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Frequency of Spontaneous Polyploids in Monoembryonic Jeju Native Citrus Species and Some Mandarin Cultivars

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Polyploids are a potentially important germplasm source in seedless citrus breeding program. Seedlessness is one of the most promising traits of commercial mandarin breeds that mandarin triploid hybrids possess permanently. The formation of new constant triploid hybrids can be recovered through diploid species hybridization from the fusion of divalent gametes at low frequencyor intra-and inter-ploidy crosses. However, extensive breeding work based on small F_1 hybrid seeds developed is impossible without a very effective aseptic methodology and ploidy event. In this study, *in vitro* embryo culture was employed to recover natural hybrids from monoembryonic diploid, open-pollinated mandarin. Flow cytometry was used to determine ploidy level. A total of 10,289 seeds were extracted from 792 fruits having approximately 13 seeds per fruit. Average frequency of small seeds developed was 7.1%, while the average frequency of small seeds per fruit were: 8.9% for 'Clementine' 10.2% for 'Harehime' 2.6% for 'Kamja' 3.1% for 'Pyunkyool' 2.8% for 'Sadookam' and 7.0% for 'Wilking' mandarin. Average size of a perfect seed was 49.52±0.07 mm^2 ('Clementine') while the small seed measured 7.95±0.04 mm^2 ('Clementine'), which was about 1/6 smaller than the perfect seed. In total, 731 small seeds were obtained and all of them contained only one embryo per seed. The efficiency of 'Clementine' was 14 times higher than 'Wilking' and more than 109 times higher than 'Pyunkyool'. The basic information on spontaneous polyploidy provides for the hybridization of constant triploids and increases the efficiency of conventional cross.

Key words : Citrus breeding, monoembryonic, embryo rescue, polyploidy, cytology

Introduction

In the genus *Citrus* such as mandarin, orange, grapefruit, lemon etc. and related genera of Aurantioideae, the diploid is a prevalent ploidy with the monoploid number x=9 [17]. Some higher euploid genotypes of *Citrus* also exist. Several haploid plants have been produced in *Citrus* [20,21,25,43,51]. Other polyploid progenies have often been emerged by ploidy event and interploid cross. Few triploids [1,12,14,19,32], tetraploids [4,6,33] and aneuploids [31] described and developed by spontaneous and artificial breeding approaches.

Citrus seeds develop through fertilization and pollination. This systematic procedure can produce both normal and abnormal seeds. Commercial varieties, such as the diploid 'Satsuma' mandarin in Jeju are classified under the seedless citrus group of pollen degeneration due to male sterility. However, all Jeju native citrus and some self-incompatible foreign varieties can produce viable pollen and form a lot of normal seeds in the non-isolated block [48]. Depending on genotypic variation and unusual environmental factors, average number of citrus seeds is less than 62 per fruit [34,56]. Commercially, excessive number of seeds can affect the consumer's acceptance of fresh fruit due to its association with unfavorable volatile compounds that strongly affect the organoleptic properties [41], and also needs additional process by inevitable ultra-filtration and adsorption [24,29,54]. Less number of seeds can mean improved quality of fresh fruits. This key to success might inherently be a sterility barrier that may be removed by stabilization of chromosome pairing in hybrids. All of triad breeds possess this characteristic permanently and a distinct commercial advantage. These genotypes such as 'Mexican' lime are

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promising [45]. Compared with the edible food crop seeds, citrus fruits' seedlessness is an excellent trait rather than a disadvantage, as the constant triploid can be propagated vegetatively [24].

Triploid genotypes can be recovered through interploid crosses. Two seedless triploid pummelo × grapefruit (*Citrus paradisi* Macf.) hybrids, derived from 2x × 4x cross, have been released [7,8,49,50]. Unfortunately, tetraploid genotypes in citrus germplasm banks are scarce, which are attributed to the limitation of artificial chromosome doubling techniques and failure of tetrad cells in meristem [27]. These restrictions had adopted various approaches to develop triploids such as regeneration through aseptic hybrid culture of endosperm [22], *in vitro* anther culture [20], and electrofusion of haploid and diploid cultivars [40]. Although the triploids are less frequently obtained, hybridized triploids arose from non-apomictic diploid parents from the chromosome doubling event in the egg cell [53].

Most of them are found in small and abnormal seeds [1,2,12,14,19]. The small seeds produced are due to the endosperm's failure to develop normally [10] and the high probability of seed germination failure under greenhouse condition. To overcome this situation, embryo rescue procedure by an aseptic condition maybe tried. Laibach [32] first demonstrated it and now it is a well-established procedure extensively used in citrus breeding [2,5,10,42, 46,47,48]. Any triploid of Jeju native citrus species has never been hitherto reported, and then comparative analysis of polyploidy event between those and foreign varieties also does not exist yet.

This study was conducted to determine the efficiency of producing spontaneous polyploids by embryo culture and to input the results of the emergence of polyploid frequency in monoembryonic diploid *Citrus* to the triploid citrus breeding program.

Materials and Methods

Plant materials

Six varieties were open pollinated in non-block area during the anthesis in May 2011. In January 2012, citrus fruits were collected and seeds were extracted. The seeds were surface-washed gently with distilled water and dried under the shade. Seeds were classified by size and developmental stage. Size (in mm^2) was measured (3100 area meter, Li-Cor, USA) and developmental stage was evaluated by morphological parameters. Perfect seeds (PS) were normal in appearance, totally filled out, and without any malformation. Undeveloped seeds (US) had incomplete development, not totally filled out, wrinkled, and with split outer integument (Fig. 1). Seeds were washed, dried, and kept in the refrigerator until the embryo rescue.

Embryo culture

Intact developed small seeds (DSS) with the aid of a stereoscopic microscope from small seeds were cultured under aseptic condition according to the methodology by Aleza et al. [2] in petri dishes containing the Murashige and Skoog [38] culture media with 50 g/l sucrose, 500 mg/l malt extract and supplemented with vitamins (100 m/gl myo-inositol, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid, 0.2 mg/l thiamine hydrochloride, 4 mg/l glycine), and 8 g/l Bacto agar (MS culture media). After germination, plants

Fig. 1. Different types of seeds obtained in open-pollinated 'Wilking' mandarin: (A) developed small seeds; (B) undeveloped seeds; and (C) developed or perfect seeds.

were transferred to Magenta GA-7 boxes containing MT basal medium [37]. Cultures were maintained at 27±1℃, 60% humidity and 16 h daily exposure to light by fluorescent lamp.

Ploidy determination

Flow cytometry, following Aleza et al [2], was used to determine ploidy level. Nuclei suspensions were made from mature leaf samples and whole seeds. About 0.5 g of leaf tissue was collected from fully or near fully expanded leaves of seedlings two months after germination. The tissue was placed in a 60×15 mm polystyrene dish with 2.5 ml nuclei stabilizing buffer (High Resolution DNA Kit Type P, solution A; Partec, Münster, Germany), chopped to a fine mash with a single-edge razor blade. The nuclei were then filtered through a 30-μm nylon filter and stained with a 4,6-diamine-2-phenylindole (DAPI) solution (High Resolution DNA Kit Type P, solution B; Partec). After a 2-min incubation period, stained samples were run in a Ploidy Analyzer (Partec, PA) flow cytometer equipped with an HBO 100-W high-pressure mercury bulb and both KG1 and BG38 filter sets. Histograms were analyzed using the dpac v 2.18 software (Partec), which determines peak position, coefficient of variation (CV), and the relative ploidy index of the samples.

Chromosomal analysis

Root tips were washed in distilled water to remove the fixative, then macerated in an enzyme mixture containing 2% Cellulase Onozuka RS, 0.75 or 1.5% Macerozyme R200 (Yakult, Japan), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd, Japan), and 1mM EDTA, pH 4.2, at 37°C for 45-60 min. Chromosomes were stained with 0.1 g/l DAPI according to Hizume [25] and observed under a microscope with fluorescence and with a DAPI filter cassette.

Results and Discussion

Seed classification and seed content per citrus varieties

The level of seed formation and frequency of polyploids were derived from monoembryonic diploid breeds and open-pollinated native germplasm (Table 1, 2). A total of 792 fruits were used, from which a total of 10,289 seeds were obtained giving approximately 13 seeds per fruit. Average number of the seeds per fruit ranged from 4 - 24 in different mandarin hybrids. Male sterile variety, 'Harehime', contained the least number of seeds, while the Jeju native variety 'Sadookam' contained fertile pollens had the most number of seeds. The different number of normal seeds brings out a genotypic variation in *Citrus* [34,56]. The seeds obtained had textile seed coat without differentiation of seed size. The seeds affect or reduce fruit quality due to the unpleasant aroma or the bitterness they impart in citrus juices [41,48]. The total average frequency of DSS was 7.1%, while the frequency of DSS in the total seeds obtained was highest in 'Harehime' at 10.2%, followed by 'Clementine' with 8.9%, 'Wilking' with 7.0%, 'Pyunkyool' with 3.1%, 'Sadookam' with 2.8%, and the Jeju native variety, 'Kamja' had the least at 2.6%. 'Clementine' contained 1.6 DSS per fruit, which was higher than that of 'Harehime', 'Kamja', 'Pyunkyool', and 'Wilking' (Table 2). 'Fortune' mandarin had 2.6 plants per fruit by Aleza et al. [1]. These different results might be dependent on the species used. The frequency of small seeds was much higher when trifoliate orange was used as the male genitor than when Kawano natsudaidai was used [41].

Table 1. Type and size of seeds obtained in 'Wilking' and 'Clementine' by open pollination, number and ploidy level of plants recovered

Type of seeds	No. of seeds	Average area (mm^2)	No. of obtained plants	Ploidy level [x]		
				2n	3n	4n
'Wilking'						
PS	100	52.72±9.29	100[z]	100	-	-
DSS	129	19.72±5.92	125[y]	-	17	108
'Clementine'						
PS	100	49.52±0.07	100[z]	100	-	-
DSS	37	7.95±0.04	37[y]	8	29	-

[z] Germinated in glass house.
[y] Embryo rescue.
[x] Ploidy analysis by young leaf sample.

Table 2. Occurrence of small seeds and spontaneous polyploids in open pollinated fruits of monoembryonic mandarin hybrids

Seed parent	No. of fruit	No. of seed set				No. of seeds per fruits	No. of DSS per fruit	Ploidy level in DSS						Frequency of Ploid in DSS (%)			No. of triploid per fruit
		DSS (%)	PS (%)	US (%)	Total			No. of identified seed [z]			No. of identified plant [y]						
								2n	3n	4n	2n	3n	4n	2n	3n	4n	
Originated from Algeria																	
'Clementine'	220	359(8.9)	2757(68.7)	898(22.4)	4014	18.2	1.6	47	212	43	8	29	-	16.2	71.1	12.7	1.09
Originated From Japan																	
'Harehime'	216	92(10.2)	483(53.3)	331(36.5)	906	4.2	0.4	29	12	-	24	5	-	75.7	24.3	-	0.07
Originated from South Korea																	
'Kamja'	49	21(2.6)	770(94.0)	28(3.4)	819	16.7	0.4	-	-	-	14	5	-	73.7	26.3	-	0.10
'Pyunkyool	89	35(3.1)	826(73.0)	271(23.9)	1132	12.7	0.4	4	-	-	22	1	-	96.3	3.7	-	0.01
'Sadookam'	15	10(2.8)	320(88.9)	30(8.3)	360	24.0	0.7	6	1	-	3	-	-	90.0	10.0	-	0.06
Originated from USA																	
'Wilking'	203	214(7.0)	1781(58.2)	1063(34.8)	3058	15.0	1.1	-	-	84	-	17	108	-	8.1	91.9	0.08
Total	792	731(7.1)	6937(67.4)	2621(25.5)	10289	13.2	0.9	86	225	127	71	57	108	23.3	41.8	34.9	0.35

[z] Developed small seed sample used for ploidy analysis.
[y] Leaf sample from obtained plant by embryo rescue used for ploidy analysis.

Size and the average frequency of developed small seed in different varieties

The average size of normal seeds in 'Clementine'was 49.52±0.07 mm^2, while small seeds was about 1/6 of the normal seeds at 7.95±0.04 mm^2 (Table 2). This finding is possible because triploids were obtained from small seeds, about 1/3 to 1/6 of the normal seeds, yielding mostly diploids [14]. Average normal seed size of 'Wilking' was 52.72±9.29 mm^2, whereas the small seeds were 19.72±5.92 mm^2. Seed size differs depending on species. Based on the same size index, small seeds were obtained in other varieties (Fig. 1). The reduced seed size of underdeveloped seeds might be due to unreduced gamete during endosperm formation and seed set. Esen and Soost [14] demonstrated that triploid embryos were obtained from unreduced egg cell. They also proposed that fertilization of the two unreduced polar nuclei with a reduced sperm resulted in a pentaploid endosperm with genomic ratio of a 4 maternal:1 paternal, which is usually responsible for reduced seed size.

Unreduced egg cell may be achieved from apospory wherein meiosis is completely bypassed or from diplospory or the production of unreduced eggs by either meiotic-like division or restitution meiosis [3,30,39]. Luro et al. [34] proposed that by molecular marker analysis, diploid egg cell results from the abortion of a reductional division in the second division restitution (SDR) for 'Clementine' (*Citrus clementina* Hort. ex Tan.). This hypothesis is consistent with the previous proposal of Esen et al. [15], whereas Chen et al. [9] proposed that unreduced egg cell was produced from the first meiotic division restitution (FDR) in sweet orange [*C. sinensis* (L.) Osb.]. Recently, Cuenca et al. [11] confirmed that all triploid hybrids obtained in the progenies of 2x × 2x crosses with 'Fortune' mandarin as female genitor were derived from unreduced egg cell by simple sequence repeat (SSR) marker analysis. A 2n megaspore commonly forms in various other species of Dandelion [51], Solanum [54] and Vaccinium [36].

Embryo culture and germination rate

All normal seeds with well formed type germinated and verified as diploid hybrids without any chromosome chimera by only one peak existed (Fig. 2). Poor seed germination might likely be due to abnormal endosperm formation. No small seed germinated in glass house. Aleza et al. [2] showed that efficient breeding programs based on shriveled caryopses type of hybridization required very effective methodologies for embryo rescue. Embryos were cultured from the 129 DSS in 'Wilking' mandarin by open pollination and were cultured in vitro. A total of 125 plantlets were obtained, of which 17 were triploid and 108 were tetraploids (Table 2). But Oiyama and Kobayashi [42] obtained pentaploid (2n=5x=45) seedlings from some small seeds of diploid × diploid crosses using monoembryonic 'Miyauchi iyokan' (*C. iyo* Hort. ex Tanaka) as the female diploid genitor. However, the seedlings were not vigorous. Aleza et al. [1] obtained four pentaploids from 'Clemenules' clementine and

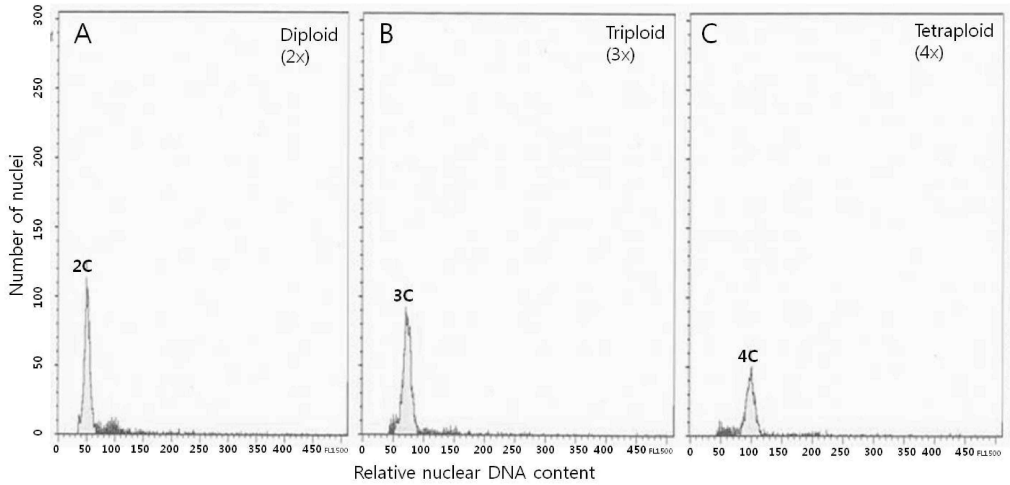

Fig. 2. Flow cytometry analysis of nuclear DNA from developed leaves of: diploid 'Clementine' (A); triploid 'Harehime' tangor (B); and tetraploidy 'Clementine' (C) by open pollination.

'Fortune' mandarin. It therefore implies that ploidy events depend on the citrus species. Although no conclusive evidence found, less number of triploids in 'Wilking' mandarin might be from bigger seed size because it is out-of-range (1/3 to 1/6). In particular, There were a lot of tetrapoids occur in diploid 'Wilking' variety which it might be unreduced gamete both male and female genitor. Most of the natural tetraploid lines arise from apomictic genotypes [2] mainly from unreduced gametes fertilized by diploid pollen [13] or from chromosome doubling in nucellar tissue [17,28]. This mode of ploid behavior in polyembryonic *Citrus* seems to be climate-dependent evolution. However, our seeds from monoembryonic ones by pollination meant not to be derivative from somatic tissues. That hybridization of 2n gametes might be a prevalent phenomenon for polyploidy event in mandarin species. Recently Song et al [48] described that a tetaraploid from monoembryonic citrus species emerges in 2n × 2n cross.

Aleza et al [2] in 2011, performed *in vitro* culture of more than 4,100 embryos rescued from small but well developed seeds from 44 different hybridizations. About 236 plantlets were obtained from DSS in seven varieties by embryo culture. The embryos from the DSS were well-developed, with two cotyledons, and well-formed embryonary axis (Fig. 3). This systematic germination process was observed in all six breeds and well-cultured triploid plants collected from embryos rescued from the small developed seeds. The polyploids obtained may also provide promising materials for future genetic and cytogenetic studies.

The DSS from open-pollinated 'Wilking' mandarin were cultured in vitro with and without double-layer testa. The intact seed germinated slower than the seed without teguments. The germination percentage of isolated embryos was 125/129 or 96.9%, which was higher than the ones obtained by Aleza et al [1] at 42/50 or 84%. Embryo rescue can be done to recover desirable polyploid seedlings in the breeding program. Results of embryo cultured (Fig. 3), plant regeneration, ploidy level, and transfer to artificial soil of plants recovered in open pollinations are shown in Table 2. From the 242 in-vitro cultured embryos, plants were recovered. Average germination percentage was 96% using the embryo culture tool, fluctuating between 80% for 'Pyunkyool' and 100% for 'Clementine', 'Harehime' and 'Sadookam'. Aleza et al [1] described how the seedless triploid progenies were recovery from 2x × 2x hybridizations by embryo rescue under aseptic conditions and verified by flow cytometry.

Frequency of triploids in monoembryonic varieties and emergent polyploidy

On the frequency of polyploidy level verified by flow cytometry, 41.8%, were triploids, 23.3% were diploids, and 34.9% were tetraploids (Table 3). From the 236 plants rescued, 71 were diploids (32.6%), 57 were triploids (24.1%), and 108 were tetraploids (45.7%) as verified by flow cytometry. The average percent survival at transplant phase was approximately 90% and the plants obtained were generally robust and vigorous (Fig. 3).

Recovery efficiency in citrus triploid plants was calculated as the number of triploid plants per harvested fruit.

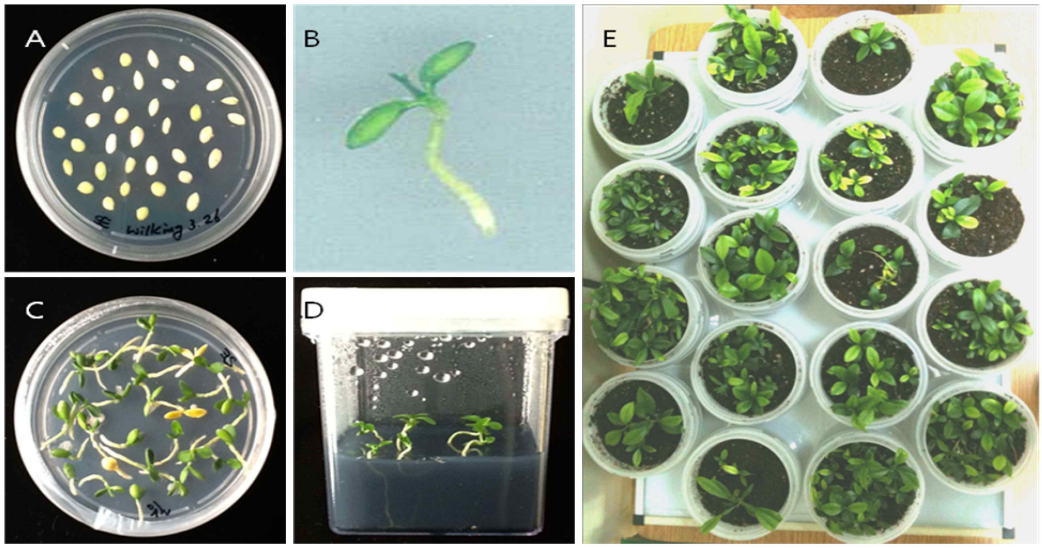

Fig. 3. (A) Small seeds of 'Wilking' mandarin with one embryo; (B and C) Germinated embryos of 'Wilking' mandarin rescued from small seeds; (D) - *In-vitro* triploid plant obtained by open-pollination; (E) - Triploid and tetraploid plants obtained from 2x - 2x crosses, transplanted and cultivated in plastic pots.

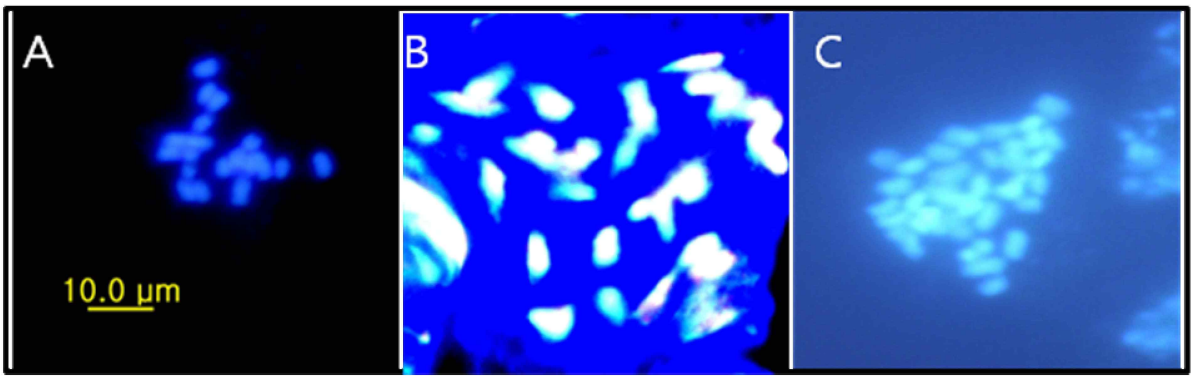

Fig. 4. DAPI-stained chromosomes from root tip of diploid 'Clementine' (A), triploid 'Harehime' (B), and tetraploid 'Clementiene'(C), which occurred spontaneously in some mandarin hybrids.

Triploids hybrids were obtained mostly from 'Clementine' (Table 2), at an average of 0.35% triploid hybrids per fruit. In Jeju native mandarin, the efficiency varied from 0.10% ('Kamja') to 0.01% ('Pyunkyool'), and 0.06% ('Sadookam'). 'Pyunkyool' mandarin had low efficiency, with only 0.01% triploid hybrids per fruit. The efficiency of 'Clementine' was 14 times higher than 'Wilking' tangor and more than 109 times higher than 'Pyunkyool' (Table 3). Aleza et al [1] indicated that genetic and probably phenotypic distances between triploid hybrids were shorter when the female parent produces diploid gametes, than when the male parent produces haploid gametes. Different results in the frequencies might also be related to specific external factors such as temperature and light intensity, depending on cultivar traits. Plant position or direction of fruit on the plant was not considered on seed number and its development as reported by Fatima et al [16] in *Citrus* [4]. Luro et al [35] also indicated that environmental factors dramatically affect the number of recovered triploids.

Confirming triad chromosomes in spontaneous triploids

At heterotypic metaphase during meiosis of 'Clementine' had 18 chromosomes as its diploid number (Fig. 4). Subgenus *Citrus* and *Papeda* had 2n=18 chromosomes [55]. At mitotic phase, 36 chromosomes were recognizable by

chromosome doubling in F_1 of 'Clementine'. 'Harehime' F_1 had 27 chromosomes as its triploid number, containing nine extra chromosomes. As the detailed description of chromosome number was nothing but a mere repetition, only diploid and tetraploid 'Clementine' and triploid 'Harehime' were shown here (Fig. 4).

In conclusion, six open-pollinated citrus varieties used obtained a total of 10,289 seeds with 731 DSS (7.1%). In addition, 236 plants obtained through embryo culture produced 57 triploid and 108 tetraploid progenies. Promising triploids might be obtained by embryo culture and tetraploid lines may be obtained for future triploid citrus breeding program.

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References

1. Aleza, P., Jua´rez, J., Cuenca, J., Ollitrault, P. and Navarro, L. 2010. Recovery of citrus triploid hybrids by embryo rescue and flow cytometry from 2x × 2x sexual hybridisation and its application to extensive breeding programs. *Plant Cell Rep.* **29,** 1023-1034.
2. Aleza, P., Froelicher, Y., Schwarz, S., Agustı´, M., Herna´ndez M., Juárez J., Luro, F., Morillon, R., Navarro, L. and Ollitrault, P. 2011. Tetraploidization events by chromosome doubling of nucellar cells are frequent in apomictic citrus and are dependent on genotype and environment. *Ann. Bot.* **108**, 37-50.
3. Asker, S. E. and Jerling, L. 1992. Apomixis in plants. CRC Press, Boca Raton, Ann Arbor, London, Tokyo.
4. Barrett, H. C. and Hutchison, D. J. 1978. Spontaneous tetraploidy in apomictic seedlings of Citrus. *Econ. Bot.* **32**, 27-45.
5. Bhojwani, S. S. and Razdan, M. K. 1983. Plant tissue culture: Theory and Practices, Developments in Crop Sciences, 5. Elsevier, Amsterdam.
6. Cameron J. W. and Burnett, R. H. 1978. Use of sexual tetraploid seed parents for production of triploid citrus hybrids. *HortScience* **13,** 167-169.
7. Cameron, J. W. and Frost, H. B. 1968. Genetic, breeding and nucellar embryony. pp. 325-370, In Reuther, W., Batchelor L. D. and Webber, H. J. (eds.), The citrus industry, Vol. 1. Riverside, CA: University of California.
8. Cameron, J. W. and Soost, R. K. 1969. Characters of new populations of Citrus polyploids, and the relation between tetraploidy in the pollen parent and hybrid tetraploid progeny. pp. 199-205, In Chapman, H. D. (ed.), Proceedings of the International Citric Symposium, Vol. 1, University of California at Riverside.
9. Chen, Z. J. 2007. Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. *Ann. Rev. Plant Biol.* **58,** 377-406.
10. Cocking, E. C. 1986. The tissue culture revolution. pp. 3-20, In Withers, L. A. and Anderson, P. G. (eds.), Plant Tissue Culture and its Agric. Application. Butterworth.
11. Cuenca, J., Froelicher, Y., Aleza, P., Jua´rez, J., Navarro, L. and Ollitrault, P. 2011. Multilocus half tetrad analysis and centromere mapping in Citrus: evidence of SDR mechanism for 2n mega gametophyte production and evidence partial chromosome interference in mandarin cv Fortune. *Heredity* **107,** 462-470.
12. Esen, A. and Soost, R. K. 1971. Unexpected triploids in Citrus: their origin, identification and possible use. *J. Hered.* **62,** 329-333.
13. Esen, A. and Soost, R. K. 1972. Tetraploid progenies from 2x X 4x crosses of Citrus and their origin. *J. Amer. Soc. Hort. Sci,* **97,** 410-414.
14. Esen, A. and Soost, R. K. 1973. Precocious development and germination of spontaneous triploid seeds in Citrus. *J. Hered.* **64,** 147-154.
15. Esen, A., Soost, R. K. and Geraci, G. 1979. Genetic evidence for the origin of diploid mega-gametophytes in citrus. *J. Hered.* **70**, 5-8.
16. Fatima, B., Usman, M., Khan, I. A., Khan, M. S. and Khan, M. M. 2010. Exploring citrus cultivars for underdeveloped and shriveled sees: A valuable resource for spontaneous polyploidy. *Pak. J. Bot.* **42**, 189-200.
17. Frost, H. B. and Soost, R. K. 1968. Seed reproduction, development of gametes and embryos. pp. 290-324, In:Reuther, W, Batchelor, L. D., Webber, H. B., (eds.), The citrus industry, vol. 2. Berkeley: University of California.
18. Frost, H. B. 1925. The chromosomes of Citrus. J. *Washington Acad. Sci.* **15,** 1-3.
19. Geraci, G., Esen, A. and Soost, R. K. 1975. Triploid progenies from 2x - 2x crosses of Citrus cultivars. *J. Hered.* **66,** 177-178.
20. Germanà, M. A. and Chiancone, B. 2001. Gynogenetic haploids of citrus after in vitro pollination with triploid pollen grains. *Plant Cell Tiss. Org.* **66,** 59-66.
21. Germanà, M. A., Wang, Y. Y., Barbagallo, M. G., Iannolino, G., and Crescimanno, F. G. 1994. Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and Citrus reticulata Blanco. J. *Hort. Sci.* **69,** 473-448.
22. Gmitter, F. J. J., Ling, X. B. and Deng, X. X. 1990. Induction of triploid Citrus plants from endosperm calli in vitro. *Theor. Appl. Genet.* **80,** 785-790.
23. Guo, Y., Zhao Y., Li, K., Liu, Z., Lin, H., Guo, X. and Li,

C. 2011. Embryo rescue of crosses between diploid and tetraploid grape cultivars and production of triploid plants. *Afr. J. Biotechnol.* **10**, 19005-19010.
24. Hernandez, E., Couture, R., Rouseff, R., Chen, C. S. and Barros, S. 1992. Evaluation of ultrafiltration and adsorption to debitter grapefruit juice and grapefruit pulp wash. *J. Food Sci.* **57,** 664-670.
25. Hidaka, T., Yamada, Y. and Shichijo, T. 1979. *In vitro* diffenentiation of halpoid plants by anther culture in *Poncirus trifoliata* (L.) Raf. *Jpn. J. Breeding* **29,** 248-254.
26. Hizume, M. 1991. Analysis of plant chromosomes using a fluorescent banding method. *Plant Cell Tech.* **3,** 78-83 (in Japanese, with English abstract).
27. Iwamasa M., Nito, N. and Ling, J. T. 1988. Intra and intergeneric hybridization in the orange subfamily, Auranthioideae. *Proc. Int. Soc. Citriculture* **6**, 123-130.
28. Kobayashi, S., Ieda, I. and Nakatani, M. 1981. Role of the primordium cell in nucellar embryogenesis in citrus. In: Proceedings of 4th International Citrus Congress. Tokyo: *Proc. Int. Soc. Citriculture* 44-48.
29. Kola, O. Kaya, C., Duran, H. and Altan, A. 2010. Removal of limonin bitterness by treatment of ion exchange and adsorbent resins. *Food Sci. Biotechnol.* **19,** 411-416.
30. Koltunow, A. M. 1993. Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. *Plant Cell* **5,** 1425-1437.
31. Krug, C. A. 1943. Chromosome numbers in the subfamily Aurantioideae with special reference to the genus Citrus. *Bot. Gaz.* **48,** 602-611.
32. Lapin, W. K. 1937. Investigations on polyploidy in Citrus work. *All-Union Scientific Research Institute Humid Subtropics,* **1,** 1-68.
33. Laibach, F. 1925. Das Taubwerden von Bastardsamen and die kunstliche Aufzucht fruh absterbender Bastardembryonen. *Z. Bot.* **17,** 417-459.
34. Liu, Y. Z., Ma, X. T., Zhang, H. Y., Peng, S. and Deng, X. X. 2004. Status quo of Ponkan (*Citrus reticulata* Blanco) fruit quality and quality comparison between several production areas in China. *Acta Hortic. Sin.* **31,** 584-588.
35. Luro, F., Maddy, F., Ollitrault, P. and Rist, D. 2000. Identification of 2n gamete parental origin and mode of nuclear restitution of spontaneous triploid Citrus hybrids. *Proc. Int. Soc. Citriculture* 168-169.
36. Lyerene, P. M., Vorsa, N. and Ballington, J. R. 2003. Polyploidy and sexual polyploidization in the genus Vaccinium. *Euphytica* **133,** 27-36.
37. Murashige, T. and Tucker, D. P. H. 1969. Growth factor requirement of citrus tissue culture. pp. 1155-1161, In Chapmam, H. D. (ed.), Proc. First Int. Citrus Symp. Vol. 3, University of California, Riverside,
38. Murashige, T. and Skoog, R. 1962. Arevised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15,** 473-497.
39. Nogler, G. A. 1984. Gametophytic apomixis. pp. 475-518, In Johri, B. M. (ed.), *Embryology of Angiosperms*. Springer, Berlin Heidelberg New York.
40. Ollitrault, P., Dambier, D., Vanel, F. and Froelicher, Y. 2000. Creation of triploid Citrus hybrids by electrofusion of haploid and diploid protoplasts. *Acta Hortic.* **535,** 191-197.
41. Ollitrault, P., Luro, F. and Yamamoto, M. 2007. Seedlessness and ploidy manipulations pp. 197-218, In Khan, I. A. (ed.), *Citrus Genetics, Breeding and Biotechnology.* CAB International UK.
42. Oiyama, I. and Kobayashi, S. 1990. Polyembryony in undeveloped monoembryonic diploid seeds crossed with a citrus tetraploid. *Hortscience* **25,** 1276-1277.
43. Oiyama, I. and Kobayashi, S. 1993. Haploids obtained from diploid × triploid crosses of citrus. *J. Japan Soc. Hort. Sci.* **62,** 89-93.
44. Peloquin, S. J., Boiteaux, L. S., Simon, P. W. and Jansky, S. H. 2008. A chromosome-specific estimate of transmission of heterozygosity by 2n gametes in potato. *J. Hered.* **99**, 177-181.
45. Raza, H., Khan, M. M. and Khan, A. A. 2003 Seedlessness in Citrus. *Int. J. Agri. Biol.* **5**, 388-391.
46. Sen-Cai, Z. 1981. Thirty years achievements in citrus varietal improvement in China. *Proc. Int. Soc. Citriculture* **1,** 51-55.
47. Soares, F., Wdos, S. and Vasque Araujo, J. E., de Cunhe, M. A. P., de Cunha Sobrinho, A. P. and Passos, O. S. 1992. Degree of polyembryony, size and survival of the zygotic embryo in citrus. *Proc. Int. Soc. Citriculture* **1,** 135-738.
48. Song, K. J., Kim, S. B., Park, J. H., Oh, E. U., Lee, K. G., Kim, D. W., Kang, J. H., Kim, J. S., Oh, J. H. and Gmitter, F. G. 2011. Frequency and growth characteristics of polyploids occurred spontaneously in some mandarin hybrids. *Kor. J. Hort. Sci. Technol.* **29**, 617-622.
49. Soost, R. K. and Cameron, J. W. 1980. 'Oroblanco', a triploid pummelo-grapefruit hybrid. *HortScience* **15,** 667-669.
50. Soost, R. K. and Cameron, J. W. 1985. 'Melogold', a triploid pummelo-grapefruit hybrid. *HortScience* **29,** 1134-1135.
51. Toolapong, P. and Komatsu, H. 1996. Triploids and haploid progenies derived from small seeds of Banpeiyu, a pummelo, crossed with Ruby Red grapefruit. *J. Japan Soc. Hort. Sci.,* **65,** 255-260.
52. Van Dijk, P. J. and Bakx-Schotman, J. M. T. 2004. Formation of unreduced megaspores (diplospory) in apomictic dandelions (*Taraxacum officinale* s.l.) is controlled by a sex specific dominant locus. *Genetics* **166,** 483-492.
53. Vardi, A. 1992. Conventional and novel approaches to citrus breeding. *Proc. Int. Soc. Citriculture* **1,** 39-43.
54. Wethern, M. 1991. Citrus debittering with ultrafiltration/adsorption combined technology. pp. 48-46. In: 37th Annual Citrus Engineering Conference. March 21. American Society of Mechanical Engineers, Lake Alfred, FL, USA.
55. Yamamoto, M., Abkenar, A. A., Matsumoto. R., Nesumi, H., Yoshida, T., Kuniga, T., Kubo, T. and Tominaga, S. 2007. CMA Banding Patterns of Chromosomes in Major Citrus Species. *J. Japan Soc. Hort. Sci.* **76**, 36-40.
56. Yamamoto, M., Yamada, Y., Matsumoto, R., Ikemiya, H. and Okudai, N. 1992. Inheritance of seed number in citrus. *Bul. Fruit Tree Res. Sta.* **23**, 47-56.

초록 : 단배성 제주 재래귤 및 만다린잡종에서 자연 발생적인 배수체의 발생 빈도

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배수체는 무핵 감귤 육종 프로그램 있어서 잠재적으로 매우 중요한 유전자원이다. 무핵성은 만다린 품종이 갖추어야 할 가장 유망한 형질 중 하나이고 3배체는 영구적으로 무핵이다. 새로운 3배체 잡종은 이종속간 배수체의 교배 또는 저빈도의 2배성 배우체 형성과 합성 같은 행동에 기인된 2배체 이종간 교배로도 이룰 수 있다. 그러나 소립의 F_1 합성 종자로 발달된 이 같은 형태에 기초한 육종은 효과적인 무균적 배 적출 및 배양법 없이는 불가능하다. 본 연구에서, 무균의 배배양을 이용하여 단배의 2배체 제주 재래귤과 만다린잡종에서 방임 수분되어 자연적으로 발생한 소립종자로부터 유묘를 얻었고 배수 검정기를 이용하여 배수 정도를 검정하여 다양한 수준의 배수체를 획득하였다. 총 792 과실을 이용하여 10,289 개의 획득된 종자를 분석하여 과실당 평균 13개의 종자가 함유됨을 알 수 있었다. 정상종자와 유사한 형태로 발달된 소립종자의 과실 내 평균 함유율은 7.1%였다. 과실당 소립종자의 평균 출현 빈도는 '클레멘타인', '하레히메', '감자', '편귤', '사두감' 그리고 ' 월킹' 순으로 각각 8.9, 10.2, 2.6, 3.1, 2.8, 그리고 7.0% 였다. 종자 크기가 조사된 '클레멘타인' 품종 내 정상 종자의 평균 크기는 49.52±0.07 mm^2 였고 반면에 소립종자는 7.95±0.04 mm^2로 전자보다 1/6배 더 작았다. 이러한 분류 기준으로 총 731 개의 소립종자를 획득하였다. 이들 소립종자 중 일부는 무균의 배 배양이 수행되었고 기내 발아되어 회복된 모든 개체들은 단 하나의 배가 확인되었고 건전하게 발육하였다. 3배체 발생 빈도 비교에 있어서, '클레멘타인'은 '월킹'과 제주 재래귤인 '편귤' 품종에 비해 각각 14배와 109배 높게 나타난 바 전자는 3배체 발생이 높음을 알 수 있었다. 자연 발생적인 배수체에 관한 기초 정보는 무핵 감귤 개발을 위해 3배체 고정종 합성에 활용될 수 있으며 그 효율 증진에도 기여할 것이라 여겨진다.