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Generation of Nonaploid Persimmons (*Diospyros kaki* Thunb.) by Embryo Culture of Imperfect Seeds Derived from a Cross between 'Fuyu' and 'Taishuu'

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We obtained nonaploid plants by embryo culture of imperfect seeds derived from a cross between 'Fuyu' and 'Taishuu', both of which are commercially important persimmon cultivars. Furthermore, we aimed to clarify the origin of nonaploid plants derived from imperfect seeds. Of 1078 seeds, 68 were imperfect; this accounted for 6.3% of the total number. Ten of the 68 seeds germinated into seedlings, and two produced abnormal plantlets without meristems at both the shoot apex and root tip. The remaining eight produced normal-appearing plantlets with normal growth. Two seedlings were recovered from the abnormal plantlets *via* callus, and grew vigorously in the greenhouse. Cytogenetic analysis confirmed that these two seedlings were nonaploids. Parental analysis using four simple sequence repeats (SSR) markers, D.CT-13, 24, 61 and 179, showed that each nonaploid seedling had alleles originating from the parents, indicating that they were generated by syngamy. The two nonaploid seedlings had alleles of 222 bp at D.CT-61, which is peculiar to the pollen parent, 'Taishuu', whereas they did not have an allele of 136 or 140 bp at D.CT-179, which is peculiar to the seed parent, 'Fuyu'. These results suggest that they might be derived from fertilization of a reduced female gamete with an unreduced male gamete.

Key Words: embryo culture, meiosis, pollination-constant non-astringent (PCNA), sexual polyploidy, unreduced gamete.

Introduction

Many Japanese persimmon (*Diospyros kaki* thunb.) cultivars are hexaploid (2n = 6X) (Namikawa and Higashi, 1928); however, some nonaploid cultivars (2n =9X) such as 'Hiratanenashi' and its bud sport (Zhuang et al., 1990) are increasingly grown for commercial culture, because of their seedlessness and vigorous growth. Since these nonaploid cultivars are astringent types, the astringency must be removed before consumption, which shortens shelf life (Ishimaru et al., 2001). If non-astringent types of nonaploid cultivars could be generated, breeding could achieve seedless cultivars with longer shelf life. Inter-crossing between pollination-constant non-astringent (PCNA) genotypes is a certain method of breeding PCNA-type Japanese persimmon, because the PCNA genotype is recessive to non-PCNA genotypes (Ikeda et al., 1985). 'Fuyu' is a major PCNA persimmon cultivar in Japan with a good eating quality and high yield (Itamura et al., 2005; Yamada, 1994). 'Taishuu' is also a PCNA persimmon cultivar producing large fruit with an excellent taste, and the production area reached around 100 ha in 2003 (Yamane et al., 2001; Yamada, 2006); therefore, we considered that they are important parent cultivars for PCNA persimmon breeding.

Sugiura et al. (2000) reported that pollinating hexaploid cultivars with sorted unreduced pollen followed by embryo rescue culture would be useful for breeding nonaploid seedless cultivars; however, this method would be impractical for pollen with a low germination rate, such as pollen of 'Taishuu' (Chijiwa et al., 1997), because the hydration sorting process further lowers the pollen germination rate (Yamada and Tao, 2007). Triploid offspring have been derived from imperfect seeds crossed between diploid and diploid in some citrus species (Esen and Soost, 1971; Oiyama and Okudai, 1983). Sugiura et al. (2000) also reported that nonaploid persimmons were derived from imperfect seeds by crossing a hexaploid cultivar with sorted unreduced pollen. Since the poor development of seeds

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is thought to be caused by an imbalance of maternal genomes and the paternal genome ratio (2 : 1) in the endosperm (Bretagnolle and Thompson, 1995; Esen and Soost, 1971; Sanford, 1983; Sugiura et al., 2000), the formation of imperfect seeds seems to be related to polyploidy variation. If the imperfect seeds of Japanese persimmon are related to polyploidy variation, utilization of the imperfect seeds obtained from hexaploid cultivars pollinated with natural pollen of hexaploid cultivars may be useful for nonaploid seedless cultivar breeding.

In this study, we aimed to obtain nonaploid persimmons from imperfect seeds by embryo culture using a cross between 'Fuyu' and 'Taishuu', both of which are hexaploid cultivars, and furthermore to clarify the origin of nonaploid embryos from imperfect seeds.

Materials and Methods

Occurrence of imperfect seeds obtained from 'Fuyu' pollinated with pollen of 'Taishuu'

Prebagged female flowers of 'Fuyu' persimmon were pollinated with pollen of 'Taishuu' that contained unreduced hexaploid pollen at a rate of approximately 1% in 2001. Fifty fruits were harvested at 180 days after pollination, and a total 166 seeds were collected and weighed. Seeds that were less than 13 mm in longitudinal length were defined as imperfect seeds in this study (Fig. 1).

Embryo culture of imperfect seeds

In 2001 and 2002, a total of 303 fruits of 'Fuyu' pollinated with pollen of 'Taishuu', which contained approximately 1% unreduced hexaploid pollen, were harvested at 70–90 days after pollination and the seeds were aseptically removed from the fruits. Imperfect seeds were cut into halves and cultured on MS medium (Murashige and Skoog, 1962) with nitrogen reduced to half-strength (1/2 N MS) supplemented with 5 μM t-zeatin and 500 mg·L^{-1} yeast extract under a 16-h photoperiod at 25°C. After two or three months of culture, the obtained plantlets were transferred to 1/2 N MS medium without plant growth regulators (PGRs). Plantlets that had no meristem at both the shoot apex and root tip were induced to form a callus on 1/2 N MS medium supplemented with 10 μM t-zeatin and 1 μM Indoleacetic Acid (IAA) under dark conditions. For adventitious bud formation, calli were transferred to 1/2 N MS medium supplemented with 10 μM t-zeatin and 1 μM IAA under a 16-h photoperiod. Adventitious buds were planted on shoot elongation medium consisting of 1/2N MS with 5 μM t-zeatin. After elongation, shoots were cut into 2–3 cm lengths, the basal ends of shoots were soaked in 1.25 mM Indolebutyric Acid (IBA) for 30 s, and then these micro cuttings were placed on 1/2N MS without PGRs to promote rooting (Tao et al., 1988). They were cultured under dark conditions for 10 days and then cultured under a 16-h photoperiod. All culture media contained 3% sucrose and were adjusted to pH 5.8. Rooted plantlets were transferred to plastic pots with a mixture of peat moss: vermiculite: zeolite (1 : 1 : 1), and were acclimatized and grown in a greenhouse (Tao et al., 1988).

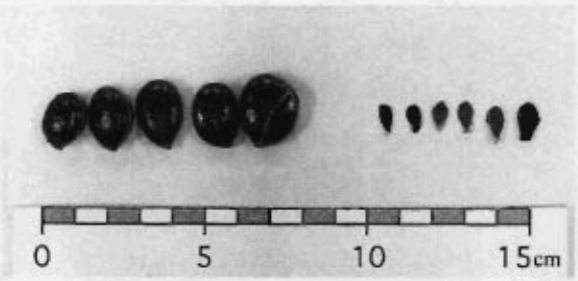

Fig. 1. Comparison of perfect and imperfect seeds obtained from 'Fuyu' pollinated with pollen of 'Taishuu'. Left: fully developed perfect seeds. Right: poorly developed imperfect seeds, each less than 13 mm in longitudinal length.

Flow cytometric analysis and chromosome counting

Relative DNA contents of the seedlings derived from imperfect seeds were determined using a ploidy analyzer (PA Partec Co., Germany) following the manufacturer's instructions. 'Fuyu' and 'Hiratanenashi' were used as reference standards of hexaploids (2n = 6X = 90) and nonaploids (2n = 9X = 135), respectively. The chromosome numbers of seedlings were also counted using enzyme macerating methods (Zhuang et al., 1990).

Parentage analysis by SSR markers

Total DNA was extracted from young leaf tissue using the CTAB method (Doyle and Doyle, 1987). For parental analysis of the two nonaploid seedlings derived from imperfect seeds and their parents, 'Fuyu' and 'Taishuu', SSR analysis was conducted using four SSR markers, D.CT-13, 24, 61, and 179 according to Wakisaka et al. (2003). PCR amplification was performed in a 20 μL of reaction mixture containing 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each forward primer labeled with a fluorescent chemical (NED) and unlabeled reverse primer, 0.5 μg of genomic DNA, and 0.5 unit of Taq polymerase (Takara, Japan). PCR products were separated and detected using an ABI 310 sequencer (PE Applied Biosystems, USA). The size of the amplified bands was calculated based on an internal standard DNA with GeneScan software (PE Applied Biosystems).

Results

Imperfect seeds obtained from 'Fuyu' pollinated with pollen of 'Taishuu'

The weight distribution of the seeds is presented in Figure 2. The distribution curve was divided into two groups; the major group ranged from 0.75 to 2.3 g, while the minor group ranged from 0.01 to 0.07 g. All imperfect seeds, which had longitudinal lengths less than 13 mm, belonged in the minor group. The mean weight of the imperfect seeds was 0.03 g, which was approximately

1/40 that of the perfect seeds (1.35 g).

Embryo culture of imperfect seeds

Overall, 1078 seeds were obtained, of which 68 were imperfect (6.3%) (Table 1). Of the 68 imperfect seeds, 10 germinated into seedlings. Two of these produced abnormal plantlets that lacked a cotyledon, and had no meristem at both the shoot apex and root tip (Fig. 3A). The remaining eight seedlings produced plantlets with a normal appearance and normal growth. The two seedlings, No. 1 and No. 2, were recovered from abnormal plantlets as complete plants *via* the callus, and grew vigorously in the greenhouse (Fig. 3B–D).

Flow cytometric analysis and chromosome counting

The relative DNA content of the two seedlings recovered from abnormal plantlets was the same as that of the nonaploid reference, while those derived from normal plantlets was the same as that of the hexaploid reference (Fig. 4). One hundred thirty five chromosomes were observed in the two seedlings derived from abnormal plantlets (2n = 9X), while 90 were observed in the normal plantlets (2n = 6X) (Fig. 5). These results indicated that the two seedlings recovered from abnormal plantlets were nonaploids, whereas those derived from normal plantlets were hexaploids.

Parentage analysis by SSR marker

The alleles of the two nonaploid seedlings and their parents generated from four simple sequence repeats (SSR) loci are shown in Table 2. Two of the four SSR loci did not show polymorphisms among the two seedlings and their parents, i.e. the two nonaploid seedlings and their parents had alleles of 163 bp at D.CT-13 and 116 bp at D.CT-24, respectively. Another two SSR loci showed polymorphisms among the two seedlings and their parents, i.e. 'Taishuu' and the two seedlings had alleles of 200, 206, and 222 bp at D.CT-61, whereas 'Fuyu' had 200 and 206 bp. On the other hand, 'Fuyu' had alleles of 130, 136, 140, 151, and 162 bp at D.CT-179, whereas neither 'Taishuu' nor seedling No. 2 had alleles of 136 and 140 bp. Seedling No. 1 also did not have the 140 bp allele that is peculiar to 'Fuyu'. No discrepancy was confirmed in the inheritance of alleles among the parents, 'Fuyu', 'Taishuu' and the two seedlings at the four SSR loci.

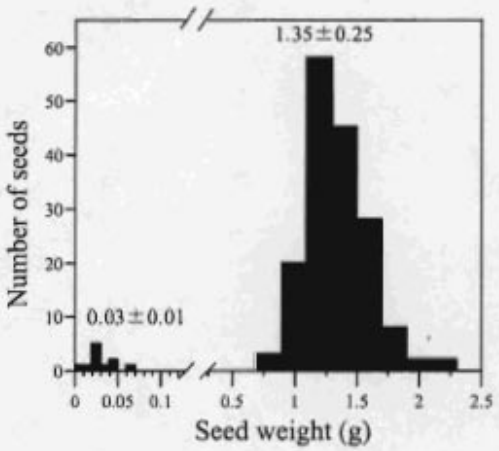

Fig. 2. Seed weights obtained from 'Fuyu' pollinated with pollen of 'Taishuu'. Seeds were harvested at 180 days after pollination. Mean weights ± SD of imperfect and perfect seeds are indicated.

Discussion

The weight distribution of the imperfect seeds was clearly different from that of the perfect seeds. Polyploidy variations, such as triploid, were derived from imperfect seeds in some citrus species. These seeds can be identified visually by their size, as they are approximately one-sixth to one-third that of normal seeds based on seed weight (Esen and Soost, 1971; Oiyama and Okudai, 1983). A similar polyploidy variation,

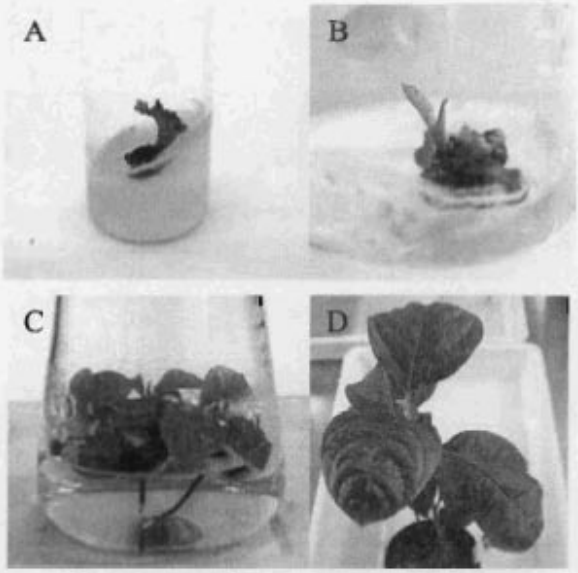

Fig. 3. Plant regeneration from abnormal plantlets derived from imperfect seeds. A: Plantlet lacking a cotyledon, and with no meristem at the shoot apex and root tip. B: Adventitious buds from callus. C: Rooting from shoots. D: Regenerated plant growing in greenhouse.

Table 1. Number of perfect and imperfect seeds from a cross between 'Fuyu' and 'Taishuu', and ploidy levels of the seedlings derived from imperfect seeds.

Number of fruits	Number of seeds			Number of seedlings		
	Perfect	Imperfect	Total	Nonaploid	Hexaploid	Total
303	1010	68	1078	2	8	10

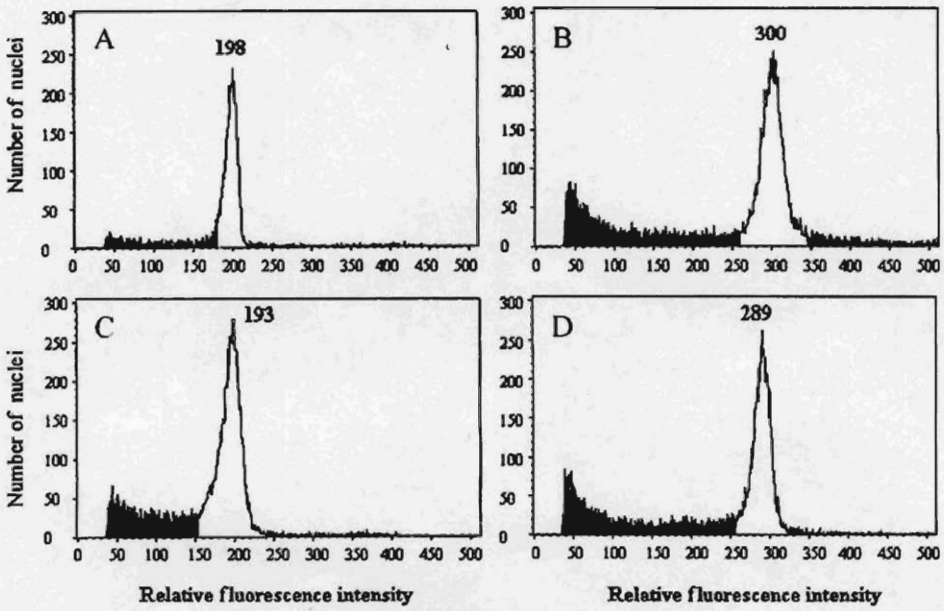

Fig. 4. Flow cytometric histograms of relative fluorescence intensity in the nuclei of 'Fuyu', 'Hiratanenashi', and seedlings derived from imperfect seeds from a cross between 'Fuyu' and 'Taishuu'. A: 'Fuyu', hexaploid reference standard (2n = 6X = 90). B: 'Hiratanenashi', nonaploid reference standard (2n = 9X = 135). C: Seedling derived from imperfect seed that developed into a normal-appearing plantlet. D: Seedling No. 1 recovered from an abnormal plantlet derived from imperfect seed. Numbers in frames indicate relative fluorescence value of the peak.

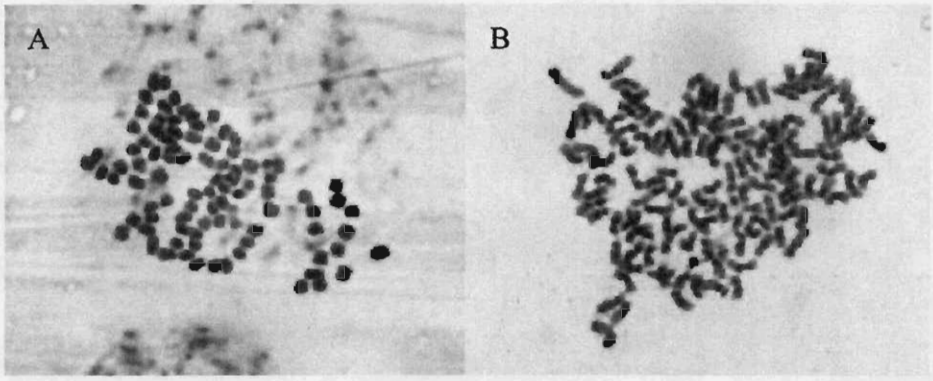

Fig. 5. Chromosomes of seedlings derived from imperfect seeds of the cross 'Fuyu' and 'Taishuu'. A: Seedling derived from imperfect seed that developed into a normal plantlet (90 chromosomes; 2n = 6X). B: Seedling No. 1 recovered from abnormal plantlet derived from imperfect seed (135 chromosomes; 2n = 9X).

Table 2. Alleles of nonaploid seedlings and their parents at four SSR loci.

Cultivar and seedling	SSR markers			
	D.CT-13	D.CT-24	D.CT-61	D.CT-179
Fuyu	163	116	200/206	130/136B/140B/151/162
Taishuu	163	116	200/206/222A[z]	130/151/162
Seedling No. 1	163	116	200/206/222A	130/136B/151/162
Seedling No. 2	163	116	200/206/222A	130/151/162

[z] A and B indicate alleles peculiar to 'Taishuu' and 'Fuyu', respectively.

nonaploid offspring derived from imperfect seeds obtained by inter-crossing between hexaploid persimmons, was observed in this study. Regeneration *via* the callus was necessary to form complete plantlets, because nonaploid plantlets derived from imperfect seeds lacked meristems. Since nonaploid embryos derived from the cross of a hexaploid cultivar with unreduced hexaploid pollen also produced abnormal plantlets, e.g., lacking a

cotyledon (Sugiura et al., 2000), it was considered that embryos with ploidy variations from imperfect seeds tend to grow abnormally and/or stop growing in an early stage of embryogenesis. On the other hand, hexaploid plants from imperfect seeds grow normally without regeneration through callus formation. It is unclear why the seeds of hexaploid plants stopped growing at an early stage, but it was thought that the embryo grew normally without stopping embryogenesis. After planting in the greenhouse, the two nonaploid seedlings derived from abnormal plantlets grew vigorously. This vigorous growth may have been caused by the polyploidy observed in triploids (Sanford, 1983) and/or by their juvenility. Further comparisons with the hexaploid parents are needed to clarify this point.

Since both nonaploid seedlings generated in this study had alleles originating from their parents, they were generated by syngamy, not apomixis. Both male and female unreduced gametes have been observed in many plant species (Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003). In addition, various types of 2n gametes, such as first division restitution (FDR), second division restitution (SDR) and post-meiotic restitution (PMR) gametes, exist (Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003). In this study it was unclear which type of unreduced gamete occurred, partly because only four SSR markers were used; however, it was confirmed that FDR of the female gamete did not occur, because seedlings No. 1 and No. 2 did not have alleles of 136 or 140 bp at D.CT-179, which are peculiar to the seed parent, 'Fuyu'. The two seedlings may have been derived from fertilization of a reduced female gamete with an unreduced male gamete, since they had an allele of 222 bp at D.CT-61, which is peculiar to the pollen parent, 'Taishuu'. As unreduced male gametes of Japanese persimmon were considered to be a type of FDR (Zhuang, 1990; Yamada, 2007), the unreduced male gamete probably arose from FDR in this study. More SSR makers for Japanese persimmon are needed to clarify this point.

In Japanese persimmon, unreduced hexaploid pollen was produced naturally at various frequencies depending on the cultivar (Sugiura et al., 2000). When using 'Taishuu' as a pollen parent, artificial pollination using hydrate-sorted unreduced pollen must be difficult, because it has a low occurrence of unreduced pollen, low yield of pollen and low germination rate (Chijiwa et al., 1997). In this study, we showed that nonaploid plants could be derived from imperfect seeds by crossing a hexaploid cultivar with natural pollen of a hexaploid cultivar. These results suggest that imperfect seeds are a useful breeding material for generating nonaploid seedless persimmon cultivars. Yamada et al. (2003) reported that unreduced pollen was increased by low temperatures before blooming. Efficient polyploidy breeding will be possible even in cultivars with a low occurrence of unreduced pollen, such as 'Taishuu', if unreduced hexaploid pollen is induced artificially.

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