrus cultivar in Korea.

Plant breeding aims to introduce useful traits which are mainly lacking in the existing cultivars and might be derived from closely related species. It has been achieved by selection of sexual hybrids obtained from crossing or mutants induced naturally. However, breeding by artificial crossing is impossible in the case of sexual incompatibility which greatly hinders the transfer of useful genetic traits. However, the various problems that have hampered conventional breeding methods have gradually been overcome as a consequence of on-going developments in in vitro breeding techniques such as somatic hybridization. In particular, somatic hybridization has been successfully applied in the case of citrus fruits species or related species (Domitique et al., 2011). The production of somatic hybrids through protoplast fusion has become an important tool for developing interspecific of intergeneric genotypes and tetraploids which are difficult to be achieved in conventional breeding.

When protoplasts are fused, not only nuclei are fused but also organelles such as chloroplasts and mitochondria may be co-existed and then allotetraploids are produced.. These plants have tendency to express intermediate types of the parental species from which the corresponding traits are derived or express new traits (Eeckhaut et al., 2013). Therefore, the applicability of these plants as a breeding

material has been widely recognized (Sattler et al., 2015). Also, some cases have reported to produce cybrids or cytoplasmic hybrids having a fused nucleus originated from both parents and containing solid organelles such as chloroplasts or mitochondria originated from individual parent. However, there has not yet been reported to phenotypes of these cybrids.

In addition, the characteristic that has been a particular focus in recent citrus breeding programs is the seedless trait, which is a favorable fruit characteristics in the citrus market (Grosser and Gmitter, 2011). Seedless hybrids can be bred through triploid breeding, and triploids can be produced by diploid × tetraploid or tetraploid × diploid crosses. Therefore, protoplast-fused tetraploids have become an important breeding material for the development of seedless cultivars by the sexual hybridization.

Consequently, in this study, two citrus species that are difficult to produce sexual hybrids because of polyembryony and different flowering time, the navel orange and kumquat, were selected and protoplast-fused to develop intergeneric citrus that have the edible peel trait of kumquat and the high soluble solid content flesh of sweet oranges. Molecular and phenotypical characterization of protoplast-fused plants were subsequently investigated.

MATERIALS AND METHODS

Plant Materials

In this study, we performed protoplast fusion using embryogenic callus and leaf cells obtained from the Giljun navel orange and Jagsil kumquat (*Fortunella japonica*), respectively. To obtain embryogenic callus from the nucellus, young immature ovules of navel orange were cultured in vitro, whereas in order to ontain leaf cells of kumquat, the seeds of kumquat were sown in vitro.

Protoplast Fusion and Plant Regeneration

Overall protoplast fusion was performed according to the method described by Grosser and Gmitter (1990) with slight modification. Embryogenic callus of Giljun navel orange (1 to 2 g) was placed in a 60 × 15 mm Petri dish, to which 1.5 mL of enzyme solution (2% Cellulase RS, 2% Macerase, 0.7 M mannitol, 24 mM $CaCl_2$, 0.92 mM NaH_2PO_4, and 6.15 mM MES) and 1.5 mL of 0.6 M BH_3 medium were added, followed by culturing in an incubator (WiseCube, Fuzzy Control System) at 29℃ and 30 rpm for over 9 hours.

Leaves of Jangsil kumquat were collected from in vitro culture, cut into 1 ‐ 2 mm sections, and placed in an Erlenmeyer flask, to which 3 mL of the aforementioned enzyme solution and 8 mL of 0.6 M BH_3 medium were added, followed by treatment under 50 kPa vacuum conditions for 15 minutes to facilitate effective enzyme penetration. Thereafter, the leaf explants were cultured in an incubator for at least 9 hours to obtain protoplasts. After culturing for 9 hours, callus and leaf explants masses were filtered through a stainless steel with 45μm pore size to remove leaf cell debris and callus lumps. The filtered solution was centrifuged for 10 minutes, and the supernatant was removed. The remaining pellet was then gently resuspended in 5 mL of 25% sucrose solution, followed by the careful addition of 2

mL of 13% mannitol solution, such that the mannitol and sucrose solutions did not mix for concentration gradient. Following centrifugation for 10 minutes, a protoplast band was formed at the boundary between the sucrose and mannitol solutions, which was carefully removed using a pipette, mixed with the BH_3 medium, and centrifuged. To the protoplast pellets obtained in the purification step, 10 volumes of BH_3 medium were added and mixed well.

Six drops of the resulting protoplast mixture were placed in a 60 × 15 mm Petri dish, followed by addition of the PEG solution to surround the rim. After 15 minutes, A+B solution (0.4 M glucose, 66 mM $CaCl_2 \cdot 2H_2O$, 10 % DMSO and 0.3 M glycine) was added to surround the rim, followed 15 minutes later by the addition of the BH_3 medium, and after a further 10 minutes, the surrounding solution was removed. This washing process was repeated a further two times, after which 15 drops of BH_3 medium was added, followed by thorough mixing.

After protoplast fusion, cell culture was initiated with a solid medium containing 0.146 M EME and maltose at 25℃ in dim light condition. When embryos had started to form, 1:1:1 medium (0.6 M BH_3, 0.6 M EME with sucrose and 0.146 M EME with sucrose) was added to induce embryo development, and when circular embryos had developed, 1:2 medium (0.6 M BH_3 and 0.146 M EME with maltose) on a solid medium containing 0.146 M EME and maltose was added for plant development. When the plants had grown to a certain extent in tissue culture room, they were transferred into greenhouse for further development. Subsequently, the plants were acclimated after being transferred to sterilized bed soil, which had initially been soaked with distilled water and then sterilized in an autoclave (HB-506; Han Beak Scientific Co.) at 121℃ for 10 minutes.

Determination of Tetraploids and Allotetraploids.

Flow cytometry (Am Flugplatz 13 02828; Sysmex Partec GmbH, Goerlitz, Germany) was used to determine the polyploidy of the plants. Sections of plant leaves (1.5 cm × 1.5 cm) were placed in a Petri dish, to which 0.5 mL of nuclei extraction buffer was added. Thereafter, the leaf material was cut into sections using single-edge blades (Dorco), to which 2 ml of staining buffer was added. The mixture was then filtered through a net and analyzed by flow cytometry.

Allotetraploids containing the nuclear DNAs of Giljun navel orange and Jangsil kumquat were further screened by simple sequence repeat (SSR) analysis after determinig the protoplast-fused tetraploids by flow cytometry. A primer of C.S SSR 66 that could detect an SSR sequence specific to the nuclear DNAs of both Giljun navel orange and Jangsil kumquat was selected from a hundred primers using clementine-derived ESTs. The nucleotide sequence of C.S SSR 66 is shown in Table 1. DNA was amplified using a PCR instrument (Veriti 96-well Thermal Cycler; Applied Biosystems), and PCR products were analyzed using an electrophoresis apparatus (QIAxcel Advanced System, Qiagen, Germany). PCR reaction was conducted in a condition as initial pre-denaturation at 95℃ for 15min, followed by 24 cycles of denaturation at 95℃ for 20s, annealing at 55℃ for 40s, and extension at 72℃ for 1min, additional 15 cycles of denaturation at 95℃ for 30s, annealing at 53℃ for 45s, and extension at 72℃ for 1min, and a final extension at 72℃ for 15 min.

Table 1. Primer sequences used in this study.

Organelle	Primer	Sequence (5'→3')
Nucleus	C.S.SSR66-R	CATCACCACCACAGCAACAA
	C.S.SSR66-L	TTCAGGTGAAAGCCCCTCT
Mitochondria	18S rRNA	GTGTTGCTGAGACATGCGCC
	5S rRNA	ATATGGCGCAAGACGATTCC
Chloroplast	NTCP9-R	CTTCCAAGCTAACGATGC
	NTCP9-L	CTGTCCTATCCATTAGACAATG

Molecular Characterization of Allotetraploids

An 18S rRNA-5S rRNA primer, a universal primer that can be used to identify mitochondrial DNA, was selected (Al-Janabi et al., 1994). DNA was PCR-amplified and treated with the restriction enzyme Tas Ⅰ at 65℃ for 5 minutes, followed by CAPS analysis following the method described Cheng et al. (2003) with a slight modification. The cytoplasmic genetic pattern was confirmed by determining the origin of mitochondria in the cytoplasm. A cpSSR primer that can identify the chloroplast DNA of navel orange and kumquat (Bryan et al., 1999) were used for determination of chloroplast DNA of protoplast-fused plants.

Phenotypic Characterization of Allotetraploids

Plants that were identified as allotetraploids were transferred into soil in a greenhouse after acclimatization and the scions of the acclimatized allotetraploids were grafted onto 2-year-old trifoliate orange root stocks. Subsequently, the leaf morphology of Giljun navel orange, Jangsil kumquat, and allotetraploids produced by protoplast fusion were evaluated. Four allotetraploids among 16 plants produced by protoplast fusion between Giljun navel orange and Jangsil kumquat had fruit sets in 2017 and fruit characteristics, including fruit size, weight, presence of seeds, soluble solids content, and acidity were determined.

RESULTS AND DISCUSSION

Production of Allotetraploids

Protoplast-fused plants were obtained via PEG-mediated fusion of protoplasts derived from the embryogenic callus of Giljun navel orange and the leaves of Jangsil kumquat. With the gradual development of protoplast-fused plants, green- or brown-colored spherical embryos were formed. The immature embryos then developed into matured ones with unevenly shaped cotyledons, some of which were regenerated into normal plants (Fig. 1).

Polyploidy level for plants derived from protoplast fusion between Giljun navel orange and Jangsil kumquat was determined by an analysis of flow cytometry (Fig. 2). Total of 16 tetraploids were identified by ploid analysis.

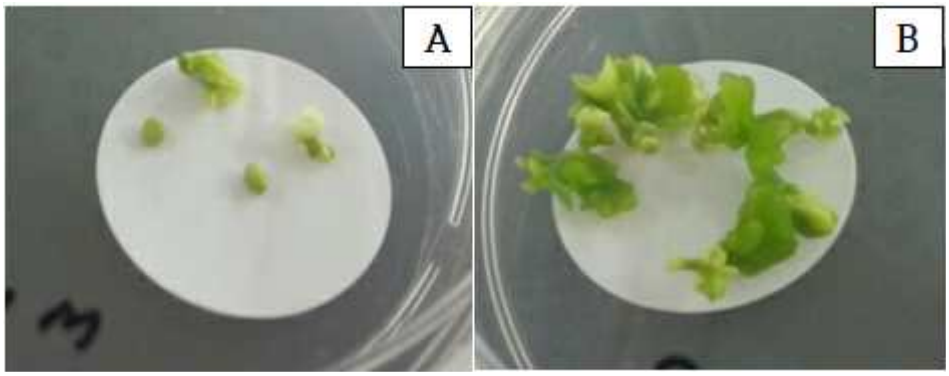


Fig. 1. Embryos (A) and mature embryos with developed cotyledons (B) derived from protoplast fusion.

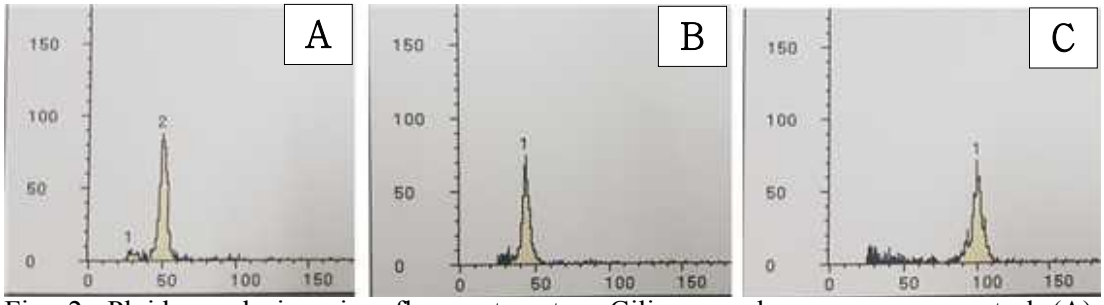


Fig. 2. Ploidy analysis using flow cytometry. Giljun navel orange as a control (A), Jangsil kumquat as a control (B), tetraploid plant derived from protoplast fusion between Giljun navel orange and Jangsil kumquat (C).

Nuclear DNA had been extracted from 16 protoplast-fused plants and amplification by PCR was performed using the C.S SSR 66 primer. Different products were accordingly amplified from the nuclear DNA of Giljun navel orange and Jangsil kumquat (Fig. 3). All of the 16 protoplast-fused tetraploids showed amplified products specific to Giljun navel orange and Jangsil kumquat, confirming that these tetraploids were allotetraploids containing nuclear DNA derived from the both of Giljun navel orange and Jangsil kumquat. The amplification products were further analyzed for more accurate confirmation, and representative chromatogram peaks were examined (Fig. 4). A peak at 148 bps was observed for the nuclear DNA of Giljun navel orange and peaks at 107, 116, and 129 bps were observed for the nuclear DNA of Jangsil kumquat. The nuclear DNA of the allotetraploids exhibited peaks at 107, 116, 129, 148, and 157 bps, thus confirming that the allotetraploids contained the nuclear DNA of both Giljun navel orange and Jangsil kumquat.

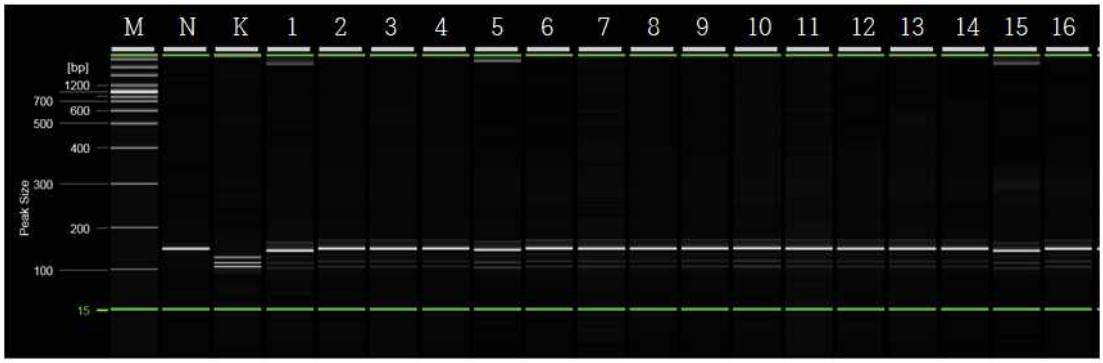


Fig. 3. Banding patterns of DNA amplified using the C.S SSR 66 primer. Nuclear DNA SSR analysis of regenerated plants from Giljun navel orange and Jangsil kumquat. M; size marker, N; Giljun navel orange, K; Jangsil kumquat, lane 1-16; allotetraploids.

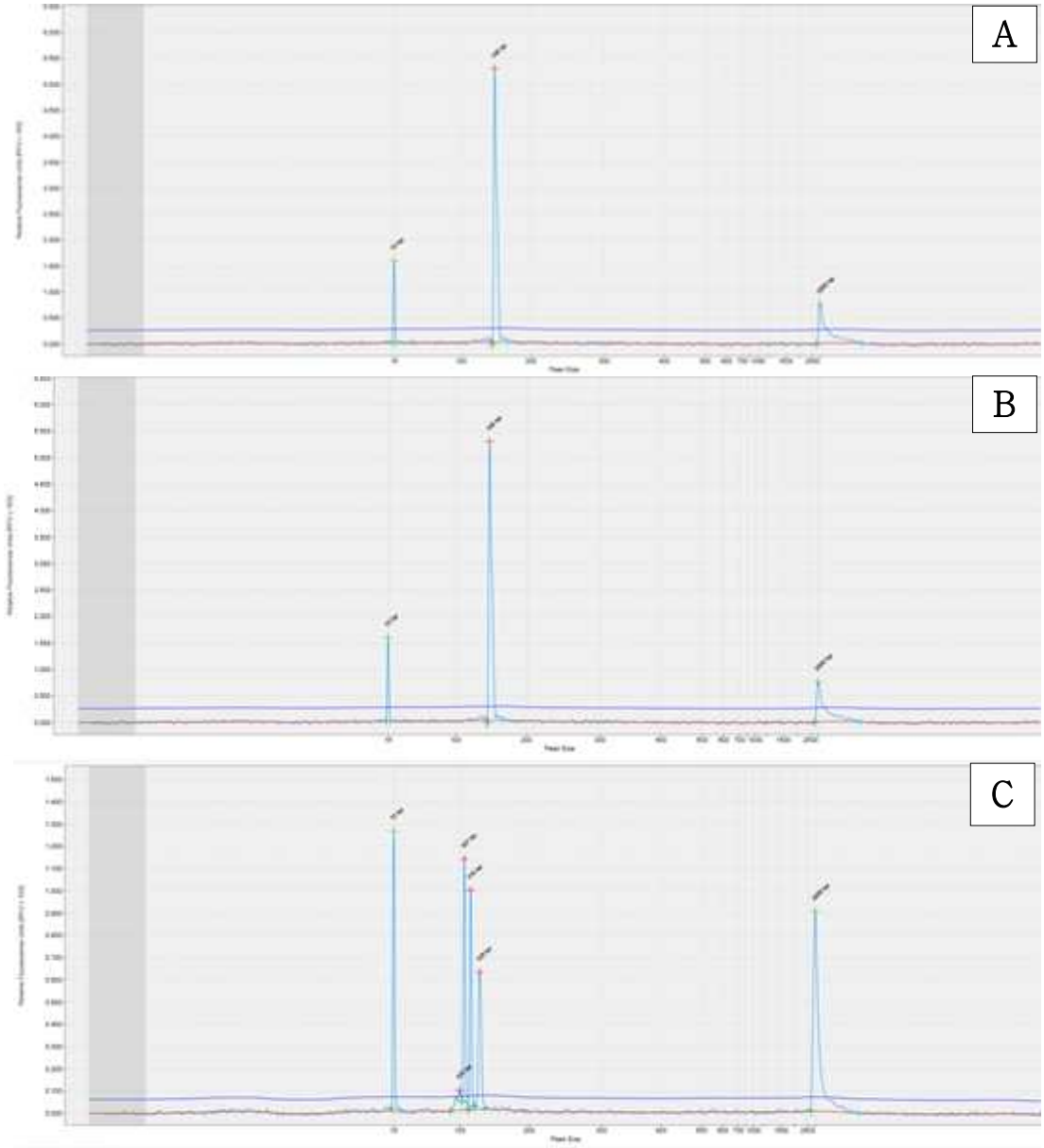


Fig. 4. Chromatograms obtained by performing PCR using a primer for detecting a nuclear DNA-specific SSR marker (C.S. SSR 66). A; Giljun navel orange, B; Jangsil kumquat, C; an allotetraploid.

Molecular Characterization of Allotetraploids

To detect mtDNA-specific sequences, CAPS analysis was performed using an 18S rRNA-5S rRNA primers and digestion with the restriction enzyme TasI, and accordingly obtained different amplification products from the mitochondrial DNA of Giljun navel orange and Jangsil kumquat. According to Cheng et al. (2003), allotetraploids have the same banding pattern as the embryogenic callus parent, and consistently all 16 allotetraploids examined in the present study showed the same amplification products as Giljun navel orange, thus confirming that they contained the mitochondrial DNA of Giljun navel orange (Fig. 5). Likewise, chromatograms (Fig. 6) of the allotetraploids also showed the same peaks sizes as those of Giljun navel orange (a difference of 2 to 4 bps was considered to be insignificant and was identical for all amplification products).

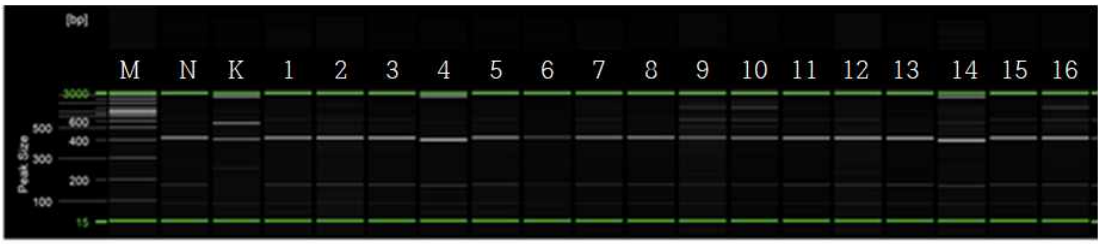


Fig. 5. Mitochondrial DNA CAPS analysis of regenerated plants from Giljun navel orange and Jangsil kumquat using the universal primer 18S rRNA-5S rRNA and digestion with TasI. M; size marker, N; Giljun navel orange, K; Jangsil kumquat, lnae 1-16; allotetraploids.

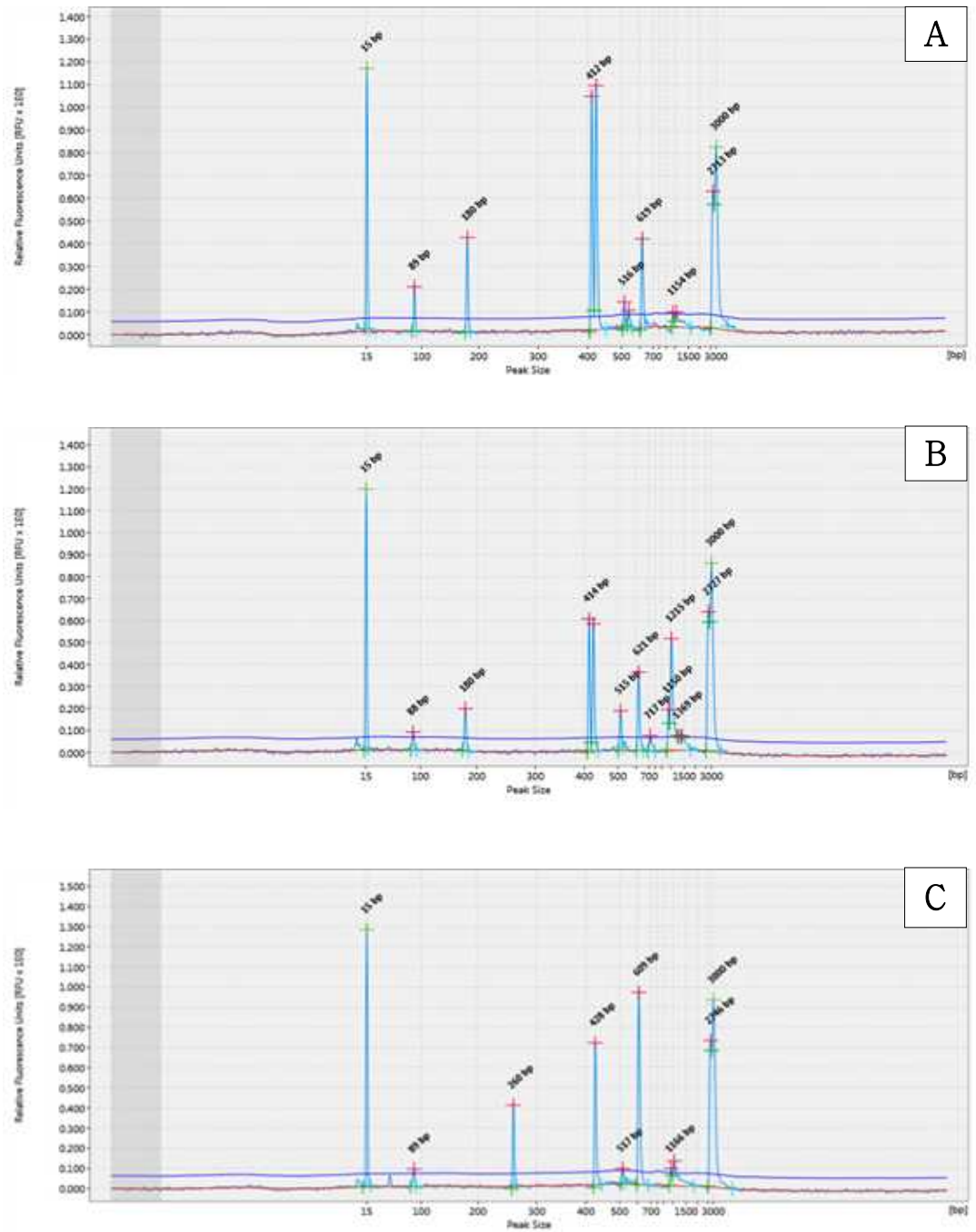


Fig. 6. Chromatograms obtained by performing PCR using a primer for detecting a mitochondrial DNA-specific CAPS marker (18S rRNA-5S rRNA and digestion with TasI). A; Giljun navel orange, B; Jangsil kumquat, C; an allotetraploid plant.

To detect chloroplast DNA-specific sequences, cpSSR analysis was performed using the universal primer NCTP9, which amplified different products from the chloroplast DNAs of Giljun navel orange and Jangsil kumquat. Among the 16 allotetraploids analyzed, 12 plants (from lane 2 to lane 14) showed amplification products of the same size as those of Giljun navel orange, whereas 4 plants (from lane 15 to lane 18) showed amplification products of the same size as those of Jangsil kumquat (Fig. 7). The chromatogram peaks showed the same results as those obtained using electrophoresis (Fig. 8). Although allotetraploids containg the chloroplast DNA of Giljun navel orange (the embryogenic callus parent) were produced three times more than those containing the chloroplast DNA of Jangsil kumquat (the leaf cell-derived parent) in this study, the result showed that the chloroplasts of allotetraploids produced by the protoplast fusion might be derived from ether parent. This result was consistent with previous reports indicating that the chloroplast genome in somatic hybridized plants may be derived from either parent (Grosser et al., 1996; Moreira et al., 2000).

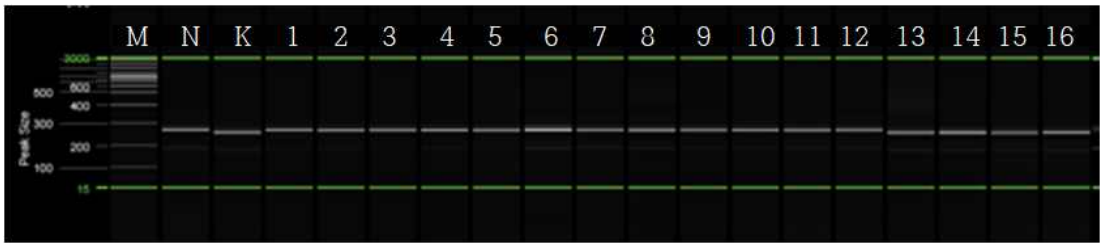
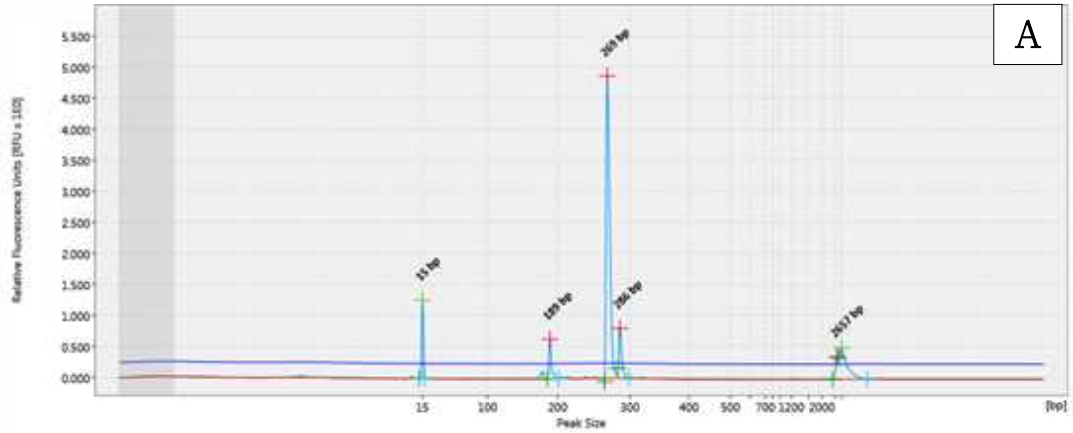
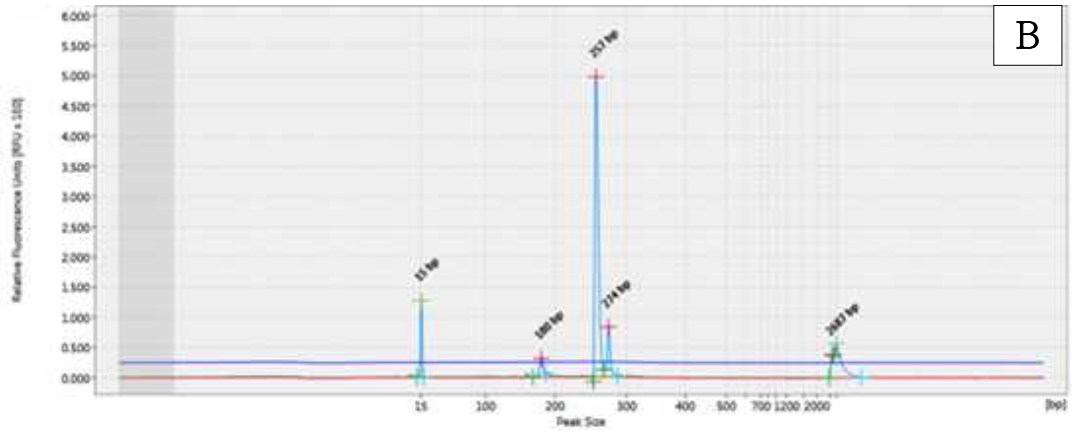
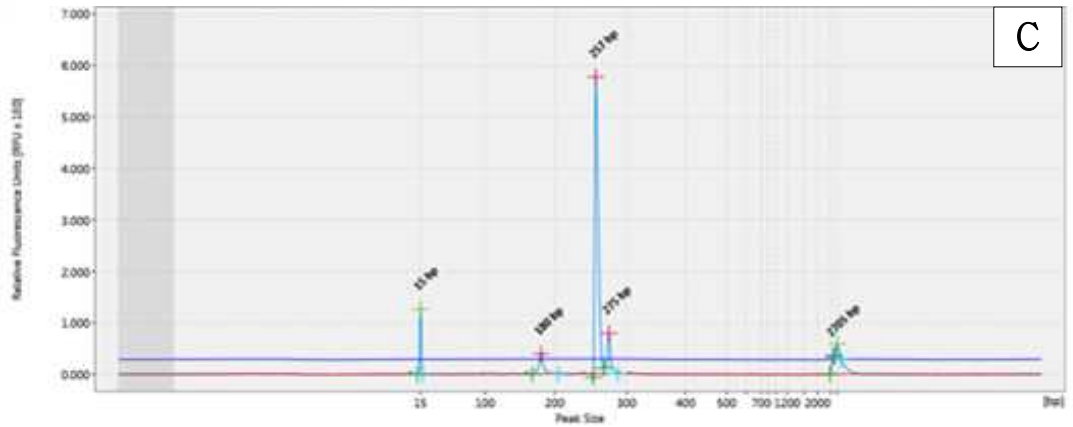


Fig. 7. Chloroplast DNA SSR analysis of regenerated plants from Giljun navel orange and Jangsil kumquat using the primer NTCP9. M; size marker, N; Giljun navel orange, K; Jangsil kumquat, lane 1-16, allotetraploid plants.







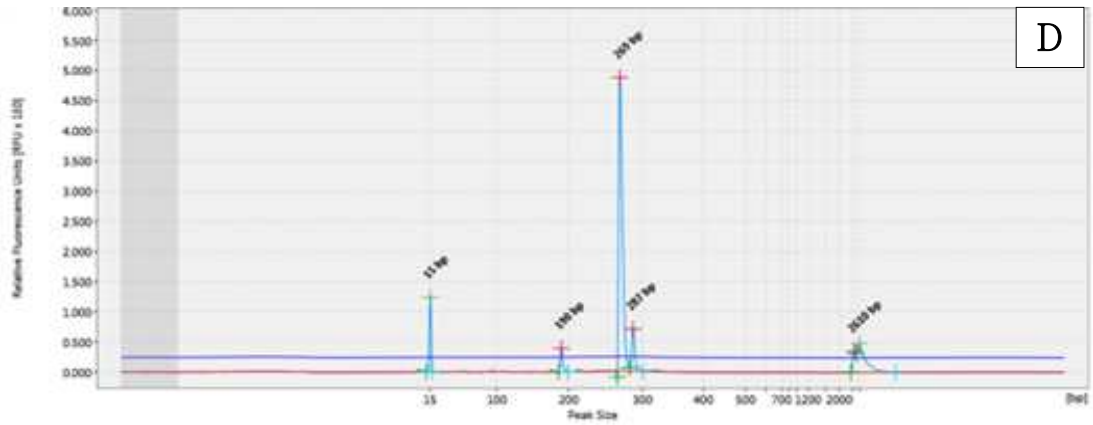


Fig. 8. Chromatograms obtained by performing PCR using a primer for detecting a Chloroplast DNA-specific SSR marker (NTCP9). A; Giljun navel orange, B; Jangsil kumquat, C; an allotetraploid containing chloroplast DNA from Giljun navel orange, D; an allotetraploid containing chloroplast DNA from Jangsil kumquat.

Phenotypic Characterization of Allotetraploids

A comparison of the leaves of Giljun navel orange, Jangsil kumquat, and protoplast-fused plants revealed that the leaf length of protoplast-fused plants (ranging from 58 mm to 124 mm) was shorter than that of Giljun navel orange (101.2 mm), but longer than that of Jangsil kumquat (50.2 mm) (Fig. 9 and Table 2). The leaf width of the protoplast-fused tetraploids (ranging from 41 to 82 mm) was wider than that of both Giljun navel orange and Jangsil kumquat (both diploids). This result was in accord with the previous reports by Guerra et al. (2014) and Oh et al. (2014) indicating that the leaf width of a tetraploid is wider than that of a diploid. The ratio of leaf length and width ranged from 1.3 to 0.6 in allotetraploids. Compared those with both parents of Giljun navel orange (2.4) and Jangsil kumquat (1.7), all protoplast-fused plants showed lower length/width ratio due to large width which might be caused by tetraploid characteristic. The leaf thickness of all protoplast-fused plants were similar to that of both parents, which was consistent with the study (Oh et al., 2014).

The leaf shape of Giljun navel orange and Jangsil kumquat was spindle, but that of all protoplast-fused plants were obovate, which might be from tetraploid characteristic. The small petiole wings existed in navel orange, not in kumquat. However, all protoplast-fused allotetraploids didn't have petiole wings being same to kumquat.

Fig. 9. Shape of the leaves of Giljun navel orange (left), Jangsil kumquat (center) and allotetraploids (right).

Table 2. Leaf characteristics of Giljun navel orange, Jangsil kumquat and allotetraploid plants.

Accession	Length (mm)	Width (mm)	Length/Width	Thickness (mm)	Shape	Pinnate
Giljun navel orange	101.2±8.3[x] ab[y]	42.0±2.7 g	2.4±0.1 a	0.5±0.1 e	Spindle	existence
Jangsil kumquat	50.2±3.3 h	30.4±2.4 h	1.7±0.2 b	0.7±0.2 bcd	Spindle	nonexistence
Fn1	96.4±11.9 abc	67.4±7.7 ab	1.4±0.1 gf	0.6±0.1 bcde	Obovate	nonexistence
Fn2	77.7±8.8 gf	50.9±6.1 f	1.6±0.2 cde	0.7±0.1 bcd	Obovate	nonexistence
Fn3	100.0±8.0 ab	70.7±3.7 a	1.4±0.0 gf	0.7±0.1 ab	Obovate	nonexistence
Fn4	86.0±9.8 def	63.0±9.4 bc	1.3±0.1 g	0.7±0.1 bcd	Obovate	nonexistence
Fn5	86.3±4.4 def	60.6±5.7 cd	1.4±0.1 efg	0.6±0.1 bcde	Obovate	nonexistence
Fn6	88.6±13.8 cde	62.7±9.4 bc	1.4±0.1 gf	0.6±0.1 cde	Obovate	nonexistence
Fn7	92.9±8.9 bcd	62.8±5.2 bc	1.5±0.1 defg	0.7±0.1 bc	Obovate	nonexistence
Fn8	104.0±10.8 a	66.8±4.5 ab	1.6±0.1 cd	0.8±0.1 a	Obovate	nonexistence
Fn9	88.6±5.2 cde	55.9±3.1 def	1.6±0.1 bc	0.6±0.0 bcde	Obovate	nonexistence
Fn10	80.9±8.2 efg	57.4±5.1 cde	1.4±0.1 gf	0.7±0.1 bcd	Obovate	nonexistence
Fn11	96.3±14.2 abc	60.8±9.7 cd	1.6±0.1 bc	0.7±0.2 bcd	Obovate	nonexistence

Fn12	84.3±6.2	def	60.4±3.5	cd	1.36±0.1	gf	0.61±0.1	cde	Obovate	nonexistence
Fk1	81.9±4.8	ef	55.7±5.6	def	1.48±0.1	defg	0.58±0.1	de	Obovate	nonexistence
Fk2	84.5±13.0	def	57.8±7.3	cd	1.44±0.1	defg	0.6±0.1	cde	Obovate	nonexistence
Fk3	81.9±5.8	ef	54.8±5.5	def	1.52±0.1	cdef	0.7±0.1	bc	Obovate	nonexistence
Fk4	72.9±9.1	g	51.6±6.0	ef	1.46±0.1	gf	0.6±0.1	cde	Obovate	nonexistence

[x]Mean ± standard error

[y]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

The flowering time of the allotetraploids was mid-May, which was similar to that of Giljun navel orange (Table 3). The shape of the flowers was also similar to that of Giljun navel orange. It is considered that among the genes regulating the flowering of Giljun navel orange and Jangsil kumquat, those which promotes early flowering time in Giljun navel orange might have been expressed in the allotetraploids. However, it was not clearly investigated and then further studies are evoked in this regard.

Table 3. Flowering time and flower characteristics.

Accession	Flowering time	Anther color	Pollen fertility
Giljun navel orange	May	White	Sterile
Jangsil kumquat	July	Yellow	Fertile
H	May	Yellow	Sterile
I	May	Yellow	Sterile
J	May	Yellow	Sterile
P	May	Yellow	Sterile

[x]Mean ± standard error

[y]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

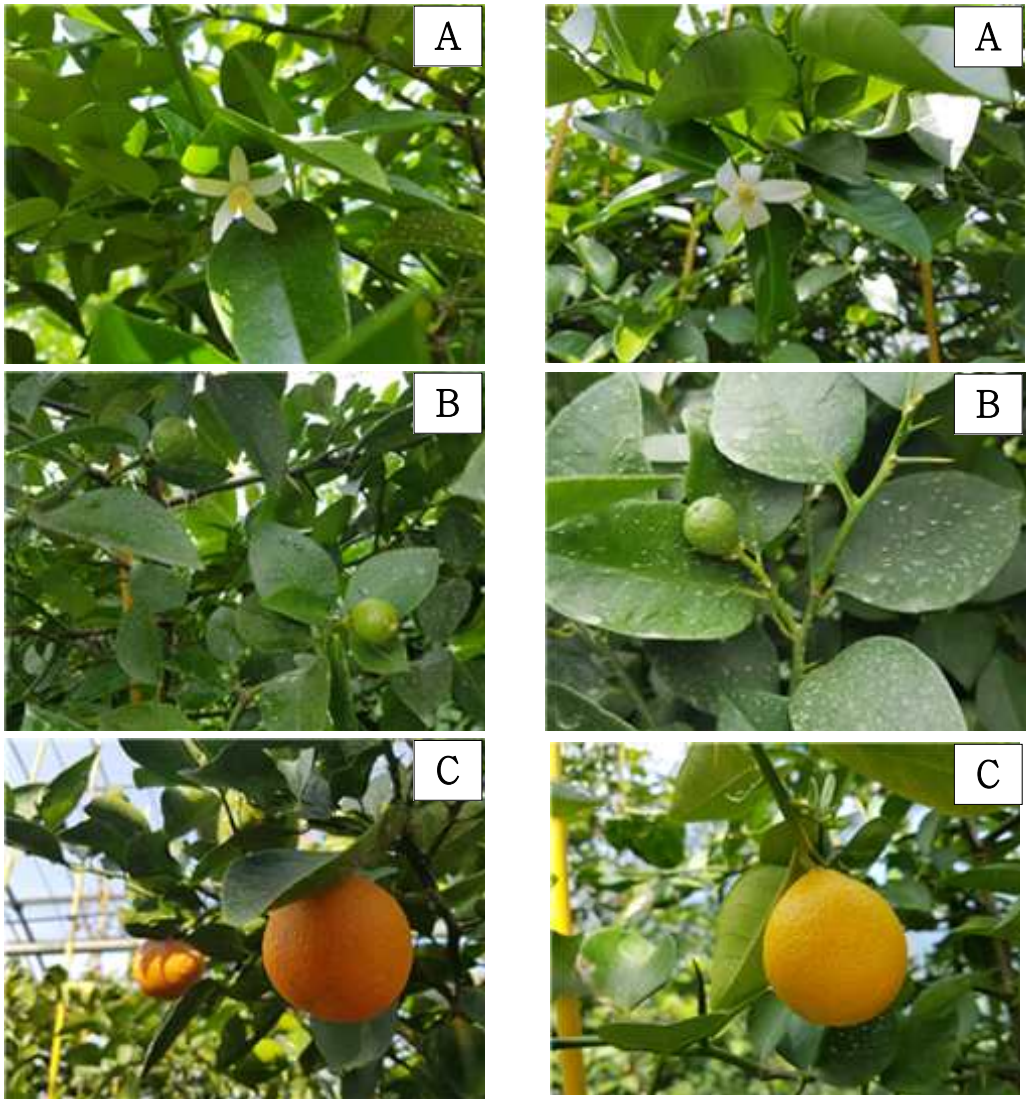


Fig. 10. Flowering and fruit set of protoplast fusion plants. A; flowers. B; immature fruit, C; mature fruit.

The fruit of allotetraploids showed intermediate size and weight between those of Giljun navel orange and Jangsil kumquat. Flesh weight of allotetraploids was similar to that of Jangsil kumquat, but peel thickness of allotetraploids was similar to that of Giljun navel orange. Acidity of allotetraploids was also intermediate between those of fruits in Giljun navel orange and Jangsil kumquat. The number of segments per fruit from allotetraploids was 5 to 8, which was similar to that of Jangsil kumquat. However, the number of seeds and soluble solids were not significantly different from those of Giljun navel orange and Jangsil kumquat (Fig. 11, Table 4). This result was in accord with the previous reports by Bassene et al. (2008) indicating that due to differences in mitochondria, the sugar content was no significant difference in cybrid and diploid plants. Therefore, this study indicated that we might produce allotetraploids having the advantages associated with Giljun navel orange and Jangsil kumquat, but lacking their disadvantages despite an exsitence of a little wide variation. Thus, we believe that the allotetraploid plants generated in this study will be applicable as breeding materials for developing a new variety of interspecific or intergeneric hybrids. Furthermore, these plants may be useful as breeding material for producing seedless triploid citrus fruits.

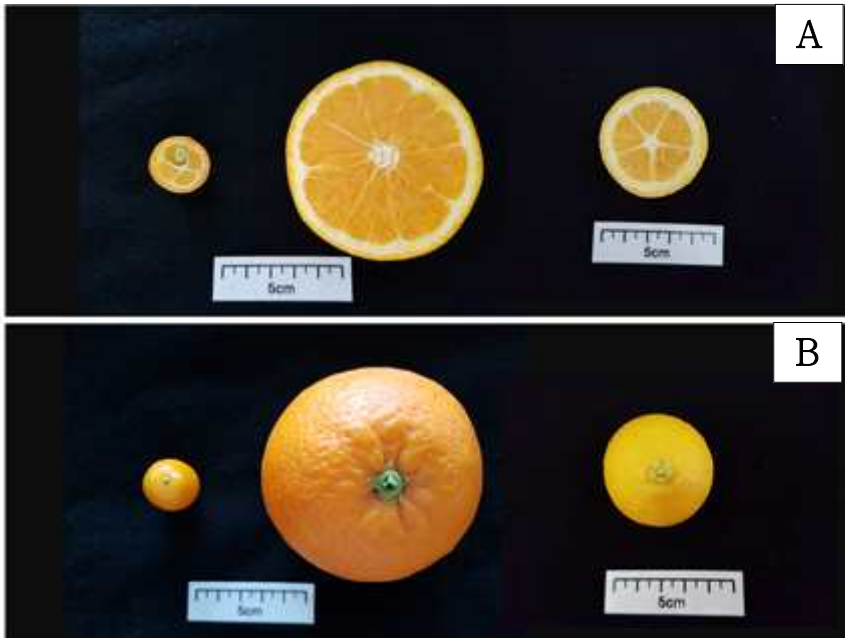


Fig. 11. Cross-section (A) and external view (B) of fruit morphology. Left; Jangsil kumquat, Center; Giljun navel orange, Right; allotetraploid derived from Giljun navel orange and Jangsil kumquat.

Table 4. Analysis of fruit characteristics.

Accession	Width (mm)	Height (mm)	Weight (g)	Flesh weight (g)
Giljun navel orange	80.5±3.2[x] a[y]	80.5±3.2 a	261.7±30.2 a	194.1±25.9 a
Jangsil kumquat	29.0±1.0 d	29.0±1.0 d	18.0±1.4 c	10.5±1.0 b
Fk1	54.6±0.0 b	54.6±0.0 b	78.3±0.0 b	44.8±0.0 b
Fk2	50.4±0.0 b	50.4±0.0 b	61.3±0.0 bc	41.3±0.0 b
Fk3	42±0.0 c	42.0±0.0 c	33.9±0.0 bc	18.6±0.0 b
Fk4	56.2±5.2 b	56.2±5.2 b	85.3±18.1 b	50.4±5.7 b

Accession	Peel thickness (mm)	Segments no.	Seeds no.	Soluble solids (°Brix)	Acidity (%)
Giljun navel orange	20.7±1.7 a	12.4±0.6 a	0.0±0.0 a	10.9±0.4 a	1.57±0.2 d
Jangsil kumquat	9.2±0.7 c	6.6±0.9 bc	1.4±1.1 a	12.3±0.7 a	5.2±0.1 a
Fk1	17.5±0.0 ab	8.0±0.0 b	0.0±0.0 a	11.3±0.0 a	3.5±0.0 b
Fk2	15.0±0.0 b	8.0±0.0 b	1.0±0.0 a	12.2±0.0 a	3.7±0.0 b
Fk3	16.9±0.0 b	5.0±0.0 c	1.0±0.0 a	11.5±0.0 a	2.8±0.0 c
Fk4	17.8±1.8 ab	8.0±0.0 b	1.5±2.1 a	12.1±0.4 a	3.5±0.4 b

[x]Mean ± standard error
[y]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

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ABSTRACT IN KOREAN

본 연구는 원형질 융합으로 생성된 이질 4배체의 식물 특성과 과실 특성을 평가하기 위하여 수행되었다. 높은 당도의 특성을 나타내는 네블 오렌지와 껍질 채 먹을 수 있는 특성을 지닌 금감을 실험 재료로 하였고, Polyethylene Glycol (PEG)을 이용한 방법으로 원형질 융합체를 생성하였다. 분자적 특성 검정을 위하여 배수성 검정기를 통해 16개체가 4배체임을 확인하였고, SSR 마커를 이용하여 16개체 모두 두 종류의 핵이 융합된 이질 4배체임을 판별하였다. 또한 미토콘드리아와 엽록체의 유래 여부도 확인하였다. 잎 표현형은 사배체의 엽 폭이 다른 두 이배체 보다 넓은 것으로 나타났고, 사배체의 엽 두께는 두 이배체 품종과 유사 하였다. 길전 오렌지는 익엽이 존재하나 금감에는 존재하지 않았는데, 이질 사배체의 잎에서는 금감과 마찬가지로 익엽이 발견되지 않았다. 과실 표현형의 특성 또한 조사하였다. 과실의 크기, 무게 및 산 함량은 두 이배체 품종의 중간적인 수치를 나타내었다. 이질 사배체의 과육중은 금감의 수치와 유사하였지만 껍질의 두께는 길전 오렌지와 유사하였다. 하지만 종자의 개수나 당도는 두 품종과 유의한 차이를 나타내지 않았다. 이러한 결과는 감귤 원형질 융합의 기초적 자료가 될 수 있으며 이질 사배체는 감귤 육종 프로그램에 사용될 수 있을 것이다.

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석사학위를 하면서 대학원에서의 수업뿐만 아니라 직장에서의 연구 활동도 함께 해 나가느라 때로는 시간에 쫓겨 힘이 들 때도 있고 어려운 일도 있었지만, 이렇게 논문을 마무리하는 시점에서 되돌아보니 모든 나날들이 저에겐 값진 시간이었던 것 같습니다. 늘 조언을 아끼지 않고 응원해주며, 직장에서나 가정에서나 가장 큰 힘이 되어 주는 사랑하는 남편에게 감사의 인사를 전하고 싶습니다. 또한 어디에 있든 마음으로 항상 응원해 주고 격려해 주는 사랑하는 가족들에게도 감사의 인사를 전합니다. 그리고 언제나 삶의 활력소가 되어주는 나의 지섭, 다원이 에게도 늘 고맙고 사랑한다는 말을 전합니다.

실험을 수행할 때나 논문을 쓸 때, 바쁜 와중에도 항상 도움을 많이 주셨던 감귤연구소의 모든 분들 모두 고맙습니다. 감귤 육종 연구를 함에 있어 항상 많은 도움을 주시는 윤수현 실장님께도 존경하는 마음을 담아 감사의 말씀을 드립니다. 특히 저에게 아낌없는 도움을 준 육종연구실 식구들에게도 감사의 말을 전합니다. 연구소에 고마운 분들을 모두 적을 순 없지만 모두 감사하다는 말을 전합니다.

지면으로 미처 언급하지 못했지만, 항상 저를 아끼고 격려해 주셨던 모든 분들께 감사하다는 말씀을 전합니다. 이번 논문을 통해 연구자로써 한층 성장하고 더 나아가 감귤 육종 분야에서 꼭 필요한 존재가 되도록 하겠습니다.

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김 민 주 올림